AKAP-Lbc is a novel member of the A-kinase anchoring protein (AKAPs) family, which functions as a cAMP-dependent protein kinase (PKA)-targeting protein as well as a guanine nucleotide exchange factor (GEF) for RhoA. We recently demonstrated that AKAP-Lbc Rho-GEF activity is stimulated by the α-subunit of the heterotrimeric G protein G12, whereas phosphorylation of AKAP-Lbc by the anchored PKA induces the recruitment of 14-3-3, which inhibits its GEF function. In the present report, using co-immunoprecipitation approaches, we demonstrated that AKAP-Lbc can form homo-oligomers inside cells. Mutagenesis studies revealed that oligomerization is mediated by two adjacent leucine zipper motifs located in the C-terminal region of the anchoring protein. Most interestingly, disruption of oligomerization resulted in a drastic increase in the ability of AKAP-Lbc to stimulate the formation of Rho-GTP in cells under basal conditions, suggesting that oligomerization maintains AKAP-Lbc in a basal-inactive state. Based on these results and on our previous findings showing that AKAP-Lbc is inactivated through the association with 14-3-3, we investigated the hypothesis that AKAP-Lbc oligomerization might be required for the regulatory action of 14-3-3. Most interestingly, we found that mutants of AKAP-Lbc impaired in their ability to undergo oligomerization were completely resistant to the inhibitory effect of PKA and 14-3-3. This suggests that 14-3-3 can negatively regulate the Rho-GEF activity of AKAP-Lbc only when the anchoring protein is in an oligomeric state. Altogether, these findings provide a novel mechanistic explanation of how oligomerization can regulate the activity of exchange factors of the Dbl family.

Compartmentalization of signaling molecules through association with anchoring and scaffolding proteins is a mechanism that ensures specificity of transduction events involved in cellular regulation. A-kinase anchoring proteins (AKAPs) are a family of scaffolding proteins that compartmentalize the cAMP-dependent protein kinase (PKA) at precise subcellular sites in close proximity to its physiological substrates (1). Each AKAP contains a conserved amphipathic helix of 14–18 residues that binds to the regulatory subunit dimers of the PKA holoenzyme (2–4) and displays a unique targeting motif that directs PKA-AKAP complexes to specific subcellular sites (5). Another fundamental role of AKAPs is to assemble signaling complexes by associating with multiple enzymes such as kinases, phosphatases, and other regulatory proteins. By simultaneously interacting with multiple signaling enzymes, AKAPs can integrate diverse transduction pathways that coordinately regulate the function of specific cellular substrates (5, 6).

Recently, we identified a novel member of the AKAP family, termed AKAP-Lbc, that functions as a type II PKA anchoring protein as well as a guanine nucleotide exchange factor (GEF) for RhoA (7), a small GTP-binding protein of the Ras family that controls fundamental cell processes such as cell cycle progression, gene transcription, remodeling of the actin cytoskeleton, and cytoexcision (8). AKAP-Lbc belongs to the Dbl family of GEFs, which all share a Dbl homology (DH) domain and an adjacent pleckstrin homology (PH) domain (9). The DH domain is responsible for the guanine nucleotide exchange activity, whereas the PH domain regulates subcellular localization of Rho-GEFs or is implicated in the binding pocket for Rho-GTPases (8). A truncated form of AKAP-Lbc missing the entire N-terminal and C-terminal regions, called Onco-Lbc, was originally isolated as an oncogene from myeloid leukemia patients and shown to represent a constitutively active Rho-GEF (10).

The Rho-GEF activity of AKAP-Lbc can be strongly enhanced by the α-subunit of the heterotrimeric G protein G12 that is activated following the stimulation of G protein-coupled receptors that couple to G12 by serum or lysophosphatidic acid (7). In the absence of activating stimuli, AKAP-Lbc is maintained in an inactive state through the association with 14-3-3. The recruitment of 14-3-3 to AKAP-Lbc is induced by the phosphorylation of serine 1565 located within the 14-3-3-binding site of the anchoring protein by the PKA holoenzyme anchored to AKAP-Lbc (11, 12).

Many members of the Dbl family of GEFs are maintained in a basal inactive conformation by intramolecular interactions involving the DH and PH domains as well as regulatory sequences. Such interactions have been proposed to block the access of Rho GTPases to the DH domain and/or suppress the GEF activity of the exchange factor (13). Recent studies now demonstrate that the activity of Dbl family members can also be regulated through oligomerization. The functional role of this intermolecular interaction has been established only for a small number of them, including Ras GRF1 and Ras GRF2 (14), Dbl (15), α- and β-Pix (16, 17), as well as p115-Rho-GEF, LARG, and PDZ-Rho-GEF (18, 19). Oligomerization of Ras GRF, Dbl, and β-Pix is required for the efficient execution of
the exchange reaction (14, 15), whereas oligomerization of p115-Rho-GEF, LARG, and PDZ-Rho GEF has been shown to negatively regulate the GEF activity (18, 19). Most interestingly, recent evidence suggests that oligomerization can also regulate the specificity of GEFs toward Rho GTPases. This mechanism was recently described for α-Pix, which can adopt a dimeric conformation that selectively activates Rac and a monomeric conformation that activates both Rac and Cdc42 (16). Although it appears that oligomerization can affect the functional role of AKAP-Lbc can form homo-oligomers through a leucine zipper motif located in the C-terminal region of the anchoring protein. We found that disruption of oligomerization strongly enhances the basal Rho-GEF activity of AKAP-Lbc, suggesting that oligomerization maintains the anchoring protein in a basal inactive state. Most importantly, we also show that oligomerization maintains AKAP-Lbc in a conformation that can be regulated by 14-3-3, as shown by the fact that oligomerization-deficient mutants of AKAP-Lbc are completely resistant to the inhibitory effect of 14-3-3. These findings provide a molecular explanation for the functional role of oligomerization of DBL family GEFs.

MATERIALS AND METHODS

Expression Constructs—The constructs encoding the FLAG-AKAP-Lbc and the FLAG-AKAP-Bc deletion mutant missing the first 1922 residues (FLAG-AKAP-BCΔN-term) were described previously (11). The deletion mutant of FLAG-AKAP-Bc missing residues 2337–2817 (FLAG-AKAP-BcΔC-term) was generated by subcloning a fragment excised from the AKAP-Lbc-(1923–2336)–pEGEX4T1 construct, which contains a stop codon at position 2336, at PspI/NotI into the FLAG-AKAP-Lbc construct.

The coiled coil region included between residues 2573 and 2687 was deleted from FLAG-AKAP-Lbc (FLAG-AKAP-Lbc ACC) as well as from FLAG-tagged and GFP-tagged AKAP-Lbc fragments encompassing residues 1923–2817 (1923–2817 ΔCC) by using standard PCR techniques. cDNA fragments encoding amino acids 1–503, 504–1000, and 1001–1387, 1388–1520, 1521–2000, 2001–2589, and 2566–2698 of AKAP-Lbc were PCR-amplified from the full-length AKAP-Lbc pEGFP vector and subcloned in the pFLAGCMV6 vector, to generate protein fragments fused with the FLAG epitope. AKAP-Lbc fragments encompassing residues 1–503, 504–1000, and 1001–1387, 1388–1520, 1521–2000, 2001–2589, and 2566–2698 were subcloned at SalI/KpnI.

For the mapping of the oligomerization site, different leucine and valine residues included in the putative leucine zipper motifs of AKAP-Lbc were substituted to alanine into the AKAP-Lbc-(1923–2187)–pFLAGCMV6 vector by PCR-directed mutagenesis using the Hot Star DNA polymerase (Qiagen). The mutants generated are the following: L216A, L2623A, and V2630A, L2637A and L2644A, L2658A, and L2665A. The oligomerization-deficient mutant of AKAP-Lbc (FLAG-AKAP-Lbc LZm) was generated by subcloning a PCR fragment amplified from the LZ mutant 7-pFLAGCMV6c construct at PspI/NotI into the FLAG-AKAP-Lbc construct. The rhotekin Rho-binding domain (RBD)-pEGEX1 construct was a generous gift of Dr. Hitoshi Kurose (Fukuoka, Japan).

Expression and Purification of Recombinant Proteins in Bacteria—GST fusion proteins of the RBD of rhotekin and RhoA were expressed using the bacterial expression vector pGEX4T1 in the BL21DE3 strain of Escherichia coli and were purified. To induce the expression of the GST-RBD fusion proteins, exponentially growing bacterial cultures were incubated 4 h at 37 °C with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer B (50 mM Tris, pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% (w/v)/Triton X-100, 1 mM PMSF, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin), sonicated, and centrifuged at 38,000 × g for 30 min at 4 °C. After incubating the supernatants with the glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, the resin was washed five times with 10 volumes of buffer A. The protein content of the beads was assessed by Coomassie Blue staining of SDS-polyacrylamide gels. Beads were used immediately for rhote kin RBD pulldown assay.

For the production of purified GST-RhoA, exponentially growing bacterial cultures were incubated 4 h at 37 °C with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer B (50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM DT and stored at −80 °C. The protein content of eluates was assessed by Coomassie Blue staining of SDS-polyacrylamide gels.

Cell Culture and Transfections—HEK-293 were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) 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 encoding the full-length AKAP-Lbc, HEK-293 cells were transfected at 80% confluency in 100-mm dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were grown for 48 h in DMEM supplemented with 10% fetal calf serum before harvesting. The total amount of transfected DNA was 6 μg/100-mm dish for the FLAG-tagged AKAP-Lbc fragments, 12 μg/100-mm dish for the FLAG-AKAP-Lbc pEGFP constructs, and 24 μg/100-mm dish for the full-length FLAG-AKAP-Lbc constructs.

Immunoprecipitation Experiments—For co-immunoprecipitation experiments, cells were lysed in 1 ml of buffer C (20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v)/Triton X-100, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF). Cell lysates were incubated 6 h at 4 °C on a rotating wheel and then centrifuged at 100,000 × g for 30 min at 4 °C. The supernatants were mixed with 20 μl of a glutathione-Sepharose 4B beads (Sigma) to immunoprecipitate overexpressed FLAG-tagged AKAP-Lbc constructs. Following a brief centrifugation on a bench-top centrifuge, the pellet elutes were washed five times with buffer C and proteins eluted in SDS-PAGE sample buffer (65 mM Tris, pH 6.8, 2% SDS, 5% glyc erol, 5% β-mercaptoethanol) by boiling samples for 5 min at 95 °C. Eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and analyzed by Western blotting.

SDS-PAGE and Western Blotting—Samples denatured in SDS-PAGE sample buffer were separated on acrylamide gels and electroblotted onto nitrocellulose membranes. The blots were incubated with primary antibodies onto horseradish-conjugated secondary antibodies (Amersham Biosciences) as indicated previously (11). The following affinity-purified primary antibodies were used for immunoblotting: mouse monoclonal anti-FLAG (Sigma, 4.9 mg/ml, 1:2000 dilution), mouse monoclonal anti-GFP (Roche Applied Science, 400 μg/ml, 1:1500 dilution), mouse monoclonal anti-RhoA (Santa Cruz Biotechnology, 1:250 dilution), and rabbit polyclonal anti-14-3-3 (Santa Cruz Biotechnology, 1:250 dilution).

GDP/GTP Exchange Assay—The exchange assays were performed as described previously (20). A 2-μg portion of recombinant RhoA was incubated for 5 min in 60 μl of loading buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2 mM DTT, 100 μM AMP-PNP and 10 μM GDP) at room temperature. MgCl₂ was then added to a final concentration of 5 mM, and the incubation was continued for an additional 15 min. To initiate the exchange reaction, protein aliquots (20 μl) of GDP-loaded GT Pases were mixed at room temperature with 80 μl of reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 100 μM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 5 μM [32P]GTPcS (11,000 cpm/mmol)) containing immunoprecipitated FLAG-AKAP-Lbc or FLAG-AKAP-Lbc LZ mutant. Aliquots (15 μl) of samples were taken at various time points and added to 10 ml of ice-cold phosphate-buffered saline. Bound and free nucleotides were separated by filtration through BA85 nitrocellulose filters. The amount of bound radioactivity was measured by liquid scintillation counting.
Homo-oligomerization Regulates AKAP-Lbc Function

RESULTS

The C-terminal Region of AKAP-Lbc Negatively Regulates Basal Rho-GEF Activity—We have demonstrated recently that AKAP-Lbc displays a low basal Rho-GEF activity in serum-starved cells, which can be significantly enhanced by the deletion of the N-terminal region of the anchoring protein upstream of the DH domain. This suggested that inhibitory determinants located in the N-terminal sequence maintain AKAP-Lbc in an inactive state in the absence of external activating stimuli (7, 11). On the other hand, our previous results could not clearly determine whether the C-terminal region downstream of the PH domain also regulates the function of AKAP-Lbc, because we found that the truncated form of AKAP-Lbc missing the N-terminal regulatory region displays a basal constitutive Rho-GEF activity comparable with that of a deletion mutant of AKAP-Lbc missing both N and C termini (7).

Therefore, in order to determine precisely the functional role of the C-terminal region, we assessed the Rho-activating properties of a deletion mutant of AKAP-Lbc missing only the C terminus. AKAP-Lbc as well as its deletion forms AKAP-Lbc \(\Delta\)Nterm and AKAP-Lbc \(\Delta\)Cterm, missing the N-terminal 1922 residues and the C-terminal 481 residues, respectively, were overexpressed in HEK-293 cells, and their basal Rho-GEF were activities assessed by using the rhotekin pulldown assay after 24 h of serum starvation (Fig. 1). Most interestingly, the C-terminal deletion mutant of AKAP-Lbc displayed a significant 4-fold higher basal Rho-GEF activity as compared with wild type AKAP-Lbc (Fig. 1B, top panel, lane 4). This activity is comparable with that of the N-terminal deletion mutant (Fig. 1B, top panel, lane 3), suggesting that both the N and C termini of the AKAP-Lbc contribute to maintain the basal Rho-GEF activity low in the absence of external activation stimuli. In order to determine whether the higher basal activity induced by N- and C-terminal deletions could be attributed to an increased association between AKAP-Lbc and RhoA, we determined the ability of RhoA to co-immunoprecipitate with wild type AKAP-Lbc, AKAP-Lbc \(\Delta\)Nterm, or AKAP-Lbc \(\Delta\)Cterm. As shown in Fig. 1C, both truncation mutants displayed a stronger interaction with endogenous RhoA under basal unstimulated conditions as compared with wild type AKAP-Lbc (Fig. 1C, middle panel, lanes 3 and 4). These findings strongly suggest that the C-terminal region of AKAP-Lbc, included between residues 2337 and 2817, negatively regulates the basal Rho-GEF activity by inhibiting the binding of RhoA to AKAP-Lbc.

AKAP-Lbc Undergoes Homo-oligomerization through Its C-terminal Region—Recent evidence suggests that GEFs of the Dbl family can be maintained in a basal inactive conformation by intramolecular interactions between regulatory sequences and the GEF module (DH and PH domains) (13). Such interactions have been proposed to modulate the function of the DH domain.

In order to assess whether the C-terminal region of AKAP-Lbc included between amino acids 2337 and 2817 could establish intramolecular interactions with other domains of AKAP-Lbc, such as the N-terminal regulatory region or the GEF module, we generated a series of FLAG-tagged AKAP-Lbc fragments encompassing residues 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336 and 2337–2817, which we expressed in HEK-293 cells, and their basal Rho-GEF activities assessed by using the rhotekin pulldown assay after 24 h of serum starvation (Fig. 1).
Homo-oligomerization Regulates AKAP-Lbc Function

1–10), a strong association was observed between FLAG- and GFP-tagged 2337–2817 fragments (Fig. 2B, middle panel, lane 12). These findings suggest that the C-terminal region of AKAP-Lbc can undergo homo-oligomerization.

Homo-oligomerization Occurs through Leucine Zipper Motifs Located in the Coiled Coil Region of AKAP-Lbc—To identify the oligomerization domain within the C-terminal region of AKAP-Lbc, we generated FLAG- and GFP-tagged AKAP-Lbc fragments encompassing residues 1923–2817, 1923–2336, 2337–2817, 1923–2589, and 1923–2698 (Fig. 3A), and we co-expressed them in HEK-293 cells. Overexpressed FLAG-tagged AKAP-Lbc fragments were immunoprecipitated using anti-FLAG antibodies, and the presence of associated GFP-tagged fragments was assessed using anti-GFP antibodies. Fragments encompassing residues 1923–2817, 2337–2817, and 1923–2698 retained the ability to undergo oligomerization (Fig. 3B, middle panel, lanes 12 and 14). These results strongly suggest that the coiled coil region of AKAP-Lbc is necessary and sufficient for the homo-oligomerization process.

Analysis of the primary sequence between residues 2566–2698 revealed the presence of two leucine zipper motifs encompassing residues 2616–2644 and 2658–2679, respectively (Fig. 4A). Based on this observation and on the fact that leucine zippers often function as protein-protein interaction motifs, we investigated the possibility that these motifs could mediate the oligomerization of AKAP-Lbc. We generated FLAG fusions of the 1923–2817 fragment of AKAP-Lbc in which valine 2630 as well as leucines 2616, 2623, 2637, 2644, 2658, 2665, 2672, and 2679 were substituted by alanine in different combinations (Fig. 4B). The different FLAG-tagged fragments were expressed in HEK-293 cells in combination with the GFP-tagged 1923–2817 fragment. Overexpressed FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibodies, and the presence of associated GFP-tagged 1923–2817 fragment was assessed using anti-GFP antibodies. As shown in Fig. 4C the mutation of leucines 2616, 2623, and valine 2630 (LZ mutant 1), leucines 2637 and 2644 (LZ mutant 2), and leucines 2658 and 2665 (LZ mutant 3) reduced the homo-oligomerization of the 1923–2817 fragments, whereas a fragment encompassing residues 2566–2698 retained the ability to form oligomers (Fig. 3B, middle panel, lanes 12 and 14). These results strongly suggest that the coiled coil region of AKAP-Lbc is necessary and sufficient for the homo-oligomerization process.
fragment of AKAP-Lbc by 50–60% (Fig. 4, C, middle panel, lanes 3–5, and D) whereas the substitution of leucines 2672 and 2679 (LZ mutant 4) had no effect (Fig. 4, C, middle panel, lane 6 and D). A 90% reduction in oligomerization could be observed after the substitution of all five leucines and valines included in the first leucine zipper (LZ mutant 5), whereas mutation of all four leucines of the second leucine zipper (LZ mutant 6) could inhibit the formation of oligomers only by 50% (Fig. 4, C, middle panel, lanes 7 and 8, and D). Finally, oligomerization was totally abolished after substitution of all leucines and valines of the first leucine zipper as well as leucines 2658 and 2665 of the second leucine zipper (LZ mutant 7) (Fig. 4, C, middle panel, lane 9, and D). Altogether these findings indicate that oligomerization requires the integrity of the entire leucine zipper motif encompassing residues 2616–2644, whereas the second leucine zipper is only partially involved.

To assess the contribution of the leucine zipper motifs to the oligomerization of the full-length AKAP-Lbc, we overexpressed the FLAG-tagged forms of AKAP-Lbc and of its mutants AKAP-Lbc ΔCC (missing the entire coiled coil region) and AKAP-Lbc LZm (in which leucines 2616 to 2665 were mutated to alanine) in HEK-293 cells in combination with AKAP-Lbc-GFP. The FLAG-tagged proteins were immunoprecipitated by using anti-FLAG antibodies, and the presence of associated GFP-tagged AKAP-Lbc was assessed using anti-GFP antibodies (lower panel). Results are representative of three independent experiments. IB, immunoblot.

Homo-oligomerization Regulates AKAP-Lbc Function

To investigate the functional role of AKAP-Lbc oligomerization, we determined whether disruption of the oligomerization domain could affect the ability of AKAP-Lbc to activate Rho in a cellular system. We overexpressed wild type AKAP-Lbc and its mutants AKAP-Lbc ΔCC and AKAP-Lbc LZm in HEK-293 cells, and we assessed their ability to activate RhoA using the rhodopsin pulldown assay. Most interestingly, both the ΔCC and
serine 1565 by the anchored PKA. Stimulation of the cAMP pathway by forskolin strongly activates anchored PKA, induces 14-3-3 recruitment, and inhibits AKAP-Lbc Rho-GEF activity (11). Dominant negative 14-3-3 proteins completely abolish forskolin-mediated AKAP-Lbc inhibition suggesting that this inhibitory effect is entirely mediated by 14-3-3 (11).

To determine whether the inhibitory function of 14-3-3 requires AKAP-Lbc oligomerization, we assessed whether the disruption of the oligomerization domain could prevent the inhibitory effect of forskolin on AKAP-Lbc Rho-GEF activity. AKAP-Lbc as well as its mutants LZm and S1565A (which is impaired in its ability to bind 14-3-3) were overexpressed in HEK-293 cells, and their ability to activate RhoA was assessed by using the rhotekin pulldown assay. As shown previously (11), treatment of cells with 10% serum strongly stimulated the Rho-activating effect of AKAP-Lbc as compared with untreated cells (Fig. 7, A, upper panel, lanes 4 and 5, and B), and this effect was totally abolished by forskolin treatment (Fig. 7, A, upper panel, lane 6, and B). In contrast, the S1565A mutant of AKAP-Lbc that fails to interact with 14-3-3 displays an increased basal Rho-GEF activity and is resistant to the forskolin-mediated inhibition (Fig. 7, A, upper panel, lanes 10–12, and B) suggesting that 14-3-3 maintains AKAP-Lbc inactive under basal conditions and that the inhibitory effect of forskolin requires the recruitment of 14-3-3 to AKAP-Lbc (11).

**Homo-oligomerization Regulates AKAP-Lbc Function**

**FIG. 6.** Homo-oligomerization maintains AKAP-Lbc inactive under basal conditions. A, HEK-293 cells expressing empty FLAG vector (lane 1) or FLAG-tagged AKAP-Lbc (lane 2), AKAP-Lbc (lanes 3), or AKAP-Lbc LZm (lane 4) were serum-starved for 24 h. Cell lysates were incubated with GST-RBD beads. The bound RhoA was detected using a monoclonal anti-RhoA antibody (upper panel). The amounts of total RhoA and FLAG-tagged proteins in the cell lysates were assessed using monoclonal antibodies against RhoA (middle panel) and FLAG, respectively (lower panel). The quantitative analysis of the GTP-RhoA associated with RBD beads was obtained by densitometry. The RhoA bound to RBD (upper panel) was normalized to the total RhoA content of cell extracts (middle panel). Results are expressed as the mean ± S.E. of three independent experiments. *, HEK-293 cells expressing FLAG-tagged AKAP-Lbc (lane 2), AKAP-Lbc ΔCC (lane 3), or AKAP-Lbc LZm (lane 4) were serum-starved for 24 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates (IP) and of the cell extracts were revealed using anti-FLAG monoclonal antibodies to detect FLAG-tagged AKAP-Lbc, AKAP-Lbc ΔCC, and AKAP-Lbc LZm (upper panel) or anti-RhoA monoclonal antibodies to detect co-immunoprecipitated RhoA (middle and lower panels). The quantitative analysis of the RhoA co-immunoprecipitated with the wild type or mutant forms of AKAP-Lbc was obtained by densitometry. The amount of RhoA in the immunoprecipitates was normalized to the RhoA content of cell extracts. Results are the mean ± S.E. of three independent experiments. IB, immunoblot.

**FIG. 7.** Homo-oligomerization is required for the regulation of AKAP-Lbc mediated by PKA and 14-3-3. A, HEK-293 cells expressing FLAG-tagged AKAP-Lbc (lanes 4–6), AKAP-Lbc-LZm (lanes 7–9), and AKAP-Lbc S1565A (lanes 10–12) were serum-starved for 24 h, and then treated for 1 h in the absence (−) or presence of 10% fetal calf serum (S) or 10% fetal calf serum + 50 μM forskolin (S + FSK). Cell lysates were incubated with GST-RBD beads. The bound RhoA was detected with a monoclonal anti-RhoA antibody (upper panel). The amounts of total RhoA and FLAG-tagged AKAP-Lbc in the cell lysates were assessed using monoclonal antibodies against RhoA (middle panel) and FLAG (lower panel), respectively. B, quantitative analysis of the GTP-RhoA associated with RBD beads was obtained by densitometry. The RhoA bound to RBD (upper panel) was normalized to the RhoA content of cell extracts (middle panel). Results are expressed as mean ± S.E. of three independent experiments. Statistical significance was analyzed by paired Student’s test. *, p < 0.05 as compared with Rho-GTP levels measured in untreated cells expressing FLAG-AKAP-Lbc. $p < 0.05$ as compared with Rho-GTP levels measured in forskolin-treated cells expressing FLAG-AKAP-Lbc. IB, immunoblot.

LZm mutants of AKAP-Lbc displayed basal Rho-GEF activities 4–5-fold higher than wild type AKAP-Lbc (Fig. 6A, upper panel, lanes 3 and 4). These basal activities are comparable with that observed with the deletion mutant of AKAP-Lbc missing the entire C-terminal region (Fig. 1B). Most interestingly, AKAP-Lbc ΔCC and AKAP-Lbc LZm also displayed an increased ability to co-immunoprecipitate with endogenous RhoA from serum-starved cells (Fig. 6B, middle panel, lanes 3 and 4), strongly suggesting that oligomerization maintains AKAP-Lbc in a low activity state by inhibiting its association with RhoA in vivo. Based on these findings, the hypotheses that oligomerization might maintain AKAP-Lbc in an inactive conformation that would mask the DH domain or might be necessary to promote the recruitment of negative regulatory proteins that can inhibit the Rho-GEF activity of AKAP-Lbc inside cells.

**Homo-oligomerization Is Required for the Regulation of AKAP-Lbc by PKA and 14-3-3**—We demonstrated previously that AKAP-Lbc is maintained in a basal inactive state through the association with 14-3-3, which is recruited to the anchoring protein in response to the phosphorylation of AKAP-Lbc on serine 1565 by the anchored PKA. Stimulation of the cAMP pathway by forskolin strongly activates anchored PKA, induces 14-3-3 recruitment, and inhibits AKAP-Lbc Rho-GEF activity (11). To determine whether the inhibitory function of 14-3-3 requires AKAP-Lbc oligomerization, we assessed whether the disruption of the oligomerization domain could prevent the inhibitory effect of forskolin on AKAP-Lbc Rho-GEF activity. A, HEK-293 cells expressing FLAG-tagged AKAP-Lbc (lanes 4–6), AKAP-Lbc-LZm (lanes 7–9), and AKAP-Lbc S1565A (lanes 10–12) were serum-starved for 24 h, and then treated for 1 h in the absence (−) or presence of 10% fetal calf serum (S) or 10% fetal calf serum + 50 μM forskolin (S + FSK). Cell lysates were incubated with GST-RBD beads. The bound RhoA was detected with a monoclonal anti-RhoA antibody (upper panel). The amounts of total RhoA and FLAG-tagged AKAP-Lbc in the cell lysates were assessed using monoclonal antibodies against RhoA (middle panel) and FLAG (lower panel), respectively. B, quantitative analysis of the GTP-RhoA associated with RBD beads was obtained by densitometry. The RhoA bound to RBD (upper panel) was normalized to the RhoA content of cell extracts (middle panel). Results are expressed as mean ± S.E. of three independent experiments. IB, immunoblot.

**FIG. 6.** Homo-oligomerization maintains AKAP-Lbc inactive under basal conditions. A, HEK-293 cells expressing empty FLAG vector (lane 1) or FLAG-tagged AKAP-Lbc (lane 2), AKAP-Lbc (lane 3), or AKAP-Lbc LZm (lane 4) were serum-starved for 24 h. Cell lysates were incubated with GST-RBD beads. The bound RhoA was detected using a monoclonal anti-RhoA antibody (upper panel). The amounts of total RhoA and FLAG-tagged proteins in the cell lysates were assessed using monoclonal antibodies against RhoA (middle panel) and FLAG, respectively (lower panel). The quantitative analysis of the GTP-RhoA associated with RBD beads was obtained by densitometry. The RhoA bound to RBD (upper panel) was normalized to the total RhoA content of cell extracts (middle panel). Results are expressed as the mean ± S.E. of three independent experiments. *, HEK-293 cells expressing FLAG-tagged AKAP-Lbc (lane 2), AKAP-Lbc ΔCC (lane 3), or AKAP-Lbc LZm (lane 4) were serum-starved for 24 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates (IP) and of the cell extracts were revealed using anti-FLAG monoclonal antibodies to detect FLAG-tagged AKAP-Lbc, AKAP-Lbc ΔCC, and AKAP-Lbc LZm (upper panel) or anti-RhoA monoclonal antibodies to detect co-immunoprecipitated RhoA (middle and lower panels). The quantitative analysis of the RhoA co-immunoprecipitated with the wild type or mutant forms of AKAP-Lbc was obtained by densitometry. The amount of RhoA in the immunoprecipitates was normalized to the RhoA content of cell extracts. Results are the mean ± S.E. of three independent experiments. IB, immunoblot.
Homo-oligomerization Regulates AKAP-Lbc Function

Remarkably, the AKAP-Lbc LZm mutant also displayed an increased resistance to forskolin-mediated inhibition (Fig. 7, A, upper panel, lanes 7–9, and B), which was comparable with that observed with the 14-3-3 binding deficient mutant of AKAP-Lbc (Fig. 7, A, upper panel, lanes 10–12, and B). These results provide evidence that 14-3-3 can exert its inhibitory function on AKAP-Lbc only when the anchoring protein is oligomeric.

DISCUSSION

Members of the Dbl family of guanine nucleotide exchange factors play a crucial role in the transduction of signals leading to the activation of Rho. Because of their implication in diverse physiological processes such as growth and development, cell migration, skeletal muscle formation, and neuronal axon guidance, the mechanisms involved in their regulation have been intensively investigated (8, 13). A growing number of studies indicate that several exchange factors of the Dbl family adopt an inactive conformation prior to their activation by upstream signals through the formation of intramolecular or intermolecular interactions (13). These binding events regulate the GEF activity by masking the interaction site for Rho GTPases on the GEF molecule or by modifying the subcellular localization of the exchange factor. Intramolecular inhibitory interactions can occur between DH and PH domains, as shown for the exchange factors Vav and Sos1 (21, 22), or between regulatory regions and the DH-PH module, as shown for Vav and Dbl (23, 24). On the other hand, exchange factors can also form oligomers through direct interaction or establish intermolecular associations with modulatory proteins through regulatory domains (13). In the present report, we describe the finding that AKAP-Lbc forms homo-oligomers in mammalian cells. Most interestingly, we found that oligomerization is required to maintain AKAP-Lbc in a basal inactive state in the absence of upstream activating stimuli. This inhibitory effect could be explained by the fact that oligomerization maintains AKAP-Lbc in a conformation that can be inhibited by PKA and 14-3-3.

Guanine nucleotide exchange factors of the Dbl have been shown to oligomerize through a variety of protein domains. Our findings indicate that oligomerization of AKAP-Lbc is mediated by two adjacent leucine zipper motifs located in a coiled coil region within the C terminus of AKAP-Lbc (Fig. 4). Similar to our observation, other Rho GEFs have been shown to undergo oligomerization through C-terminal coiled coil regions or leucine zippers. This is the case for the exchange factors α-PIX (16), β-PIX (17), and Bcr (25, 26) as well as for the Rho-specific GEFs p115-RhoGEF, LARG and PDZ-Rho-GEF undergo oligomerization through a C-terminal coiled coil domain (19). In other cases, oligomerization has been shown to occur through an interaction between DH domains as shown for RasGRF1, Ras GRF2, and Dbl (14, 15).

The fact that GEF oligomerization can occur through structurally and functionally different domains suggests that this intermolecular interaction might mediate different functional effects. Here we show that oligomerization inhibits the GEF function of AKAP-Lbc, as shown by the fact that disruption of oligomerization significantly increased the basal Rho GEF activity of anchoring protein inside cells (Fig. 6). Similar to our observation, Chikumi et al. (19) demonstrated that oligomerization negatively regulates the activity of the Rho-specific exchange factors p115-Rho-GEF, LARG, and PDZ-Rho-GEF. In contrast, Dbl family members that are interacting through their DH domain require oligomerization to efficiently activate Rho GTPases, as shown by the fact that inhibition of oligomerization diminishes the GEF activity of Dbl for Cdc42 and Rho (15) and that of RasGRF1 and Ras GRF2 for Ras (14). Most interestingly, the role of oligomerization can differ even between highly similar GEFs such as α-PIX and β-PIX. In fact, whereas β-PIX requires oligomerization to activate Rac and Cdc42 (17), the oligomerization state of α-PIX controls its specificity toward Rho GTPases, the dimers being selective for Rac and the monomers for both Rac and Cdc42 (16).

Whereas oligomerization of Dbl family members has been shown to play a crucial role in the modulation of their GEF activity and specificity toward Rho GTPases, a mechanistic explanation of how this intermolecular interaction might regulate the GEF function is still missing. We demonstrated previously that activation of PKA by forskolin induces the recruitment of 14-3-3 to AKAP-Lbc and the inactivation of the anchoring protein (11, 12). We now show that PKA and 14-3-3 can negatively regulate AKAP-Lbc only when the anchoring protein is oligomeric, as demonstrated by the fact that the oligomerization-deficient mutant of AKAP-Lbc is completely resistant to the inhibitory effect of forskolin, which is entirely mediated by 14-3-3 (Fig. 7). Based on these findings, we tested the possibility that 14-3-3 might associate only with the oligomeric form AKAP-Lbc and that disruption of oligomerization would prevent 14-3-3 binding. However, our experiments rule out this hypothesis because we could show that both the wild type and the oligomerization-deficient forms of AKAP-Lbc bind 14-3-3 in a similar manner (results not shown). Therefore, AKAP-Lbc oligomerization is not required for 14-3-3 binding. This suggests that although 14-3-3 can associate with AKAP-Lbc independently of oligomerization, it can exert its inhibitory action only when the anchoring protein adopts an oligomeric conformation.

The recent structural analysis of 14-3-3 revealed its dimeric structure (27, 28). Although both monomeric and dimeric forms of 14-3-3 have been shown to associate with cellular proteins (29, 30), our previous results (11) show that AKAP-Lbc can only associate with the dimeric 14-3-3 in a PKA-dependent manner. Therefore, one can speculate that two halves of the 14-3-3 dimer might associate with the two molecules of the AKAP-Lbc dimer. In this configuration, 14-3-3 binding might induce a conformational constraint that could mask the DH domain of AKAP-Lbc and inhibit its interaction with Rho. Based on these observations, we propose a model in which AKAP-Lbc oligomers are maintained inactive through the association with a 14-3-3 dimer.

In line with our findings, recent evidence suggests that oligomerization of the Rho-specific exchange factors p115-Rho-GEF, LARG, and PDZ-Rho-GEF might play a crucial role in the recruitment of inhibitory proteins that would negatively regulate their GEF activity (19). This model was based on the observation that oligomerization negatively regulates the basal Rho-GEF activity of the exchange factors inside cells but not in vitro, where cellular regulatory components are absent. Therefore, it appears that oligomerization can regulate the basal activity of Dbl family GEFs by maintaining the exchange factors in a conformation that can be regulated by modulatory proteins.

Recent evidence indicates that the dimerization state of Dbl family GEFs can be modulated by regulatory proteins. In fact, it has been demonstrated that the dimer-monomer equilibrium of the exchange factor α-PIX can be regulated by a protein complex formed by the protein kinase PAK and the β-subunits (Gβγ) of the heterotrimeric G protein G. This complex, which is assembled upon release of Gβγ from the G protein, interacts with the α-PIX dimer and induces its dissociation.

Because oligomerization maintains AKAP-Lbc inactive, one can speculate that upstream activating signals might enhance AKAP-Lbc activity by promoting the dissociation of AKAP-Lbc oligomers. Knowing that AKAP-Lbc can be activated by the α-subunit of the heterotrimeric G protein G13, one possibility would be that AKAP-Lbc oligomerization might be regulated by...
Homo-oligomerization Regulates AKAP-Lbc Function

G_{o12} either directly or through downstream effector proteins. In conclusion, our findings have several implications. First, they demonstrate that AKAP-Lbc is maintained in a basal inactive state through homo-oligomerization. Second, they provide a mechanistic hypothesis for the inhibitory role of oligomerization by showing that PKA and 14-3-3 can negatively regulate AKAP-Lbc Rho-GEF activity only when the anchoring protein is in an oligomeric form. Finally, they contribute to the elucidation of the role of oligomerization in the regulation of Dbl family guanine nucleotide exchange factors.

Acknowledgments—We acknowledge Prof. Susanna Cotecchia for helpful discussions and suggestions and for the critical reading of the manuscript, Monique Nenniger-Tosato and Liliane Abuin for excellent technical assistance, and Dr. Laura Stanasila for the critical reading of the manuscript.

REFERENCES

1. Michel, J. J., and Scott, J. D. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 235–257
2. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R. G., and Scott, J. D. (1991) J. Biol. Chem. 266, 14188–14192
3. Newlon, M. G., Roy, M., Hausken, Z. E., Coghlan, V., Scott, J. D., and Jennings, P. A. (1999) Nat. Struct. Biol. 6, 222–227
4. Newlon, M. G., Roy, M., Hausken, Z. E., Scott, J. D., and Jennings, P. A. (1997) J. Biol. Chem. 272, 23637–23644
5. Wong, W., and Scott, J. D. (2004) Nat. Rev. Mol. Cell. Biol. 12, 959–970
6. Bauman, A. L., and Scott, J. D. (2002) Nat. Cell Biol. 4, E203–E206
7. Diviani, D., Soderling, J., and Scott, J. D. (2001) J. Biol. Chem. 276, 44247–44257
8. Etienne-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
9. Schmidt, A., and Hall, A. (2002) Genes Dev. 16, 1587–1609
10. Zheng, Y., Olson, M. F., Hall, A., Cerione, R. A., and Toksoz, D. (1995) J. Biol. Chem. 270, 9031–9034
11. Diviani, D., Abuin, L., Cotecchia, S., and Pansier, L. (2004) EMBO J. 23, 2811–2820
12. Jin, J., Smith, F. D., Stark, C., Wells, C. D., Fawcett, J. P., Kulkarni, S., Metalnikov, P., O’Donnell, P., Taylor, P., Taylor, L., Zouman, A., Woodgett, J. R., Langeberg, L. K., Scott, J. D., and Pawson, T. (2004) Curr. Biol. 14, 1436–1450
13. Zheng, Y. (2001) Trends Biochem. Sci. 26, 724–732
14. Anborgh, P. H., Qian, X., Papageorge, A. G., Vass, W. C., DeClue, J. E., and Lowy, D. R. (1999) Mol. Cell. Biol. 19, 4611–4622
15. Zhu, K., Debreceeni, B., Bi, F., and Zheng, Y. (2001) Mol. Cell. Biol. 21, 425–437
16. Feng, Q., Baird, D., and Cerione, R. A. (2004) EMBO J. 23, 3492–3504
17. Kim, S., Lee, S. H., and Park, D. (2001) J. Biol. Chem. 276, 10551–10556
18. Eisenhaure, T. M., Francis, S. A., Willison, L. D., Coughlin, S. R., and Lerner, D. J. (2003) J. Biol. Chem. 278, 30975–30984
19. Chikumi, H., Barac, A., Behbahani, B., Gao, Y., Teramoto, H., Zheng, Y., and Gutkind, J. S. (2004) Oncogene 23, 233–240
20. Zheng, Y., Hart, M. J., and Cerione, R. A. (1995) Methods Enzymol. 256, 77–84
21. Han, J., Luby-Phelps, K., Dav, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Palck, J. R., White, M. A., and Brock, D. (1998) Science 279, 558–560
22. Nimnuan, A. S., Yatsula, B. A., and Bar-Sagi, D. (1998) Science 279, 560–563
23. Aghazadeh, B., Lowry, W. E., Huang, X. Y., and Rosen, M. K. (2000) Cell 102, 625–633
24. Bi, F., Debreceeni, B., Zhu, K., Salani, B., Eva, A., and Zheng, Y. (2001) Mol. Cell. Biol. 21, 1463–1474
25. McWhirter, J. R., Galasso, D. L., and Wang, J. Y. (1993) Mol. Cell. Biol. 13, 7387–7395
26. Zhang, X., Subrahmanyam, R., Wong, R., Gross, A. W., and Ren, R. (2001) Mol. Cell. Biol. 21, 840–853
27. Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) Nature 376, 191–194
28. Xiao, B., Smerdon, S. J., Jones, D. H., Dodson, G. G., Soneji, Y., Aitken, A., and Gamblin, S. J. (1995) Nature 376, 188–191
29. Tzivion, G., and Avruch, J. (2002) J. Biol. Chem. 277, 3061–3064
30. Yaffe, M. B. (2002) FEBS Lett. 513, 53–57