

# Microtubule-Associated Protein 1B-Light Chain 1 Enhances Activation of Rap1 by Exchange Protein Activated by Cyclic AMP but Not Intracellular Targeting

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## ABSTRACT

We have previously demonstrated that EPAC1 interacts with light chain (LC) 2 of microtubule-associated protein (MAP) 1A. In the present study, we investigated whether the structurally related LC1 of MAP1B also interacts with EPAC1. We demonstrate that LC1 copurifies with EPAC1 from extracts of PC-12 cells, using cyclic AMP-agarose. Using recombinant LC1 and LC2 in pull-down and solid phase binding assays, we demonstrate direct interaction with a glutathione S-transferase-fusion of the cyclic AMP-binding (CAMP) domain of EPAC1. We also tested whether LC1 directed intracellular targeting of EPAC1 through its interaction with the CAMP domain. EPAC1 was found to be in the soluble and particulate, nuclear/perinuclear fractions of cells. We found that the catalytic (CAT) domain of EPAC1, and not the CAMP domain, was responsible for recruit-

ment to the nuclear/perinuclear fraction of cells. The targeting sequence responsible was located between amino acids 764 and 838 of EPAC1. Overexpression of an isolated CAT domain in COS1 cells was found to displace endogenous EPAC1 from the nuclear/perinuclear fraction, thereby inhibiting EPAC-activated Rap1 in this compartment. In contrast, LC1 was not able to compete for the binding of EPAC1 to this fraction. LC1, however, was able to enhance interaction of EPAC1 with cyclic AMP and heightened the ability of EPAC to activate Rap1. Antibody disruption of EPAC1/LC1 interaction in PC-12 cells ablated the ability of cyclic AMP to activate Rap1. LC1 is therefore not involved in intracellular targeting of EPAC1, but it is rather a molecular chaperone of EPAC activity toward Rap1.

Cyclic AMP is a pivotal second messenger that regulates a diverse range of key cellular processes encompassing central metabolic events, including gluconeogenesis, glycogenolysis, and lipogenesis; cardiac and smooth muscle contraction; secretory processes; ion channel conductance; learning and memory; cell growth and differentiation; and apoptosis (Beavo and Brunton, 2002). Ligand binding to the seven-transmembrane domain class of G protein-coupled receptors causes activation of heterotrimeric G protein  $G_s\alpha$ , which stimulates one or more isoforms of adenylyl cyclase, to cata-

lyze production of cyclic AMP. Intracellular levels of cyclic AMP are then regulated through the action of cyclic AMP phosphodiesterases (PDEs), which degrade cyclic AMP to 5'AMP (Houslay, 1998). Given the importance of cyclic AMP in regulating physiological processes, it is perhaps not surprising that a wide range of disease states are linked to improper regulation of the cyclic AMP signaling system (Spiegel et al., 1993). For example, selective inhibitors of cyclic AMP-specific PDEs have anti-inflammatory and antidepressant properties (Houslay et al., 1998; Conti and Jin, 1999) and overproduction of cyclic AMP leads to endocrine hyperplasia and hyperfunction in many endocrine glands, including gonads, adrenal cortex, thyroid, and pituitary somatotrophs (Spiegel et al., 1993).

Understanding the mechanisms by which the cyclic AMP

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**ABBREVIATIONS:** PDE, phosphodiesterase; PKA, protein kinase A; GEF, guanine nucleotide exchange factor; EPAC, exchange protein directly activated by cyclic AMP; CAMP, cyclic AMP-binding; CAT, catalytic; REM, Ras exchange motif; DEP, Dishevelled; PIPES, 1,4-piperazinediethanesulfonic acid; Egl-10, and pleckstrin; AKAP, A-kinase anchoring protein; LC, light chain; MAP, microtubule-associated protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; MES, 2-(N-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GSH, glutathione; NLS, nuclear localization signal; 8-CPT-2Me-cyclic AMP, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3'-5'-cyclic monophosphate.

signal is transduced in cells is clearly critical to elucidating the cellular processes leading to disease. In the past, the actions of cyclic AMP in most cells were thought to be manifest by protein phosphorylation events catalyzed by cyclic AMP-dependent protein kinase (PKA) (Engh and Bossemeyer, 2001). However, it is now clear that not all of the signaling actions of cyclic AMP are mediated by PKA (de Rooij et al., 1998; Kawasaki et al., 1998). One recently discovered, PKA-independent mechanism of cyclic AMP action is through direct activation of the small GTPase Rap1, by a family of guanine nucleotide exchange factors (GEFs) called cyclic AMP-GEFs or exchange protein directly activated by cyclic AMP (EPAC) (de Rooij et al., 1998; Kawasaki et al., 1998).

EPACs are multidomain proteins containing an autoinhibitory cyclic AMP-binding (CAMP) domain that inhibits the catalytic (CAT) region (Bos et al., 2001). Direct interaction of the CAMP domain in EPAC with cyclic AMP facilitates GEF activation by relieving autoinhibition of the CAT region (de Rooij et al., 1998; Kawasaki et al., 1998). Our recent findings suggest that the regulation of EPAC activity may be more complicated than this, involving interaction with ancillary proteins. The recruitment of signaling proteins to specific complexes within cells underpins the functioning of most, if not all, signaling pathways. The machinery that underpins this for cyclic AMP is now becoming apparent (Rubin, 1994; Houslay and Milligan, 1997; Colledge and Scott, 1999). Thus, adenylyl cyclase and  $G_s\alpha$  can be localized to discrete plasma membrane regions. Gradients of cyclic AMP are then established through PDE action, with anchored species presumed to tailor localized cyclic AMP gradients. PKA populations localized at specific intracellular sites, by binding to anchor proteins (AKAPs), sample these gradients to provide a compartmentalized response (Rubin, 1994; Colledge and Scott, 1999). From our investigations, we have identified EPAC as a cyclic AMP-activated enzyme that is also regulated by protein complex formation (Gupta and Yarwood, 2005).

Using a combination of yeast two-hybrid analysis and co-immunoprecipitation, we have identified the light chain (LC) 2 of microtubule-associated protein (MAP) 1A as a novel protein-binding partner for EPAC1 and EPAC2 (Magiera et al., 2004). The MAP1A/LC2 gene is organized to encode a precursor polypeptide that undergoes proteolytic processing to generate the final 2556 amino acid MAP1A heavy chain and 249 amino acid LC2 polypeptide (Noiges et al., 2002). Using deletion analysis and GST-protein chimeras of individual EPAC1 domains, we have identified the CAMP domain of EPAC as being responsible for mediating interaction with LC2 (Magiera et al., 2004). Once bound to the CAMP domain, LC2 seems to increase the affinity of EPAC1 for cyclic AMP, thereby sensitizing catalytic activity toward Rap1 to lower concentrations of cyclic AMP (Gupta and Yarwood, 2005).

LC2 belongs to a family of MAP light chains, including LC1 and LC3, which seem to serve as a "scaffold" or "adaptor" proteins, facilitating the stable interaction of MAPs with other signal transduction components or structural proteins (Noiges et al., 2002). In the present study, we identify the 250 amino acid, C-terminal light chain of MAP1B, LC1, as a novel protein interaction partner for EPAC1. We also address the function of the EPAC1/LC1 interaction in terms of directing

intracellular targeting of EPAC1 and the ability of EPAC to activate Rap1.

## Materials and Methods

**Materials.** 8-(6-Aminoethyl)amino-2'-O-methyladenosine-3',5'-cyclic monophosphate agarose and 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cyclic AMP) were purchased from Biolog Life Science Institute (Bremen, Germany). Monoclonal anti-FLAG antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-LC1 polyclonal antibody and bacterial expression vectors expressing poly-histidine-tagged MAP1B-LC1 and MAP1A-LC2 (pMT22NHis and pAH1NH, respectively) were generously provided by Prof. F. Propst (University of Vienna, Vienna, Austria).

**Purification of GST-Fusion Proteins.** Vectors (pGEX-6P-1) expressing GST-fusions of individual EPAC1 domains in bacteria were generated previously as described in Magiera et al. (2004). GST-fusions of the CAT domain (amino acids 619–881), CAMP domain (amino acids 199–316), Dishevelled, Egl-10, and pleckstrin (DEP) domain (amino acids 74–140), and Ras exchange motif (REM) domain (amino acids 345–410) were then purified using glutathione-Sepharose as described previously (Yarwood et al., 1999).

**Generation of Expression Vectors.** The myc-EPAC1-FLAG expression vector, which expresses full-length EPAC1 simultaneously tagged with myc and FLAG epitopes, was constructed using the p3xFLAG-myc-CMV-26 vector backbone (Sigma-Aldrich), as described previously (Gupta and Yarwood, 2005). Point mutation of putative nuclear localization signals was done with the Quick Change mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. EPAC1-CAT, which expresses a myc- and FLAG-tagged CAT domain (EPAC1 catalytic domain; amino acids 619–881) was generated by cloning a PCR-amplified fragment from the full-length EPAC1 open reading frame, using primers 5'-CTGCTGAATTCCTGAGTGCCAAAGGACCTG (forward) and 5'-GACAAGTCTAGATCATGGCTCCAGCTCTCG (reverse). This fragment was then cloned into the EcoRI and XbaI sites of p3xFLAG-myc-CMV-26. Truncates of the CAT domain were cloned by PCR into the EcoRI and XbaI sites of p3xFLAG-myc-CMV-26 using EPAC1-CAT as the template. The CAT reverse primer and the following forward primers were used: CAT2, 5'-CCGAATTCACAGCTGCTCAGGAAGTTC; CAT7, 5'-CCGAATTCAAAGCTCTCCCTCTCTGTC; and CAT14, 5'-CCGAATTCAGCGAGGATTTCCACATGC. EPAC1-CAMP, which expresses a myc- and FLAG-tagged EPAC1 cyclic AMP binding domain (amino acids 199–316), was generated by PCR amplification from full-length EPAC1 cDNA using primers forward, 5'-CTGCTGAATTCCTGACATCAAGGCTGTGG and reverse, 5'-GACAAGTCTAGATTTCTCCAGCCGATGGTCTTTGCC. The amplified CAMP fragment was then subcloned into the EcoRI and XbaI sites of p3xFLAG-myc-CMV-26.

**Bacterial Expression and Purification of Recombinant LC1 and LC2.** Poly-histidine-tagged recombinant LC1 and LC2 were expressed in *Escherichia coli* BL21, induced by isopropyl  $\beta$ -D-thiogalactoside as described previously (Noiges et al., 2002) and purified by affinity chromatography on  $Ni^{2+}$  columns, according to the manufacturers' instructions (Novagen, Madison, WI; QIAGEN GmbH, Hilden, Germany). Recombinant proteins were solubilized, bound to, and eluted from the column in the presence of 6 M guanidine hydrochloride. After purification, recombinant proteins were dialyzed at 4°C against 50 mM MES, pH 6.6, for LC1, or PEM (100 mM PIPES, 1 mM EDTA, and 1 mM  $MgSO_4$ ) containing 40 mM L-arginine, for LC2.

**Antibody Production.** Specific polyclonal antibodies against EPAC1 were generated and affinity purified by Alpha Diagnostic International, Inc. (San Antonio, TX) using the synthetic EPAC1 peptide spanning residues 41–60, (C)DFSESELEQASTERVLAGR. Polyclonal antibodies against the DEP, REM, CAMP, and CAT domains of EPAC1 were generated by Diagnostics Scotland (Carlisle,

Scotland) using fusion protein immunogens formed between GST and the individual PCR-amplified domains as described previously (Gupta and Yarwood, 2005).

**Cell Culture and Transfections.** COS1 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and a 2% (v/v) penicillin-streptomycin solution (Sigma-Aldrich). PC-12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum (Sigma-Aldrich), 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and a 2% (v/v) solution of penicillin-streptomycin (Sigma-Aldrich). Transfection of COS1 cells was carried out using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate (Boehringer Ingelheim GmbH, Ingelheim, Germany) using the manufacturer's protocols.

**Cyclic AMP Pull-Down Assays.** Increasing concentrations of GST-CAMP were incubated in the presence or absence of 10  $\mu$ g of purified recombinant LC1 or LC2. Cyclic AMP-agarose slurry (50  $\mu$ l) was then added and incubated for 1 h. Cyclic AMP-agarose with bound GST-CAMP was then recovered by centrifugation (30 s; 13,000g), washed three times in PBS, and then subject to SDS-PAGE. For pull-down assays from COS1 and PC-12 cells, 500  $\mu$ g of cell lysate was incubated with or without 10  $\mu$ l of anti-DEP, anti-REM, anti-CAMP, or anti-CAT antibody for 30 min at 4°C. Cyclic AMP-agarose (50  $\mu$ l) was then added, and the lysates were further incubated for 1 h. The pellets were washed three times with PBS, and the protein was eluted in sample buffer and subjected to SDS-PAGE.

**[<sup>3</sup>H]Cyclic AMP Binding Assay.** [<sup>3</sup>H]Cyclic AMP (40  $\mu$ M) was diluted in 50 mM potassium phosphate buffer, pH 7.0, containing 2 M NaCl, 4 mM EDTA, 20 mM benzamidine-HCl, and 10 mM magnesium sulfate and incubated with 1  $\mu$ g of GST-CAMP with either 1  $\mu$ g of either LC1 or LC2 protein on ice for 1 h. Twenty microliters of prewashed glutathione beads was then added and incubated further for 1 h on ice. Radioactivity was recovered by GSH elution of beads and quantitated by liquid scintillation counting.

**Protein Interaction Assays Using Reacti-Bind Glutathione-Coated Plates.** Five micrograms of poly-His-tagged LC1 or LC2 was serially diluted in dilution buffer (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and then added to Reacti-Bind glutathione-coated plates that had previously been incubated GST or GST-CAMP. Protein complex formation was determined by addition of anti-poly-His monoclonal antibody for 1 h at room temperature followed by alkaline phosphatase-conjugated anti-mouse IgG for a further hour at room temperature. Immunoreactivity was visualized with the BCIP Microwell 2 component phosphatase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) following the manufacturer's instructions and quantified using a microplate reader set at 620 nm.

**Cell Fractionation.** Cytoplasmic and nuclear/perinuclear fractions were prepared as described previously (Rundell et al., 2004), using the nuclear extract kit (catalog no. 40010) from Active Motif Europe (Rixensart, Belgium), according to the manufacturer's instructions. In brief, cells were collected in ice-cold PBS in the presence of phosphatase inhibitors and then resuspended in hypotonic buffer to weaken cell membranes. Next, addition of detergent promoted the leakage of cytoplasmic proteins. Centrifugation produced a particulate nuclear fraction, which was solubilized in lysis buffer containing protease inhibitor cocktail.

**Rap Activation Assays.** Five hundred micrograms of COS or PC-12 cell lysate was incubated with either 10  $\mu$ l of either anti-REM, anti-DEP, anti-CAMP, or anti-CAT antibody for 30 min followed by incubation for 30 min with 8CPT-2Me-cyclic AMP (50  $\mu$ M). Thereafter, the lysates were incubated with 50  $\mu$ l of RalGDS-RBP-GST for 30 min followed by 50  $\mu$ l of GSH-Sepharose for a further hour, as described previously (McPhee et al., 2000). Recovered precipitates were washed three times and then proteins were eluted with sample buffer.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting.** Samples were resuspended in Laemmli buffer and subjected to SDS-PAGE and immunoblotting as described previously (Yarwood et al., 1999). Nitrocellulose membranes were blocked in 5% (w/v) low-fat milk powder in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 30 min at room temperature. Membranes were then incubated with the appropriate antibody diluted in 1% (w/v) low-fat milk powder in Tris-buffered saline/Tween 20 [Tris-buffered saline plus 0.1% (v/v) Tween 20] for 16 h at 4°C. Detection of the bound antibody was with anti-mouse IgG peroxidase or anti-rabbit IgG peroxidase secondary antibodies (Sigma-Aldrich) incubated for 90 min and the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoblots were routinely scanned and subject to densitometric analysis using NIH Image (<http://rsb.info.nih.gov/nih-image/Default.html>). In some cases, scanned immunoblot images had their total contrast autoadjusted using Photoshop (Adobe Systems, Mountain View, CA). In all cases, the results from this adjustment remained an accurate representation of the original data.

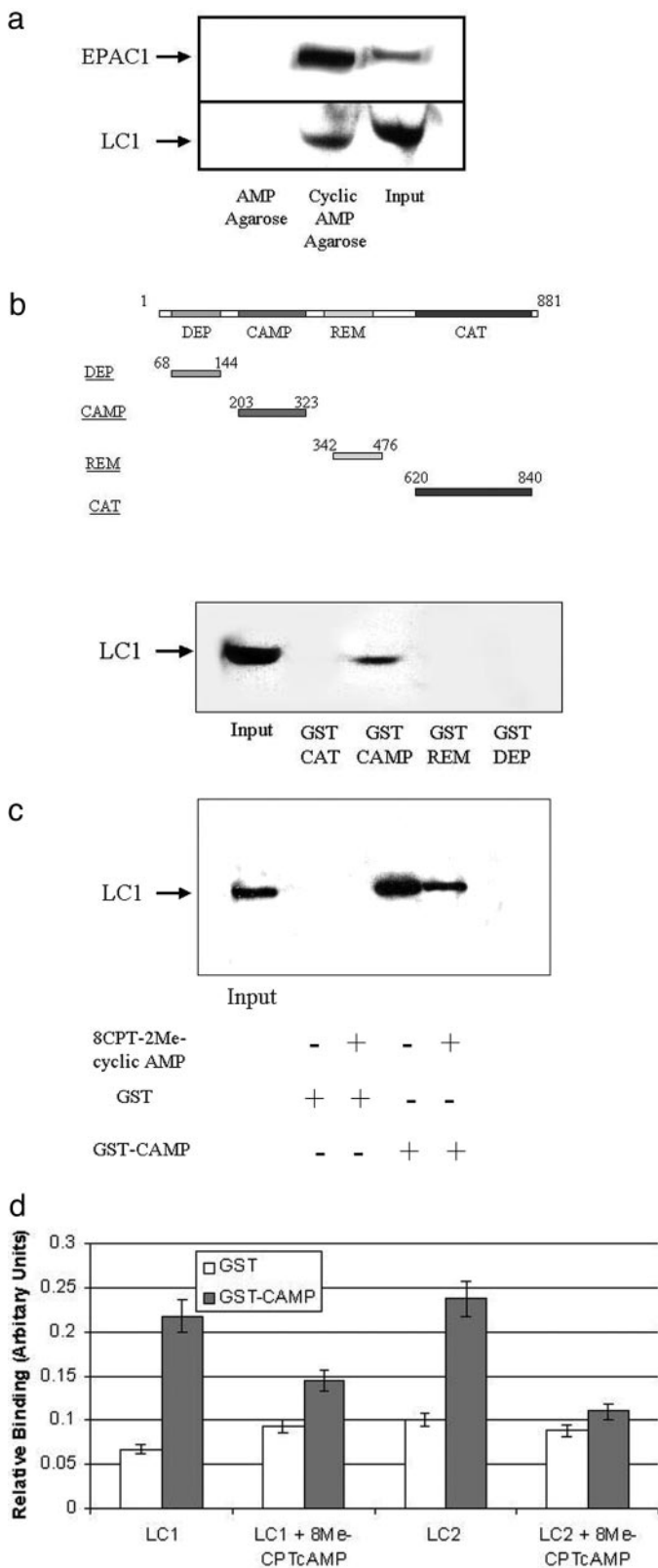
**Immunocytochemistry.** Cells were washed with PBS at 4°C, 36 h after transfection with myc-EPAC1-FLAG, and then fixed in a 4% (w/v) paraformaldehyde solution. Cells were then permeabilized in a 0.5% Triton X-100 solution in PBS for 5 min at 4°C and blocked with goat serum [0.1% (v/v) in PBS] and fish gelatin [0.2% (w/v) in PBS] for 30 min at room temperature. The cells were then coincubated for 2 h at room temperature with anti-FLAG polyclonal antibody, at a dilution of 1:250 in block solution. Cells were then washed three times in PBS and then incubated for 1 h at room temperature with anti-rabbit fluorescein isothiocyanate conjugate (Vector Laboratories, Peterborough, UK) diluted 1:200 in block solution. Cells were washed and mounted using Immu-Mount (Shandon Products, Cheshire, UK) and visualized by confocal microscopy.

## Results

**LC1 Interacts with the CAMP Domain of EPAC1.** We had previously demonstrated that the light chain of MAP1A (LC2) interacted with EPAC1 (Magiera et al., 2004). Given that LC2 is homologous to LC1 within the C-terminal segment of the protein (Noiges et al., 2002), we decided to check whether LC1 also interacted with EPAC1. To do this, we took advantage of the ability of EPAC1 to specifically interact with cyclic AMP-agarose. We purified EPAC1 from PC-12 cell lysates with cyclic AMP-agarose and immunoblotted eluates for the presence of LC1 (Fig. 1a). We found that LC1 did indeed copurify with EPAC1 on cyclic AMP-agarose, but it did not purify with AMP-agarose (Fig. 1a). We were able to purify approximately 90% of EPAC1 from cell lysates with cyclic AMP and found this to be associated with approximately 20 to 30% of cellular LC1. LC1 therefore exists as a complex with EPAC1 in PC-12 cells. This is only the second report of a protein capable of interaction with EPAC.

To identify where LC1 interacted within the EPAC1 protein, we used a panel of EPAC1 subdomains expressed and purified from bacteria as GST-fusion proteins (Fig. 1b). These GST fusion proteins were used in pull-down assays from PC-12 cell lysates (Fig. 1b). We found that LC1 copurified with the cyclic AMP-binding domain of EPAC1 (GST-CAMP; Fig. 1b) but did not interact with the REM (GST-REM), DEP (GST-DEP) or catalytic domain (GST-CAT) of EPAC1 (Fig. 1b). Our previous findings with LC2 also demonstrated that it specifically interacted with the EPAC1 CAMP domain in pull-down assays (Magiera et al., 2004). The mode of interaction of LC1 and LC2 with EPAC1 may therefore be very similar.





**Fig. 1.** MAP1B LC1 interacts with the CAMP domain of EPAC1. **a**, PC-12 cells extracts (500  $\mu$ g) were incubated with either AMP-agarose or cyclic AMP-agarose beads. Eluates were then subjected to SDS-PAGE and immunoblotting with specific antisera to EPAC1 and LC1. **b**, individual EPAC1 DEP (amino acids 68–144), REM (amino acids 342–476), CAMP (amino acids 203–323), and CAT (amino acids 620–840) domain GST fusion proteins were purified from bacteria using glutathione-Sepharose. The position of the domains in the primary structure of EPAC1 is shown

In experiments so far, we had used PC-12 cell lysates as a source of LC1 protein. We could not be certain, however, that the interaction between LC1 and the CAMP domain of EPAC1 was direct. To address this, we decided to use a pure source of recombinant LC1 and LC2 in protein interaction assays with purified GST-CAMP. Poly-histidine-tagged LC1 and LC2 were expressed in bacteria and purified using nickel resin. Using these recombinant proteins, we found that LC1 directly interacted with GST-CAMP, but not GST, in pull-down assays (Fig. 1c). Approximately 80% of input LC1 protein was found to precipitate with GST-CAMP. Coincubation of binding reactions with 8-CPT-2Me-cyclic AMP (50  $\mu$ M), a cyclic AMP analog that specifically activates EPAC (Enserink et al., 2002), was found to slightly reduce the direct interaction between LC1 and CAMP (Fig. 1c). This suggests that LC1 preferentially interacts with CAMP in the empty, noncyclic AMP-bound form. We next compared the direct interaction between LC1 and CAMP with the interaction between LC2 and CAMP in a solid phase binding assay. GST or GST-CAMP was allowed to adhere to glutathione-coated 96-well plates and then incubated with His-tagged LC1 or LC2. The amount of LC1 or LC2 bound was then determined using an anti-poly-histidine antibody. We found that both LC1 and LC2 directly interacted to a similar degree with GST-CAMP but not GST (Fig. 1d). Moreover, incubation with 8CPT-2Me-cyclic AMP reduced the binding of LC1 and LC2 to CAMP, again to a similar degree (Fig. 1d). Together, these results demonstrate that both LC1 and LC2 can directly interact with the CAMP domain of EPAC1. In addition, both LC1 and LC2 preferentially bind to CAMP in the noncyclic AMP-bound form.

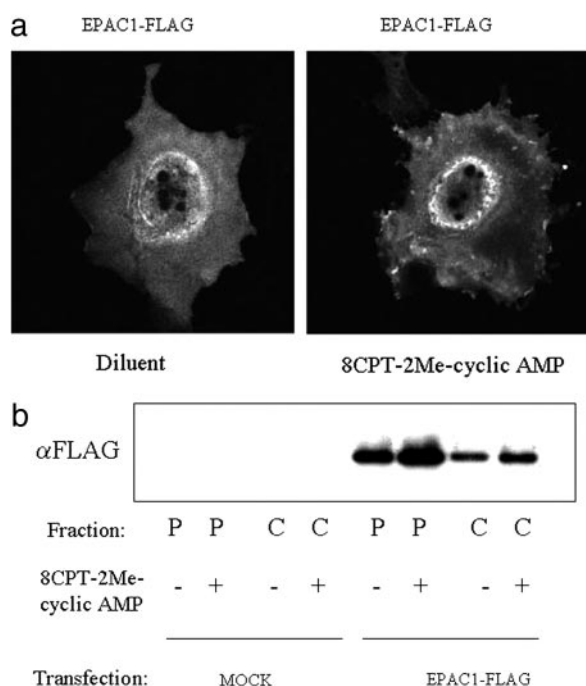
**The CAT Domain, and Not the CAMP Domain, Is Responsible for Intracellular Targeting of EPAC1.** The fact that LC1 and LC2 interact with the CAMP domain of EPAC1 raises the question as to whether these interactions contribute to intracellular targeting of EPAC1. This is based on the premise that interaction of the CAMP domain (R-subunit) of PKA with AKAP family proteins leads to the tethering of the inactive PKA holoenzyme to discrete intracellular sites and organelles (Wong and Scott, 2004). The interaction between LC1 and LC2 and the CAMP domain of EPAC1 could therefore be analogous to the interaction between AKAPs and the R-subunit of PKA. To date little work has been done on the intracellular targeting of EPAC proteins. It has been reported that EPAC1 is tethered to the nuclear membrane and mitochondria of cells during interphase (Qiao et al., 2002). During metaphase EPAC1 disassociates from the nuclear membrane and localizes to the mitotic spindle and centrosomes (Qiao et al., 2002). The DEP domain

diagrammatically (top). PC-12 lysates were precipitated with GST-DEP, GST-REM, GST-CAMP, or GST-CAT. Cell lysates and precipitates were then separated by SDS-PAGE, followed by immunoblotting with the LC1 polyclonal antibody. **c**, purified, recombinant His-tagged LC1 was incubated with GST or GST-CAMP, in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). Protein complexes were then isolated with glutathione-Sepharose, separated by SDS-PAGE, and then immunoblotted with an anti-poly-His antibody. In all cases, recombinant LC1 specifically interacted with GST-CAMP. **d**, glutathione-coated enzyme-linked immunosorbent plates were coated with either GST or GST-CAMP. Wells were then incubated with purified, recombinant LC1 or LC2 in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). The amount of LC1 and LC2 that had bound to individual wells was determined using an anti-poly-His antibody as described under *Materials and Methods*.

seems to be responsible for membrane association EPAC1, whereas the mitochondrial-targeting sequence is found in the N terminus.

In agreement with Qiao et al. (2002), we found that immunofluorescent analysis of overexpressed, FLAG-tagged version of EPAC1 in COS1 cells demonstrated that although EPAC1 immunoreactivity was found throughout cells, there was distinct association of EPAC1 with the perinuclear region of cells, which was manifest as a bright punctate ring (Fig. 2a). Consistent with these findings, separation of cells into a cytoplasmic and nuclear/perinuclear pellet fraction (Rundell et al., 2004) demonstrated that EPAC1 was localized to both the soluble and pellet fractions (Fig. 2b). We have previously verified that both nuclear and perinuclear proteins were present in our particulate fractions, which indicates that the fraction is composed of nuclear and perinuclear components (Rundell et al., 2004). We next tested whether stimulation with cyclic AMP effected the intracellular distribution of EPAC1 in cells. However, stimulation of cells with 8CPT-2Me-cyclic AMP had little effect on the intracellular distribution of EPAC1 detected by either immunofluorescence (Fig. 2a) or fractionation (Fig. 2b). These results argue against a role of the EPAC1 CAMP regulating intracellular targeting of EPAC1.

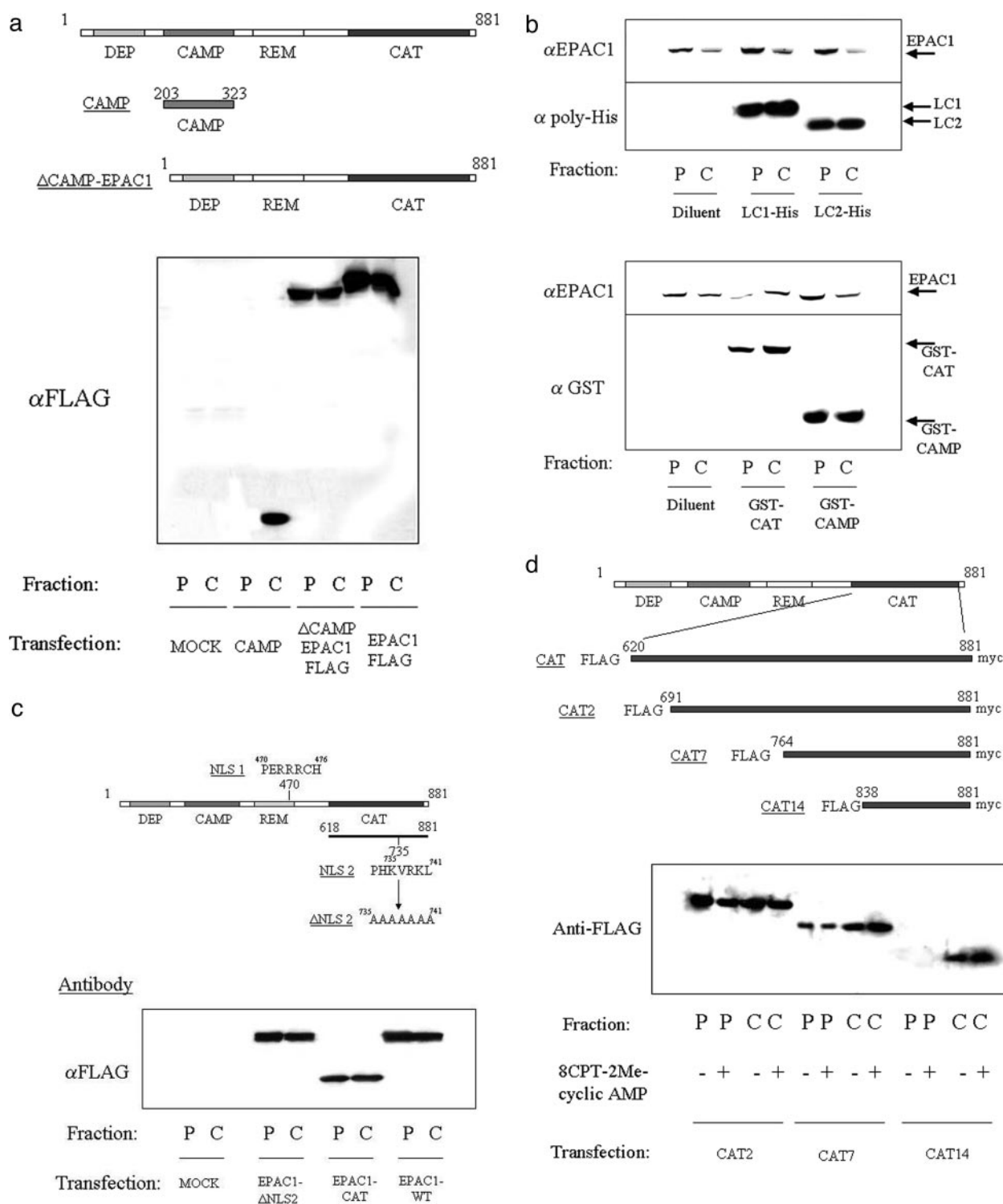
To analyze the involvement of the CAMP domain in an-



**Fig. 2.** EPAC1 is localized to nuclear/perinuclear regions in COS1 cells. **a**, COS1 cells were transfected with myc-EPAC1-FLAG and stimulated in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). Cells were then probed with an anti-FLAG polyclonal antibody and then secondary labeled with an anti-rabbit IgG-fluorescein isothiocyanate conjugate, to detect myc-EPAC1-FLAG. A series of 0.25- $\mu$ m optical sections were then captured using a laser scanning confocal microscope. EPAC1 immunoreactivity was clearly present in a bright ring around to nucleus. Incubation with 8CPT-2Me-cyclic AMP had little effect on the distribution of EPAC1 in cells. **b**, nuclear/perinuclear and cytoplasmic fractions were prepared from COS1 cells that had been transfected with myc-EPAC1-FLAG and then stimulated with or without 50  $\mu$ M 8CPT-2Me-cyclic AMP. Fractions were immunoblotted with an anti-FLAG antibody to detect transfected EPAC1. Immunoreactivity was found associated with both fractions, and 8CPT-2Me-cyclic AMP treatment had little effect on the distribution of EPAC1 between each fraction.

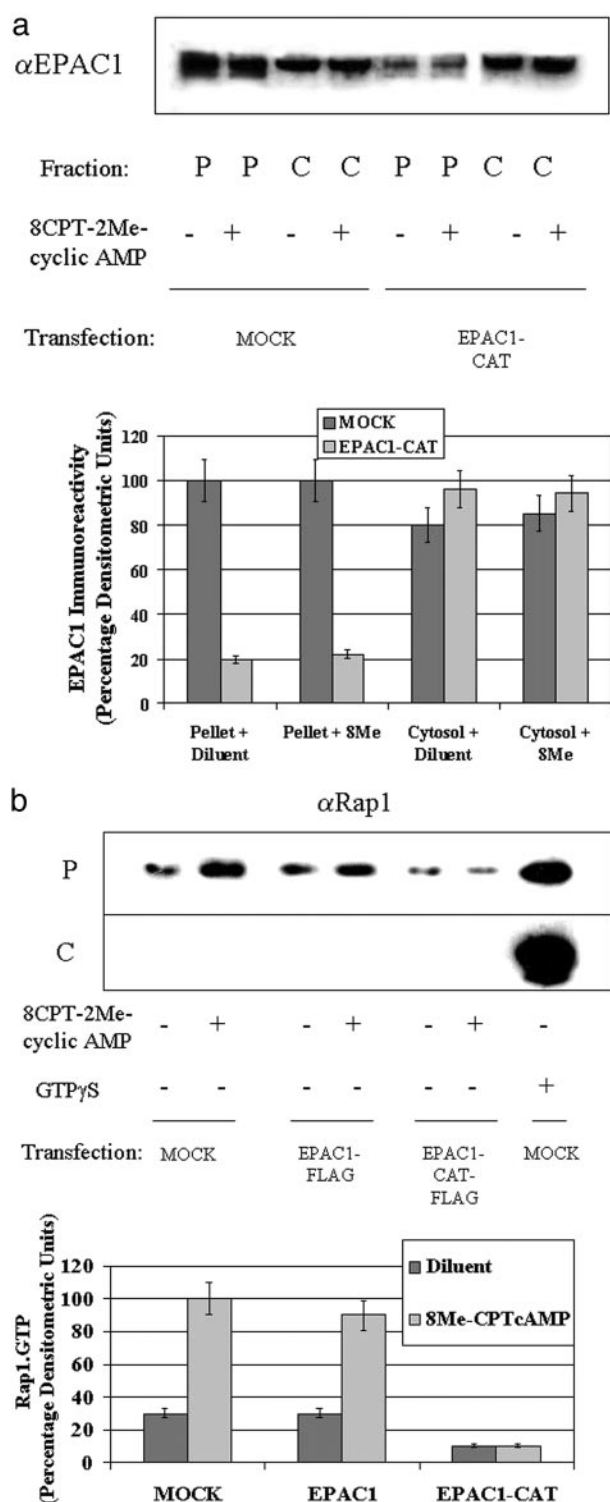
choring EPAC1 in cells, we expressed a FLAG-tagged version of EPAC1 that lacked a CAMP domain ( $\Delta$ CAMP-EPAC1-FLAG) and a FLAG-tagged CAMP domain (EPAC1-CAMP) in COS1 cells (Fig. 3a). Cells were then separated into cytoplasmic and nuclear/perinuclear fractions and immunoblotted with anti-FLAG epitope antibodies. Results showed that the isolated CAMP domain was expressed solely in the cytoplasm of cells and did not demonstrate appreciable association with particulate fractions (Fig. 3a). Consistent with this,  $\Delta$ CAMP-EPAC1-FLAG, which lacks a CAMP domain, remained associated with particulate fractions and showed a very similar distribution to full-length EPAC1 (Fig. 3a). These results suggest that the CAMP domain is not required for nuclear/perinuclear targeting of EPAC1 in COS1 cells and that targeting sequences must be present elsewhere in the EPAC1 protein. If the CAMP domain is not required for intracellular targeting of EPAC1 this suggests that association of EPAC1 with LC1 or LC2 will also have little effect on the tethering of EPAC1 to intracellular compartments. Therefore, incubation of cell lysates with either recombinant LC1 or LC2, before separation into cytoplasmic of nuclear/perinuclear fractions, did not effect the distribution of endogenous EPAC1 in COS1 cells (Fig. 3b). This is despite the fact that both LC1 and LC2 readily associate with nuclear/perinuclear pellet fractions after incubation (Fig. 3b). In contrast, incubation of cell lysates with a GST fusion of the EPAC1 catalytic domain (GST-CAT), but not GST-CAMP, led to a reduction of the amount of EPAC1 associated with nuclear/perinuclear pellet fractions (Fig. 3b).

Together, these results suggest that intracellular targeting of EPAC1 to the nuclear/perinuclear fraction of cells occurs independently of the CAMP domain and may involve the CAT domain. Moreover, association of LC1 or LC2 with the CAMP domain has little effect on the intracellular distribution of EPAC. The question remains, however, as to why EPAC1 associates with the nuclear/perinuclear region of cells and what are the targeting determinants? We first checked the involvement of putative nuclear localization signals (NLSs) in EPAC1. It is unlikely that NLSs are required for nuclear entry of EPAC1 because it seems that the breakdown of the nuclear envelope during the cell cycle is a prerequisite for nuclear entry, rather than entry through nuclear pores (Qiao et al., 2002). However, EPAC1 does contain two putative NLSs, NLS1 and NLS2 (Fig. 3c). Removal of NLS1 by truncating EPAC1 up until the CAT domain had little effect on EPAC1 targeting in COS1 cells, because the CAT domain alone was sufficient for association with the nuclear/perinuclear fraction (Fig. 3c). NLS2 is found within the CAT domain, however alanine substitution of this NLS in full-length EPAC1 (EPAC1- $\Delta$ NLS2) also did not effect nuclear-perinuclear association of EPAC1 (Fig. 3c). Together, these results demonstrate that the EPAC1 CAT domain is sufficient for association with the nuclear/perinuclear region of COS1 cells, independently of NLSs. This is confirmed by further deletion analysis of the CAT domain (Fig. 3d). N-terminal truncates of the CAT domain demonstrated that the region between amino acids 764 and 838, which lacks NLS2 found between amino acids 735 and 741, is required for association of the CAT domain with the nuclear/perinuclear fraction of COS1 cells (Fig. 3d). In addition, there may be an involvement, to a lesser extent, of amino acids 691–764 be-



**Fig. 3.** The EPAC1 CAT domain, but not the CAMP domain, is responsible for nuclear/perinuclear targeting in COS1 cells. **a**, COS1 cells were transfected with myc-EPAC1-FLAG, EPAC1-CAMP (which expresses an isolated EPAC1 cyclic AMP binding domain), or myc-ΔCAMP-EPAC1-FLAG (which expresses EPAC1 lacking a CAMP domain). Cells were then separated into nuclear/perinuclear and cytosolic fractions and immunoblotted with anti-FLAG antibodies. In all cases, both myc-EPAC1-FLAG and myc-ΔCAMP-EPAC1-FLAG were present in particulate and soluble fractions, whereas EPAC1-CAMP was exclusively soluble. **b**, cell lysates were prepared from COS1 cells and then incubated with 10 μg of recombinant, His-tagged LC1 or LC2 (top) or 10 μg of GST-CAMP or GST-CAT (bottom), before being separated into nuclear/perinuclear and cytosolic fractions. The distribution of endogenous EPAC1 between the two fractions was monitored with an anti-EPAC1 polyclonal antibody. Recombinant LC1 and LC2 were detected with an anti-poly-His antibody whereas GST-CAMP and GST-CAT were detected with an anti-GST antibody. **c**, COS1 cells were transfected with myc-EPAC1-FLAG, EPAC1-CAT (which expresses an isolated EPAC1 catalytic domain) or myc-ΔNLS2-EPAC1-FLAG (which expresses EPAC1 lacking a PHKVRKL putative nuclear localization signal). Cells were then separated into nuclear/perinuclear and cytosolic fractions and immunoblotted with an anti-FLAG antibody to detect transfected species. **d**, COS1 cells were transfected with CAT2, CAT7 and CAT14, which are myc- and FLAG-tagged truncations of the EPAC1 catalytic domain as indicated in the diagram (top). Cytosolic and nuclear/perinuclear fractions were then prepared from cells, and these were immunoblotted with an anti-FLAG antibody.





**Fig. 4.** Displacement of endogenous EPAC1 inhibits Rap1 activation in the nuclear/perinuclear fraction of COS1 cells. **a**, COS1 cells were transfected with or without EPAC1-CAT and then stimulated in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). Nuclear/perinuclear (pellet) and cytosolic fractions were prepared and endogenous EPAC was detected with an anti-EPAC1 antibody. Expression of EPAC1-CAT was found to displace endogenous EPAC1 from pellet fractions. Results are displayed in the bottom panel as a histogram of densitometric values (percentage) from three separate experiments. **b**, cells were transfected with EPAC1-CAT or myc-EPAC1-FLAG and then stimulated in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). The amount of GTP-bound Rap1 was then measured in nuclear/perinuclear (P) and cytosolic (C) fractions using GST-RalGDS-RBD as described under *Materials and*

cause there is reduced binding of this truncate compared with full-length CAT (Fig. 3d).

To test whether the CAT domain is sufficient for nuclear/perinuclear targeting of EPAC1, we overexpressed the CAT domain in COS1 cells and measured the subcellular localization of endogenous EPAC1 in these cells by immunoblotting (Fig. 4a). We found that in cells expressing EPAC1-CAT, there was a significant reduction in the amount of endogenous EPAC1 associated with nuclear/perinuclear fractions (Fig. 4a). Overexpression of the CAT domain seems, therefore, to be competing for EPAC1 binding sites in this fraction. This demonstrates that the CAT domain is involved in the anchoring of EPAC1 to nuclear/perinuclear regions in these cells.

The reason why EPAC1 might be associated with this fraction was revealed when we measured Rap1.GTP in cytoplasmic and nuclear/perinuclear fractions (Fig. 4b). We found that stimulation of cells with 8CPT-2Me-cyclic AMP (50  $\mu$ M) led to a large increase in Rap1.GTP associated with the nuclear/perinuclear fraction of COS1 cells, but not in the cytoplasmic fraction (Fig. 4b). This is despite the fact that the cytoplasm of these cells does contain Rap1, which is readily activated by incubation of cell lysates with the non-hydrolyzable GTP analog guanosine 5'-O-(3-thio)triphosphate (Fig. 4b). These results are consistent with previous fluorescence resonance energy transfer analysis of EPAC activation in COS1 cells, which demonstrated that the majority of EPAC activity was localized to the perinuclear region of cells after cyclic AMP stimulation (Ohba et al., 2003). Overexpression of EPAC1 did not provoke an enhancement of basal or stimulated Rap1-bound GTP levels (Fig. 4b), indicating that the binding sites for EPAC1 in the nuclear/perinuclear fraction were saturated by endogenous EPAC1. However, overexpression of the catalytically dead CAT domain, which displaces endogenous EPAC1 from this fraction (Fig. 4a), was found to inhibit both basal and 8CPT-2Me-cyclic AMP-stimulated GTP-binding by Rap1 in a dominant-negative manner (Fig. 4b). These results suggest that the CAT domain anchors EPAC1 to the nuclear/perinuclear fraction of COS1 cells to allow the activation of a compartmentalized pool of Rap1 in response to elevations in intracellular cyclic AMP.

**LC1 Enhances Cyclic AMP Binding to the CAMP Subunit of EPAC1 and Potentiates Rap1 Activation.** In an attempt to analyze the effect of direct interaction of LC1 with EPAC1 (Fig. 1, b–d) on the function of EPAC1, we have eliminated a role of LC1 in controlling the intracellular targeting to the nuclear/perinuclear fraction of cells. Rather, this seems to be mediated through the CAT domain of EPAC1. We have demonstrated previously that transfection of cells with LC2 enhances the ability of endogenous or transfected EPAC1 to interact with cyclic AMP-agarose (Gupta and Yarwood, 2005). This correlated with an enhancement of 8CPT-2Me-cyclic AMP to provoke Rap1 activity in cells (Gupta and Yarwood, 2005). We therefore decided to check whether LC1 could enhance cyclic AMP binding to the

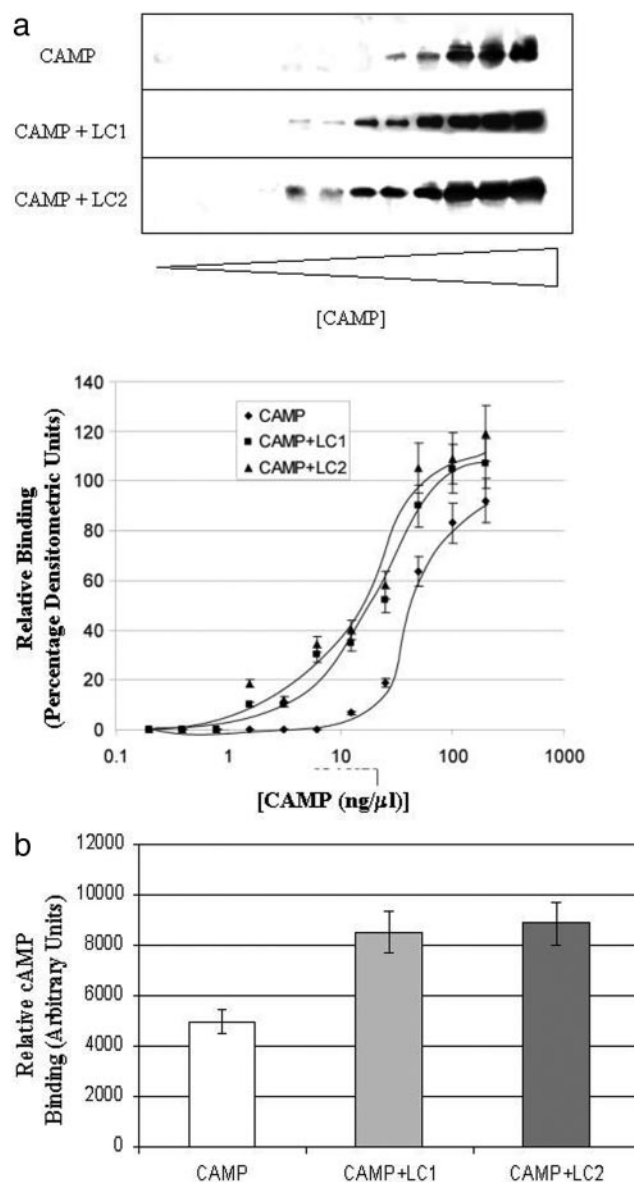
**Methods.** Densitometric values were measured for three separate experiments, and these results are presented as percentages (bottom). In all cases, Rap1 activity was only increased in nuclear/perinuclear after 8CPT-2Me-cyclic AMP treatment, and this was diminished upon transfection with EPAC1-CAT. Guanosine 5'-O-(3-thio)triphosphate was used to show that Rap1 could be activated in cytosolic fractions.

EPAC1 CAMP domain. In this case, rather than use a transfection strategy, which, it could be argued, may not result in direct interaction between LC1 and EPAC1, we instead used purified recombinant proteins. We coincubated recombinant LC1 and LC2 with purified GST-CAMP under conditions that provoked direct interaction between the LCs and CAMP (Fig. 1). We then tested the ability of GST-CAMP to interact with cyclic AMP immobilized on agarose (Fig. 5a). We found that increasing the concentration of GST-CAMP led to a dose-dependent increase in GST-CAMP binding to cyclic AMP-agarose (Fig. 5a). Coincubation with either LC1 or LC2 increased the ability of GST-CAMP to interact with cyclic AMP-agarose by approximately half an order of magnitude. To obtain independent confirmation that cyclic AMP binding to CAMP was enhanced by LC1 and LC2, we measured direct binding of [ $^3$ H]cyclic AMP to GST-CAMP (Fig. 5b). Binding reactions were carried out in the presence of 40  $\mu$ M [ $^3$ H]cyclic AMP because this is the concentration at which half-maximal binding of cyclic AMP to EPAC1 has been reported previously (Bos et al., 2001). Coincubation with either recombinant LC1 or LC2 lead to increased cyclic AMP binding in these experiments by between 1.5- and 2-fold (Fig. 5b). It seems therefore that direct interaction of LC1 and LC2 with the CAMP domain of EPAC1 causes a conformational change that enhances the ability of the cyclic AMP binding pocket to interact with cyclic AMP.

We next tested whether LC1 enhanced the ability of EPAC to activate Rap1. This was based on the idea that if LC1 were to enhance the ability of EPAC to interact with cyclic AMP, then it might improve the efficiency of Rap1 activation. We incubated cell lysates from COS1 cells, which do not express LC1 or LC2 (results not shown) with recombinant LC1 or LC2 (Fig. 6a). We then stimulated endogenous EPAC with 8CPT-2Me-cyclic AMP (50  $\mu$ M) and measured Rap1 activation as described under *Materials and Methods*. We found that in the absence of LC1 and LC2 that 8CPT-2Me-cyclic AMP had no appreciable effect on endogenous Rap1 activity in these cells (Fig. 6a). However, in cell extracts incubated with LC1 or LC2, we found that both basal and 8CPT-2Me-cyclic AMP-stimulated Rap1 levels were dramatically increased (Fig. 6a). It seems therefore that both LC1 and LC2 may be acting as biological enhancers or "chaperones" of EPAC activity toward Rap1, by directly interacting with the EPAC CAMP domain.

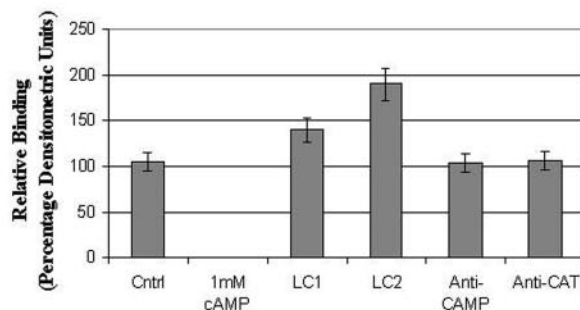
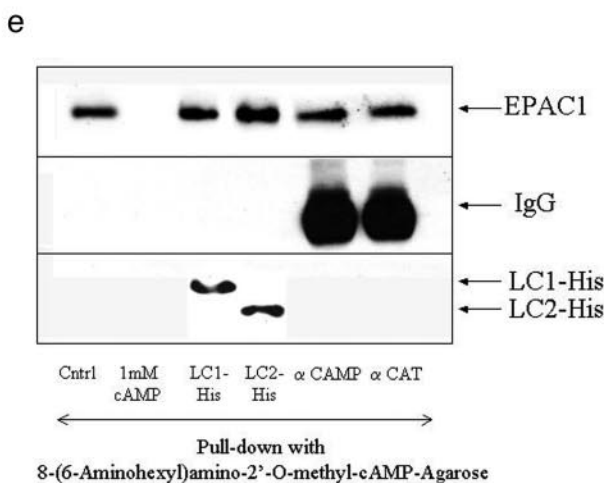
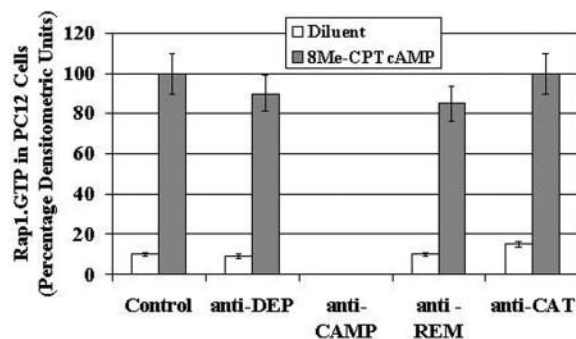
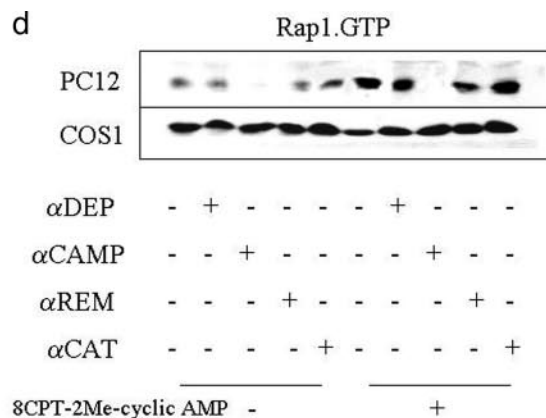
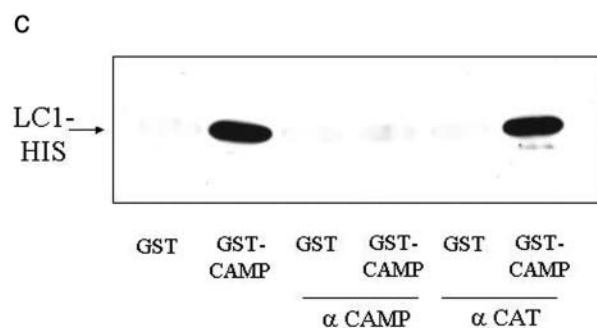
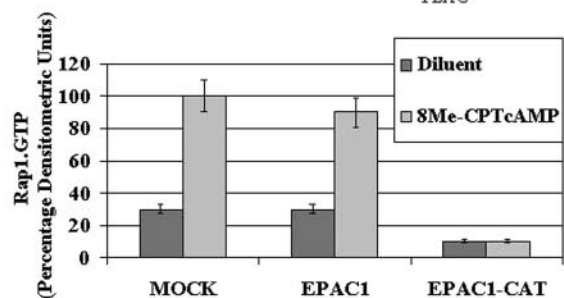
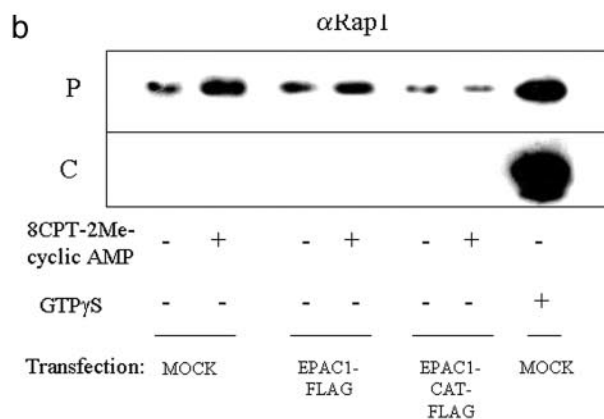
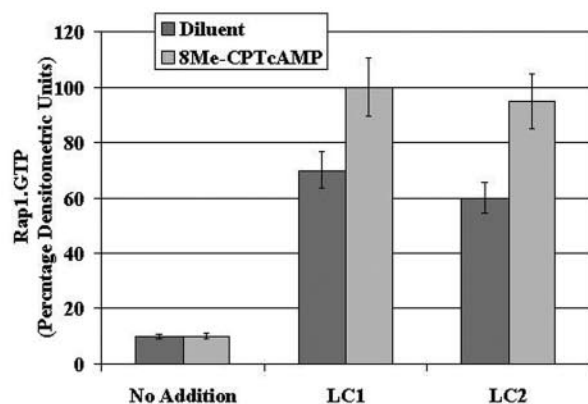
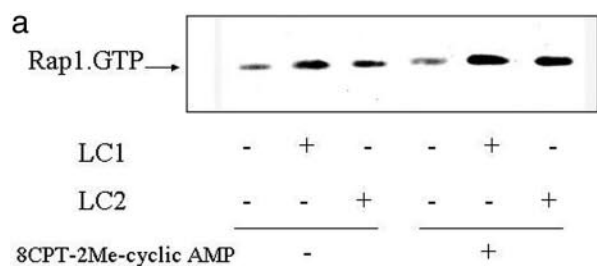
To test whether endogenous LC1 enhances EPAC activity, we applied an antibody disruption protocol to interfere with LC1 binding to EPAC and tested whether this disrupted Rap1 activation by 8CPT-2Me-cyclic AMP. We used a panel of antibodies raised against the individual EPAC1 domains (DEP, CAMP, REM, and CAT) and tested whether these interfered with endogenous LC1 interaction with EPAC1 (Fig. 6b). Cell extracts from PC-12 cells, which express LC1, and COS1 cells, which do not express LC1, were incubated in the presence or absence of each of these antibodies (Fig. 6b). EPAC1 was then isolated from cells using cyclic AMP-agarose, and the presence of LC1 in precipitates was detected using an anti-LC1 antibody (Fig. 6b). As expected, EPAC1 was recoverable from both COS1 and PC-12 cell lysates, but LC1 was only present in precipitates from PC-12 cells (Fig. 6b). Incubation with the individual antibodies did not affect the amount of EPAC1 recovered from either COS1 or PC-12 cells (Fig. 6b). It was found, however, that the anti-CAMP

antibody, apparently by disrupting the EPAC1/LC1 interface, completely inhibited the association of LC1 with EPAC1 precipitates (Fig. 6b). Incubation with anti-DEP, anti-REM, or anti-CAT antibodies had little effect on the association between EPAC1 and LC1 (Fig. 6b). To further confirm that the CAMP antibody disrupted interaction between LC1 and the EPAC1 CAMP domain, we used recombinant forms of LC1 and CAMP (Fig. 6c). LC1 interacted with GST-CAMP in pull-down assays; however, this association was completely disrupted by coincubation with the anti-CAMP, but not the anti-CAT, antibody. This demonstrates that the anti-CAMP



**Fig. 5.** LC1 enhances cyclic AMP binding to EPAC1 CAMP. a, purified, recombinant His-tagged LC1 and LC2 were incubated with various concentrations of GST-CAMP. GST-CAMP was then purified by pull-down with cyclic AMP-agarose beads. Beads were boiled in sample buffer, separated by SDS-PAGE, and then immunoblotted with an anti-CAMP antibody. Experiments were carried out three times with similar results. Percentage densitometric results from the three experiments are plotted as means  $\pm$  S.E.M. (bottom). b, purified GST-CAMP was incubated with 40  $\mu$ M [ $^3$ H]cyclic AMP and then isolated with GSH-Sepharose. GST-CAMP was then eluted with GSH, and the radioactivity associated with eluates was measured by scintillation counting. Results are means  $\pm$  S.E.M. for three separate experiments.





antibody is an effective reagent for disrupting interaction between LC1 and the EPAC1 CAMP domain.

We next checked whether disruption of LC1 interaction with EPAC1 affected the binding of GTP to Rap1 in response to 50  $\mu$ M 8CPT-2Me-cyclic AMP (Fig. 6d). COS1 and PC-12 cell lysates were incubated with the individual antibodies and then stimulated with 8CPT-2Me-cyclic AMP (50  $\mu$ M; Fig. 6d). No increase in Rap1 GTP loading was noted in COS1 cell extracts after incubation with antibodies or stimulation with 8CPT-2Me-cyclic AMP (50  $\mu$ M), presumably because LC1 is not expressed in this cell type (Fig. 6d). 8CPT-2Me-cyclic AMP, however, did provoke an increase in GTP-bound Rap1 in PC-12 cell extracts (Fig. 6d). Incubation with the anti-CAMP antibody was found to completely ablate both basal and 8CPT-2Me-cyclic AMP-stimulated Rap1.GTP levels in PC-12 cell extracts, whereas the anti-DEP, anti-REM, and anti-CAT antibodies exerted little effect. To exclude the possibility that the anti-CAMP antibody is simply competing for the binding of 8CPT-2Me-cyclic AMP to its binding pocket, we carried out further control experiments (Fig. 6e). From the structure of 8CPT-2Me-cyclic AMP, it is known that the 2'-O-methyl group is responsible for the specificity of the analog to EPAC compared with protein kinase A (Bos et al., 2001). We therefore tested whether interaction of EPAC1 with 2Me-cyclic AMP, immobilized on agarose, was effected by LC1, LC2, or the anti-CAMP and anti-CAT antibodies (Fig. 6e). We found that neither LC1, LC2, nor the anti-CAMP and anti-CAT antibodies prevented 2Me-cyclic AMP from interacting with the cyclic AMP binding pocket on EPAC1 (Fig. 6e). The sole effect of the anti-CAMP antibody in Fig. 6, b and d, therefore is to disrupt interaction of LC1 with the EPAC1 CAMP domain. LC1 therefore seems to enhance EPAC activity toward Rap1 through direct interaction with the EPAC CAMP domain.

## Discussion

We have identified MAP1B LC1 as a novel protein interaction partner for EPAC1. This is only the second report of the involvement of EPAC1 in specific protein interactions. Our experiments led us to the finding that LC1 interacts directly with the CAMP domain of EPAC1. This concurs with our finding for MAP1A LC2, which we also found to interact specifically with the CAMP domain of EPAC1 (Magiera et al., 2004). Comparison of the primary structure of LC1 and LC2 reveals significant amino acid sequence homology in the C termini of the proteins, whereas the N termini are very

dissimilar (Langkopf et al., 1992). This suggests the mode of interaction of LC1 and LC2 with EPAC1 CAMP may be similar and may involve the C terminus of the proteins. In addition, although the N termini of LC1 and LC2 differ in their amino acid composition, both proteins have been reported to interact with microtubules through this domain (Noiges et al., 2002). This suggests that both LC1 and LC2 have the capability of recruiting EPAC1 to microtubules. Indeed, EPAC1 has been shown to coimmunoprecipitate with tubulin (Gupta and Yarwood, 2005) and removal of the CAMP domain of EPAC1 prevents association with cytoskeletal structures in cells (Magiera et al., 2004). Because of the amino acid variation in their microtubule binding domains, the mode of interaction of LC1 and LC2 with microtubules may differ. This suggests that the identity of the microtubule-adaptor protein, whether LC1 or LC2, may have a particular effect on the function of EPAC1 recruited to microtubules.

The fact that LC1 and LC2 interact with the CAMP domain of EPAC1 led us to speculate that, akin to interactions between AKAPs and the R-subunit of PKA, LC1 might be involved in the intracellular targeting of EPAC1. Subcellular targeting of signaling molecules is a possible mechanism to achieve biological specificity, by maintaining discrete subcellular localization. Therefore, correct targeting of EPAC has been shown to be essential for activating the downstream effector Rap1 (Mei et al., 2002). From immunofluorescent imaging and cell fractionation, we found that a significant amount of endogenous and transfected EPAC1 was found associated with the nuclear/perinuclear fraction of cells. We were excited to find that specific activation of EPAC with the cyclic AMP analog 8CPT-2Me-cyclic AMP led to activation of Rap1 in this fraction of cells, but not in the soluble fraction of the cells. Therefore, specific association of EPAC with the particulate fraction of cells leads to compartmentalized activation of Rap1. We found that protein interaction with the EPAC1 CAMP domain with proteins such as LC1 is probably not responsible for targeting of EPAC to this cell compartment, however, because deletion of the CAMP domain did not cause a redistribution of EPAC. Moreover, the CAMP domain when individually expressed in cells was found to be predominantly soluble.

Further truncation analysis of EPAC1 revealed that the CAT domain was, in fact, responsible for targeting of EPAC1 through interactions occurring between amino acids 764 and 838 of EPAC1. This is the first occasion that the CAT domain of EPAC has been implicated in intracellular targeting of

**Fig. 6.** Interaction with LC1 enhances EPAC1 activity toward Rap1. a, cell lysates from COS1 cells were incubated with purified, recombinant LC1 or LC2 and then stimulated with 8CPT-2Me-cyclic AMP. The amount of GTP-bound Rap1 was then measured using GST-RalGDS-RBP as described under *Materials and Methods*. Densitometric results from three separate experiments are shown as a histogram (bottom). b, cell extracts from COS1 cells (top) or PC-12 cells (bottom) were incubated with anti-DEP, anti-CAMP, anti-REM, or anti-CAT antibodies in the presence or absence of 8CPT-2Me-cyclic AMP (50  $\mu$ M). EPAC1 was then purified from extracts using cyclic AMP-agarose and immunoblotted with anti-EPAC1 and anti-LC1 antibodies. In every case, anti-CAMP antibodies prevented association between EPAC1 and LC1 in PC-12 cell extracts. c, purified, recombinant His-tagged LC1 was incubated with GST or GST-CAMP in the presence or absence of anti-CAMP or anti-CAT antibodies. Protein complexes were then isolated with glutathione-Sepharose, separated by SDS-PAGE, and immunoblotted with an anti-poly-His antibody. In all cases, recombinant LC1 specifically interacted with GST-CAMP, and this interaction was disrupted by incubation with anti-CAMP, but not anti-CAT antibodies. d, PC-12 (top) and COS1 (bottom) cell extracts were incubated with anti-DEP, anti-CAMP, anti-REM, or anti-CAT antibodies and then stimulated with 8CPT-2Me-cyclic AMP (50  $\mu$ M). Rap1 activity was then measured in cell extracts using GST-RalGDS-RBP as described under *Materials and Methods*. A histogram of percentage densitometric values from three individual experiments is presented. In all cases, incubation with the anti-CAMP antibody inhibited Rap1 activity in PC-12 (which express LC1), but not COS1 (which do not express LC1) cell extracts. e, EPAC1 was purified from COS1 cell lysates by pull-down with 8-(6-aminohexyl)-amino-2'-O-methyl-cyclic AMP agarose beads in the presence or absence of 1 mM cyclic AMP, 10  $\mu$ g of recombinant LC1 and LC2, or anti-CAT or anti-CAMP antibodies. Beads were boiled in sample buffer, separated by SDS-PAGE, and then immunoblotted with an anti-EPAC1 antibody. Experiments were carried out three times with similar results. Percentage densitometric results from the three experiments were plotted as means  $\pm$  S.E.M. (bottom).

EPAC. We also noted that two putative NLSs were not responsible for recruitment of EPAC1 to the nuclear/perinuclear fraction of cells. Although EPAC1 has been localized to intranuclear sites, this seems to be achieved during nuclear envelope breakdown during prophase/prometaphase of the cell cycle, rather than through import via nuclear pore complexes (Qiao et al., 2002). During teleophase, EPAC1 is seen to reassociate with the nuclear envelope (Qiao et al., 2002). We also found that directing EPAC1 to the nuclear/perinuclear fraction was a prerequisite for activation of Rap1 in this fraction by cyclic AMP. This is consistent with fluorescence resonance energy transfer analysis that demonstrates that Rap1 is specifically activated in the nuclear/perinuclear region of cells (Mochizuki et al., 2001). Together, these findings highlight the importance of the CAT domain in targeting EPAC1 to nuclear-associated regions in cells, which probably contributes to nuclear entry during mitosis and to signal propagation through the Rap1 pathway. The consequences of these EPAC1-mediated events for cell function remain to be determined.

Overall, our results argued against a role for LC1 in nuclear/perinuclear targeting of EPAC. We did find, however, that direct interaction of LC1 with the CAMP domain of EPAC1 had a marked effect on its ability to interact with cyclic AMP (Fig. 5a). We have previously transfected LC2 into cells and examined how the binding of endogenous or cotransfected EPAC1 to cyclic AMP as effected (Gupta and Yarwood, 2005). In this study, for the first time, we used purified recombinant forms of LC1, LC2, and CAMP and demonstrated that direct interaction between LC1 or LC2 and CAMP is sufficient to increase the affinity of CAMP for cyclic AMP (Fig. 5, a and b). This is an important finding and suggests that the EPAC1 CAMP requires the chaperone function of an ancillary protein, such as LC1 or LC2, to stably interact with cyclic AMP. This observation may go some way to explaining why EPAC has been reported to bind cyclic AMP some 10-fold less efficiently than PKA (Bos et al., 2001). LC1 and LC2 are therefore predicted to stabilize the EPAC1 CAMP domain in a conformation that interacts more readily with cyclic AMP. This seems to have functional consequences for EPAC activity toward Rap1. Incubation of cell lysates with LC1 or LC2 was found to enhance EPAC activity toward Rap1 (Fig. 6a). Moreover, disruption of EPAC1 interaction with LC1 with an anti-CAMP antibody abolished EPAC activity toward Rap1 (Fig. 6c). These results suggest that the ability of LC1 to enhance cyclic AMP interaction with EPAC1 is critical to EPAC function.

A functional role of LC1 or LC2 in EPAC/Rap1 signaling could provide an explanation for the sometimes unexpectedly low doses of 8CPT-2Me-cyclic AMP that are required for the generation of certain biological effects. The minimal dose of 8CPT-2Me-cyclic AMP required to activate Rap1 in NIH3T3 cells was initially reported to be 10  $\mu$ M (Enserink et al., 2002). However, in a report by Keiper et al. (2004) doses of 8CPT-2Me-cyclic AMP as low as 0.1  $\mu$ M were reported to activate extracellular signal-regulated kinase in human embryonic kidney 293 cells (Keiper et al., 2004). The presence of biological enhancers of EPAC function, like LC1 and LC2, in certain cell types could possibly explain this kind of sensitization of EPAC signaling. It is clear that the design of peptide

mimetics or inhibitors of EPAC interaction with LC1 or LC2 may therefore form the future basis for novel therapeutics. These could modulate the sensitivity of the cyclic AMP/EPAC pathway to elevated or diminished cellular cyclic AMP levels that occur in certain disease states.

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