The Ubiquitin-like Protein LC3 Regulates the Rho-GEF Activity of AKAP-Lbc

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AKAP-Lbc is a member of the A-kinase anchoring protein (AKAP) family that has been recently associated with the development of pathologies, such as cardiac hypertrophy and cancer. We have previously demonstrated that, at the molecular level, AKAP-Lbc functions as a guanine nucleotide exchange factor (GEF) that promotes the specific activation of RhoA. In the present study, we identified the ubiquitin-like protein LC3 as a novel regulatory protein interacting with AKAP-Lbc. Mutagenesis studies revealed that LC3, through its NH2-terminal regulatory protein interacting with AKAP-Lbc. Mutagenesis studies revealed that LC3, through its NH2-terminal regulatory region of AKAP-Lbc. Interestingly, LC3 overexpression strongly reduced the ability of AKAP-Lbc to interact with RhoA, profoundly impairing the Rho-GEF activity of the anchoring protein and, as a consequence, its ability to promote cytoskeletal rearrangements associated with the formation of actin stress fibers. Moreover, AKAP-Lbc mutants that fail to interact with LC3 show a higher basal Rho-GEF activity as compared with the wild type protein and become refractory to the inhibitory effect of LC3. This suggests that LC3 binding maintains AKAP-Lbc in an inactive state that displays a reduced ability to promote downstream signaling. Collectively, these findings provide evidence for a previously uncharacterized role of LC3 in the regulation of Rho signaling and in the reorganization of the actin cytoskeleton.

Intracellular processing of signals is coordinated by a multitude of scaffolding and adaptor proteins that assemble macromolecular transduction complexes by forming highly specific protein-protein interactions (1). By sequestering signaling enzymes to a specific subcellular environment, these proteins ensure that upon activation the enzymes are near their relevant targets. Protein kinase A anchoring proteins (AKAPs) are prototypic examples of scaffolding proteins that compartmentalize signaling complexes at precise subcellular sites (2). This group of functionally related proteins tethers the cAMP-dependent protein kinase (PKA) in close proximity to their physiological substrate(s) and favor specific PKA phosphorylation events (2, 3). Each AKAP contains a conserved amphipathic helix that binds to the regulatory subunit dimers of the PKA holoenzyme (4, 5) and displays a unique targeting motif that directs PKA-AKAP complexes to specific subcellular sites (2). Another fundamental role of AKAPs is to assemble signaling complexes by interacting with additional signaling enzymes, such as phosphatases (6, 7), phosphodies- terases (8), GTPases (9), and other protein kinases (10–13). Through modular interactions, multi-enzyme complexes are assembled at specific sites in the cell to ensure integration and processing of multiple signals that coordinate the phosphorylation and function of specific cellular substrates (3).

Evidence obtained in recent years indicates that AKAPs can coordinate the regulation of small molecular weight GTPases of the Ras superfamily. This is the case of AKAP-Lbc, which functions as a guanine nucleotide exchange factor (GEF) for RhoA (9), a GTP-binding protein that controls a variety of cellular processes, such as gene transcription, cell cycle progression, cytokinesis, cell growth, and remodeling of the actin cytoskeleton (14). AKAP-Lbc belongs to the Dbl family of GEFs, which are characterized by the presence of a Dbl homology (DH) domain and an adjacent pleckstrin homology domain (15). The DH domain is responsible for the guanine nucleotide exchange activity, whereas the pleckstrin homology domain regulates the subcellular localization of Rho-GEFs or is implicated in the binding pocket for Rho-GTPases (15). Activation of AKAP-Lbc Rho-GEF activity occurs in response to the stimulation of G protein-coupled receptors linked to the heterotrimeric G protein G12 by agonists or by serum (9).

In recent years, AKAP-Lbc has been shown to play a role in various pathologies. On one hand, a deleted form of AKAP-Lbc missing the entire NH2-terminal and COOH-terminal regulatory regions and displaying constitutive Rho-GEF activity was originally isolated as an oncogene from myeloid leukemia patients (16). On the other hand, in cardiomyocytes, AKAP-Lbc has been shown to assemble a macromolecular signal transduction complex that mediates cardiomyocyte hypertrophy, a pathophysiological condition that is associated with heart failure (17, 18). Interestingly, deletion of the NH2-terminal regulatory region from AKAP-Lbc strongly increases its hypertrophic activity (17). Collectively, these findings indicate that unregulated AKAP-Lbc GEF activity can produce deleterious effects. They also strongly suggest that the regulatory elements located outside of the GEF region of AKAP-Lbc play a critical role in the modulation of the signaling properties of the anchoring protein. Therefore, identifying the molecular mechanisms that affect AKAP-Lbc activity will importantly contribute to...
our understanding of how this anchoring protein modulates pathophysiological responses.

In the present study, using a yeast two-hybrid approach, we identified the ubiquitin-like protein LC3 as a novel regulatory protein interacting with AKAP-Lbc. LC3 was originally identified as a subunit of the neuronal microtubule-associated proteins MAP1A and MAP1B in rats (19, 20). However, experimental evidence collected over the last 10 years demonstrates that LC3 is expressed in many mammalian tissues, where it participates in autophagy, a cellular process that targets cytosolic proteins and organelles to the lysosome for degradation in response to nutrient starvation as well as cellular and tissue remodeling (21, 22). During autophagy, LC3 is targeted to autophagosomes. These membrane structures engulf cytosolic components and subsequently fuse with the lysosome, where the sequestered material is degraded and recycled (23–25). Because most studies have focused on the role of LC3 in autophagy, its role in non-autophagic cellular signaling pathways is poorly documented. Here we provide evidence that binding of LC3 to AKAP-Lbc inhibits its ability to activate RhoA and to promote RhoA-dependent reorganization of the actin cytoskeleton. These results strongly suggest that LC3 plays a previously unappreciated role in the modulation of the Rho signaling pathway.

EXPERIMENTAL PROCEDURES

Expression Constructs—AKAP-Lbc fragments encoding amino acids 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336, and 2337–2817 were PCR-amplified from the AKAP-Lbc pGEX4T1 vector (9) and subcloned at EcoRI/SalI into pLexA, pEGFPN1 vector (9) and subcloned at EcoRI/SalI into pLexA, and the pFLAG-CMV6 vectors to generate protein fragments fused with LexA, GST, and the FLAG epitope, respectively.

The full-length cDNA encoding human LC3 (isoform A) was PCR-amplified from library clones isolated in the yeast two-hybrid screen and subcloned at EcoRI/Sall in pACT2 (Clontech), pEGFPc2 (Clontech), pFLAGCMV6c (Sigma), and pET30a (Novagen) vectors to generate fusion proteins with the FLAG epitope, the histidine tag, GAL4, and GFP at the NH2 terminus, respectively. The NH2-terminal (LC3 N-term) and COOH-terminal (LC3 C-term) domains of LC3 were PCR-amplified from the LC3-pFLAGCMV6 vector and subcloned at EcoRI/Sall into the pFLAGCMV6, pET30a, and pEGFPc2 vectors to generate fusion proteins with the FLAG epitope, the histidine tag, and GFP at the NH2 terminus, respectively.

For mapping the two LC3-binding sites on AKAP-Lbc, fragments encoding amino acids 1–100, 101–200, 201–300, 301–400, 401–503, 504–1000, 1001–1387, 1388–1450, 1450–1550, and 1470–1550 were PCR-amplified from the AKAP-Lbc-pEGFPN vector and subcloned at EcoRI/Sall into the pGEX4T1 vector to construct fusion proteins with GST. FLAG-tagged AKAP-Lbc deletion mutants missing one (AKAP-Lbc Δ1 and AKAP-Lbc Δ2) or both (AKAP-Lbc Δ1,2) LC3 binding domains were generated by deleting the regions encoding amino acids 301–360 and 1450–1513, individually or in combination, by standard PCR-directed mutagenesis, using the FLAG-AKAP-Lbc vector (26) as a template. The GFP-tagged AKAP-Lbc Δ1,2 mutant was generated by excising a SacII/BspEI fragment from the FLAG-AKAP-Lbc Δ1,2 construct and by subcloning it into the AKAP-Lbc-pEGFP vector. The FLAG-RhoA T19N construct was generated by PCR-amplifying the entire coding sequence of RhoA T19N from the RhoA T19N-pCMV5 vector and by subcloning it at EcoRI/Sall into the pFLAGCMV6c vector.

Yeast Two-hybrid Screening—The yeast strain L40 was transformed with the AKAP-Lbc-(1–503)-pLexA plasmid encoding a fragment of AKAP-Lbc encompassing residues 1–503 fused to LexA. Clones were selected and subsequently transformed with 250 μg of a human heart Matchmaker cDNA library in the pACT2 vector (Clontech). Of four million double transformants, 15 exhibited moderate to strong growth on histidine-deficient plates. The library plasmids isolated from positive clones were cotransformed in the L40 strain with either the original bait vector or the empty pLexA plasmid, and the specificity of the interactions was confirmed by growth on histidine-deficient plates as well as by β-galactosidase activity (Yeast Protocols Handbook; Clontech).

Expression and Purification of Recombinant Proteins in Bacteria—GST fusion proteins of AKAP-Lbc, LC3 and the Rho binding domain (RBD) of Rhotekin were expressed using the bacterial expression vector pGEX-4T1 in the BL21DE3 strain of Escherichia coli and purified. Exponentially growing bacterial cultures were incubated for 16 h at 16 °C with 1 mM isopropl-1-thio-β-d-galactopyranoside and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer A (20 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1% (w/v) Triton-X-100, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 38,000 × g for 30 min at 4 °C. After incubating the supernatants with glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C, the resin was washed five times with 10 bed volumes of buffer A and stored at 4 °C. Beads containing GST-RBD were used immediately for the Rhotekin RBD pull-down assay.

His6-tagged fusion proteins of LC3, LC3 N-term, and LC3 C-term were expressed using the bacterial expression vector pET30 in BL21DE3 bacteria and purified. Bacterial extracts containing His6-tagged fusion proteins were prepared in buffer B (20 mM Hapes, pH 7.8, 500 mM NaCl, 10 mM imidazole, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin). After a 1-min sonication, the lysates were centrifuged at 38,000 × g for 30 min at 4 °C. The His6-tagged fusion proteins were purified by incubating the supernatant with nickel-nitrotriacetic acid chelating resin (Amersham Biosciences) for 1 h at 4 °C. The His6-tagged fusion proteins were eluted from the resin with 20 mM Hapes, pH 7.8, 500 mM NaCl, 300 mM
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imidazole, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml peptatin for 1 h at room temperature, dialyzed, and stored at −20 °C. The protein content of the eluates was assessed by Coomassie staining of SDS-polyacrylamide gels.

Cell Culture and Transfections—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and gentamycin (100 μg/ml) and transfected at 50–60% confluence in 100-mm dishes using the calcium-phosphate method. For the overexpression of constructs containing the full-length AKAP-Lbc, HEK293 and NIH3T3 cells were transfected at 80% confluence in 100- or 35-mm dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were grown for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum before harvesting. The total amount of transfected DNA was 10–24 μg/100-mm dish and 1–4 μg/35-mm dish.

GST Pull-down and Immunoprecipitation Experiments—For in vitro GST pull-downs, increasing concentrations (0.1 nM, 1 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, and 10 μM) of bacterially purified His6-tagged LC3, LC3 N-term, and LC3 C-term were incubated with glutathione-Sepharose beads (Amersham Biosciences) coupled to GST or to different GST-fused AKAP-Lbc fragments in 0.5 ml of buffer C (20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 4 h at 4 °C. The beads were then washed five times with buffer C containing 300 mM NaCl, resuspended in SDS-PAGE sample buffer (65 mM Tris, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, pH 6.8), and boiled for 3 min at 95 °C. Eluted proteins were analyzed by SDS-PAGE and Western blotting. For GST pull-down experiments performed from cell lysates, HEK293 cells expressing various constructs grown in 100-mm dishes were lysed in 1 ml of buffer C and centrifuged at 100,000 × g for 30 min at 4 °C. Glutathione-Sepharose beads (Amersham Biosciences) coupled to the different GST fusion proteins were incubated with 1.5 mg of proteins derived from the cell lysates in a total volume of 1 ml overnight at 4 °C. The beads were then washed five times with buffer C, and the proteins were eluted and analyzed as indicated above.

For immunoprecipitation experiments, HEK293 cells expressing various constructs grown in 100-mm dishes were lysed in 1 ml of buffer C. Cell lysates were incubated 2 h at 4 °C on a rotating wheel. The solubilized material was centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatants were incubated for 4 h at 4 °C with 20 μl of anti-FLAG M2 affinity resin (Sigma) to immunoprecipitate overexpressed FLAG-tagged proteins. Following a brief centrifugation on a benchtop centrifuge, the pelleted beads were washed five times with buffer C and twice with PBS, and proteins were eluted in SDS-PAGE sample buffer by boiling samples for 3 min at 95 °C. Eluted proteins were analyzed by SDS-PAGE and Western blotting. Immunoprecipitations of endogenous AKAP-Lbc from HEK293 cells were performed as previously indicated (26) using anti-AKAP13 antibodies from Bethyl Laboratories. Immunoprecipitation of endogenous AKAP-Lbc from HEK293 cell lysates was performed as indicated previously (9), using 4 μg of affinity-purified rabbit polyclonal anti-AKAP13 antibodies (Bethyl Laboratories).

SDS-PAGE and Western Blotting—Samples denatured in SDS-PAGE sample buffer were separated on acrylamide gels and electrophoretically transferred onto nitrocellulose membranes. The blots were incubated with primary antibodies and horseradish-conjugated secondary antibodies (Amersham Biosciences), as indicated previously (9). The following affinity-purified primary antibodies were used for immunoblotting: rabbit polyclonal anti-AKAP-Lbc (1 mg/ml, 1:1000 dilution) (9), rabbit polyclonal anti-AKAP-Lbc (1:1000 dilution; Bethyl Laboratories), mouse monoclonal anti-FLAG (4.9 mg/ml, 1:2000 dilution; Sigma), mouse monoclonal anti-GFP (400 μg/ml, 1:500 dilution; Roche Applied Science), rabbit polyclonal anti-GFP (400 μg/ml, 1:1000 dilution; Roche Applied Science), mouse monoclonal anti-RhoA (200 μg/ml, 1:250 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-LC3 (1:200 dilution; a generous gift from Dr. Kominami), and mouse monoclonal anti-histidine tag (100 μg/ml, 1:1000 dilution; Qiagen).

Rhotekin RBD Pull-down Assay—HEK293 cells grown in 100-mm dishes were transfected with 16 μg of the cDNAs encoding the various FLAG-AKAP-Lbc constructs in the absence or presence of 8 μg of the cDNAs encoding FLAG-LC3 or FLAG-LC3 C-term. 24 h after transfection, cells were incubated in Dulbecco’s modified Eagle’s medium without serum for an additional 24 h. Cells were then treated for 15 min with 10% fetal calf serum and lysed in RBD lysis buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% (w/v) Triton X-100, 0.1% sodium deoxycholate, 30 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride). Lysates were subjected to centrifugation at 38,000 × g for 10 min at 4 °C and incubated with 60 μg of RBD beads for 1 h at 4 °C. Beads were then washed three times with RBD buffer without sodium deoxycholate, resuspended in SDS-sample buffer, and analyzed by SDS-PAGE.

Fluorescence Microscopy—NIH3T3 cells grown on coverslips were transfected at 40% confluence using Lipofectamine 2000 with 0.5 μg of the cDNAs encoding AKAP-Lbc-GFP or AKAP-Lbc Δ1,2-GFP in the absence or presence of 0.5 μg of the vectors encoding FLAG-LC3 or FLAG-LC3 C-term; washed twice with PBS; fixed for 10 min in PBS, 3.7% formaldehyde; and permeabilized for 5 min with 0.2% (w/v) Triton X-100 in PBS. Cells were blocked for 30 min in PBS, 1% bovine serum albumin and subsequently incubated for 1 h with a 1:2000 dilution of the anti-FLAG monoclonal antibody. Cells were then washed three times with PBS and incubated for 1 h with a Cy5-conjugated anti-mouse secondary antibody (Jackson Immunoresearch) and Texas Red phalloidin (Molecular Probes). Immunofluorescent staining as well as direct fluorescence of GFP was visualized using a Zeiss Axiohot fluorescence microscope. Cy5 and Texas Red staining were detected using the following sets of filters: Cy5 (excitation 640/30 nm; emission 690/50 nm); Texas Red (excitation 565/30 nm; emission 620/60 nm).
Identification of LC3 as a novel protein that directly interacts with AKAP-Lbc. A, schematic representation of the protein domain organization of AKAP-Lbc. The ankyrin repeats (ANK), the PKA binding domain (PKA), the 14-3-3 binding site, the DH and pleckstrin homology domains, and the leucine zipper (LZ) motif are shown. The AKAP-Lbc fragment included between amino acids 1 and 503 that was used in the yeast two-hybrid screening and the region encompassing residues 1388–1922 are indicated. B, yeast two-hybrid screening. The AKAP-Lbc-(1–503)-pLexA plasmid was transformed into the L40 yeast strain in combination with either empty pACT2 vector or with pACT2 containing the cDNA encoding LC3. Transformants were plated on His− plates to select for histidine prototrophy (top). Quantitative analysis of the interaction between the AKAP-Lbc fragment and LC3 was performed using the liquid β-galactosidase assay (bottom). C, bacterially purified His6-tagged LC3 (100 nM) was incubated with glutathione-Sepharose beads coupled to 2 μg of GST alone or GST-fused AKAP-Lbc fragments encompassing amino acids 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336, and 2337–2817. Associated His6-tagged LC3 was detected using anti-LC3 polyclonal antibodies, whereas those transformed with the empty pACT2 vector did not.

RESULTS

Identification of LC3 as a Protein Directly Interacting with AKAP-Lbc—We have previously shown that truncation of the NH2-terminal region of AKAP-Lbc can significantly increase its basal Rho-GEF activity in the absence of external activating stimuli (9, 27). This suggested that inhibitory determinants located in the NH2-terminal sequence of AKAP-Lbc maintain the Rho-GEF activity of the anchoring protein low under basal conditions. To identify potential regulatory proteins interacting with this inhibitory region, a fragment of AKAP-Lbc encompassing residues 1–503 (Fig. 1A) was used as bait to screen a human heart cDNA library using the yeast two-hybrid system. Two independent positive clones encoding the ubiquitin-like protein LC3 (isoform A) were identified from 4 × 106 cotransformants, as assessed by their ability to grow in the absence of histidine and to produce β-galactosidase. To confirm these interactions, the L40 yeast strain was transformed with the bait plasmid expressing the 1–503 fragment of AKAP-Lbc (AKAP-Lbc-(1–503)-pLexA) in combination with either the pACT2 vector containing the LC3 cDNA (LC3-pACT2) or with the empty pACT2 vector. As shown in Fig. 1B, yeasts transformed with the LC3-pACT2 construct were able to grow in the absence of histidine (top) and to produce β-galactosidase (bottom), whereas those transformed with the empty pACT2 vector did not.

To assess whether the association between LC3 and AKAP-Lbc occurs through a direct interaction or whether it is mediated through another protein, we monitored the ability of six purified GST-tagged AKAP-Lbc fragments encompassing residues 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336, and 2337–2817 to associate with purified His6-tagged LC3 using an in vitro pull-down assay. Interestingly, our results indicate that LC3 can directly bind the AKAP-Lbc fragments 1–503 (Fig. 1C, top, lane 3) but also reveal a second interaction with the AKAP-Lbc fragment encompassing residues 1388–1922 (Fig. 1C, top, lane 6). Control experiments showed that the six AKAP-Lbc fragments used in this study did not interact with an unrelated His6-tagged protein (His6-Rac1) (results not shown). These results strongly suggest that the LC3 can directly interact with AKAP-Lbc through two distinct binding domains.

LC3 Interacts with AKAP-Lbc inside Cells—To determine whether LC3 can associate with the full-length AKAP-Lbc inside cells, we performed co-immunoprecipitation experiments from HEK293 cells that were transiently transfected with the empty FLAG vector or the FLAG-tagged AKAP-Lbc in combination with GFP or GFP-LC3 (Fig. 2A). After immunoprecipitating the anchoring protein using anti-FLAG antibodies, we performed co-immunoprecipitation experiments from HEK293 cells that were transiently transfected with the empty FLAG vector or the FLAG-tagged AKAP-Lbc in combination with GFP or GFP-LC3 (Fig. 2A). After immunoprecipitating the anchoring protein using anti-FLAG antibo-
ies, anti-GFP antibodies were used to immunoblot the immunoprecipitated samples. The Western blots revealed that GFP-LC3 could specifically co-immunoprecipitate with AKAP-Lbc, whereas no bands were immunoprecipitated from cells expressing GFP-LC3 or FLAG-AKAP-Lbc alone (Fig. 2A, top). Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and of the cell extracts were revealed using anti-GFP polyclonal antibodies to detect the GFP-tagged AKAP-Lbc fragments (Fig. 2B, top and middle) or anti-FLAG monoclonal antibodies to detect FLAG-LC3 (bottom). C, schematic representation of the AKAP-Lbc fragments used for the mapping of the second LC3-binding site. The minimal binding site (residues 1450–1470) is boxed. D, HEK293 cells were transfected with FLAG-tagged LC3 in combination with either GFP alone or the different GFP-tagged fragments of AKAP-Lbc encompassing residues 1388–1450, 1388–1513, 1388–1470, 1388–1450, 1428–1550, and 1450–1470. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and of the cell extracts were revealed as described in B. The results shown are representative of three independent experiments. E, schematic representation of the AKAP-Lbc mutants carrying deletions in the LC3 binding sites. F, extracts from HEK293 cells expressing the different FLAG-tagged AKAP-Lbc constructs indicated in E in combination with either GFP or GFP-LC3 were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates were revealed using anti-GFP polyclonal antibodies to detect GFP-LC3 or anti-FLAG monoclonal antibodies to detect the FLAG-tagged AKAP-Lbc constructs (bottom). The expression of GFP and GFP-tagged LC3 in cell lysates was assessed using polyclonal antibodies against GFP (middle). The results are representative of three independent experiments.
3B, top) and the second binding site to residues 1450–1470 (Fig. 3D, top). These experiments indicate that LC3 associates with AKAP-Lbc with molecular determinants that are included in two distinct regions of the AKAP-Lbc molecule.

To determine the affinity of LC3 for these two sites, we performed in vitro pull-down experiments by incubating increasing concentrations of purified His6-tagged LC3 ranging from 0.1 nm to 10 μm with various GST-tagged AKAP-Lbc fragments encompassing the LC3 binding domains. Binding of His6-LC3 was assessed by immunoblotting. Our results indicate that LC3 interacts with nanomolar affinity with both LC3 binding sites (supplemental Fig. S2, A–F). Calculated dissociation constant ($K_d$) values were included between 42 and 66 nM for the interaction with the first LC3 binding site (supplemental Fig. S2, A–C) and between 66 and 74 nM for the interaction with the second site (supplemental Fig. S2, D–F). Interestingly, deletion of the residues between amino acids 340 and 360 within the first binding site reduced the binding affinity by more than 10-fold ($K_d = 766$ nM), whereas deletions within residues 301–340 completely disrupted the interaction with LC3 (supplemental Fig. S2, A–C). Similarly, deletion of residues 1450–1470 within the second LC3 binding site dramatically compromised the interaction (supplemental Fig. S2, D–F).

To assess the relative contribution of the two LC3 binding domains to the interaction between full-length AKAP-Lbc and LC3, we generated AKAP-Lbc deletion mutants in which the two LC3 binding sites were deleted independently (AKAP-Lbc Δ1 and AKAP-Lbc Δ2) or in combination (AKAP-Lbc Δ1,2) (Fig. 3E). The FLAG-tagged forms of wild type AKAP-Lbc and of its different deletion mutants were overexpressed in HEK293 cells in combination with either GFP or GFP-LC3 and immunoprecipitated using anti-GFP antibodies. The presence of associated GFP-LC3 was assessed by immunoblot using anti-GFP antibodies. As shown in Fig. 3F, the deletion of individual LC3 interaction sites reduced the interaction of AKAP-Lbc with LC3 of about 40% (Fig. 3F, top, lanes 5 and 7), whereas the deletion of both binding domains completely abolished the ability of the anchoring protein to form stable complexes with LC3 (Fig. 3F, top, lane 9). This suggests that the two LC3 binding sites contribute equally to the interaction of full-length AKAP-Lbc with LC3.

The presence of two LC3 binding sites on AKAP-Lbc raises the question of how LC3 interacts with the anchoring protein. One possibility is that each site can interact with one LC3 molecule. Alternatively, one can also conceive that a single molecule of LC3 could coordinate the two binding sites on AKAP-Lbc. To address this issue, liquid phase competition binding assays were performed by simultaneously incubating recombinant GST-LC3 coupled to Sepharose beads with extracts from HEK293 cells expressing the GFP-tagged AKAP-Lbc fragment 301–400 (containing the first LC3 binding site) as well as with lysates transfected with increasing amounts of the FLAG-tagged fragment 1388–1493 (encompassing the second LC3 binding site). Binding of the AKAP-Lbc fragments to GST-LC3 was assessed by immunoblotting. Interestingly, increased binding of the 1388–1493 fragment (supplemental Fig. S3A, lanes 5–8) corresponded to a decreased interaction of the 301–400 fragment (supplemental Fig. S3B, lanes 5–8) with the LC3 fusion protein (supplemental Fig. 3E). These data indicate that the two interaction sites located within the NH2-terminal region of AKAP-Lbc bind LC3 in a mutually exclusive manner and, therefore, suggest that each site on AKAP-Lbc interacts with a single LC3 molecule.

Identification of the Binding Site for AKAP-Lbc on LC3—The recent determination of the solution structure of LC3 (isoform A) using NMR spectroscopy revealed the presence of two distinct structurally organized domains: a carboxyl-terminal subdomain that is constituted by a ubiquitin-folded core composed of 5 β-strands and 2 α-helices and an amino-terminal tail of 28 amino acids composed of 2 α-helices (28) (Fig. 4A). The ubiquitin fold contains the interaction sites for the enzymes ATG7 and ATG3, which play a role in the activation and the transfer of LC3 to autophagosomes, whereas the amino-terminal tail has been proposed to bind tubulin (28).

In order to determine which of these two main regions of LC3 participates in the interaction with AKAP-Lbc, we determined the ability of wild type LC3 as well as of its deletion.
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mutant missing the entire NH2-terminal tail (FLAG-LC3 C-term) to associate with the two LC3-binding regions on AKAP-Lbc. FLAG-tagged LC3 and LC3 C-term were expressed in HEK293 cells in the presence or absence of GFP-tagged AKAP-Lbc fragments encompassing residues 301–400 and 1450–1513, respectively. LC3 proteins were immunoprecipitated from cell lysates using anti-FLAG antibodies, and the presence of associated GFP-tagged AKAP-Lbc fragments was detected using anti-GFP antibodies. As shown in Fig. 4B, deletion of the NH2-terminal region strongly inhibited the ability of LC3 to interact with the two binding sites on AKAP-Lbc (Fig. 4B, top, lanes 7 and 8).

To provide direct evidence that the NH2-terminal portion of LC3 contains the determinants required for AKAP-Lbc binding, we determined whether FLAG-AKAP-Lbc could interact with GFP fusion proteins of wild type LC3 as well as of its isolated NH2-terminal (GFP-LC3 N-term) and COOH-terminal (GFP-LC3 C-term) domains. As shown in Fig. 4C, the NH2-terminal tail of LC3, but not its COOH-terminal domain, retained the ability to coimmunoprecipitate with AKAP-Lbc (Fig. 4C, top, lanes 5 and 6), suggesting that the main structural requirements involved in the recognition of AKAP-Lbc are located within the first 28 residues of LC3. To confirm this view and to provide a quantitative assessment of the affinity of the interaction between the NH2-terminal domain of LC3 and AKAP-Lbc, we performed in vitro pull-down experiments by incubating increasing concentrations of purified His6-tagged LC3, LC3 N-term, and LC C-term with GST-tagged AKAP-Lbc constructs. As shown in Fig. 4C, the LC3 fragment missing the NH2-terminal α-helical domain (LC3 C-term) displayed only marginal association with AKAP-Lbc (supplemental Fig. S4, A–C).

LC3 Inhibits AKAP-Lbc Rho-GEF Activity inside Cells—Based on the observation that LC3 interacts with the NH2-terminal region of AKAP-Lbc, which was previously shown to negatively regulate the Rho-GEF activity of the anchoring protein, we investigated the hypothesis that LC3 binding to AKAP-Lbc might influence its ability to promote RhoA activation.

To test this hypothesis, we initially assessed the ability of LC3 to modulate RhoA activation in HEK293 cells using the Rhotekin pull-down assay. As shown in Fig. 5, the overexpression of wild type FLAG-tagged LC3 reduced by 37% the ability of serum (S) to induce Rho-GTP formation (Fig. 5, A (first panel, lane 4) and B) as compared with cells that did not overexpress LC3 (Fig. 5, A (first panel, lane 2) and B). Interestingly, the overexpression of FLAG-tagged LC3 C-term, which is unable to associate with AKAP-Lbc, had no effect on serum-induced RhoA activation (Fig. 5, A (first panel, lane 6) and B), suggesting that LC3 might inhibit Rho signaling through the association with endogenous AKAP-Lbc.

To directly investigate this possibility, we monitored the impact of FLAG-LC3 and FLAG-LC3 C-term overexpression on the ability of recombinant FLAG-AKAP-Lbc to promote the activation of RhoA in HEK293 cells. We previously showed that AKAP-Lbc displays low basal activity in serum-starved cells and that this activity can be enhanced by treatment of cells with serum (26). Accordingly, treatment of cells with 10% serum strongly stimulated the RhoA-activating effect of AKAP-Lbc as compared with untreated cells (Fig. 5, top (first panel, lanes 7 and 8) and bottom). Interestingly, LC3 overexpression inhibited basal and serum-induced AKAP-Lbc Rho-GEF activity by 45 and 59%, respectively (Fig. 5, top (first panel, lanes 9 and 10) and B), whereas overexpression of LC3 C-term had no effect (Fig. 5, top (first panel, lanes 11 and 12) and bottom). These results strongly suggest that the activity of AKAP-Lbc is strongly

![FIGURE 5. LC3 inhibits the Rho-GEF activity of AKAP-Lbc.](image_url)
inhibited by LC3 and that this inhibition requires physical interaction between the two signaling proteins.

The Inhibitory Effect of LC3 Requires Two Intact Binding Sites on AKAP-Lbc—Our results indicate that AKAP-Lbc contains two interaction sites that equally contribute to the binding to LC3 (Fig. 3F). To assess which of these two sites contributes to the inhibitory effect of LC3 on AKAP-Lbc, we determined the ability of LC3 to inhibit the Rho-GEF activity of AKAP-Lbc Δ1, AKAP-Lbc Δ2, and AKAP-Lbc Δ1,2, which are lacking the first, the second, or both LC3 binding domains, respectively. FLAG-tagged AKAP-Lbc constructs were overexpressed in HEK293 cells alone or in combination with FLAG-LC3, and the ability of AKAP-Lbc to promote RhoA activation was measured using the Rhotekin pull-down assay.

Interestingly, AKAP-Lbc Δ1, AKAP-Lbc Δ2, and AKAP-Lbc Δ1,2 displayed basal Rho-GEF activities higher than wild type AKAP-Lbc (Fig. 6, A (top, lanes 9 and 10, 13 and 14, and 17 and 18) and B), suggesting that binding of endogenous LC3 maintains AKAP-Lbc in a state of reduced activity. Remarkably, although the Rho-GEF activity of wild type AKAP-Lbc could be strongly inhibited by LC3 overexpression (Fig. 6, A (top, lanes 7 and 8) and B), all three AKAP-Lbc deletion mutants were completely resistant to LC3-mediated inhibition both under basal conditions and in the presence of serum (Fig. 6, A (top, lanes 11 and 12, 15 and 16, and 19 and 20) and B). This suggests that LC3 needs to physically interact with two binding sites on AKAP-Lbc in order to inhibit its Rho-GEF activity.

LC3 Inhibits the Interaction between AKAP-Lbc and RhoA—In order to determine whether the reduction of AKAP-Lbc Rho-GEF activity observed upon binding of LC3 could be attributed to the inhibition of the ability of the anchoring protein to recruit RhoA, we determined the effect of LC3 overexpression on the formation of complexes between endogenous AKAP-Lbc and RhoA. AKAP-Lbc was immunoprecipitated from HEK293 cells expressing either FLAG-LC3 or FLAG-LC3 C-term using affinity-purified rabbit polyclonal anti-AKAP-Lbc antibodies and the presence of associated RhoA revealed by Western blot using monoclonal anti-RhoA antibodies. Interaction between AKAP-Lbc and RhoA was detected with either the empty pFLAG vector (lanes 1–4), FLAG-AKAP-Lbc (lanes 5–8), FLAG-AKAP-Lbc Δ1 (lanes 9–12), FLAG-AKAP-Lbc Δ2 (lanes 13–16), or FLAG-AKAP-Lbc Δ1,2 (lanes 17–20) in combination with either the empty pFLAG vector (lanes 1 and 2, 5 and 6, 9 and 10, 13 and 14, and 17 and 18) or FLAG-tagged LC3 (lanes 3 and 4, 7 and 8, 11 and 12, 15 and 16, and 19 and 20). Cells were serum-starved for 24 h and incubated in the absence (−) or in the presence (+) of serum for 15 min. Cell lysates were incubated with GST-RBD beads. The bound RhoA was detected with a monoclonal anti-RhoA antibody (top). The relative amount of total RhoA, FLAG-LC3, and FLAG-AKAP-Lbc constructs in the cell lysates were assessed using monoclonal antibodies against RhoA (middle) and the FLAG epitope (bottom), respectively. B, quantitative analysis of the GTP-RhoA associated with RBD beads was obtained by densitometry. The RhoA bound to RBD (top) was normalized to the RhoA content of cell extracts (middle). Results are expressed as mean ± S.E. of five independent experiments. Statistical significance was analyzed using a Kruskal-Wallis test, followed by Mann-Whitney U tests with the Bonferroni correction. *, p < 0.05 as compared with Rho-GTP levels measured in serum-starved cells transfected with FLAG-AKAP-Lbc alone. §, p < 0.05 as compared with Rho-GTP levels measured in serum-treated cells transfected with FLAG-AKAP-Lbc alone. #, p < 0.05 as compared with Rho-GTP levels measured in serum-starved cells expressing FLAG-AKAP-Lbc alone.

To confirm these results, we determined whether overexpression of LC3 could inhibit the interaction between AKAP-Lbc and a GDP-locked mutant of RhoA (RhoA T19N), which fails to undergo Mg2+−dependent activation and which therefore binds activated Rho-GEFs in a stable manner. HEK293 cells were transfected with the FLAG-tagged RhoA mutant in combination with GFP, GFP-LC3, or GFP-LC3 C-term. Interaction between endogenous AKAP-Lbc and FLAG-RhoA T19N was assessed by coimmunoprecipitation. As shown in supplemental Fig. S5, serum stimulation increased the interaction between AKAP-Lbc and RhoA T19N 6.5-fold (supplemental Fig. S5, A (top, lanes 1 and 2) and B). Importantly, LC3 overexpression but not that of LC3 C-term could reduce the association between AKAP-Lbc and FLAG-RhoA T19N both under basal conditions and in serum-stimulated cells (supplemental Fig. S5, A (top, lanes 3–6) and B). These results strongly suggest that LC3 inhibits the Rho-GEF activity of AKAP-Lbc by impairing the formation of stable complexes between the anchoring protein and RhoA. Based on these findings, one can raise the hypothesis that LC3 might maintain AKAP-Lbc in an inactive conformation that would limit the access of RhoA to the DH domain.

LC3 Inhibits the Ability of AKAP-Lbc to Induce Actin Stress Fiber Formation—In order to determine the cellular consequences of the inhibition of AKAP-Lbc Rho-GEF activity medi-
ated by LC3, we initially measured the effect of LC3 overexpression on the ability of AKAP-Lbc to induce the formation of actin stress fibers in NIH3T3 fibroblasts. GFP-tagged AKAP-Lbc was expressed in fibroblasts in the presence or absence of FLAG-LC3 or FLAG-LC3 C-term. Actin fibers were visualized by staining cells using Texas Red-conjugated phalloidin. As previously shown (9), AKAP-Lbc promoted efficient formation of actin stress fibers in fibroblasts (Fig. 8A, top). Interestingly, these effects were abolished by the overexpression of LC3 (Fig. 8A, middle), but not by LC3 C-term, which does not bind AKAP-Lbc (Fig. 8A, bottom). The ability of LC3 to inhibit the formation of actin stress fibers induced by AKAP-Lbc was entirely dependent upon the interaction of LC3 with the anchoring protein, as shown by the fact that LC3 was unable to inhibit the appearance of actin fibers induced by AKAP-Lbc Δ1,2, which lacks both LC3 binding sites (Fig. 8B). Overall, these findings strongly suggest that, by inhibiting AKAP-Lbc Rho-GEF activity, LC3 can negatively regulate the Rho signaling pathway leading to the reorganization of the cellular actin cytoskeleton.

DISCUSSION

Guanine nucleotide exchange factors of the Dbl family are multifunctional transduction molecules that integrate and coordinate signals from multiple cell surface receptors in order to precisely modulate the activation of small molecular weight
GTPases of the Rho family in response to various extracellular signals (15, 29). Because of their implication in several physiological functions, such as growth and development, skeletal muscle formation, neuronal axon guidance, and immune cell formation, and in pathophysiological processes, including cancer development (29, 30) and cardiac remodeling processes associated with heart hypertrophy (17, 18), the mechanisms involved in their regulation have been intensively investigated. A growing number of studies now indicate that the activation state of several exchange factors of the Db family can be affected by modulatory proteins, which, by interacting with regulatory sequences located on the GEF molecule, can enhance or repress the nucleotide exchange activity. Our current findings indicate that the ubiquitin-like protein LC3 interacts with the RhoA-selective exchange factor AKAP-Lbc. Interestingly, LC3 binding strongly reduces the ability of RhoA to interact with AKAP-Lbc, and, as a consequence, it profoundly impairs the Rho-GEF activity of the anchoring protein and its ability to promote cytoskeletal rearrangements associated with formation of actin stress fibers.

Although in recent years many efforts have been made to elucidate the role of LC3 in cell autophagy (25, 31), very little evidence is currently available for the implication of this protein in other cellular processes. Our current findings indicate that LC3 can bind and inhibit AKAP-Lbc function provide evidence for a previously uncharacterized role of this ubiquitin-like protein in the regulation of Rho signaling. In line with our current results, it has been shown that binding of LC3 to the exchange factor SOS-1 can suppress its ability to activate the GTPase Rac1 and, as a consequence, to induce the formation of membrane ruffles in COS-7 cells (32). Therefore, it appears that LC3 could play an important role in modulating the activity of Dbl family GEFs in other cellular processes. Our current findings indicate that LC3 can bind and inhibit AKAP-Lbc function provide evidence for a previously uncharacterized role of this ubiquitin-like protein in the regulation of Rho signaling. In line with our current results, it has been shown that binding of LC3 to the exchange factor SOS-1 can suppress its ability to activate the GTPase Rac1 and, as a consequence, to induce the formation of membrane ruffles in COS-7 cells (32). Therefore, it appears that LC3 could play an important role in modulating the activity of Dbl family GEFs in other cellular processes.

Evidence collected over recent years indicates that members of the Db family can be maintained in an inhibited conformation by several mechanisms, including the formation of intramolecular interactions between the GEF module and regulatory regions, the formation of homo- or hetero-oligomers (15, 29), and the formation of macromolecular complexes with regulatory proteins (15, 29). In particular, the regulatory protein 14-3-3 has recently emerged as an important negative modulator of the GEF activity of several Dbl family GEFs, including AKAP-Lbc (26, 33), GEF-H1 (34), and β-1-Pix (35). In this respect, we have shown that 14-3-3 can be recruited to AKAP-Lbc in response to the activation of anchored PKA and the phosphorylation of serine 1565 within the NH$_2$-terminal regulatory region of the anchoring protein (26). This regulatory mechanism reduces the activity of AKAP-Lbc in response to the elevation of the cellular concentration of cAMP. These findings, together with our current results that identify LC3 as a novel regulator of AKAP-Lbc, indicate that AKAP-Lbc can integrate multiple inhibitory pathways that modulate its ability to promote RhoA activation. Recently, the solution structure of LC3 has been solved (28). It appears that LC3 is organized into two spatially distinct domains, including an amino-terminal α-helical region of 28 residues and a carboxyl-terminal ubiquitin core (28). Our results indicate that the structural determinants involved in the binding to AKAP-Lbc are located within the NH$_2$-terminal extension of LC3 (Fig. 4), a region previously proposed to bind tubulin in neurons (28), and, more recently, to interact with the polyubiquitin-binding protein p62 (36). Interestingly, this latter protein has been shown to bind LC3 through a domain of about 20 amino acids containing negatively charged residues (36). Alignment of the amino acid sequence corresponding to this region with the sequence of the two LC3 binding sites identified on AKAP-Lbc failed to reveal any primary sequence conservation, suggesting that LC3 recognizes distinct motifs on AKAP-Lbc and p62. Therefore, it appears that although the ubiquitin fold of LC3 is mainly involved in the interaction with proteins involved in the processing and the targeting of LC3 to autophagosomes, its NH$_2$-terminal extension can specify additional LC3 functions, such as microtubule targeting (28) and Rho signaling inhibition.

Our mapping studies revealed the presence of two LC3 binding sites located within the NH$_2$-terminal regulatory region of AKAP-Lbc (Fig. 3), raising the question of how LC3 binds and inhibits the Rho-GEF activity of the anchoring protein. We could show that a single LC3 molecule cannot simultaneously bind the two sites on AKAP-Lbc (supplemental Fig. S3), suggesting that each site can interact with one LC3 molecule. Interestingly, the inhibitory action of LC3 on the Rho-GEF activity of AKAP-Lbc requires the presence of two intact binding sites on the anchoring protein (Fig. 6). Collectively, these findings infer that AKAP-Lbc Rho-GEF activity is inhibited by the binding of at least two LC3 molecules.

It has been recently shown that the LC3 homolog GABARAP (GABA(A) receptor-associated protein) can assume a dimeric conformation that is required for its binding to microtubules and GABA receptors (37). This raises the intriguing possibility that LC3 might form dimers that could coordinate the two binding sites on AKAP-Lbc. In line with this hypothesis, we have observed that FLAG- and GFP-tagged LC3 are able to coimmunoprecipitate from HEK-293 cell lysates, suggesting that LC3 can form oligomers inside cells (results not shown). In this configuration, LC3 binding could promote a conformational change that impacts the Rho-GEF activity of AKAP-Lbc.

In an effort to define the molecular basis of the inhibitory effect of LC3 on AKAP-Lbc Rho-GEF activity and based on the evidence that LC3 was originally identified as a subunit of the neuronal MAP1 protein complex (19, 20), we initially considered the possibility that LC3 could promote AKAP-Lbc inhibition by targeting the anchoring protein to the microtubule cytoskeleton. Microtubule targeting has been shown to influence the activity of two Dbl family exchange factors, including GEF-H1 (38, 39), which is inhibited following microtubule binding, and PDZ-Rho-GEF, which is maintained in an inactive conformation through an interaction with the light chain 2 of MAP1 in neurons (40). However, our results indicate that AKAP-Lbc is distributed within the cytoplasm and does not colocalize with microtubules either when expressed alone or in combination with LC3 (results not shown).

We could subsequently show that the reduction of AKAP-Lbc Rho-GEF activity induced by LC3 is paralleled by a significant decrease in the amount of RhoA associated with the anchoring protein (Fig. 7). This suggests that LC3 reduces the
LC3 Inhibits AKAP-Lbc Rho-GEF Activity

Rho-GEF activity by interfering with the interaction between AKAP-Lbc and RhoA. Based on this observation, one possibility is that LC3 binding could induce a conformational change that would either mask the DH domain or reduce the affinity of the DH domain for RhoA. A similar but not identical mechanism has been proposed to describe the inhibitory action of LC3 on the Rac-GEF activity of SOS-1 (32). In this case, binding of LC3 to a site within the Dbl homology domain has been shown to competitively inhibit the interaction between Rac1 and SOS-1 (32). This suggests that LC3 can affect the GEF activity of guanine nucleotide exchange factors of the Dbl family either by binding directly to the DH domain or by interacting with regulatory regions.

In conclusion, our findings have several implications. First, they demonstrate that the ubiquitin-like protein LC3 is a negative modulator of AKAP-Lbc Rho-GEF activity. Second, they provide a mechanistic explanation for this inhibitory role by showing that binding of two LC3 molecules on the NH2-terminal regulatory region of AKAP-Lbc inhibits the interaction between the anchoring protein and RhoA. Finally, they provide evidence for a previously uncharacterized role of LC3 in the regulation of Rho signaling.

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