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Modulation of Rho Guanine Exchange Factor Lfc Activity by Protein Kinase A-Mediated Phosphorylation▽

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Lfc is a guanine nucleotide exchange factor (GEF) for Rho that demonstrates an unusual ability to associate with microtubules. While several phosphorylated residues have been detected in the Lfc polypeptide, the mechanism(s) by which phosphorylation regulates the exchange activity of Lfc remains unclear. We confirm that Lfc is a phosphorylated protein and demonstrate that 14-3-3 interacts directly and in a phosphorylation-dependent manner with Lfc. We identify AKAP121 as an Lfc-binding protein and show that Lfc is phosphorylated in an AKAP-dependent manner by protein kinase A (PKA). Forskolin treatment induced 14-3-3 binding to Lfc and suppressed the exchange activity of wild-type Lfc on RhoA. Importantly, a mutant of Lfc that is unable to associate with 14-3-3 proteins was resistant to inhibition by forskolin. Tctex-1, a dynein motor light chain, binds to Lfc in a competitive manner with 14-3-3.

RhoGTPases are key regulators of transcription, cell cycle progression, and the organization of the microtubule and actin cytoskeletons. By cycling between active GTP-bound and inactive GDP-coupled states, these enzymes behave as molecular switches. The activation state of RhoGTPases is governed by the balance between the activities of GTPase-activating proteins (GAPs) and guanine exchange factors (GEFs). While the hydrolysis of GTP to GDP by RhoGTPases is enhanced by RhoGAPs, RhoGEFs mediate the exchange of GDP for GTP.

Characterized by tandem Dbl homology (DH) and pleckstrin homology (PH) domains, the Dbl family represents the largest group of RhoGEFs. The DH domain mediates binding to inactive GTPases and catalyzes the exchange of GDP for GTP. The role of the PH domain is less well defined and may facilitate the interaction of some RhoGEFs with the plasma membrane and cooperate with the DH domain in activating RhoGTPases (45). In addition to the DH-PH core, many RhoGEFs also possess extended N and/or C termini with negative regulatory functions. Thus, a number of RhoGEFs are constitutively activated by N- or C-terminal truncation (21, 34, 36). Moreover, N and C termini frequently mediate interactions with other proteins, thereby functioning to integrate several signaling pathways. The regulator of G protein signaling (RGS) homology domain-containing RhoGEFs, p115RhoGEF (35), LARG (50), and PDZ-RhoGEF (25), for instance, can bind directly to and be activated by the Gα subunits of heterotrimeric G proteins. Nearly 40% of human Dbl family RhoGEFs contain C-terminal PDZ binding motifs, suggesting that interactions with PDZ domain-containing proteins represent a common mechanism for controlling RhoGEF localization and activity (26). A number of RhoGEFs possess unrelated domains in addition to the tandem DH-PH core, thus allowing the enzymes to nucleate unique signaling networks. For instance, mammalian Son-of-sevenless (Sos) can coordinate the activities of both Rac and Ras by virtue of both a tandem DH-PH cassette and a RasGEF homology domain (12, 42). Kalirin and Trio have separate functional GEF domains for Rho and Rac in addition to a C-terminal serine/threonine kinase domain with unknown function (3, 15, 16); reviewed in reference 6. Finally, A-kinase anchoring protein (AKAP)-Lbc, a splice variant of the RhoGEF proto-Lbc, possesses a PKA-anchoring domain in addition to tandem DH and PH domains and functions both as an AKAP and exchange factor for Rho (20).

Lbc's first cousin (Lfc) is a Rho-specific exchange factor (28, 37, 44) and shares more than 40% sequence identity with proto-Lbc at the protein level. It initially was identified as a C-terminally truncated protein with a capacity to induce focus formation in NIH 3T3 fibroblasts (55). Lfc, also known as GEF-H1 or ARHGEF2, has the unusual ability to associate with microtubules (5, 7, 28, 37, 44), and we have demonstrated a requirement for the enzyme in prometaphase spindle assembly and orientation (5). Recently, we have shown that Lfc is required for the genesis of neurons from precursors in the embryonic murine cortex and is required to determine the orientation of mitotic precursor cell divisions in vivo (27). Lfc has been shown to play a role in cytokinesis in HeLa cells (8) and has emerging roles in the regulation of paracellular permeability (2, 7, 29) and in the disassembly of apical junctions (48). The overexpression of Lfc induces the assembly of stress fibers (9, 37, 54), while the depletion of Lfc protein expression is associated with an inability of cells to reorganize the actin cytoskeleton in response to lysophosphatidic acid (LPA),

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thrombin, or nocodazole (9). Given this diversity of functions, the localization and activity of Lfc is likely to be tightly controlled in the cell. Indeed, several Lfc-interacting and regulatory proteins have been identified. Lfc is negatively regulated by its interaction with microtubules and may mediate cross-talk between the microtubule and actin cytoskeletons (37). The adaptor protein cingulin binds to and inhibits Lfc at epithelial cell tight junctions, thereby downregulating RhoA activity and cell proliferation in confluent cells (2). Lfc also interacts with the F-actin-binding proteins neurabin and spinophilin in dendritic spines following neuronal stimulation (46). While the significance remains unclear, Lfc is phosphorylated by PAK kinases (10, 59) at several sites and interacts with 14-3-3 proteins in a phosphorylation-specific manner (59). Moreover, Lfc recently was shown to be phosphorylated and inhibited by Aurora A/B and Cdk1/Cyclin B during mitosis (8) and activated by ERK1/2 (24).

We recently identified Tctex-1 as a novel Lfc-interacting protein. While initially characterized as a light chain of the dynein motor, several lines of evidence suggest that Tctex-1 also has dynein-independent functions in the cell. Mammalian Tctex-1 was independently identified as AGS2, an activator of G protein signaling (AGS) protein, and shown to bind directly to Gβ (47, 51). Tctex-1 also associates with several receptors, including the Trk neurotrophin receptor (58), bone morphogenetic receptor type II (39), and the parathyroid hormone receptor (49), although the significance of these interactions is poorly understood. Accumulating evidence implicates Tctex-1 as a regulator of the actin cytoskeleton. Tctex-1 is required for actin cytoskeleton remodeling during neuritogenesis (14, 47), and the protein interacts with the F-actin and myosin-binding protein supervillin (52). In support of a role for Tctex-1 in regulating actin rearrangement, we observed that Tctex-1 overexpression suppressed Lfc-dependent stress fiber assembly (data not shown) and in developing neurons (27). Cells depleted of Tctex-1 assembled a disordered, highly branched stress fiber array in an Lfc-dependent manner, demonstrating that Tctex-1 functionally represses Lfc.

Here, we show that Lfc is a phosphorylated protein that binds to 14-3-3η at distinct sites in its N and C termini. We identify an AKAP, designated AKAP121/D-AKAP1/S-AKAP84, as an Lfc-binding protein and show that Lfc phosphorylation at two 14-3-3-binding sites is dependent on PKA. The activation of PKA by forskolin promotes the association of Lfc with 14-3-3η and inhibits the Lfc-dependent activation of RhoA. We demonstrate that the interaction between 14-3-3 and Tctex-1 is mutually exclusive and may represent one mechanism by which Lfc is regulated.

MATERIALS AND METHODS

Expression constructs. Full-length Lfc (accession no. AF177032) and Lfc truncations were cloned into pFLAG-CMV2 (Sigma). Murine 14-3-3η (accession no. U57311) was cloned into pcDNA3.1(−)B-His/Myc (Invitrogen). Wild-type Lfc, 14-3-3-binding mutants, and Tctex-1 (accession no. NM_174620) were cloned into peGFP-C1 (Invitrogen). Lfc and 14-3-3η were cloned into the BglII and AgeI sites of pCMV-HA-VN173/VC155-tubulin for biomolecular fluorescence complementation analysis. Constitutively active Gα12 (Q231L), pCMV-HA-VN173/VC155-tubulin, and pCMV-FLAG Tctex-1 constructs were kind gifts from J. Sondek (University of North Carolina at Chapel Hill), J. DeLuca (Colorado State University), and C. H. Sung (Cornell University), respectively. Site-directed mutagenesis was performed using the QuikChange XL kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Cell culture, transfection, and treatments. Rat2, NIH 3T3, and 293T cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) supplemented with 10% cosmic calf serum (HyClone). 293T and Rat2 cells were transfected using Polyfect or Effectene (Qiagen). Stable enhanced GFP (eGFP) and eGFP–Tctex-1 Rat2 cells were established by culturing transfected cells in selection medium (500 μg/ml G418) and isolating positive clones by cell sorting. All cultures were maintained in a 5% CO_2 environment at 37°C.

For cell treatments, cells were starved and treated in DMEM containing 20 mM HEPES and 0.5 mg/ml fatty acid-free bovine serum albumin (BSA; A8806; Sigma). LPA (7260; Sigma) was suspended in Hank's buffered saline solution containing 0.5 mg/ml fatty acid-free BSA and 20 mM HEPES to a stock concentration of 1 mM. Bovine thrombin (1 U/μl; 605157), calyculin A (60 μM; *Discodermia calyx* 208851), forskolin (10 mM; *Coleus forskohlii* 344270), 3-isobutyl-1-methylxanthine (IBMX; 500 mg/ml; 410957), and H89 (30 mM; 371963) were obtained from Calbiochem. Staurosporine (*Streptomyces* sp. strain S4400) (Sigma) was constituted to 0.5 mM.

Yeast two-hybrid assays. Yeast two-hybrid assays were performed essentially as previously described (17). Full-length or C-terminal Lfc (amino acids 574 to 985) was cloned into the pBTM116 vector and used to screen an EML-C1 cDNA library for Lfc-interacting proteins.

Antibodies. Western blotting and immunofluorescence were performed using the following primary antibodies: anti-FLAG (M2; F3165; Sigma), anti-His (H15; sc-803; Santa Cruz Biotechnology), anti-glutathione *S*-transferase (anti-GST; B-14; sc-138; Santa Cruz Biotechnology), anti-Gα12 (sc-409; Santa Cruz Biotechnology), anti-Myc (9E10; sc-40; Santa Cruz Biotechnology), anti-GM130 (610822; Becton Dickinson), anti-Myc (610990; Cell Signaling), and antihemagglutinin (anti-HA; H6908; Sigma). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies were from Amersham Pharmacia Biotech. Anti-14-3-3β (sc-1657; Santa Cruz), anti-AKAP121 (611573), and anti-phospho-Ser-14-3-3 binding motif (4E2; 9606; Cell Signaling Technology) also were used. Rabbit anti-phospho-S885 antibody was obtained from Cell Signaling Technology.

Immunoprecipitations, GST pulldowns, Western blotting, and phosphatase assays. For immunoprecipitations and Western blotting, cells were scraped into ice-cold lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 10 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride [PMSF] with Complete Protease Inhibitor cocktail [Roche]), and cleared extracts were incubated with protein G-Sepharose and appropriate antibodies for 2 h at 4°C. Immunoprecipitates were washed three times with wash buffer (30 mM Tris, pH 7.5, 300 mM NaCl, 5 mM NaF, and 0.1% Triton X-100), resuspended in 2× sample buffer, and boiled, and protein complexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to polyvinylidene difluoride (PVDF) membranes and immunoblotting.

Recombinant protein production and GST pulldowns. pGEX-4T3 constructs were transformed into the *Escherichia coli* strain BL21(DE3)pLYS-S (Novagen), and protein synthesis was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM and incubation for 4 h at 37°C. Recombinant proteins were dialyzed with phosphate-buffered saline (PBS) before use. Lysates from 293T cells or adult mouse brain were incubated with 5 μg purified GST-tagged proteins for 1 h, followed by incubation with glutathione-Sepharose beads for 1 h at 4°C.

To treat lysates and immunoprecipitates with phosphatase, cells were lysed in phosphatase lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8, 1% [vol/vol] NP-40, 1 mM dithiothreitol [DTT], 1 mM PMSF with Complete Protease Inhibitor cocktail [Roche]). Lysates were diluted 1:1 with 2× calf intestinal phosphatase (CIP) buffer (100 mM Tris, pH 7.5, and 2 mM $MgCl_2$) and preheated at 37°C for 10 min prior to the addition of 40 U CIP and incubation at 37°C for 30 min (11097075001; Roche Applied Science). Immunoprecipitates were washed three times with lysis buffer, followed by three washes with CIP buffer (50 mM Tris, pH 7.5, and 1 mM $MgCl_2$) and preincubation with 100 μl CIP buffer at 37°C for 10 min. Tubes were incubated for 30 min at 37°C following the addition of 20 U CIP, and beads were washed with lysis buffer.

Overlay assays. Overlay assays were performed as described previously (43), and membranes were incubated with 0.5 μM GST–Tctex-1, GST–14-3-3η, or GST for 2 h at room temperature. Overlaid proteins were detected using anti-GST antibody (sc-138; Santa Cruz).

SRE.L and RhoA-Raichu assays. RhoA-Raichu assays were performed as described previously (32, 33) using NIH 3T3 cells.

Kinase assay. 293T cells were transfected with 4 μg of pFLAG-CMV2-Lfc or pFLAG-CMV2-vector. Cells were washed with PBS and scraped into 500 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM PMSF, 2 mM sodium orthovanadate, and a protease inhibitor tablet [Roche]). Lysates were sonicated and centrifuged for 10 min at 12,500 rpm in a 4°C centrifuge. Lysates were precleared for 30 min with 25 μl of a 50% slurry of protein G-Sepharose. Half of the lysate was used for each immunoprecipitation with 1 μg of anti-Flag antibody (F3165; Sigma) or 1 μg of mouse immunoglobulin G1 isotype control and a 10-μl bed volume of protein G-Sepharose. Immunoprecipitations were performed for 3 h at 4°C. The beads were washed twice with 500 μl of lysis buffer and three times with kinase buffer (50 mM Tris, pH 7.4, 15 mM $MgCl_2$, 1 mM dithiothreitol). Ten microliters of kinase buffer alone or kinase buffer containing 60 μM H89 was added to the washed beads and left on ice for 15 min. Ten microliters of kinase buffer containing 10 μCi of [γ-^{32}P]ATP was added to the tubes, mixed, and placed in a 30°C water bath for 5 min. Reactions were stopped by adding Laemmli sample buffer and boiling samples for 3 min. Samples were run on an SDS–10% polyacrylamide gel. The gel was fixed for 30 min in a 10% acetic acid, 30% methanol solution and dried for 2 h at 80°C before being exposed to film.

RESULTS

Lfc is phosphorylated at 14-3-3 consensus sites. To elucidate mechanisms that regulate Lfc activity in vivo, we performed mass-spectrometric analysis on Lfc immune complexes derived from 293T cells overexpressing Lfc (data not shown). Both 14-3-3 and AKAP121 were identified as potential Lfc-binding proteins. Zenke et al. (59) identified a site in the C terminus of Lfc (S885) that, when phosphorylated, facilitated the interaction of Lfc with 14-3-3 proteins. To confirm that Lfc can be phosphorylated at 14-3-3-binding sites in vivo, 293T cells overexpressing FLAG-tagged Lfc were treated with vehicle or the PP1/PP2A-type Ser/Thr phosphatase inhibitor calyculin A. FLAG-Lfc immunoprecipitates then were incubated with or without CIP. Calyculin treatment was associated with the retarded migration of Lfc during SDS-PAGE, while the phosphatase treatment of Lfc immunoprecipitates abolished the migratory shift observed following calyculin treatment (Fig. 1A). To determine if calyculin could induce the 14-3-3 site-specific phosphorylation of Lfc, a polyclonal antibody that recognizes phosphorylated 14-3-3 binding motifs (CST) was used to immunoblot Lfc precipitated from calyculin-treated cells. Lfc was detected by the anti-phospho-14-3-3 substrate antibody following calyculin treatment, and this band was sensitive to CIP treatment (Fig. 1A). These data suggest that Lfc can be phosphorylated at 14-3-3 consensus sites.

To confirm that Lfc associates with 14-3-3 proteins directly and in a phosphorylation-dependent manner, we performed far-Western analysis of His-Lfc immunoprecipitates from vehicle- or calyculin-treated 293T cells. Lfc complexes or whole-cell lysates derived from untransfected cells were subjected to SDS-PAGE and transferred to PVDF, and membranes were left untreated or were treated with CIP before overlay with recombinant GST (not shown) or GST–14-3-3η (Fig. 1B). GST–14-3-3η associated directly with Lfc in a calyculin-dependent manner. The CIP treatment of immunoblots prior to overlay completely abolished the interaction of overlaid GST–14-3-3η with membrane-bound proteins. These data show that 14-3-3 proteins associate directly with Lfc in a phosphorylation-dependent manner.

We next sought to determine the 14-3-3-binding sites on Lfc and noticed the presence of two potential additional sites at T114 and T184 (http://scansite.mit.edu/) in addition to the established site at S885 (Fig. 1C). Residues T114, T184, and S885 were mutated to alanine using site-directed mutagenesis, and each mutant form of Lfc was transfected into 293T cells together with Myc–14-3-3η. Cells were left untreated or were treated with calyculin, and Myc immunoprecipitation was performed. Calyculin treatment prior to lysis markedly increased the interaction of eGFP-Lfc with Myc–14-3-3η. The phosphorylation-dependent interaction between Lfc and 14-3-3 was significantly impaired following the mutation of S885 to alanine, modestly reduced in the Lfc T114A mutant, and not at all reduced in the T184A mutant (Fig. 1D). These data show that the interaction of Lfc with 14-3-3 proteins is regulated by the phosphorylation of Lfc at T114 and S885, with the latter being the dominant regulatory site.

To determine if the direct interaction of Lfc with 14-3-3η is mediated by the three 14-3-3 consensus sites, we performed an overlay assay on the Lfc point mutants. eGFP-tagged Lfc mutants were transfected into 293T cells, and cells were treated with calyculin to promote Lfc phosphorylation. eGFP-tagged proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, and transferred to membranes for Western blotting or overlay (Fig. 1E). As expected, GST–14-3-3η associated directly and robustly with Lfc. The mutation of residue T114 or S885 to alanine markedly reduced this association, while mutation at position T184 had little effect on GST–14-3-3η binding. The binding of GST–14-3-3η to the triple mutant Lfc-AAA was further reduced compared to that of the single mutants at T114A and S885A alone. The reduced binding of 14-3-3η to the Lfc mutants was correlated with the reduced phosphorylation of Lfc, as detected by the anti-phospho-14-3-3 motif antibody (Fig. 1E and F). These results show that T114 and S885 mediate the direct binding of 14-3-3 proteins with Lfc.

To determine whether 14-3-3 and Lfc interact in living cells, we performed bifunctional complementation (30, 31) using the N-terminal fragment of Venus fluorescent protein (VN173) fused with 14-3-3η and the C-terminal fragment of Venus (VC155) fused with Lfc. VN173 and VC155 are nonfluorescent when expressed alone but exhibit fluorescence complementation when expressed together as fusions with interacting partner proteins. Venus fluorescence was not observed in cells expressing VN173–14-3-3η or VC155-Lfc fusion alone (Fig. 2A and B), whereas the coexpression of both fragments in the same cell resulted in the full fluorescence of the Venus protein (Fig. 2C). Importantly, the expression of VN–14-3-3 with the triple mutant VC–Lfc-AAA showed no fluorescence (Fig. 2D). These data support an in vivo interaction between Lfc and 14-3-3 and the idea that this interaction is contingent on sites S885 and T114.

Lfc associates with AKAP121 and is phosphorylated at 14-3-3 sites by PKA. Our mass spectrometric analysis of FLAG-Lfc immunoprecipitates identified AKAP121/D-AKAP-1/S-AKAP84 as a potential Lfc-interacting protein. To confirm this interaction, AKAP121 protein was immunoprecipitated from the lysates of NIH 3T3 fibroblasts. Endogenous Lfc protein was detected in AKAP121 immunoprecipitates but not when beads alone were incubated with lysates (Fig. 3A). AKAPs function to tether PKA to substrates by binding the RI or RII regulatory subunits of the PKA complex (56). To determine if Lfc is a substrate for PKA, we performed an in vitro kinase

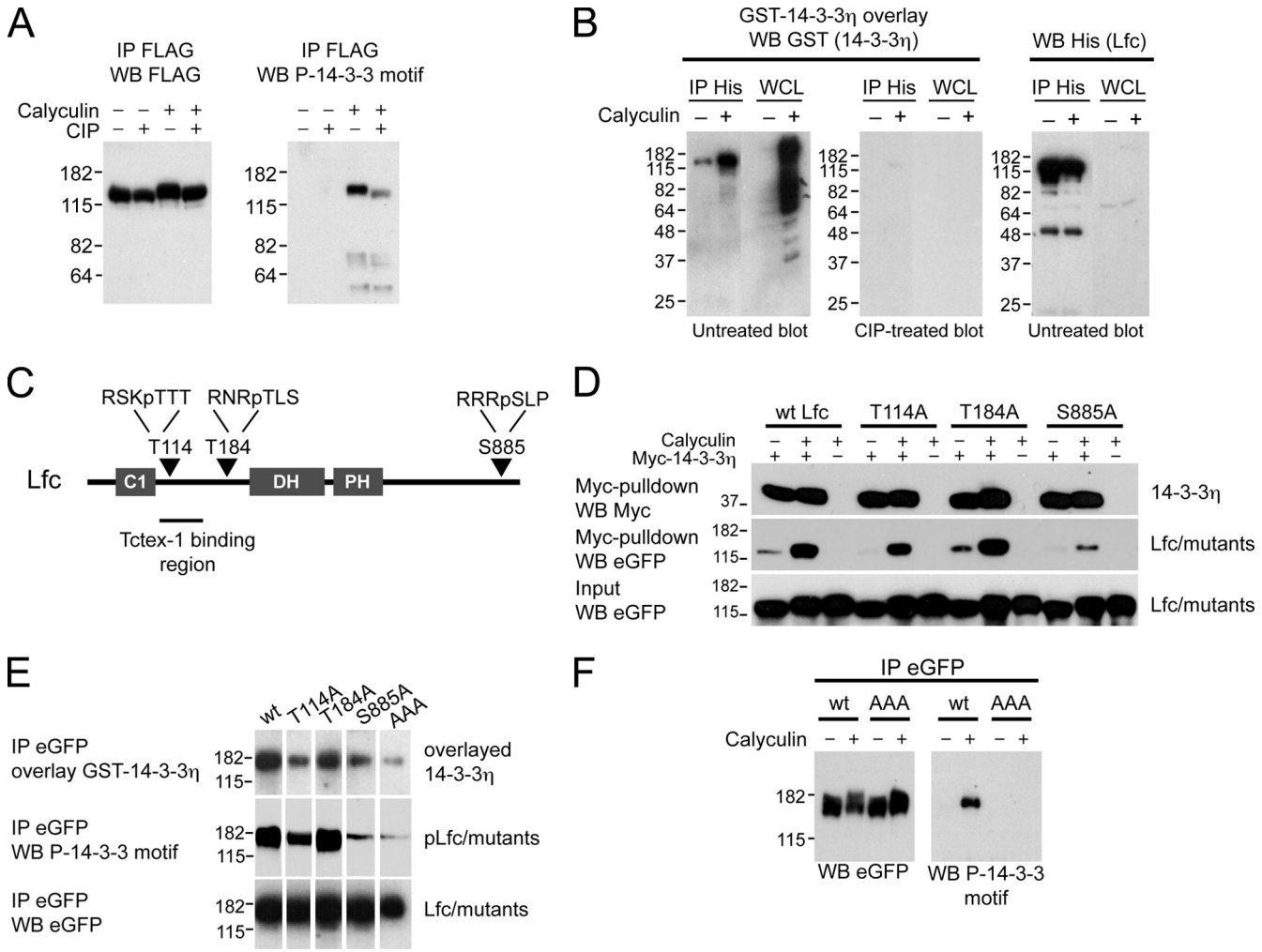

FIG. 1. Lfc is a phosphoprotein and associates directly with 14-3-3η. (A) 293T cells overexpressing FLAG-tagged Lfc were treated with calyculin or dimethylsulfoxide (DMSO) vehicle for 30 min immediately prior to lysis. Anti-FLAG immunoprecipitates (IP) were left untreated or were treated with CIP and immunoblotted for FLAG-Lfc or phosphorylated 14-3-3 binding motifs. WB, Western blotting. (B) 14-3-3η binds to Lfc directly, and the binding is phosphorylation dependent. 293T cells transfected with a His-tagged Lfc construct or untransfected cells were treated with calyculin (or vehicle) for 30 min, and immunoprecipitated His-Lfc protein or whole-cell lysate (WCL) was electrophoresed and transferred to PVDF. Membranes were incubated with or without CIP overnight prior to the overlay of recombinant GST-14-3-3η or immunoblotted for His-Lfc. (C) Domain structure of Lfc showing three predicted 14-3-3-binding sites and their amino acid sequences. The bar represents the Tctex-1-binding region (residues 87 to 151). (D) Myc-14-3-3η coimmunoprecipitation performed using lysates from 293T cells expressing eGFP-tagged Lfc, Lfc T114A, Lfc T184A, or Lfc S885A treated with calyculin or left untreated. Myc-14-3-3η complexes were electrophoresed, transferred to PVDF, and immunoblotted with anti-Myc or anti-eGFP to detect 14-3-3η and Lfc, respectively. wt, wild type. (E) Far-Western analysis of immunoprecipitated, eGFP-tagged Lfc, Lfc T114A, Lfc T184A, Lfc S885A, or Lfc-AAA prepared from calyculin-treated 293T cells. Membranes were probed with purified recombinant GST–14-3-3η (upper) or immunoblotted for phosphorylated 14-3-3 binding motifs (middle) or total eGFP-tagged Lfc protein levels (bottom). (F) Lfc is phosphorylated at sites in addition to 14-3-3 consensus sites. 293T cells transfected with eGFP-tagged Lfc or Lfc-AAA (AAA) were left untreated or were treated with calyculin. Immunoprecipitated Lfc and Lfc-AAA complexes were electrophoresed and immunoblotted for total levels of Lfc protein (anti-eGFP) and phosphorylated 14-3-3 binding motifs (anti-P-14-3-3 motif).

assay on the presumption that Lfc binds to and becomes phosphorylated by an associated kinase (Fig. 3B). FLAG-Lfc was immunoprecipitated from 293T cells treated with either vehicle or the PKA inhibitor H89 and incubated with [γ-^{32}P]ATP. The products of the reaction were resolved by SDS-PAGE and visualized by autoradiography. We observed that Lfc is an efficient substrate for a kinase present in Lfc immune complexes, and that this kinase activity is reduced by the PKA inhibitor H89. To determine if PKA phosphorylates Lfc at potential 14-3-3 sites in vivo, 293T cells expressing FLAG-Lfc were left untreated or were treated with forskolin to elevate intracellular cyclic AMP (cAMP) levels or treated with forskolin plus H89. FLAG-Lfc immune complexes then were probed with the phospho-14-3-3 motif antibody. Forskolin treatment was associated with the phosphorylation of Lfc at 14-3-3-binding sites, whereas the pretreatment of cells with H89 reduced the capacity of forskolin to stimulate Lfc phosphorylation in vivo (Fig. 3C).

We next investigated whether the phosphorylation of Lfc by PKA could regulate the association of the exchange factor with

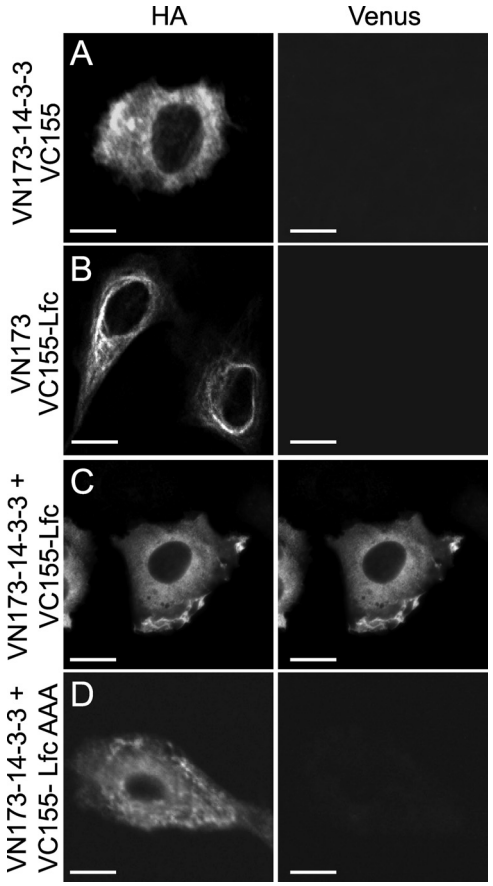

FIG. 2. Lfc interacts with 14-3-3η in cells. 14-3-3η and Lfc were cloned into pCMV-HA vectors proximal to N-terminal (VN173) or C-terminal (VC155) fragments of Venus, respectively, and were introduced into Rat2 cells. Transfected cells were imaged by confocal microscopy. (A) Coexpression of VN173–14-3-3 and VC155 (vector without insert). (B) Coexpression of VN173 (vector without insert) and VC155-Lfc. (C) Coexpression of VN173–14-3-3 and VC155-Lfc. (D) Coexpression of VN173–14-3-3 and VC155–Lfc-AAA. Bars represent 10 μM.

14-3-3 proteins. 293T cells expressing FLAG-Lfc and His-14-3-3η were treated with vehicle or forskolin or pretreated with H89 prior to forskolin treatment. FLAG-tagged Lfc immune complexes were assessed for the presence of His-14-3-3η by Western blotting. Low levels of His-14-3-3η were detected in FLAG immunoprecipitates prepared from untreated cells, while the treatment of cells with forskolin elevated 14-3-3η levels in Lfc precipitates (Fig. 3D). However, the pretreatment of cells with H89 prior to forskolin treatment completely abolished the interaction of Lfc with 14-3-3η.

To determine if an AKAP is required for the PKA-induced stimulation of 14-3-3 binding to Lfc, NIH 3T3 cells were transfected with a construct encoding the RII-binding domain of AKAP121 to uncouple Lfc from PKA by competitive inhibition for the PKA catalytic subunit. Cells were left untreated or were treated with forskolin, and 14-3-3 immune complexes were analyzed for the presence of Lfc. As expected, forskolin treatment enhanced the association of endogenous 14-3-3 with Lfc in cells overexpressing eGFP-C1 vector alone (Fig. 3E). However, forskolin was ineffective in increasing the Lfc–14-3-3 interaction in the presence of the eGFP-tagged RII domain, suggesting that the association of Lfc with an AKAP, likely AKAP121, is required for the phosphorylation of Lfc by PKA. Taken together, these data show that Lfc binds to AKAP121 and is associated with PKA activity necessary for phosphorylating Lfc at 14-3-3-binding sites.

To elucidate which 14-3-3-binding sites are phosphorylated by PKA in vivo, 293T cells expressing eGFP-tagged wild-type Lfc or a series of Lfc variants mutated at T114A, T184A, S885A, T114A/T184A, T114A/S885A, or T184A/S885A or the triple mutant Lfc-AAA were treated with forskolin to stimulate PKA activity. eGFP complexes were subjected to Western blotting with the anti-phospho-14-3-3 motif antibody. The mutation of S885 was sufficient to abrogate the forskolin-induced phosphorylation of Lfc, whereas the mutation of T114 or T184 had little effect. To confirm that S885 is targeted for phosphorylation by PKA, we utilized a phosphospecific antibody (Cell Signaling Technology) that detects the phosphorylation of Lfc at S885. 293T cells were transfected with eGFP-tagged wild-type Lfc or Lfc S885A and treated with vehicle, forskolin, or H89. eGFP-tagged complexes were immunoprecipitated from lysates and probed with the anti-phospho-S885-specific antibodies (Fig. 3G). Forskolin treatment increased Lfc phosphorylation, while the addition of H89 markedly reduced Lfc phosphorylation to below basal levels. We confirmed the specificity of this antibody by repeating the experiment with Lfc S885A and found no detectable signal under any condition. Taken together, these data demonstrate that Lfc can be phosphorylated by PKA in vivo at the major 14-3-3-binding site S885.

PKA inhibits Lfc GEF activity. We next sought to determine whether the PKA phosphorylation of Lfc can regulate the ability of Lfc to activate Rho. NIH 3T3 cells were transfected with FLAG-tagged wild-type Lfc or the triple mutant Lfc-AAA and were left untreated or were treated with forskolin and the phosphodiesterase inhibitor IBMX. To measure Rho activation by Lfc, we utilized a Rho-sensitive Raichu probe and a fluorescent resonance energy transfer (FRET)-based assay (32, 33). Both wild-type Lfc and Lfc-AAA stimulated FRET above basal levels in NIH 3T3 cells treated with IBMX alone (Fig. 4A). Following treatment with both IBMX and forskolin, however, wild-type Lfc was unable to stimulate the activation of RhoA, with FRET induction comparable to levels in cells transfected with vector alone. In contrast, forskolin was unable to repress the activity of the triple mutant Lfc-AAA. We and others have demonstrated that LPA-induced stress fibers in fibroblasts require Lfc (9 and data not shown). Since forskolin inhibits the capacity of Lfc to activate Rho, we sought to determine if stress fiber formation also is negatively regulated by forskolin. Rat2 cells were cultured in growth media in the absence of serum prior to treatment with or without forskolin. Cells were treated with LPA to induce stress fiber assembly, fixed, and stained with phalloidin. Cells grown in serum-free media assembled few stress fibers (Fig. 4B). Thick, parallel stress fibers were detected following LPA treatment. However, cells cultured in the presence of 10 μM forskolin prior to LPA treatment were unable to assemble stress fibers. To demonstrate that LPA-induced stress fiber formation was dependent on Lfc, we transfected GFP together with one of three independent small interfering RNA oligonucleotides directed against Lfc, and after 48 h we treated the cells with LPA. As

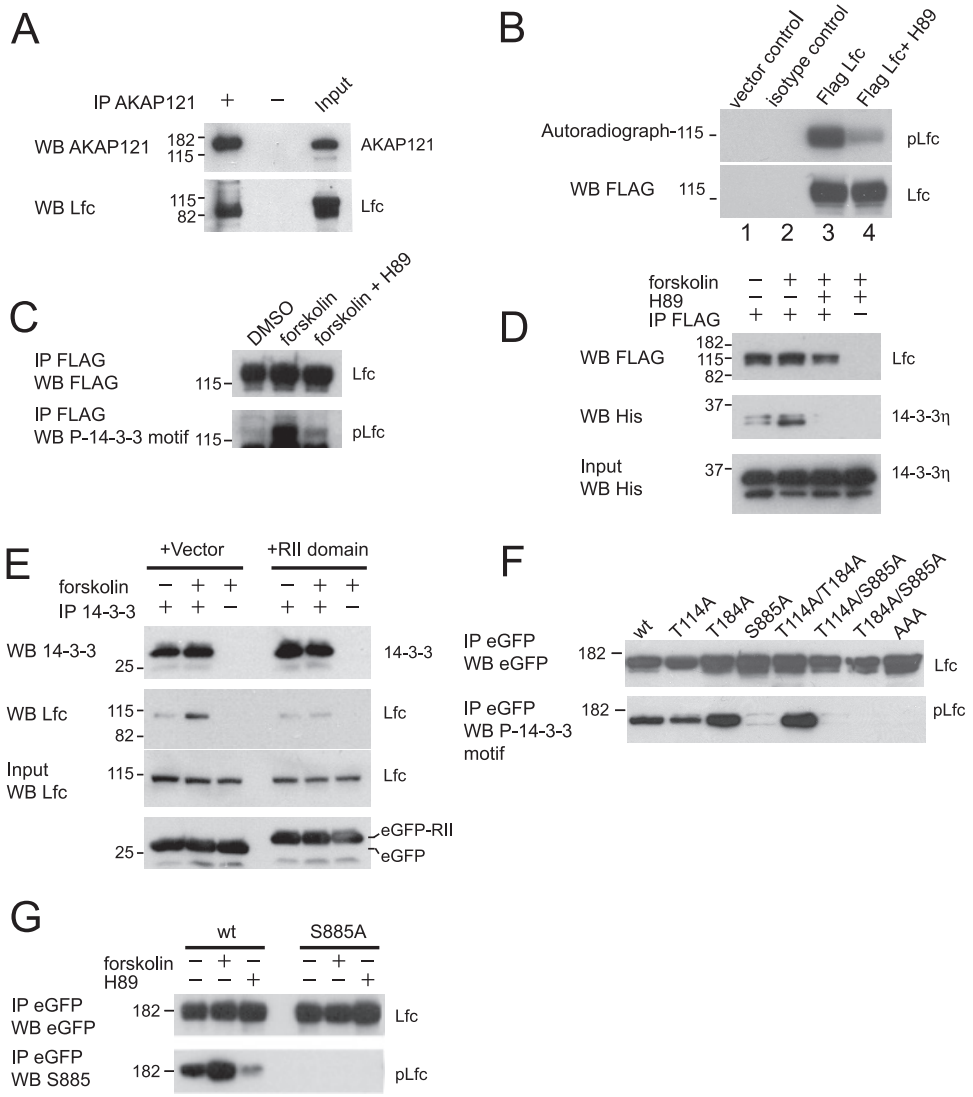

FIG. 3. Lfc associates with AKAP121 and is phosphorylated by PKA. (A) Lfc interacts with AKAP121. Endogenous AKAP121 was immunoprecipitated (IP) from NIH 3T3 cells, and immunoprecipitates were immunoblotted for AKAP121 and Lfc. WB, Western blotting. (B) Lfc immune complex kinase assay. pFLAG-Lfc was immunoprecipitated from transfected 293T cells, washed, and incubated with [γ-^{32}P]ATP. The products of the kinase assay were visualized by autoradiography. Transfected cells were treated with vehicle or the PKA inhibitor H89 prior to lysis. (C) PKA phosphorylates Lfc. 293T cells were transfected with FLAG-tagged Lfc, and cells were treated with DMSO or 30 μM H89 for 1 h prior to the addition of 10 μM forskolin for 30 min. Anti-FLAG immunoprecipitates were immunoblotted with anti-FLAG antibodies (upper) or anti-phospho-14-3-3 motifs antibodies (lower). (D) Forskolin treatment enhances 14-3-3 binding to Lfc. 293T cells were transfected with FLAG-tagged Lfc and His-tagged 14-3-3η and pretreated with DMSO vehicle or 30 μM H89 for 1 h prior to the addition of 10 μM forskolin to culture media. FLAG-tagged complexes were immunoprecipitated and subjected to Western blotting for FLAG-Lfc and the associated His–14-3-3η. (E) Forskolin-induced 14-3-3 binding to Lfc is AKAP dependent. NIH 3T3 cells were transfected with eGFP or the eGFP-tagged RII-binding domain of AKAP121 and were left untreated or treated with 10 μM forskolin for 30 min. Endogenous 14-3-3 complexes were immunoprecipitated and immunoblotted for 14-3-3 or Lfc. (F) PKA phosphorylates Lfc on residues T114 and S885. Anti-eGFP immunoprecipitates prepared from 293T cells expressing eGFP-tagged wild-type (wt) Lfc or Lfc mutants and treated with 10 μM forskolin for 30 min were immunoblotted for eGFP or anti-phospho-14-3-3 motif antibodies. (G) PKA phosphorylates Lfc at S885. 293T cells were transfected with wild-type eGFP-tagged Lfc, Lfc S885A, or Lfc T114/S885A and treated with 10 μM forskolin for 30 min or 30 μM H89 for 1 h. eGFP-tagged complexes were immunoprecipitated and subjected to Western blotting with anti-eGFP or anti-S885 Lfc antibody.

shown in Fig. 4C, transfected cells were devoid of stress fibers. In cells overexpressing Lfc, we noted that LPA-induced stress fiber formation was increased. Again, this effect could be suppressed by forskolin (Fig. 4C). Lastly, we transfected the Lfc-AAA mutant into cells and noted that while these cells form stress fibers following LPA exposure, they no longer are responsive to forskolin inhibition. Taken together, these data demonstrate that Lfc exchange activity and its capacity to induce stress fibers in vivo is negatively regulated by PKA and sites required for 14-3-3 binding.

Constitutively active Gα12 inhibits the association of 14-3-3 proteins with Lfc. Lfc modulates stress fiber and focal adhesion assembly downstream of LPA and the G-protein-coupled receptor subunits Gα12/13 (data not shown). Since the phos-

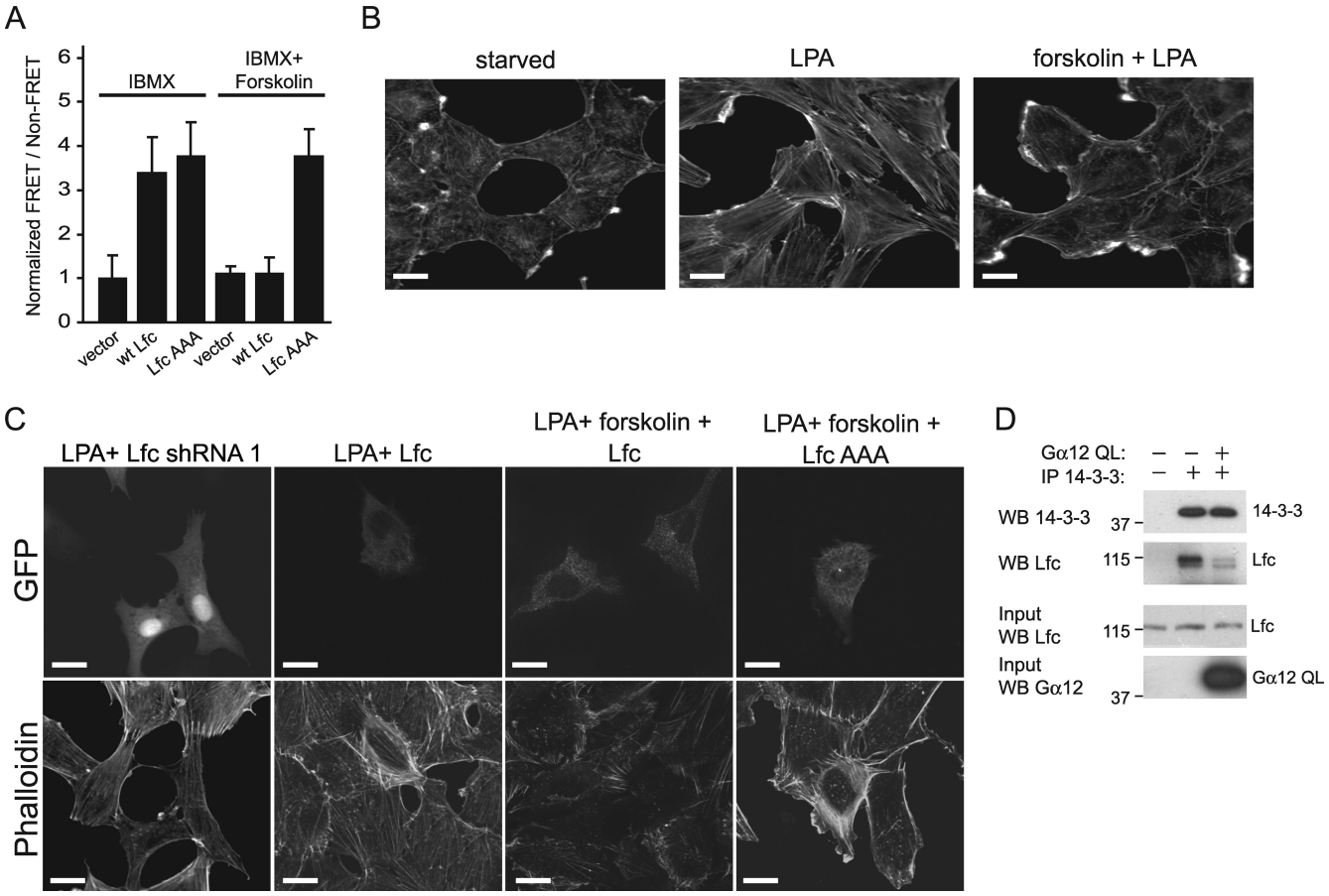

FIG. 4. PKA inhibits RhoA activation by Lfc and stress fiber assembly. (A) RhoA-Raichu assay of Lfc exchange activity. NIH 3T3 cells transfected with the Raichu probe and His-tagged wild-type (wt) Lfc or Lfc-AAA were treated with IBMX alone or IBMX and forskolin for 10 min, and lysates were prepared to measure normalized fluorescence at 528 and 480 nm. Experiments were performed three times, and results are expressed as a ratio of basal FRET ± standard errors. (B) Forskolin inhibits stress fiber assembly. Rat2 cells were grown on slides and starved of serum for 24 h. Cells were fixed or pretreated with or without 10 μM forskolin for 30 min prior to 1 μM LPA treatment for 30 min. Coverslips were stained with phalloidin to visualize actin filaments. (C) LPA signaling to the actin cytoskeleton requires active Lfc. Rat2 cells were transfected with short hairpin RNA, eGFP Lfc, or eGFP–Lfc-AAA and starved for 24 h. Cells were fixed or pretreated with 10 μM forskolin or left untreated for 30 min prior to 1 μM LPA treatment for 30 min. Coverslips were stained with phalloidin to visualize actin filaments. Bars represent 10 μM. (D) Gα12 modulates 14-3-3 binding to Lfc. Gα12 inhibits the interaction of Lfc with 14-3-3 proteins. 293T cells were transfected with pcDNA3.1 vector or pcDNA3.1-Gα12 Q231L (Gα12 QL). Endogenous 14-3-3 complexes were immunoprecipitated (IP) from lysates and separated by SDS-PAGE. After transfer to PVDF, membranes were subjected to Western blotting (WB) for 14-3-3 and Lfc.

phorylation of Lfc at 14-3-3-binding sites inhibits the activity of the exchange factor, the dephosphorylation of Lfc may be required to activate its exchange activity. We postulated that the activation of G protein-coupled pathways decreased 14-3-3 binding to Lfc. To test this hypothesis, we measured the change in Lfc bound to 14-3-3 in the absence or presence of a constitutively active mutant of Gα12. 293T cells were transfected with pcDNA3.1 vector or Gα12 (Q231L), and endogenous 14-3-3 was immunoprecipitated from lysates and probed with anti-Lfc antibodies. While Lfc was detected in immunoprecipitates derived from vector-transfected cells, the expression of Gα12 (Q231L) markedly reduced the association of Lfc with 14-3-3 proteins (Fig. 4D). This suggests that the activation of Gα12 is correlated with the activation of Lfc and the diminution of the interaction of Lfc with 14-3-3.

The Lfc–Tctex-1 interaction is regulated by phosphorylation. We have identified Tctex-1, a dynein motor light chain and an AGS2 protein(51), as an Lfc-binding protein that binds within a region encompassed by residues 87 to 151 in the N terminus of Lfc (data not shown). Since T114 is a subordinate 14-3-3-binding site that resides within the Tctex-1-binding region, we postulated that phosphorylation modulated the association of Lfc with Tctex-1. To determine if phosphorylation affected Tctex-1 binding, Rat2 cells stably expressing eGFP-tagged Tctex-1 were treated with calyculin to promote the phosphorylation of intracellular proteins (Fig. 5A). Endogenous Lfc immunoprecipitated from the lysates of calyculin-treated cells associated with less eGFP-Tctex-1 than cells treated with vehicle alone, suggesting that phosphorylation diminishes the Lfc–Tctex-1 interaction. Conversely, the treatment of 293T cells expressing FLAG-Lfc with staurosporine, a broad-specificity kinase inhibitor, enhanced the capacity for recombinant GST–Tctex-1 to precipitate FLAG-Lfc from cell lysates (Fig. 5B). These data show that the dephosphorylation of Lfc augments Tctex-1 binding, while phosphorylation antagonizes interaction with Tctex-1.

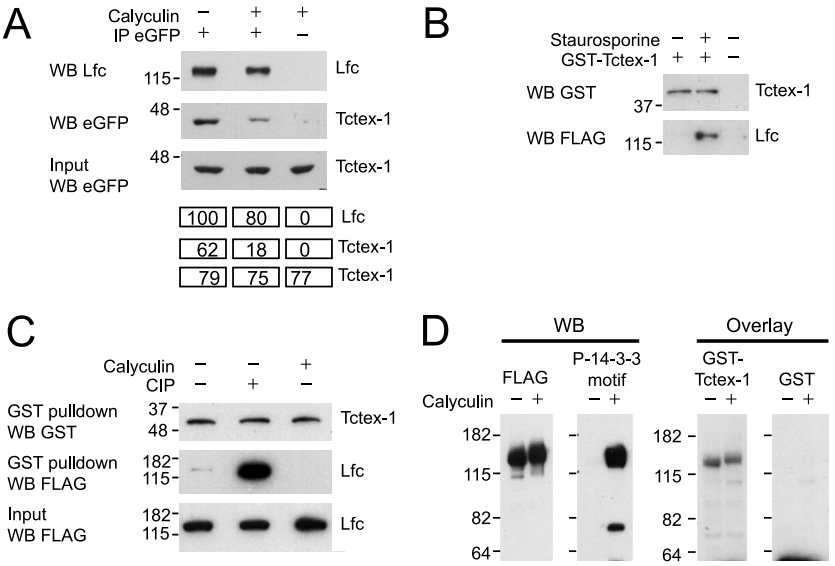

FIG. 5. Dephosphorylation of Lfc promotes Tctex-1 binding. (A) Endogenous Lfc complexes immunoprecipitated (IP) from Rat2 cells stably expressing eGFP–Tctex-1 left untreated or treated with calyculin A were immunoblotted for Lfc (top) or associated eGFP–Tctex-1 (middle). The intensity of the bands was determined by densitometry using ImageJ Prime software and are presented under the bands. WB, Western blotting. (B) Phosphorylation regulates Tctex-1 binding to Lfc. 293T cells expressing FLAG-tagged Lfc were treated with 0.5 μM staurosporine for 1 h, and lysates were incubated with immobilized GST–Tctex-1. GST complexes were immunoblotted for FLAG-Lfc and GST–Tctex-1. (C) Lfc dephosphorylation promotes Tctex-1 binding. Lysates prepared from FLAG-Lfc-transfected 293T cells were incubated with or without CIP, or cells were treated with calyculin prior to lysis. Lysates were incubated with GST–Tctex-1, and immobilized GST complexes were immunoblotted for GST–Tctex-1 (upper) and FLAG-Lfc (middle). (D) Tctex-1 overlay assay of nonphosphorylated and phosphorylated Lfc. Cells transfected with FLAG-Lfc were treated with calyculin for 15 min, and FLAG-Lfc immunocomplexes were electrophoresed and immunoblotted for total Lfc protein levels (first gel) or phosphorylated 14-3-3 binding motifs (second gel), or membranes were overlaid with purified recombinant GST–Tctex-1 (third gel) or GST alone (fourth gel). GST–Tctex-1 associated directly with FLAG-Lfc equally independent of phosphorylation status.

To clarify the role of phosphorylation in the Lfc–Tctex-1 interaction, we specifically manipulated the phosphorylation state of Lfc prior to incubation with immobilized Tctex-1 protein. Lysates from 293T cells expressing FLAG-Lfc were dephosphorylated with CIP for 30 min and incubated with recombinant GST–Tctex-1. As shown in Fig. 5C, immobilized GST–Tctex-1 precipitated markedly more FLAG-Lfc from phosphatase-treated lysates than from untreated controls. Similarly, the phosphorylation of Lfc by treating FLAG-Lfc-expressing 293T cells with calyculin prior to the incubation of lysates with GST–Tctex-1 reduced its association with Lfc to levels that were less than those of controls. These data confirm that the phosphorylation state of Lfc is inversely associated with its capacity to interact with Tctex-1.

To determine if phosphorylation directly interfered with Tctex-1 binding to Lfc, we performed the far-Western analysis of FLAG-Lfc immunoprecipitates, prepared from 293T cells left untreated or treated with calyculin for 15 min, using purified recombinant GST–Tctex-1 protein as a probe. Immunoblotting with anti-FLAG or anti-phospho-14-3-3 substrate confirmed that calyculin treatment markedly enhanced Lfc phosphorylation at 14-3-3 recognition sites (Fig. 5D, left). Far-Western analysis using GST–Tctex-1 as a probe demonstrated that Tctex-1, but not GST alone, associated directly with Lfc in a phosphorylation-independent manner (Fig. 5D, right), thus showing that Lfc phosphorylation per se does not interfere with Tctex-1 binding.

14-3-3 and Tctex-1 bind Lfc in a mutually exclusive manner. Phosphorylation represses Tctex-1 binding to Lfc while stimulating the association with 14-3-3. We next tested whether 14-3-3 directly antagonizes the interaction of Lfc with Tctex-1. We assessed the ability of 14-3-3 to disrupt the interaction between Tctex-1 with either wild-type Lfc or Lfc mutants unable to bind to 14-3-3. His–14-3-3η was transfected into 293T cells together with eGFP–Tctex-1 and either FLAG-Lfc, the mutant T114A or S885A, or the triple mutant Lfc-AAA. eGFP–Tctex-1 immunoprecipitated from the lysates of transfected cells associated with wild-type Lfc and all Lfc mutants equally in the absence of ectopically expressed 14-3-3η (Fig. 6). The cotransfection of cells with 14-3-3η significantly impaired the ability of wild-type Lfc to bind Tctex-1, whereas 14-3-3η did not suppress the association of the Lfc mutants with Tctex-1. These data demonstrate that 14-3-3 is capable of inhibiting the formation of Lfc–Tctex-1 complexes and support a model whereby Lfc associates with Tctex-1 or 14-3-3 in a mutually exclusive manner. We propose a model whereby the cAMP-dependent PKA phosphorylation of S885 creates a high-affinity 14-3-3-binding site within the carboxy terminus of Lfc. 14-3-3 dimers docked at the S885 site then would be poised to bind to a lower-affinity 14-3-3 site at T114 within the N-terminal region, creating an intramolecular closed inactive configuration of the GEF and the simultaneous exclusion of Tctex-1 from the Lfc complex.

DISCUSSION

RhoGEFs play a crucial role in transducing signals to Rho-GTPases and therefore are essential for multiple coordinated

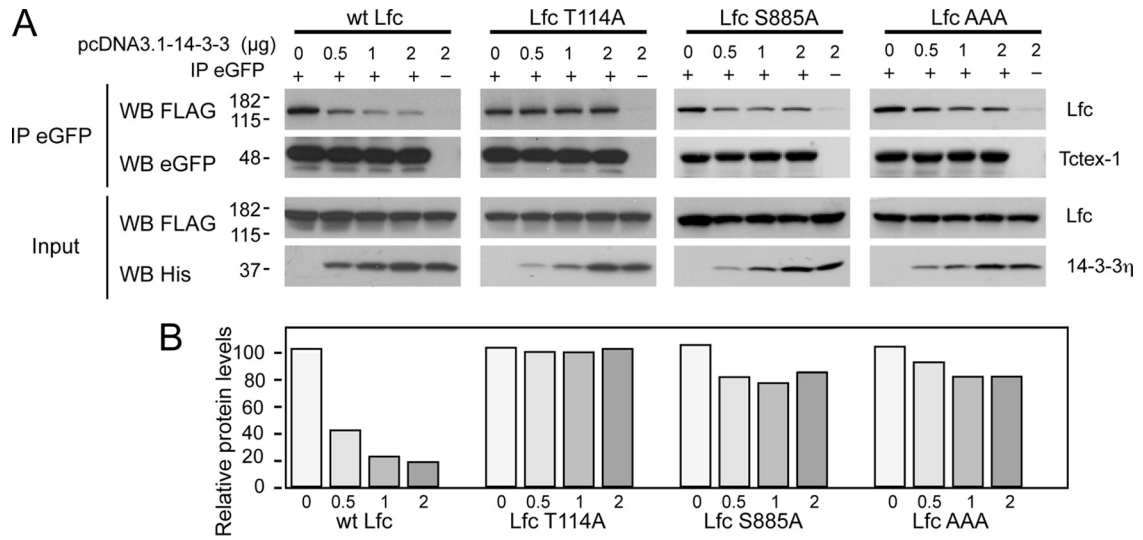

FIG. 6. 14-3-3 and Tctex-1 bind to Lfc in a mutually exclusive manner. (A) 293T cells were transfected with FLAG-tagged Lfc, Lfc T114A, Lfc S885A, Lfc-AAA, and eGFP-tagged Tctex-1 in the presence of increasing amounts of His-tagged 14-3-3η. eGFP immunocomplexes and input lysates were immunoblotted for FLAG-Lfc, eGFP–Tctex-1, and His-14-3-3η. IP, immunoprecipitation; WB, Western blotting; wt, wild type. (B) The intensity of the bands of variant FLAG-tagged Lfc from panel A were determined (by densitometry using ImageJ Prime software) by dividing the observed signal of each band with that of input FLAG-tagged Lfc.

cellular processes. To ensure the correct spatial and temporal activation of pathways downstream of Rho, the localization and activity of RhoGEFs is tightly controlled within the cell. Lfc is regulated by several mechanisms, which include sequestration and inhibition by microtubules, inhibition by the tight junction protein cingulin, and Tctex-1, an inhibitor of Lfc-dependent actin cytoskeletal reorganization. Here, we show that the Lfc exchange activity is negatively regulated by cAMP, and that the Lfc-dependent induction of actin stress fibers by LPA can be repressed by the pharmacologic elevation of cAMP levels. PKA may regulate RhoA signaling at several levels. PKA can directly phosphorylate RhoA, which inhibits its association with Rho guanine dissociation inhibitors (22, 38). PKA also can phosphorylate Gα13 to destabilize its association with Gβγ, thereby preventing the G-protein-coupled receptor-mediated activation of RhoA (40). We therefore cannot discount the contribution of suppressed RhoA and Gα13-coupled signaling to the failure of forskolin-treated cells to assemble stress fibers. However, the failure of forskolin to suppress stress fiber formation by the triple mutant Lfc-AAA suggests that Lfc is an important target of PKA in regulating actin structures.

We have demonstrated that PKA activity is physically associated with Lfc and that PKA can phosphorylate S885, the dominant 14-3-3-binding site on Lfc. We have identified AKAP121/D-AKAP-1 as the AKAP that recruits PKA to Lfc. This mode of GEF-negative regulation is reminiscent of AKAP-Lbc, which incorporates an AKAP domain directly within its primary amino acid polypeptide sequence and is phosphorylated and inactivated by tethered PKA in vivo (18, 20, 33). Distinctly from AKAP-Lbc, Lfc binds to AKAP121/D-AKAP-1 through noncovalent interactions.

We show that 14-3-3η associates directly with Lfc at one dominant site, S885, and a second ancillary site at T114. While this study focused on the interaction between 14-3-3η and Lfc, it is likely that several 14-3-3 protein family members are competent to interact with Lfc, including γ, ε, and ζ (33, 41, 59). Zenke et al. (59) showed that the phosphorylation of S885 by PAK1 induces the association of Lfc with 14-3-3 proteins. However, the authors were unable to detect changes in Lfc activity when Cos1 cells expressing wild-type Lfc were cotransfected with constitutively active PAK1. Birkenfeld et al. (8) observed an increase in the exchange activity of CIP-treated Lfc in vitro. Nevertheless, the effect of in vivo kinases on Lfc activity or function was not assessed. Our data demonstrate that the phosphorylation of Lfc by PKA stimulates the association of Lfc with 14-3-3 proteins and negatively regulates the ability of the exchange factor to activate RhoA.

Several other RhoGEFs interact with 14-3-3 proteins. The phosphorylation-independent interaction of 14-3-3η and 14-3-3ε with p190RhoGEF may control the nucleocytoplasmic shuttling and antiapoptotic properties of the exchange factor (57, 60). The oligomerization of β_1Pix is required for the enzyme to exhibit exchange activity toward Rac, and β_1Pix dimers can be inhibited by a PKA-mediated interaction with 14-3-3β (11). In contrast, the homodimerization of AKAP-Lbc negatively regulates its exchange activity (4). Like β_1Pix, the activity of AKAP-Lbc is suppressed by PKA-dependent 14-3-3 binding (18), and AKAP-Lbc monomers are resistant to inhibition by 14-3-3 (4), suggesting that 14-3-3 can negatively regulate the exchange activity of AKAP-Lbc only when in an oligomeric state (19). It has been proposed that the association of 14-3-3 proteins with AKAP-Lbc dimers induces a conformational change that prohibits interaction with Rho (19). Since a number of RhoGEFs have been shown to homo- and heterodimerize (4, 11, 13, 21, 23, 61), this may represent a common mechanism for the regulation of Dbl family RhoGEFs.

Our data reveal that 14-3-3 proteins regulate Lfc in two ways. First, the phosphorylation of Lfc by PKA at 14-3-3 consensus sites suppresses its activity. It is possible that Lfc forms

homodimers that are regulated by 14-3-3 proteins, similarly to β_1Pix and AKAP-Lbc. Alternatively, since 14-3-3 proteins can oligomerize and bind to sites in both the N and C termini of Lfc, 14-3-3 proteins may force the exchange factor into a closed conformation that precludes an interaction with Rho. Several RhoGEFs are regulated by intramolecular interactions that dictate the accessibility of the DH domain to RhoGTPases. For example, the interaction of the N terminus of Vav with the DH domain of the molecule inhibits exchange activity and is relieved upon the phosphorylation of Y174 by Syk and Src family tyrosine kinases or the deletion of N-terminal residues (1). AKAP-Lbc originally was identified as an N- and C-terminally truncated, constitutively active protein in myeloid leukemia patients and was termed onco-Lbc (53), and the deletion of either N- or C-terminal residues from AKAP-Lbc enhances RhoA binding and confers comparable increases in exchange activity (18, 20). Likewise, Lfc was cloned as a C-terminally truncated protein that could transform NIH 3T3 fibroblasts, suggesting that the C terminus of full-length Lfc contains a negative regulatory element (28, 55). Cells expressing a mutant of Lfc that lacks N-terminal amino acids assemble more numerous and thicker stress fibers than those expressing wild-type Lfc, and these cells are resistant to the inhibition of thrombin-induced stress fiber assembly by Tctex-1 (data not shown). These observations suggest that Lfc mutants that are unable to bind to either 14-3-3 or Tctex-1 are constitutively activated and suggest that Lfc activity is dually inhibited by these proteins.

We have demonstrated that Lfc is a cAMP-regulated RhoGEF. PKA is coupled to Lfc through the AKAP protein AKAP121/D-AKAP-1, resulting in phosphorylation at S885, a high-affinity 14-3-3-binding site. The pharmacologic induction of cAMP inhibits Lfc-associated GEF activity in cells and suppresses Lfc-dependent stress fiber formation in fibroblasts. Moreover, Lfc mutants that are unable to bind 14-3-3 no longer are sensitive to cAMP inhibition, suggesting that 14-3-3 binding is essential for maintaining Lfc in an inhibited state. Currently, we are examining the role of the cAMP-dependent regulation of Lfc activity during mitosis and in cortical neuron progenitors where Lfc has an essential function.

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