Supplementary Material

Materials and Methods

Expression constructs

cDNA fragments encoding residues 1-503, 504-1000, 1001-1387, 1388-1922, and 1923-2817 of AKAP-Lbc were PCR amplified from the AKAP-Lbc pEGFP vector (Diviani *et al*., 2001) and subcloned at EcoRI/SalI in pLexA, pGEX4T1 and pEGFPN3 plasmids to construct fusion proteins with LexA, GST, and GFP, respectively. Fragments encompassing residues 1388-1493, 1494-1593, 1594-1693, 1694-1793 and 1794-1922 were PCR amplified from the AKAP-Lbc 1388-1922-pEGFP vector and subcloned at EcoRI/SalI into pEGFPN3 to fuse GFP at the C-terminus. The S1655A mutations were introduced into the AKAP-Lbc pEGFP plasmid by standard PCR directed mutagenesis using the Hot Star DNA polymerase (Qiagen).

The Flag-tagged AKAP-Lbc construct was generated by subcloning a Flag epitope PCR amplified from the pFlagCMV6 vector (Sigma) at KpnI at the N-terminus of the AKAP-Lbc sequence. The S1565A and A1251P/I1260P mutations were introduced into the Flag-AKAP-Lbc vector by standard PCR directed mutagenesis using the Hot Star DNA polymerase (Qiagen).

The full-length cDNAs encoding human 14-3-3 β, ε and ζ were PCR amplified from library clones isolated in the yeast two-hybrid screen and subcloned at EcoRI-SalI in pFlagCMV6, pET30a, pGEX4T1 and pEGFPN3 to generate fusion proteins with the Flag-tag, His_6-tag and GST at the N-terminus and GFP at the C-terminus, respectively. The dominant negative mutant (R56A/R60A) and the dimerization deficient mutant (E5K/L12AE to Q12QR/Y82Q/K85N/E87Q) of 14-3-3β were generated by standard PCR directed mutagenesis using the 14-3-3β-pFlagCMV6 vector as a template. The Rhotekin Rho-binding domain (RBD)-pGEX4T1 construct was a generous gift of Dr. Hitoshi Kurose (Fukuoka, Japan).

Expression and purification of recombinant proteins in bacteria

GST-tagged fusion proteins of AKAP-Lbc, 14-3-3, and the Rho binding domain (RBD) of Rhotekin were expressed using the bacterial expression vector pGEX4T1 in the BL21DE3 strain of *Escherichia coli* and purified. To induce the expression of AKAP-Lbc and 14-3-3 fusion proteins, exponentially growing bacterial cultures were incubated 16 hours at 16 C with 1mM IPTG, and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer A (20mM Tris pH 7.4, 100mM NaCl, 5mM $MgCl_2$, 1% (w/v) Triton-X-100, 1mM benzamidine, 2μg/ml leupeptin, 2μg/ml pepstatin), sonicated and centrifuged at 38,000 x g for 30 min at 4 ˚C. After incubating the supernatants with glutathione Sepharose beads (Pharmacia) 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 pulldown assay.

His_6-tagged fusion proteins of AKAP-Lbc and 14-3-3 were expressed using the bacterial expression vector pET30 in BL21DE3 bacteria and purified. Bacterial extracts containing His_6-tagged fusion proteins were prepared in buffer B (20mM Hepes pH 7.8, 500mM NaCl, 10mM imidazole, 1mM benzamidine, 2μg/ml leupeptin, 2μg/ml pepstatin). After a 1 min sonication, the lysates were centrifuged at 38,000 x g for 30 min at 4 ˚C. The His_6-tagged fusion proteins were purified by incubating the supernatant with Nickel-NTA chelating resin (Amersham Pharmacia Biotech) for 1 h at 4 ˚C. The resin was then washed 5 times with 10 bed volumes of buffer B and stored at 4 ˚C. His_6-tagged fusion proteins were eluted from the resin with 20mM Hepes pH 7.8, 500mM NaCl, 300 mM imidazole, 1mM benzamidine, 2μg/ml leupeptin, 2μg/ml pepstatin for 1h at room temperature, dialyzed and stored at –20 ˚C. The protein content of the eluates was assessed by Coumassie staining of SDS-PAGE gels.

Cell culture and transfections

HEK293 and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamycin (100 μg/ml) and transfected at 50-60% confluency in 100 mm dishes using or the calcium-phosphate method. For the overexpression of constructs containing the full-length AKAP-Lbc, HEK293 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 of 10 to 16 μg/100 mm dish.

GST-pulldown and immunoprecipitation experiments

For GST pulldowns, HEK293 cells expressing the various constructs grown in 100 mm dishes were lysed in 1ml of buffer C (20mM Tris pH 7.4, 150mM NaCl, 1% (w/v) Triton-X-100, 5μg/ml aprotinin, 10μ mg/ml leupeptin and 1 mM PMSF) and centrifugation at 100,000 x g for 30 min at 4˚C. Glutathione Sepharose beads 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 resuspended in SDS-PAGE sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blotting.

For immunoprecipitation experiments, HEK-293 cells expressing various constructs grown in 100 mm dishes were lysed in 1 ml of buffer C. Cell lysates were incubated 6 h at 4˚C on a rotating wheel. The solubilized material was centrifuged at 100,000 x g for 30 min at 4˚C and the supernatants were incubated overnight at 4˚C with 20µl of anti-FlagM2 affinity resin (Sigma) to immunoprecipitate overexpressed Flag-AKAP-Lbc proteins. Following a brief centrifugation on a bench-top centrifuge, the pelleted beads were washed five times with buffer C, twice with PBS and proteins eluted in SDS-PAGE sample buffer (65 mM Tris, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, pH 6.8) by boiling samples for 3 min at 95°C. Eluted proteins were analysed by SDS-PAGE and Western blotting. Immunoprecipitations of AKAP-Lbc from HeLa cells were performed as previously indicated (Diviani *et al.*, 2001).

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 and horseradish-conjugated secondary antibodies (Amersham) as previously indicated(Diviani *et al.*, 2001). The following affinity purified primary antibodies were

used for immunoblotting: rabbit polyclonal anti-AKAP-Lbc (1mg/ml, 1:1000 dilution) (ref.), mouse monoclonal anti-Flag (Sigma, 4.9 mg/ml, 1:2000 dilution), mouse monoclonal anti-GFP (Roche, 400μg/ml, 1:500 dilution), mouse monoclonal anti-RhoA (Santa Cruz, 1:250 dilution), mouse monoclonal anti-RII (Transduction Laboratories, 1:500 dilution), rabbit polyclonal anti-14-3-3 β, (Santa Cruz, 1:250 dilution), mouse monoclonal anti-14-3-3 β (Santa Cruz, 1:250 dilution).

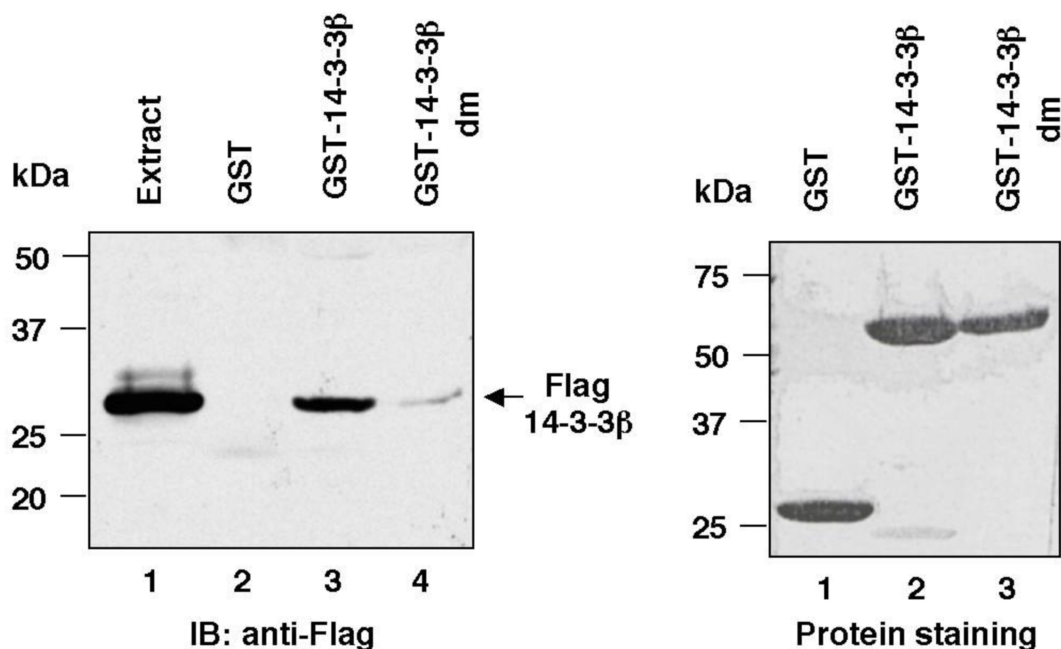
Solid phase overlay assay

Nitrocellulose filters as well as peptide arrays were incubated with TBS-Tween containing 5% (w/v) non-fat dry milk and 1% BSA for 1 h at room temperature and with 50nM of His_6-tagged 14-3-3 β, ε or ζ in TBS-Tween containing 5% non-fat dry milk and 0.1% BSA for 16 h at room temperature. After three washes in TBS-Tween, the blots were incubated for 2 hours with HRP-conjugated S-protein (Jackson Labs, 1:5000 dilution) in TBS-Tween containing 5% (w/v) non-fat dry milk. Membranes were then washed three times in TBS-Tween and subjected to autoradiography.

Fluorescence microscopy

Cells grown on coverslips were transfected at 40% confluency using Lipofectamine 2000, washed twice with PBS then fixed for 10min in PBS/ 3.7% formaldehyde and permeabilized for 5 min with 0.2% (w/v) Triton-X-100 in PBS. Cells were blocked for 30min in PBS/1% BSA, then either incubated for 1hr with 1:2000 dilution of anti-Flag monoclonal antibody followed by 1hr in FITC conjugated anti-mouse secondary antibody (Jackson ImmunoResearch), and Texas-red phalloidin (Molecular Probes). The cells were mounted using Prolong (Molecular Probes). Immunofluorescent staining was visualized using a Zeiss Axiophot fluorescence microscope.

Supplementary figure 1



B

AKAP-Lbc GFP | Flag-14-3-3 WT + AKAP-Lbc GFP | Flag-14-3-3 dm + AKAP-Lbc GFP

- FSK FSK PKI$_{myr}$ - FSK FSK PKI$_{myr}$ - FSK FSK PKI$_{myr}$

kDa

250
150

IP: anti-Flag
IB: anti-GFP

37
25
20

IP: anti-Flag
IB: anti-Flag

250
150

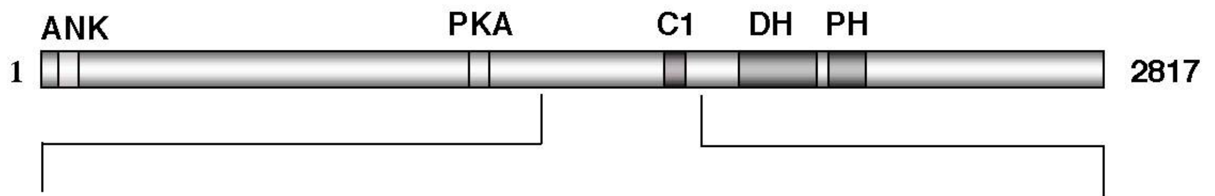
Cell extacts
IB: anti-GFP

1 2 3 4 5 6 7 8 9

Figure S1. AKAP-Lbc interacts with dimeric 14-3-3 in a PKA-dependent manner. **A)** Extracts from HEK-293 cells overexpressing Flag-14-3-3β were incubated with glutathione-sepharose beads coupled to GST alone, GST-14-3-3β and GST-14-3-3β dm. Associated Flag-14-3-3β was detected using anti-Flag monoclonal antibodies (left panel). A control protein staining indicating the expression level of the GST, GST-14-3-3β and GST-14-3-3β dm is shown (right panel). **B)** HEK-293 cells were transfected with the cDNAs encoding AKAP-Lbc-GFP alone (lanes 1-3) or in combination with the plasmid encoding Flag-14-3-3β (lanes 4-6) or Flag-14-3-3β dm (lanes 7-9). After a 24h serum starvation, cells were treated for 1 h in the absence or presence of 10μM forskolin (FSK) or 10μM forskolin + 20μM of myristoylated PKI 5-24 peptide (FSK + PKI_{myr}). Cell lysates were subjected to immunoprecipitation with anti-Flag monoclonal antibodies. Western blots of the immunoprecipitates were revealed using anti-GFP monoclonal antibodies to detect AKAP-Lbc-GFP (upper panel) or anti-Flag monoclonal antibodies to detect Flag-14-3-3β constructs (middle panel). The expression of AKAP-Lbc-GFP in the cell lysates was assessed using monoclonal antibodies against GFP (lower panel). Results are representative of three independent experiments.

Supplementary figure 2

A



B

C

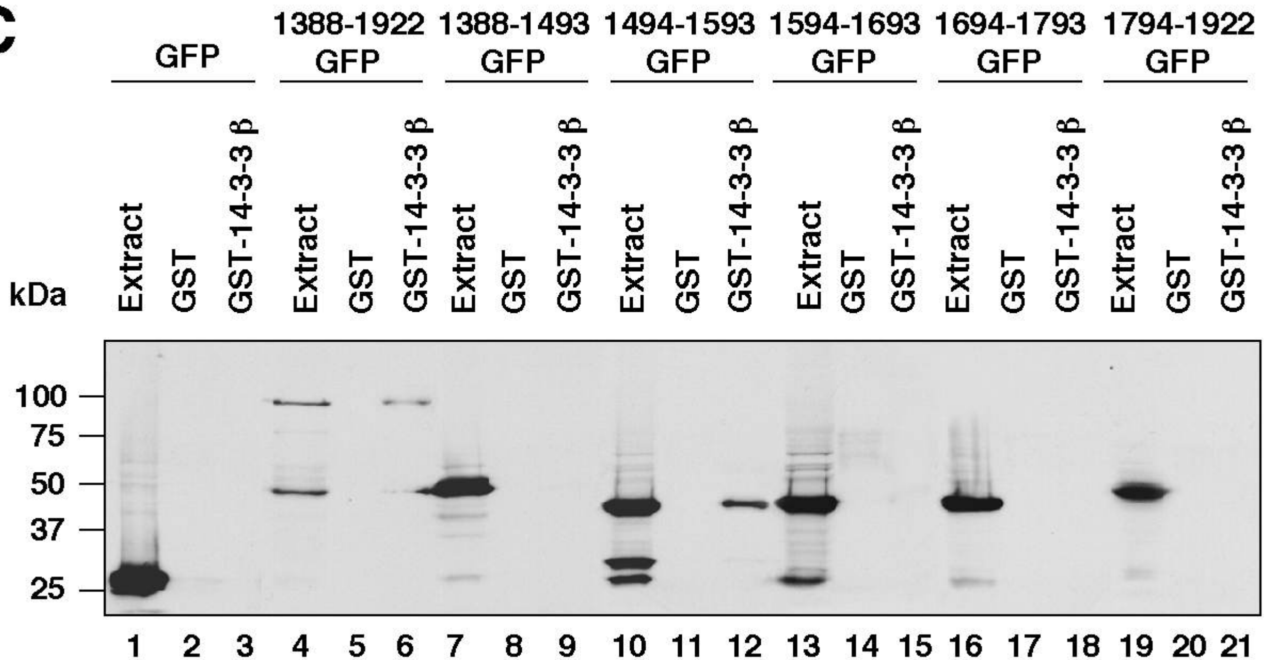


Figure S2. Mapping of the binding site for 14-3-3 on AKAP-Lbc. A) Domain organization of AKAP-Lbc. The ankyrin repeats (ANK), the PKA-binding domain (PKA), the C1 homology region (C1), the Dbl (DH) and pleckstrin (PH) homology domains are shown. **B)** Schematic of the GFP-tagged AKAP-Lbc fragments used for the mapping of the 14-3-3 binding site. For each fragment, the position of the first and last amino acid is indicated. **C)** Extracts from HEK-293 cells expressing either GFP or GFP-tagged AKAP-Lbc fragments indicated in B) were incubated with glutathione-sepharose beads coupled to GST alone or to GST-tagged 14-3-3β. The GFP-tagged AKAP-Lbc fragments eluted from the beads were detected by Western blotting using anti-GFP monoclonal antibodies. The results are representative of three independent experiments.

Supplementary figure 3

A

GFP | GFP + Flag-14-3-3β R56A/R60A | AKAP-Lbc-GFP | AKAP-Lbc-GFP + Flag-14-3-3β R56A/R60A

kDa

- S S+ FSK - S S+ FSK - S S+ FSK - S S+ FSK

25
20
Rho-GTP

25
20
Total RhoA

250
AKAP-Lbc GFP

37
25
Flag-14-3-3 R56A/R60A

1 2 3 4 5 6 7 8 9 10 11 12

B

Basal
Serum
Serum + FSK

Rho-GTP (% of control)

1000
800
600
400
200
0

*
§

GFP
GFP + Flag-14-3-3β R56A/R60A
AKAP-Lbc-GFP
AKAP-Lbc-GFP + Flag-14-3-3β R56A/R60A

Figure S3. A Dominant negative mutant of 14-3-3β upregulates AKAP-Lbc Rho-GEF activity. A) HEK-293 cells were transfected with the cDNAs encoding GFP (lanes 1-3) or AKAP-Lbc-GFP (lanes 7-9) alone or in combination with the plasmid encoding Flag-14-3-3β R56A/R60A (lanes 4-6 and 10-12). After a 24h serum starvation, cells were treated for 1 h in the absence or presence of 10% fetal calf serum (S) or 10% fetal calf serum + 10μM forskolin (S +FSK). Cell lysates were incubated with a GST fusion protein of the RBD of Rhotekin. The bound RhoA was detected by immunoblot with a monoclonal anti-RhoA antibody (upper panel). The relative amount of total RhoA, and transfected GFP, AKAP-Lbc-GFP and Flag-14-3-3β R56A/R60A in the cell lysates were assessed using monoclonal antibodies against RhoA (middle panel), GFP (middle panel) and the Flag epitope (lower panel), respectively. **B)** Quantitative analysis of the total amount GTP-RhoA associated with RBD beads was obtained by densitometry. For these experiments, the RhoA bound to RBD (upper panel) was normalized to the total RhoA content of cell extracts (middle panel). Results are expressed as mean ± S.E of four independent experiments. Statistical significance was analyzed by paired Student's test. * $p < 0.05$ as compared with Rho-GTP levels measured untreated cells expressing AKAP-Lbc GFP. § $p < 0.05$ as compared with Rho-GTP levels measured forskolin treated cells expressing AKAP-Lbc GFP.

Supplementary figure 4

A

kDa	Empty vector			Flag-AKAP-Lbc			Flag-AKAP-Lbc S1565A		
	-	S	S+ FSK	-	S	S+ FSK	-	S	S+ FSK

250

IP: anti-Flag
IB: anti-Flag

50
37
25
20

IP: anti-Flag
IB: anti-RhoA

25
20

IB: anti-RhoA

1 2 3 4 5 6 7 8 9

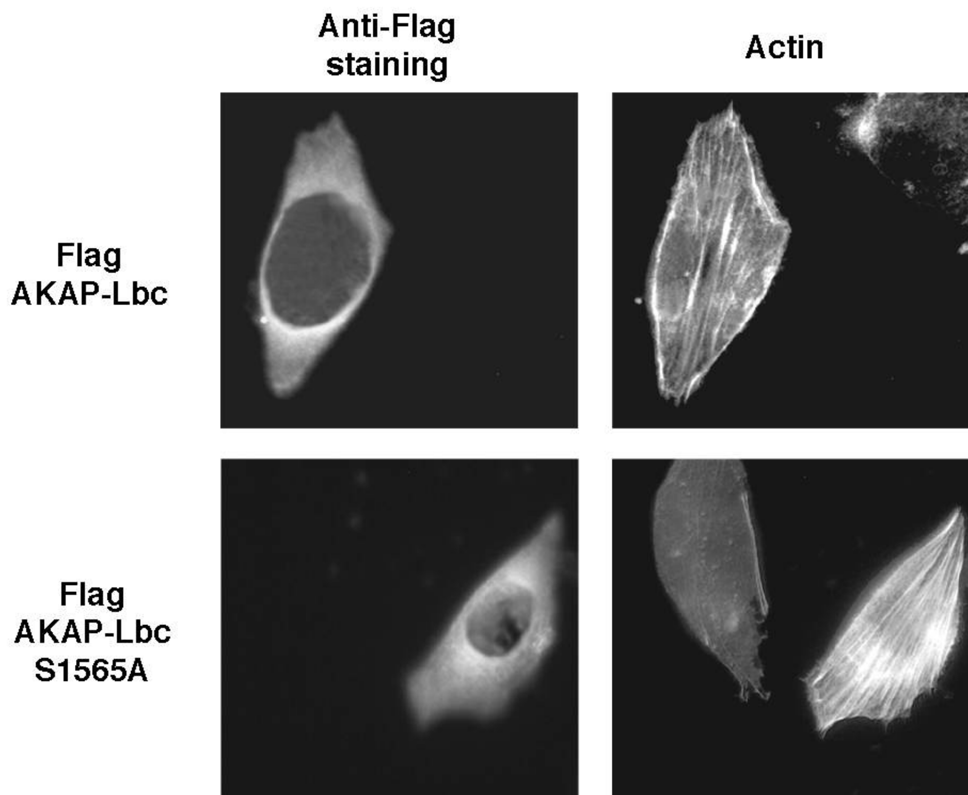


Figure S4. 14-3-3β inhibits the interaction between AKAP-Lbc and RhoA. A) HEK-293 cells were transfected with the empty pFlag-cmv6 vector (lanes1-3) or with the plasmids encoding Flag-AKAP-Lbc (lanes 4-6) or its mutant Flag-AKAP-Lbc S1565A (lanes 7-9). After a 24h serum starvation, cells were treated for 1 h in the absence or presence of 10% fetal calf serum (S) or 10% fetal calf serum + 10μM forskolin (S +FSK). Cell lysates were subjected to immunoprecipitation with anti-Flag monoclonal antibodies. Western blots of the immunoprecipitates were revealed using anti-Flag monoclonal antibodies to detect the Flag-AKAP-Lbc constructs (upper panel) or anti-RhoA polyclonal antibodies to detect RhoA (middle panel). The relative amount of RhoA in the cell lysates was assessed using polyclonal antibodies against RhoA (lower panel). Results are representative of three independent experiments. **B)** NIH-3T3 fibroblasts were transfected with cDNAs encoding for wild type Flag-AKAP-Lbc or its mutant Flag-AKAP-Lbc S1565A. After a 24 h serum starvation cells were fixed, permeabilized, and incubated with anti-Flag monoclonal antibodies as well as FITC-conjugated anti-mouse secondary antibodies to detect cells expressing the Flag-AKAP-Lbc constructs (left panels), and stained using Texas-red phalloïdin to detect actin (right panels).