RasGRP3 is an exchange factor for Ras-like small GTPases that is activated in response to the second messenger diacylglycerol. As with other diacylglycerol receptors, RasGRP3 is redistributed upon diacylglycerol or phorbol ester binding. Several factors are important in determining the pattern of translocation, including the potency of the diacylglycerol analog, the affinity of the receptor for phospholipids, and in some cases, protein-protein interactions. However, little is known about the mechanisms that play a role in RasGRP3 redistribution aside from the nature of the ligand. To discover potential protein binding partners for RasGRP3, we screened a human brain cDNA library using a yeast two-hybrid approach. We identified dynein light chain 1 as a novel RasGRP3-interacting protein. The interaction was confirmed both in vitro and in vivo and required the C-terminal domain encompassing the last 127 amino acids of RasGRP3. A truncated mutant form of RasGRP3 that lacked this C-terminal domain was unable to interact with dynein light chain 1 and displayed a dramatically altered subcellular localization, with a strong reticular distribution and perinuclear and nuclear localization. These findings suggest that dynein light chain 1 represents a novel anchoring protein for RasGRP3 that may regulate subcellular localization of the exchange factor and, as such, may participate in the signaling mediated by diacylglycerol through RasGRP3.

RasGRP3 is one of the members of the RasGRP family, a novel group of exchange factors that catalyzes the formation of the active, GTP-bound form of Ras-like small GTPases (1-4). RasGRP3 has the broadest substrate selectivity of all the RasGRP family members and is able to activate H-Ras, R-Ras, and Rap1 (3,5). An important aspect of RasGRP3 regulation is its binding to the second messenger diacylglycerol. This binding is usually accompanied by subcellular redistribution of RasGRP3, which is believed to contribute to co-localization with its substrates (6). In fact, subcellular redistribution in response to diacylglycerol and its ultrapotent analogs, the phorbol esters, is one of the hallmarks of activation of diacylglycerol receptors, such as Protein Kinase C (PKC) (7). The process of translocation for a given receptor is orchestrated by a combination of factors, among them the nature and lipophilicity of the ligand (8) and the affinity for phospholipids like phosphatidylserine (9,10). The interaction of the receptor with specific intracellular proteins can also participate in the translocation events. The role of an adaptor protein as a raft or shuttling molecule has been shown in the case of PKC and its adaptor protein RACK (11). It is tempting to speculate that distinct adaptor proteins participate in translocation of the different diacylglycerol receptors, among them RasGRP3. The identification of such adaptors for RasGRP3 should provide insights into the mechanisms involved in RasGRP3 regulation and the subsequent activation of downstream signaling cascades. To this end, we performed a yeast two-hybrid screen to identify proteins that interact with RasGRP3 using a cDNA library from human brain, a tissue where RasGRP3 is expressed (3).

We have identified the dynein light chain 1 (DLC1, also known as LC8) as a novel RasGRP3-interacting protein. DLC1 is a component of cytoplasmic dynein, a molecule involved in cytoskeleton-mediated motility and intracellular transport events (12). Recent studies have shown that DLC1 interacts with a variety of proteins, many of them involved in cell survival and...
apoptosis, such as BimL (13), neuronal nitric oxide synthase (14), Ikbα (15), and Pak1 (16).

In this study, we demonstrated that RasGRP3 specifically interacted with DLC1 both in vitro and in vivo and that the interaction occurred through the C-terminal region of RasGRP3 located after the C1 domain and EF hands, which are responsible for binding to diacylglycerol and calcium, respectively. Moreover, a truncated form of RasGRP3 that lacked the ability to interact with DLC1, displayed a strikingly different subcellular localization from that of full-length RasGRP3. Taken together, these findings provide evidence of a novel interaction between RasGRP3 and DLC1 and suggest that DLC1 could represent an anchoring protein that participates in the subcellular localization of RasGRP3.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents** – CHO-K1 cells were cultured in Dulbecco’s modified essential medium (Invitrogen, Carlsbad, CA) containing 2 mM GlutaMAX-I, 100 I.U./ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, Carlsbad, CA), and supplemented with 10% fetal bovine serum (Biomeda, Foster City, CA). Saccharomyces cerevisiae strains AH109 (MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1-UAS-GAL1-TATA- HIS3 GAL2-UAS-GAL2-TATA-ADE2 URA3::MEL1-UAS-MEL1-TATA-lacZ), Y187 (MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ met1 gal80Δ URA3::GAL1-UAS-GAL1-TATA-lacZ), and Y187[pTD1-1] were obtained from BD Biosciences Clontech (Palo Alto, CA). Anti-DLC1 rat monoclonal antibody was raised against recombinant mouse DLC1 (Axxora, San Diego, CA). Anti-FLAG M2 mouse monoclonal antibody and anti-FLAG rabbit polyclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO).

**Expression Constructs** - pGBK7 (TRP1, 2μ) Gal4p DNA-binding domain vector was obtained from BD Biosciences Clontech (Palo Alto, CA). pACT2B2 (LEU2, 2μ) Gal4p activation domain vector was reconstituted by deleting the 2.1-kb BgIII fragment, including the SV40 large T-antigen and an HA epitope, from pTD1-1. pGBK7-RasGRP yeast two-hybrid bait plasmids, for expression of the different RasGRP proteins fused to the Gal4p DNA-binding domain, were prepared by standard molecular biology procedures using the following cDNAs: rat RasGRP1, human RasGRP2 (short spliced version, isoform 2), and human RasGRP3 (KIAA0846). RasGRP3EC (aa 408-564 of RasGRP3) and RasGRP3CS (aa 408-691 of RasGRP3) yeast two-hybrid bait plasmids were generated by introducing the corresponding cDNAs into pGBK7. pACT2-K6, containing a hybrid cDNA insert that includes the full-length coding sequence of cytoplasmic dynein light chain DLC1 (NCBI Accession # NM_003746) downstream of a mitochondrial genomic sequence and poly(A) tail, was isolated through screening of a pretransformed adult human brain cDNA library for clones displaying a positive interaction with pGBK7-RasGRP3 in a yeast-two hybrid assay (see below). The DLC1 cDNA was isolated from pACT2-K6 through PCR amplification of the open reading frame and cloned into a pACT2 (LEU2, 2μ) Gal4p activation domain vector plasmid to create pACT2-DLC1. DLC1 was subcloned into pGEX-2T (Amersham Biosciences, Piscataway, NJ) for expression of a glutathione S-transferase (GST)-DLC1 fusion protein in E. coli. Mammalian expression constructs for N-terminally FLAG-tagged proteins were generated by subcloning the corresponding RasGRP cDNAs into pCMV-Tag2A/B/C expression vectors (Stratagene, La Jolla, CA). Plasmids for expression of N-terminally green fluorescent protein (GFP)-tagged proteins were generated using the pEGFP-C1 vector (BD Biosciences, Palo Alto, CA). Correct sequence, orientation, and reading frame of inserts were verified by DNA sequencing at the University of Hawaii at Manoa by the Greenwood Molecular Biology Facility, CVRC/CRCRH DNA Sequencing/Genotyping Facility, or Center for Genomics, Proteomics, and Bioinformatics Research Initiative.

**Yeast Transformations and Two-Hybrid Screening** - Plasmids were transformed into yeast strains using a lithium acetate transformation procedure as outlined in the BD Biosciences Clontech MATCHMAKER GAL4 Two-Hybrid
System 3 and Libraries User Manual (BD Biosciences, Palo Alto, CA). The pGBKT7-RasGRP3 yeast two-hybrid bait plasmid was transformed into reporter strain AH109 to create AH109 [pGBKT7-RasGRP3]. An adult human brain cDNA library pretransformed in yeast strain Y187 (BD Biosciences Clontech Pretransformed Human Brain MATCHMAKER cDNA library; BD Biosciences, Palo Alto, CA) was screened in a yeast two-hybrid assay through large-scale mating to AH109 [pGBKT7-RasGRP3] following manufacturer’s instructions. Positive clones were selected on medium lacking adenine, histidine, leucine, and tryptophan to identify diploids containing a pACT2-cDNA library plasmid and the pGBKT7-RasGRP3 plasmid and expressing the ADE2 and HIS3 two-hybrid reporter genes. Positive clones were further screened for expression of an additional reporter gene, MEL1 (encoding melibiase/α-galactosidase), using the chromogenic substrate 5-Bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-gal; BD Biosciences, Palo Alto, CA). Plasmids were recovered using the YEASTMAKER Yeast Plasmid Isolation Kit (BD Biosciences, Palo Alto, CA) or Zymoprep Yeast Plasmid Minipreparation kit (Zymo Research, Orange, CA).

GST Pull-down and Co-immunoprecipitation Assays - CHO-K1 cells were transiently transfected with RasGRP plasmids using FuGENE 6 Transfection Reagent according to the manufacturer’s instructions (Roche, Indianapolis, IN). Forty-eight to seventy-two h later, cells were washed twice with ice-cold TBS (25 mM Tris-HCl, pH 7.4, 0.45% NaCl) and resuspended in 500 μl of lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% IGEPAL, 1 mM dithiothreitol, 5% glycerol, and protease inhibitors). For immunoprecipitations, the lysis buffer also included 25 mM NaF. Complete Mini, EDTA-free protease inhibitor tablets were purchased from Roche (Indianapolis, IN). Lysates were briefly vortexed, incubated on ice for 5 min, and then centrifuged at 13,000 rpm for 15 min at 4 °C. The resulting supernatants were collected for GST pull-down or co-immunoprecipitation assays.

For GST pull-down studies, a GST-DLC1 fusion protein was produced in E. coli. Cells were lysed by sonication on ice in phosphate buffered saline (PBS) in the presence of protease inhibitors, followed by addition of Triton X-100 to a 1% final concentration and subsequent incubation for 30 min on ice. The lysate was partially purified by centrifugation at 18,000 x g for 20 min at 4 °C. The resulting supernatant was used as a GST-DLC1 probe in a pull-down assay. GST alone was also produced to use as a negative control in the assay. The GST-DLC1 probe was incubated with rotation at 4 °C for 1-3 h with glutathione agarose beads (SwellGel Immobilized Glutathione Discs, Pierce, Rockford, IL) in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% IGEPAL, 1 mM dithiothreitol, 5% glycerol, and protease inhibitors). GST-DLC1-glutathione agarose beads were washed three times with lysis buffer to remove unbound GST-DLC1 and then aliquoted for incubation with cell lysates. For each cell lysate, an aliquot containing 500 μg of total protein was incubated with the bead-coupled GST-DLC1 probe at 4 °C for 1 h with rotation. After incubation, beads were washed three times with lysis buffer, resuspended in 2X Laemmli buffer containing 5% β-mercaptoethanol, and boiled for 10 min. Samples were analyzed by SDS-PAGE and immunoblotting using the anti-FLAG M2 mouse monoclonal antibody to detect FLAG-tagged RasGRP proteins. For co-immunoprecipitation assays, aliquots of cell lysates containing equal amounts (~1 mg) of total protein were incubated with 24.5 μg anti-FLAG M2 mouse monoclonal antibody overnight with rotation at 4 °C. Immunocomplexes were then precipitated by rotation for 2 h at 4 °C with 50 μl Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) that had been prewashed and resuspended in lysis buffer. Beads were washed four times with lysis buffer, resuspended in 2X Laemmli buffer containing 5% β-mercaptoethanol, and boiled for 10 min. Anti-FLAG immunoprecipitated samples and total lysates were analyzed by SDS-PAGE and immunoblotting, using anti-DLC1 rat IgM to detect endogenous DLC1 and anti-FLAG rabbit polyclonal antibody to detect FLAG-tagged RasGRP proteins.

Confocal Microscopy Studies – For the immunofluorescence studies, CHO-K1 cells were seeded onto Permanox chamber slides and transfected 18 h later with the FLAG-tagged RasGRP3 expression constructs using FuGENE 6 (Roche, Indianapolis, IN). Forty-eight h later, the cells were fixed with 3.7% neutral formaldehde at 37 °C for 10 min, followed by 5-min permeabilization with 0.2% Triton X-100. Slides
were blocked with 10% normal goat serum diluted in PBS for 1 h at room temperature, followed by a 2-h incubation with 20 μg/ml of the anti-FLAG M2 monoclonal antibody diluted in 1% normal goat serum. After extensive washes in PBS, a 1:200 dilution of Alexa Fluor 594 goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) was added to the slides in 1% normal goat serum for 1 h in the dark. Cells were washed several times in PBS, mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and viewed with a Leica TCS SP5 or a Zeiss LSM Pascal scanning confocal microscope. Cells transfected with GFP-tagged constructs were either fixed as above or used in live-cell experiments. For the latter, CHO-K1 cells were seeded onto 40-mm circular glass coverslips and transfected with the GFP constructs using FuGENE 6. Forty-eight h later, the coverslips were enclosed in a Bioptechs Focht Chamber (Bioptechs, Butler, PA), which was attached to the microscope stage of a Leica TCS SP5 confocal microscope with a custom stage adaptor, connected to a temperature controller at 37 °C, and perfused with phenol red–free medium supplemented with 8% fetal bovine serum using a Lambda microperfusion pump. A 1 μM dilution of 12-O-tetradecanoyl phorbol 13-acetate (TPA) in medium was perfused for 30 min, during which time sequential images of the same cell were collected.

**Ras-GTP Pull-Down Assay and Western Blots** - Levels of GTP-loaded Ras (Ras GTP) were measured as previously described with some modifications (6). Briefly, CHO-K1 cells were transiently transfected with RasGRP3 expression constructs for 48 h and cytoplasmic fractions were prepared to be used as lysates for the pull-down assay. To evaluate TPA-mediated activation of Ras, cells were serum-starved overnight and then treated with vehicle (DMSO) or 1 μM TPA for 30 min before harvesting them in lysis buffer for the pull-down assay. Lysates were mixed, incubated on ice for 5 min, and then clarified by centrifugation at 13,000 rpm for 15 min at 4 °C. Lysate protein (500 μg) was incubated with the Ras binding domain (RBD) of Raf-1 conjugated to glutathione S-transferase (GST-Raf-1-RBD probe) and bound to glutathione beads for 1 h with rotation in the cold. The affinity complexes were washed thrice with lysis buffer and then resuspended in Laemmli buffer containing 5% β-mercaptoethanol, boiled, and resolved on 15% acrylamide gels. Total lysate protein (25 μg) was run in parallel as control of the input of total Ras in the pull-down assay. Proteins were blotted onto nitrocellulose membranes. Immunostaining was done using an anti-Ras antibody. Rap1 pull-down assays were done using the EZ-Detect Rap1 activation Kit according to the manufacturer’s instructions (Pierce, Rockford, IL). Western blot bands were analyzed by densitometry using UNSCAN-IT gel software (Silk Scientific, Orem, UT) or Scion Image software (Scion Corporation, Frederick, MD) and statistical significance was calculated in GraphPad Prism (GraphPad Software, San Diego, CA) using nonpaired Student’s t test or one-way ANOVA followed by Tukey’s Multiple Comparison test.

**RESULTS**

**Dynein Light Chain 1 (DLC1) is a novel RasGRP3-interacting protein isolated in a yeast two-hybrid screen** - To identify binding partners of RasGRP3, we performed a yeast two-hybrid screen of a human brain cDNA library using the full-length human RasGRP3 protein as bait. One of the clones isolated from the screen contained the full-length coding sequence of DLC1 (NCBI Accession Number NM_003746) downstream of a mitochondrial genomic sequence and poly (A) tail. The DLC1 cDNA was isolated from the clone, and the two-hybrid interaction with RasGRP3 was confirmed by cotransforming the yeast reporter strain AH109 and assaying for expression of the yeast two-hybrid reporter genes ADE2 and HIS3 by growth on nutrient-deficient plates (Fig. 1A).

Since RasGRP3 shares a significant degree of sequence similarity with other members of the RasGRP family (Fig. 1B), we tested if RasGRP1 and RasGRP2 (short spliced version) could also interact with DLC1. Neither protein interacted with DLC1 in the yeast two-hybrid assay, suggesting that the association was specific for RasGRP3.

To map the region of RasGRP3 responsible for DLC1 binding, we used a truncated form of RasGRP3 consisting of C-terminal amino acids 408-691 (RasGRP3CSt) or a shorter variant...
interaction of RasGRP3 with DLC1 and further
demonstrated that RasGRP2 did not interact with
DLC1 (Fig. 2B). For comparison, we used FLAG-RasGRP2, which was
negative for interaction with DLC1 in the yeast two-hybrid screen and in the
in vitro assay. As shown in Fig. 3, endogenous DLC1 was detected
in the immunoprecipitates from cells expressing RasGRP3 (lane 2) but not in those from RasGRP2-
transfected cells (lane 1). Deletion of the C-
terminal domain of RasGRP3 (RasGRP3C) abolished the
in vivo interaction with DLC1 (Fig. 3, lane 3), while immunoprecipitation of
RasGRP3COnly demonstrated that the isolated C-
terminal region of RasGRP3 was sufficient to
coprecipitate DLC1 (Fig. 3, lane 4).

RasGRP3 and the RasGRP3ΔC mutant protein display different subcellular localization patterns - We hypothesized that if interaction with
DLC1 was an important determinant of the subcellular localization of RasGRP3, disruption of the interaction should affect RasGRP3 distribution
in the cell. Immunofluorescence analysis of CHO-K1 cells expressing either FLAG-RasGRP3 or the
FLAG-RasGRP3ΔC mutant protein, which was
defective for interaction with DLC1, revealed
significant differences in localization of these proteins. While full-length RasGRP3 mainly
localized in the cytoplasm with some perinuclear
staining, RasGRP3ΔC displayed a strong reticular
distribution and staining of the nuclear envelope
(Fig. 4A, FLAG panel). We observed the same
results using GFP-tagged proteins, in fixed as well
as in live cells (Fig. 4A, GFP-fixed and GFP-live
panels, respectively). The FLAG-RasGRP3ΔC
protein, which was able to bind to DLC1 both in vitro
and in vivo, displayed a subcellular localization
very similar to that of full-length
RasGRP3 (Fig. 4S, Supplemental Data).

Interestingly, the RasGRP3ΔC mutant protein also formed punctate and filamentous
structures inside the nucleus (Fig. 4A, right
column). Fractionation of FLAG-RasGRP3 or
FLAG-RasGRP3ΔC expressing cells into
cytoplasmic and nuclear fractions confirmed accumulation of the RasGRP3 mutant protein in the nucleus (Figure 4B).

The subcellular location of RasGRP3 should determine its accessibility to substrates and changes in response to activators such as TPA and various diacylglycerol analogs (6,19,20). To evaluate if there was a difference in the response to TPA between RasGRP3 and RasGRP3ΔC, we transfected CHO-K1 cells with the corresponding GFP-tagged constructs and examined TPA-induced translocation of the proteins in live cells by confocal microscopy. As we have seen before using HEK293 cells (6), 1 μM TPA caused a rapid redistribution of GFP-RasGRP3 mainly to internal membranes (Fig. 4C, left column). Translocation of GFP-RasGRP3ΔC in response to TPA was less evident because of the strong reticular localization of this protein under non-stimulated conditions. Nevertheless, there was some redistribution of the protein in response to TPA, particularly to the cell periphery (Fig. 4C, right column). As noted before, there was marked localization of RasGRP3ΔC to the nucleus. Interestingly, we also observed the formation of a few filamentous and punctate structures in the nucleus of RasGRP3-expressing cells upon TPA treatment (Figure 4C, left column).

The striking difference in subcellular localization between RasGRP3 and the RasGRP3ΔC mutant protein led us to investigate if there were any consequences on the downstream RasGRP3 signaling. To this end, we measured activation of one of the substrates for RasGRP3, the small GTPase Ras. In CHO-K1 cells grown in complete media, level of active Ras (RasGTP) were only slightly elevated in the RasGRP3 expressors compared to the mock (vector-transfected control) cells (Figure 5A). However, ectopic expression of the RasGRP3ΔC mutant protein caused a ≥ 2.0-fold increase in RasGTP levels compared with those of the RasGRP3 expressors and control cells (Figure 5A). We also observed a tendency for elevated RasGTP levels in RasGRP3ΔC-expressing cells under serum starvation conditions, albeit not statistically significant when compared to the levels of active Ras in control or RasGRP3-expressing cells (Figure 5B). Stimulation with 1 μM TPA further increased the levels of active, GTP-bound Ras in both RasGRP3 and RasGRP3ΔC overexpressors (Figure 5B).

Activation of the Ras-related small GTPase Rap1 in response to TPA in CHO-K1 cells was insensitive to ectopic expression of either RasGRP3 or the mutant protein (Fig. 2S, Supplemental Data).

**DISCUSSION**

In the present work, we have demonstrated that DLC1 is a novel RasGRP3-interacting protein. Preliminary mapping of the RasGRP3 domain involved in the association with DLC1 pointed at a region located within the last 127 amino acids of human RasGRP3. Interestingly, we identified a TQT motif at amino acids 615-617 within this region. The (R/K)XTQT consensus motif, where X is any amino acid, is one of the motifs reported to be responsible for DLC1-binding (17). The TQT motif present in RasGRP3 lacks the (R/K) amino acid, but contains an alanine immediately before the threonine, similar to a AATQT site identified in p53-binding protein 1, another DLC1-interacting molecule (18).

A RasGRP3 mutant protein lacking the C-terminal domain, including the TQT consensus motif, failed to interact with DLC1 both in vitro and in vivo in mammalian cells. This C-terminal region does not contain any other known domains, repeats, or motifs and is located after the C1 domain and EF hands of RasGRP3. In addition to loss of DLC1 binding, this RasGRP3 mutant protein displayed a dramatically different subcellular localization compared to full-length RasGRP3, with strong reticular partitioning and staining of the nuclear envelope. Additionally, we observed a significant nuclear accumulation of the RasGRP3 mutant protein, which formed punctate and filamentous structures. We also noticed formation of punctate nuclear structures in some of the cells transfected with full-length RasGRP3 upon activation with phorbol esters. Localization of RasGRP3 in the nucleus has not been reported before, and its significance is puzzling. However, forms of H-Ras and Rap1, which are both potential substrates of RasGRP3, have been found in the nuclei of N-nitrosodimethylamine-induced liver tumor cells and squamous cell carcinoma lines, respectively (21,22). It is interesting to note that DLC1 has been suggested to act as a chaperone for nuclear translocation of estrogen receptor in breast cancer cells (23) and for nuclear...
accumulation of p53 in response to DNA damage (18), but was also reported to function together with DLC2 to prevent nuclear localization of the brain-enriched protein PMES-2 (24). Thus, DLC1 appears to regulate the nuclear localization of certain proteins, but whether DLC1 promotes or inhibits nuclear accumulation appears to depend on its interacting partners. The unusual localization of the RasGRP3 C-terminal deletion protein in the nucleus may be related to its loss of interaction with DLC1; however, we cannot preclude the possibility that interactions with other proteins besides DLC1 could occur at the C-terminus of RasGRP3 and contribute to RasGRP3 localization.

The ectopic expression of RasGRP3ΔC increased the levels of active Ras in CHO-K1 cells. The effect of the RasGRP3ΔC mutant protein was significant in the presence of serum (complete media) compared to serum starvation conditions. One could argue that RasGRP3 mislocalization could lead to closer proximity to Ras, consequently inducing higher levels of Ras activation in response to growth factor stimulation. This finding supports the concept that localization of RasGRP3, potentially mediated in part by the interaction with DLC1, could play an important role in controlling downstream signaling pathways.

Treatment with the phorbol ester TPA, which changed the subcellular distribution of RasGRP3 and, to some extent, of the mutant protein, induced a further increase in Ras activation in both RasGRP3- and RasGRP3ΔC-expressing cells. TPA appeared to be equally potent at activating Ras through RasGRP3 and its mutant protein, in contrast with the effect of complete media. This could indicate that activation of RaGRP3 via TPA may proceed independently of its interaction with DLC1. Alternatively, TPA may dissociate the RasGRP3-DLC1 interaction as part of its mechanism of RasGRP3 activation. This dissociation would not be needed in the case of the RasGRP3ΔC mutant protein. However, preliminary experiments did not show a significant change in the amount of DLC1 co-precipitating with RasGRP3 upon TPA treatment (data not shown), arguing against the second hypothesis. Thus, the relevance of the RasGRP3-DLC1 interaction for RasGRP3 signaling appears to vary depending on the stimulus.

DLC1 is a highly conserved protein that is ubiquitously distributed among cell lines and tissues. Its ability to interact with a variety of unrelated proteins that play roles in apoptosis and proliferation suggests that DLC1 may act to bring diverse signaling molecules together. In the context of our present findings, it is tempting to speculate that DLC1 may act as an anchoring protein for RasGRP3, preventing it from adopting an active distribution in the absence of activating signals. Further experiments measuring association kinetics of the RasGRP3-DLC1 interaction in response diacylglycerol and other physiological stimuli, as well as analysis of substitutions within the TQT consensus motif in RasGRP3, are needed to more completely define the RasGRP3-DLC1 association and examine the significance of this interaction in RasGRP3 signaling and function.

REFERENCES

1. Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) Science 280(5366), 1082-1086
2. Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) Proc Natl Acad Sci U S A 95(22), 13278-13283
3. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K., and Matsuda, M. (2000) J Biol Chem 275(33), 25488-25493
4. Li, L., Yang, Y., and Stevens, R. L. (2002) Mol Immunol 38(16-18), 1283-1288
5. Rebhun, J. F., Castro, A. F., and Quilliam, L. A. (2000) J. Biol. Chem. 275(45), 34901-34908
6. Lorenzo, P. S., Kung, J. W., Bottorff, D. A., Garfield, S. H., Stone, J. C., and Blumberg, P. M. (2001) Cancer Res 61(3), 943-949
7. Ron, D., and Kazanietz, M. G. (1999) FASEB J. 13(13), 1658-1676
8. Braun, D. C., Cao, Y., Wang, S., Garfield, S. H., Min Hur, G., and Blumberg, P. M. (2005) Mol Cancer Ther 4(1), 141-150
9. Conesa-Zamora, P., Lopez-Andreo, M. J., Gomez-Fernandez, J. C., and Corbalan-Garcia, S. (2001) Biochemistry 40(46), 13898-13905
10. Cho, W. (2001) J. Biol. Chem. 276(35), 32407-32410
11. Schechtman, D., and Mochly-Rosen, D. (2001) Oncogene 20(44), 6339-6347
12. Hirokawa, N., Noda, Y., and Okada, Y. (1998) Curr Opin Cell Biol 10(1), 60-73
13. Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. (1999) Mol Cell 3(3), 287-296
14. Jaffrey, S. R., and Snyder, S. H. (1996) Science 274(5288), 774-777
15. Crepieux, P., Kwon, H., Leclerc, N., Spencer, W., Richard, S., Lin, R., and Hiscott, J. (1997) Molecular and Cellular Biology 17(12), 7375
16. Vadlamudi, R. K., Bagheri-Yarmand, R., Yang, Z., Balasenthil, S., Nguyen, D., Sahin, A. A., den Hollander, P., and Kumar, R. (2004) Cancer Cell 5(6), 575-585
17. Lo, K. W. H., Naisbett, S., Fan, J.-S., Sheng, M., and Zhang, M. (2001) J. Biol. Chem. 276(17), 14059-14066
18. Lo, K. W. H., Kan, H.-M., Chan, L.-N., Xu, W.-G., Wang, K.-P., Wu, Z., Sheng, M., and Zhang, M. (2005) J. Biol. Chem. 280(9), 8172-8179
19. Shao, L., Lewin, N. E., Lorenzo, P. S., Hu, Z., Enyedy, I. J., Garfield, S. H., Stone, J. C., Marner, F. J., Blumberg, P. M., and Wang, S. (2001) J. Med. Chem. 44(23), 3872-3880
20. Pu, Y., Perry, N. A., Yang, D., Lewin, N. E., Kedei, N., Braun, D. C., Choi, S. H., Blumberg, P. M., Garfield, S. H., Stone, J. C., Duan, D., and Marquez, V. E. (2005) J. Biol. Chem. 280(29), 27329-27338
21. Wurzer, G., Mosgoeller, W., Chabicovsky, M., Cerni, C., and Wiesierska-Gadek, J. (2001) J Cell Biochem 81(S36), 1-11
22. Mitra, R. S., Zhang, Z., Henson, B. S., Kurnit, D. M., Carey, T. E., and D'Silva, N. J. (2003) Oncogene 22(40), 6243-6256
23. Rayala, S. K., den Hollander, P., Balasenthil, S., Yang, Z., Broaddus, R. R., and Kumar, R. (2005) EMBO Rep 6(6), 538-544
24. Ninomiya, K., Ishimoto, T., and Taguchi, T. (2005) Cell Mol Neurobiol 25(5), 899-911

FOOTNOTES

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1 The abbreviations used are: PKC, protein kinase C; DLC1, dynein light chain 1; CHO-K1 cells, Chinese Hamster Ovary-K1 cells; TBS, Tris Buffered Saline; PBS, Phosphate Buffered Saline; GST, glutathione S-transferase; GFP, green fluorescent protein; SDS-PAGE, Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis; GST-Raf-1-RBD, glutathione S-transferase-Ras binding domain (RBD) of Raf-1; TPA, 12-O-tetradecanoyl-13-acetate.
FIGURE LEGENDS

FIG. 1. Identification of DLC1 as a novel RasGRP3-interacting protein. A, The yeast two-hybrid reporter strain AH109 was cotransformed with (1) pGBK7 (empty Gal4p DNA-binding domain vector, TRP1) and pACT2B2 (empty Gal4p activation domain vector, LEU2), (2) pGBK7 and pACT2-K6 (DLC1 plasmid isolated through the RasGRP3 yeast two-hybrid screen), (3) pGBK7-RasGRP3 and pACT2B2, or (4) pGBK7-RasGRP3 and pACT2-K6 (DLC1). Approximately equal numbers of cotransformed cells were plated on synthetic medium lacking leucine and tryptophan (SD-Leu-Trp) to select for cotransformants and on synthetic medium lacking adenine, histidine, leucine, and tryptophan (SD-Ade-His-Leu-Trp) to select for cotransformants in which the ADE2 and HIS3 two-hybrid reporter genes were expressed to indicate a positive interaction. Growth was recorded after 23 days. Similar results were obtained with pACT2-DLC1, which contains only the DLC1 gene from pACT2-K6; however, cells transformed with this plasmid exhibited a dramatic growth defect. B, Schematic representation of the RasGRP proteins tested in the yeast two-hybrid assay. The results of the two-hybrid assay are indicated on the right (-, no interaction; +, positive interaction).

FIG. 2. Interaction of RasGRP3 and DLC1 in vitro. A, Pull-down experiments were conducted using GST-DLC1 or GST alone on lysates from CHO-K1 cell transfected with empty vector (FLAG) or FLAG-tagged RasGRP3 (FLAG-RasGRP3) as described under “Experimental Procedures”. The precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting using an anti-FLAG antibody. B, Schematic representation of the FLAG-RasGRP fusion proteins tested in the GST-DLC1 pull-down assay (top panel). The results are summarized in the right column (-, no interaction; +, positive interaction) and are representative of three independent experiments. The results from a representative GST-DLC1 pull-down experiment are shown in the bottom panel. Total FLAG-tagged proteins are shown as input. The asterisk denotes a non-specific band recognized by the anti-FLAG antibody in the input samples. Note that the RasGRP3COnly protein exhibited two SDS-PAGE mobility forms.

FIG. 3. Association of RasGRP3 with DLC1 in vivo. CHO-K1 cells were transiently transfected with different FLAG-tagged constructs to test for co-immunoprecipitation of endogenous DLC1. After 48 h, cells were lysed and immunoprecipitation was performed with an anti-FLAG antibody as described under “Experimental Procedures”. Top panel, Schematic representation of the FLAG-RasGRP fusion proteins tested in the co-immunoprecipitation assay. The results are summarized in the right column (-, no interaction; +, positive interaction) and are representative of three independent experiments. Bottom panel, Immunoprecipitation (IP) of FLAG-tagged proteins and co-immunoprecipitation (Co-IP) of endogenous DLC1 from a representative experiment. Levels of total FLAG-tagged proteins and DLC1 were measured from aliquots of the total lysate used as input in the immunoprecipitation assay. The asterisk denotes a non-specific band recognized by the anti-FLAG antibody in the input samples.

FIG. 4. Subcellular localization of RasGRP3 and a truncated RasGRP3 mutant protein defective for DLC1 interaction. A, CHO-K1 cells were transfected with plasmids encoding RasGRP3 or the truncated RasGRP3ΔC (ΔC) mutant protein tagged with either FLAG or GFP at the N-terminus. After 48 h, cells expressing the FLAG-tagged proteins were processed for immunofluorescence as described under “Experimental Procedures” (first row, FLAG). Cells expressing the GFP fusion proteins were either fixed in neutral formaldehyde (second row, GFP-fixed) or examined in live-cell chambers at 37 °C (third row, GFP-live). Images shown are from a representative experiment. Similar results were observed in two to three independent experiments. B, CHO-K1 cells transfected with plasmids encoding FLAG-RasGRP3 (RasGRP3) or the truncated FLAG-RasGRP3ΔC (ΔC) mutant protein were fractionated into cytoplasmic (C) and nuclear (N) fractions as described under “Experimental Procedures.” Western blot is from a representative experiment (left panel). Intensities of the Western blot bands were determined by densitometry and plotted as Nuclear/Cytoplasm fractions (right panel). Data are representative of three
independent experiments and are shown as mean ± S.E.M. *, p<0.05. C, CHO-K1 cells expressing either GFP-RasGRP3 or the truncated mutant GFP-RasGRP3ΔC (GFP-ΔC) protein were perfused with 1 µM TPA for 30 minutes. Images were recorded every 20 s using a scanning laser confocal microscope. Images correspond to 0, 5, 15, and 30 min after TPA treatment and are representative of two independent experiments. The red arrows denote formation of punctate/filamentous structures in the nucleus of cells expressing GFP-RasGRP3. Results are representative of two independent experiments.

FIG. 5. Activation of Ras in response to RasGRP3 and RasGRP3ΔC. A, CHO-K1 cells transfected with either empty FLAG vector (Vector), or the FLAG-epitope tagged RasGRP3 (RasGRP3) or RasGRP3ΔC (ΔC) expression constructs, were grown in complete media for 48-72 h and harvested for a Ras pull-down assay using cytoplasmic fractions as described under “Experimental Procedures”. Levels of total Ras and the FLAG-tagged proteins were measured from aliquots of the total lysate used as input in the pull-down assay. Western blots are from a representative experiment (left panel). Intensities of the Western blot bands were determined by densitometry and results for RasGTP were normalized by the total amount of Ras in each lane (right panel). Data are representative of three independent experiments and are shown as mean ± S.E.M. *, p< 0.05 compared with FLAG and RasGRP3; one-way ANOVA followed by Tukey’s test. B, Ras pull-down assays were performed on overnight serum-starved cells treated with 1 μM TPA for 30 minutes. DMSO was used as negative control. Intensities of the Western blot bands were determined as indicated above. Left panel, Representative Western blot. Right panel, Mean ± S.E.M. of three independent experiments *, p< 0.05 compared with the corresponding negative controls; Student’s t test.
### FIGURE 1

**A**

SD-Leu-Trp

1

2

3

4

SD-Ade-His-Leu-Trp

1

2

3

4

**B**

- **Rat RasGRP1**
  - Location: 52-176-201-437-474-502
  - Length: 795 aa
  - Interaction: -

- **Human RasGRP2 (isoform 2)**
  - Location: 3-126-150-388-459-458
  - Length: 609 aa
  - Interaction: -

- **Human RasGRP3**
  - Location: 4-127-150-386-454-482
  - Length: 691 aa
  - Interaction: +

- **RasGRP3**
  - Location: 408-564
  - Length: 691 aa
  - Interaction: +

- **RasGRP3EC**
  - Location: 408-564
  - Length: 691 aa
  - Interaction: -

- **RasGRP3CSt**
  - Location: 408-691
  - Length: 691 aa
  - Interaction: +

**Interaction**

- DLC1

**QATQT**
FIGURE 2

A

|          | Input | GST | GST-DLC1 |
|----------|-------|-----|----------|
| FLAG vector | +     | -   | -        |
| FLAG-RasGRP3 | -     | +   | -        |

B

|        | Pull-down |
|--------|-----------|
| RasGRP2 |           |
| RasGRP3 |           |
| RasGRP3ΔC |         |
| RasGRP3COnly |       |

|        | Input | GST-DLC1 pull-down |
|--------|-------|--------------------|
| 1      |       |                    |
| 2      | RasGRP3 | 691                |
| 3      | RasGRP3ΔC | 549                  |
| 4      | RasGRP3COnly | 549 691            |

* Denotes the protein bands.
FIGURE 3

| Input | IP |
|-------|----|
| 1 | 2 | 3 | 4 |

**Co-IP**

| 1 | RasGRP2 | 609 | - |
| 2 | RasGRP3 | 691 | + |
| 3 | RasGRP3ΔC | 549 | - |
| 4 | RasGRP3COnly | 549 | 691 | + |

**Legend**

- **Input** and **IP** columns represent different samples.
- **Co-IP** indicates the presence (+) or absence (-) of co-immunoprecipitation.
- **FLAG** and **DLC1** are antibodies used for detection.
- **IgG** controls for non-specific binding.
- **Co-IP** shows the specific interaction captured by the co-immunoprecipitation process.
FIGURE 4

A

RasGRP3

ΔC

FLAG

GFP-fixed

GFP-live

B

RasGRP3

ΔC

C

GFP-RasGRP3

GF-ΔC

0 min

5 min

15 min

30 min

TPA (1 μM)

0 min

5 min

15 min

30 min

Nuclear/Cytoplasm fraction

0.24±0.07

0.69±0.11*

0.25

0.50

0.75

1.00

*
FIGURE 5

A

Vector RasGRP3 ΔC
RasGTP
Total Ras
FLAG

B

TPA (1 μM) - + - + - + - +
RasGTP
Total Ras
FLAG

0.0 0.5 1.0 1.5
RasGTP/Total Ras

0.0 0.5 1.0 1.5 2.0
RasGTP/Total Ras

*
The exchange factor and diacylglycerol receptor RasGRP3 interacts with dynein light chain 1 through its C-terminal domain
Sara M. Okamura, Carolyn E. Oki-Idouchi and Patricia S. Lorenzo

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