Disruption of Oligomerization Induces Nucleocytoplasmic Shuttling of Leukemia-Associated Rho Guanine-Nucleotide Exchange Factor

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Received February 13, 2007; accepted July 3, 2007

ABSTRACT

The rgsRhoGEFs comprise a subfamily of three guanine nucleotide exchange factors, which function in linking heterotrimeric G-proteins to the monomeric RhoGTPase. Here, we reveal the novel finding that oligomerization of leukemia-associated Rho-GEF (LARG) functions to prevent nucleocytoplasmic shuttling and to retain LARG in the cytoplasm. We establish that oligomerization is mediated by a predicted coiled-coil sequence (amino acids 1507–1520) in the extreme C terminus of LARG and that substitution of isoleucines 1507/1510 with alanines disrupts homo-oligomerization and leads to nucleocytoplasmic shuttling via the CRM1 nuclear transport pathway. In addition, we demonstrate that induced dimerization of an otherwise nuclear monomeric LARG mutant promotes cytoplasmic localization. Furthermore, we establish that nuclear import of monomeric LARG is mediated by the nuclear localization sequence 29PTDKKQK35 in the extreme N terminus. We propose that nucleocytoplasmic shuttling provides a mechanism for spatially regulating the activity of LARG toward its cytoplasmic targets and potentially new nuclear targets.

Rho, together with Rac and Cdc42, belongs to the Rho family of small monomeric GTPases and functions in regulating several cellular processes such as cell movement, survival, cell cycle, and gene transcription (Schmidt and Hall, 2002a; Rossman et al., 2005). Like all GTPases, Rho cycles between an inactive GDP-bound state and an active GTP-bound state. The GDP/GTP cycle of Rho is regulated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP leading to the activation of downstream effector pathways, and GTPase-activating proteins, which catalyze the hydrolysis of GTP. Rho activation is mediated by several cell-surface receptors, including many heterotrimeric G-protein-coupled receptors (Seasholtz et al., 1999; Sah et al., 2000; Fukuhara et al., 2001), growth factor receptors (Taya et al., 2001), and the semaphorin-4D receptor (Basile et al., 2004).

The oncogenic potential of Rho GTPases has been revealed by several studies (Dhanasekaran and Prasad, 1998; Fukuhara et al., 2001, 2002; Sahai and Marshall, 2002). RhoGTPases have also been shown to mediate heterotrimeric G-protein-induced cell transformation (Fromm et al., 1997). A subfamily of RhoGEFs consisting of p115RhoGEF, PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG) interact with Gα12 and Gα13, leading to Rho activation and thereby coupling heterotrimeric G-protein signaling to Rho signaling (Hart et al., 1998; Kozasa et al., 1998; Fukuhara et al., 1999, 2000; Suzuki et al., 2003). Interactions of p115RhoGEF, PRG, and LARG with Gα occur through regulator of G-protein signaling (RGS) domains, a distinct feature of the proteins in this subfamily of RhoGEFs (rgsRhoGEFs). Like all RhoGEFs, p115RhoGEF, PRG, and LARG contain a Dbl homology and a Pleckstrin homology domain, which mediate the exchange activity on Rho. In addition, LARG and PRG contain PDZ domains, which have been shown to be involved

This work was supported by grant GM62884 (to P.W.) from the National Institutes of Health.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.


The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: GEF, guanine-nucleotide exchange factor; ChFP, cherry fluorescent protein; RGS, regulator of G protein signaling; LARG, leukemia-associated Rho-guanine-nucleotide exchange factor; MLL, mixed lineage leukemia; PM, plasma membrane; NES, nuclear export sequence; NLS, nuclear localization sequence; PRG, postsynaptic density 95/disc-large/zona occludens Rho guanine-nucleotide exchange factor; SRE, serum response element; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; PBS, phosphate-buffered saline; GFP, green fluorescent protein; LMB, Leptomycin B; DAPI, 4,6-diamidino-2-phenylindole; FL, full length; FKBP, FK506 binding protein; FRB, FKBP-rapamycin binding.
in cell-surface receptor coupling (Taya et al., 2001; Yamada et al., 2005).

LARG is one of the very few RhoGEFs that have been found mutated in human cancers. The gene for LARG was identified as a fusion partner with the mixed lineage leukemia gene (MLL) in a patient with acute myeloid leukemia (Kourlas et al., 2000). MLL rearrangements are common in both primary and secondary leukemias, and recent studies have reported additional cases of MLL-LARG fusions (Shih et al., 2006; Tyybakinoja et al., 2006). The activity of the MLL-LARG has not been investigated; however, the chimeric protein lacks the N terminus of LARG, and numerous studies indicate that both the N and C termini of several Rho GEFs often contain regulatory sequences that regulate RhoGEF activity by determining intramolecular inhibitions, protein-protein interactions, and RhoGEF relocation in response to stimuli (Schmidt and Hall, 2002a). Current models on the mechanisms of function of RhoGEFs uphold a requirement for translocation to membranes or cytoskeletal structures in response to stimuli. However, studies with two Rho-specific GEFs, Net1 and Ect2, have revealed nucleocytoplasmic shuttling as a mechanism that tightly regulates their activity (Tatsumoto et al., 1999; Schmidt and Hall, 2002b).

This study provides the first insight into mechanisms regulating the subcellular distribution of LARG. We identify a coiled-coil sequence in the extreme C terminus of LARG that mediates homo-oligomerization and cytoplasmic retention, and a nuclear localization sequence (NLS) in the extreme N terminus. Our results using mutations that disrupt the oligomerization of LARG and constructs that allow for induced dimerization of LARG suggest a novel model of LARG localization, in which LARG oligomers are retained in the cytoplasm, whereas LARG monomers undergo nucleocytoplasmic shuttling. Taken together, our findings raise the interesting possibility that signaling functions of LARG may be regulated by compartmentalized localization of this protein in the nucleus and the cytoplasm.

### Materials and Methods

#### Cell Culture and Transfection.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin. Unless otherwise noted, cells were plated in 10-cm or six-well plates 24 h before transfection. Cells were transfected with either 1 μg of DNA/well of six-well plate or 9 μg of DNA in a 10-cm plate using FuGENE 6 (Roche Diagnostics, Indianapolis, IN), according to manufacturer's protocol.

#### Reagents and Expression Plasmids.

Leptomycin B was purchased from BIOMOL International (Plymouth Meeting, PA). The Argent Regulated Heterodimerization Kit was a kind gift from Aria Pharmaceuticals (Cambridge, MA). The reporter plasmid that expresses the luciferase gene under the control of serum-response element (SRE), termed pSRE-Luc, was purchased from Stratagene (La Jolla, CA).

For the construction of GFP fused to LARG FL, LARGΔC (amino acids 1–1160), LARGΔN (amino acids 359–1544), LARGΔN/C (amino acids 359–1160), LARG (amino acids 1–1240), LARG (amino acids 1–1340), LARG (amino acids 1–1440), LARG (amino acids 1–1490), forward and reverse primers containing a 5′ Xhol site and a 3′ BamHI site were used to amplify LARG from pcDNA3-MycLARG. After digestion with Xhol and BamHI, the PCR-generated fragments were ligated into pEGFP-C1. Sequential PCR was used to generate the chimeric GFP-LARGΔC, in which the N terminus of PRG has been replaced with the N terminus of LARG. The Strategy generated fragments were ligated into pIC111.

The expression vector for ChFP used to contain tandem repeat of the mCherry protein (Shaner et al., 2005) was generated in two steps. First, the mCherry coding sequence was PCR-amplified from pRSSET-B-mCherry using primers containing a 5′ NheI site and a 3′ BglII site and then subcloned into pEGFP-C1 to replace the EGFP coding sequence. Second, the mCherry coding sequence was PCR-amplified using primers containing a 5′ BglII site and a 3′ HindIII site and then subcloned into the corresponding restriction sites of pmCherry-C1 to produce pChFP2x. Complementing single-stranded oligonucleotide sequences for three repeats of the nuclear localization sequence of SV40 in tandem, SV40NLS3x, and linker with 5′ EcoRI and 3′ SaIi overhangs were annealed to generate double-stranded sequences for subcloning into pChFP2x to generate pChFP2x-SV40NLS3x, ChFP2x-LARG(29–35), ChFP2x-LARG(21–41), and ChFP2x-LARG(15–41) were constructed by subcloning double-stranded corresponding sequences and linker, with 5′ EcoRI and 3′ SaIi overhangs as generated by annealing single-stranded complementary sequences. The correct DNA sequence of the mutants was confirmed by DNA sequencing of the entire open-reading frame (Kimmel Cancer Institute Nucleic Acid Facility, Philadelphia, PA).

mCherry eDNA was provided by R. Tsien. pcDNA3-MycLARG was a gift from T. Kosaza, whereas pKH3-HA-RanWT and pKH3-HA-RanG19V were gifts from I. Macara. The HA epitope (DVPDYA)-tagged pcDNA3HA α13Yt and pcDNA3HA α1qQL were gifts from J. S. Gutkind.

#### Coimmunoprecipitations.

Cos-7 cells in 10-cm plates were transfected with the indicated constructs. Twenty-four to forty-eight hours after transfection, cells were washed twice with ice-cold PBS and lysed with 0.5 ml of lysis buffer (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 100 mM NaCl, 2.5 mM MgCl2, 1 mM EDTA, 5 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 25 mM β-glycerophosphate, and 1 mM sodium orthovanadate) on ice. After lysis, cell lysates were centrifuged at 13,000 rpm for 5 min at 4°C. A 5.75-μg sample of an anti-GFP goat polyclonal antibody (Rockland, Gilbertsville, PA) was added, and the supernatants were tumbled for 1 h at 4°C. Next, 30 μl of Protein A/G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and samples were tumbled for 2 h at 4°C. Coimmunoprecipitation samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride, and probed with 0.2 μg/ml anti-GFP rabbit polyclonal antibody (Santa Cruz Biotechnology) or with 0.04 μg/ml ChFP rabbit polyclonal antibody (Chemicon International, Temecula, CA), followed by horseradish peroxidase-conjugated anti-rabbit antibody (Promega, Madison, WI). The blots were visualized using SuperSignal West Pico (Pierce Chemical, Rockford, IL).

#### Cell Imaging.

Cos-7 cells were grown on coverslips in six-well plates and were transfected for 24 or 48 h as indicated. For the effect of Leptomycin B (LMB) on protein localization, LMB was added at 20
ng/ml for 2 h before fixation or live cell imaging. Fixation and staining have been described previously (Grabocka and Wedegaertner, 2005), but briefly, cells were fixed with 3.7% formaldehyde in PBS for 15 min, washed, and then incubated in blocking buffer containing 2.5% nonfat milk in Tris-buffered saline/1% Triton X-100. Cells were then incubated with 12CA5 mouse monoclonal antibody in blocking buffer for 1 h and then with Alexa Fluor 594 goat anti-mouse (Invitrogen, Carlsbad, CA) secondary antibody in blocking buffer for 30 min. The coverslips were then washed with Tris-buffered saline/1% Triton X-100, rinsed with distilled water, and mounted on glass slides with 20 μl of Prolong Antifade reagent (Invitrogen). For DAPI staining, coverslips were incubated with 0.1 μg/ml DAPI (Invitrogen) in PBS for 5 min after fixation or secondary antibody incubation and washes.

Representative images were acquired using an Olympus BX-61 upright microscope (Olympus, Tokyo, Japan) with an ORCA-ER (Hamamatsu, Bridgewater, NJ) cooled charge-coupled device camera controlled by Slidebook version 4.0 (Intelligent Imaging Innovations, Denver, CO). Images of fixed cells were captured with an Olympus PlanApo 60x numerical aperture 1.4 oil objective. Live cell images were captured with a water immersion objective (Olympus LUMPlanFl 60x/0.9w). Images were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

SRE-Mediated Luciferase Gene Transcription Assay. Cos-7 cells were plated in six-well plates in serum-supplemented DMEM for 24 h. Cells were then switched to serum-free DMEM and transfected with 0.05 μg of pSRE-Luc, 0.1 μg of pCMV-β-gal, pcDNA3, and GFP-LARG or GFP-PRG constructs as indicated. Twenty-four hours after transfection, cells were washed with ice-cold PBS and lysed using reporter lysis buffer according to the manufacturer’s protocol (Promega). Lysates (20 μl) were mixed with 50 μl of the luciferase substrate (Promega) at room temperature. Luciferase activity was determined by measuring luminescence intensity. The β-galactosidase activities were determined by the colorimetric method and were used to normalize transfection efficiencies. SRE readouts were also normalized for expression differences as determined by densitometry readings of Western blots for GFP-LARG or GFP-PRG probed with anti-GFP rabbit polyclonal antibody.

Results

Deletion of the C Terminus of LARG Induces Nuclear Localization. Recent work has shown that deletion of the C terminus of rgsRhoGEFs disrupts their ability to homodimerize and/or hetero-oligomerize and enhances their ability to activate Rho-mediated SRE-gene transcription (Chikumi et al., 2004). As a first step in identifying regulatory elements defining the subcellular distribution of LARG, constructs encoding GFP fused to the N terminus of LARG full-length (FL) and the C-terminal deletion LARGΔC were generated (Fig. 1A). Because of the similarity in size and function between PRG and LARG, constructs encoding GFP-PRG FL and GFP-PRGΔC were prepared (Fig. 1A). After expression in Cos-7 cells, GFP-LARG FL was found mainly in the cytoplasm (Fig. 1B, top) as indicated by the presence of a nuclear shadow and the decrease in GFP fluorescence toward the cell periphery. Myc-LARG FL, in which an Myc tag has been fused to the N terminus of LARG, and LARG FL-GFP, in which GFP has been fused to the C terminus of LARG, display identical cytoplasmic localizations (Supplementary Fig. S1). In addition, endogenous LARG also exhibits a predominantly cytoplasmic distribution (Supplementary Fig. S1). GFP-PRG FL exhibits the characteristic cytoplasmic and peri-PM localization (Banerjee and Wedegaertner, 2004), indicated by the sharp staining at the cell periphery (Fig. 1B, top) and colocalization with cortical actin revealed by phalloidin staining (data not shown) distinct from the cytoplasmic-only distribution of GFP-LARG FL. Coimmunoprecipitation studies also revealed that GFP-LARG FL, in contrast to GFP-PRG FL, does not interact with actin (data not shown). Thus, association with actin structures is not a feature of GFP-LARG FL.

We were surprised to find that GFP-LARGΔC displayed a strong nuclear localization (Fig. 1B, top) as confirmed by identification of nuclei with DAPI staining (Fig. 1B, bottom). In contrast, the C-terminal truncation of PRG did not promote nuclear localization; GFP-PRGΔC retained cytoplasmic and peri-PM staining (Fig. 1B).

Next, we examined the ability of GFP LARG FL and GFP-LARGΔC to interact with activated α13 by assaying for PM recruitment. Previous work has demonstrated that expression of a mutationally activated α13 leads to the PM translocation of wild-type LARG (Banerjee et al., 2004). A, schematic presentation of GFP-LARG FL (amino acids 1–1544), GFP-PRG FL (amino acids 1–1522), and ΔC truncation mutants (amino acids 1–1160). B, Cos-7 cells were transfected with 0.5 μg of the indicated LARG and PRG constructs and 0.5 μg of pcDNA3. Cells were fixed 48 h after transfection. Localization of GFP-LARG and GFP-PRG constructs was visualized by the intrinsic fluorescence of GFP (top), whereas nuclei were visualized by staining of chromatin with DAPI (bottom). Images are representatives of >100 cells analyzed in at least three independent experiments. Bars, 10.2 μm.

Fig. 1. Deletion of the C terminus of LARG induces its nuclear localization. A, schematic presentation of GFP-LARG FL (amino acids 1–1544), GFP-PRG FL (amino acids 1–1522), and ΔC truncation mutants (amino acids 1–1160). B, Cos-7 cells were transfected with 0.5 μg of the indicated LARG and PRG constructs and 0.5 μg of pcDNA3. Cells were fixed 48 h after transfection. Localization of GFP-LARG and GFP-PRG constructs was visualized by the intrinsic fluorescence of GFP (top), whereas nuclei were visualized by staining of chromatin with DAPI (bottom). Images are representatives of >100 cells analyzed in at least three independent experiments. Bars, 10.2 μm.
cation of an otherwise cytoplasmic p115RhoGEF (Bhattacharyya and Wedegaertner, 2000, 2003, 2005), but a similar PM recruitment of LARG has not been tested. Coexpression of α\textsubscript{13}wt (HA panel) had no effect on the localization of GFP-LARG FL or GFP-LARG\textsubscript{AC} (Fig. 1C, middle). However, expression of constitutively active HA-α\textsubscript{13}QL (Fig. 1C, left) induced PM recruitment of GFP-LARG FL or GFP-LARG\textsubscript{AC} (Fig. 1C, middle). Furthermore, α\textsubscript{13}\textsubscript{E229KQL}, a mutant of α\textsubscript{13} that we have demonstrated previously to be deficient in interacting with and inducing the PM translocation of p115RhoGEF (Grabocka and Wedegaertner, 2005), was also deficient in inducing the PM translocation of GFP-LARG FL or GFP-LARG\textsubscript{AC} (data not shown). In addition to demonstrating that both GFP-LARG FL and GFP-LARG\textsubscript{AC} redistribute in response to α\textsubscript{13}QL, the proper signaling function of the LARG constructs was confirmed. GFP-LARG FL and GFP-PRG FL activated SRE-mediated gene transcription, and C-terminal deletion results in a further 2- to 5-fold increase in activity, similar to a previous report (Supplemental Fig. S2) (Chikumi et al., 2004). Thus, the above results (Fig. 1C and Supplemental Fig. S2) suggest that GFP-LARG\textsubscript{AC} retains stability and structural integrity.

The key finding in Fig. 1 is that removal of the C-terminal 384 amino acids of LARG results in strong nuclear localization rather than the cytoplasmic localization of GFP-LARG FL. Moreover, the nuclear localization of GFP-LARG\textsubscript{AC} but not GFP-PRG\textsubscript{AC} (Fig. 1B) indicates a unique role of the C terminus of LARG in regulating subcellular localization. These results further suggest that some aspect of the C terminus of LARG functions as a cytoplasmic retention signal.

A Predicted Coiled-Coil Domain in the Extreme C Terminus of LARG Mediates Its Homo-Oligomerization. Previous work showed that the C terminus of LARG was required for its oligomerization (Chikumi et al., 2004), and results in Fig. 1 indicate that the C terminus is required for cytoplasmic retention; thus, a logical prediction is that oligomerization functions to prevent nuclear localization of LARG. A previous study (Chikumi et al., 2004) did not define the structural requirements or sequence motifs in the C terminus that mediate oligomerization of LARG; therefore, we first sought to understand the mechanism to more precisely disrupt LARG oligomerization. It is interesting that a recent report revealed that Lsc, the murine ortholog of p115RhoGEF, oligomerizes through a coiled-coil domain in its C terminus (Eisenhaure et al., 2003). COILS (Lupas et al., 1991) predicts several potential coiled-coil domains in LARG (Fig. 2A) characterized by heptad repeats (each heptad is designated by residues a–g), with hydrophobic residues a and d comprising the putative helix interface, and hydrophilic residues b, c, e, f, and g in the solvent-exposed region (Lupas, 1997). Amino acids 1507 to 1520 in the extreme C terminus of LARG seem to constitute a coiled-coil sequence of two heptad repeats (Fig. 2A). To test whether this predicted coiled-coil domain is required for oligomerization of LARG, isoleucines at positions 1507 and 1510 were substituted with alanines (termed 2IA mutation), and coimmunoprecipitation experiments were performed. Cos-7 cells expressing both GFP-LARG FL and ChFP-LARG FL, in which the mCherry fluorescent protein had been fused to LARG, were lysed, and GFP-LARG FL was immunoprecipitated with an anti-GFP antibody. (Fig. 2B). ChFP-LARG FL is detected in the GFP-LARG FL immunoprecipitate, indicating that, as expected, LARG FL homo-oligomerizes (Fig. 2B). In contrast, the 2IA mutation strongly disrupted oligomerization, as revealed by the lack of coimmunoprecipitation of ChFP-LARG FL-2IA with GFP-LARG FL-2IA (Fig. 2B). GFP-LARG FL-2IA is able to activate Rho-mediated SRE-luciferase gene transcription to levels similar to those of GFP-LARG FL (Fig. 2C), suggesting that the 2IA mutation does not disturb the structural folding LARG. In summary, the coiled-coil domain prediction and the demonstration that the point mutations of isoleucines 1507 and 1510 disrupt LARG-LARG interaction (Fig. 2, A and B) strongly suggests that the short coiled-coil domain in the extreme C terminus mediates oligomerization of LARG.

Oligomerization of LARG Determines Its Cytoplasmic Retention. To assess whether the C-terminal coiled-coil domain-mediated oligomerization regulates the cytoplasmic retention of LARG, we examined the subcellular distribution of monomeric GFP-LARG FL-2IA. We were surprised to find that the steady-state distribution of monomeric GFP-LARG FL-2IA is cytoplasmic (Fig. 3A, middle) similar to oligomeric GFP-LARG FL (Fig. 3A, top). This cytoplasmic localization of GFP-LARG FL-2IA is in contrast to the strong steady-state nuclear localization of GFP-LARG\textsubscript{AC} (Fig. 1B). However, this apparent paradox was resolved through the use of LMB, an inhibitor of the CRM1/Exportin 1 nuclear export pathway. When Cos-7 cells expressing GFP-LARG FL-2IA were treated with LMB for 2 h, GFP-LARG FL-2IA displayed a dramatic accumulation in the nucleus (Fig. 3A, middle). The effect of LMB was quantitated by scoring cells for the following subcellular distribution phenotypes: N < C for higher fluorescence intensity in the cytoplasm; N = C for equal fluorescence intensity in the nucleus and cytoplasm; and N > C for higher fluorescence intensity in the nucleus (Fig. 3B). In the absence of LMB, approximately 80% of the cells expressing GFP-LARG FL-2IA show the N < C phenotype, but treatment with LMB for 2 h drastically reduces the N < C phenotype to only approximately 10% of the cells. In addition, LMB treatment increases the N = C phenotype to approximately 80% from only approximately 20% of the cells with this phenotype in the absence of LMB. As a positive control, GFP-arrestin\textsubscript{3} showed the N > C phenotype in the majority of the cells after LMB treatment (Wang et al., 2003) compared with the predominant N < C in the absence of LMB (Fig. 3B, bottom).

In contrast to the effect of LMB on monomeric GFP-LARG FL-2IA, LMB treatment did not change the predominantly cytoplasmic distribution of GFP-LARG FL (Fig. 3B, top). It remained mostly cytoplasmic even after 16 h of LMB treatment (data not shown). Thus, GFP-LARG FL does not seem to undergo nucleocytoplasmic shuttling, at least under the conditions of these experiments. However, disruption of oligomerization of LARG by the two isoleucine-to-alanine point mutations prevents cytoplasmic retention of LARG and promotes nucleocytoplasmic shuttling, as revealed by LMB treatment (Fig. 3, A and B).

Why does GFP-LARG\textsubscript{AC} reside strongly in the nucleus but GFP-LARG FL-2IA apparently shuttles in and out of the nucleus with a strongly cytoplasmic steady-state distribution? A likely reason is that GFP-LARG\textsubscript{AC} translocates into the nucleus because of a lack of oligomerization but has a defect in nuclear export and thus shows nuclear accumula-
tion in the absence of LMB. Nuclear export is addressed below. Regardless, the key finding in this set of experiments is that disruption of oligomerization of full-length LARG allows nucleocytoplasmic shuttling.

To further test the proposal that oligomerization functions to retain LARG in the cytoplasm, we asked whether induced dimerization of a monomeric LARG would prevent its nuclear localization. We selected to induce dimerization of LARGΔC because of its readily observable strong nuclear localization using an analog of the synthetic bivalent-dimerizing ligand rapamycin and the rapamycin-binding proteins FRB and FKBP (Muthuswamy et al., 1999; Banaszynski et al., 2005; Paulmurugan and Gambhir, 2005). GFP-LARGΔC chimeras containing the FKBP protein or an FRB domain (Fig. 4A) were transfected into Cos-7 cells in the presence or absence of the rapamycin analog AP21967 (a gift of Arrad Pharmaceuticals; for more information, see Bayle et al., 2006) for 24 h. Treatment with the dimerizer AP21967 caused a strong decrease in nuclear localization (Fig. 4C). Live cells were also scored for subcellular distribution phenotypes (Fig. 4B). Treatment with AP21967 resulted in an approximately 4-fold increase in the N < C phenotype (from ~6 to ~23%) and 2-fold increase in the N = C phenotype (from ~22 to ~50%) compared with nontreated cells expressing GFP-LARGΔC-FRB and GFP-LARGΔC-FKBP (Fig. 4C). This increase in the N < C and N = C phenotypes is paralleled by the decrease in the N > C phenotype from 72% in nontreated cells to only 26% in treated cells expressing GFP-LARGΔC-FRB and GFP-LARGΔC-FKBP, when expressed individually, localize to the nucleus in the presence or absence of AP21967 (data not shown). Thus, induced dimerization of GFP-LARGΔC strongly reduces its ability to translocate to the nucleus. The nuclear GFP-LARGΔC-FRB and GFP-LARGΔC-FKBP in the

Fig. 2. Oligomerization of LARG is mediated by an extreme C-terminal coiled-coil domain. A, coils output for LARG as predicted by COILS 2.2 (MTIDK matrix, weighed) and amino acid sequence of the targeted coiled-coil. Mutated residues are indicated by asterisks (*). B, substituting isoleucines 1507/1510 with alanines disrupts oligomerization of LARG. Cos-7 cells were transfected with GFP-LARG FL and ChFP-LARG FL or GFP-LARG FL-2IA and ChFP-LARG FL-2IA, as indicated. Twenty-four hours after transfection, cells were lysed, and GFP-LARG FL or GFP-LARG FL-2IA were immunoprecipitated with a polyclonal anti-GFP antibody. Coimmunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with a polyclonal anti-ChFP antibody (anti-ChFP). Immunoblotting of the immunoprecipitates with a polyclonal anti-GFP antibody reveals similar amounts of immunoprecipitated GFP-LARG FL and GFP-LARG FL-2IA (anti-GFP IP). Cell lysates were immunoblotted with an anti-ChFP antibody (TCL, top) and an anti-GFP antibody (TCL, bottom) to determine the expression levels of the ChFP and GFP constructs, respectively. Results shown are representative of at least three independent experiments. C, monomeric GFP-LARG FL-2IA activates SRE-luciferase gene transcription similar to oligomeric GFP-LARG FL. Cos-7 cells were transfected with the indicated amounts of LARG constructs, pSRE-Luc, and pcDNA3. Cells were lysed 24 h after transfection and assayed for luciferase activity. Luciferase activity was normalized for expression differences as detected by densitometry readings of cell lysates immunoblotted with a polyclonal GFP antibody. Results are presented as -fold increase over pcDNA3-transfected cells, and are the means ± S.E from at least three independent experiments. There is no significant statistical difference between GFP-LARG FL and GFP-LARG FL-2IA.
presence of AP21967 may reflect GFP-LARGΔC-FRB or GFP-LARGΔC-FKBP present as monomers and/or dimers with a weak ability to be imported into the nucleus. In summary, these results demonstrate that whereas mutationally disrupting the ability of the GFP-LARG FL to oligomerize leads to its nucleocytoplasmic shuttling (Fig. 3, A and B), inducing dimerization of the monomeric and strongly nuclear LARGΔC leads to its retention in the cytoplasm (Fig. 4).

The N Terminus of LARG Contains a Functional NLS. Mutational disruption of oligomerization of full-length LARG allows nucleocytoplasmic shuttling, suggesting that monomeric LARG is actively imported into the nucleus. Consistent with a model in which monomeric LARG is actively imported instead of diffusing into the nucleus, nuclear import of GFP-LARGΔC requires the Ran GTPase, as shown by inhibition of nuclear localization when a dominant-interfering Ran mutant is expressed (Supplemental Fig. S3B), and FRAP analysis (Supplemental Fig. S3A) indicates that nuclear import seems to be a slow process.

Thus, we asked whether LARG contains a functional NLS. The classic NLS consists of either a short basic stretch of amino acids rich in lysines and arginines or two basic stretches separated by approximately 10 nonconserved residues (bipartite NLS) (Jans et al., 2000). PSORTII predicts three NLSs in LARG: a seven-amino acid stretch 29PTD-KKQK35; a four-amino acid stretch 450KRRP453; and a bipartite NLS 593KRRGFPSILGPPRRPSR569 (Nakai and Horton, 1999). PRG and p115RhoGEF are also predicted to contain nuclear localization signals. A comparison of the location of the NLS of LARG, PRG, and p115RhoGEF, reveals the extreme N-terminal NLS of LARG as unique. This, together with our observation that PRGΔC (Fig. 1A) and p115RhoGEFΔC (Bhattacharyya and Wedegaertner, 2003), unlike LARGΔC (Fig. 1A), localize to the cytoplasm, led us to hypothesize that the N terminus of LARG carries a functional NLS. Thus, we deleted both the N and C termini of LARG generating a construct, GFP-LARGΔN/ΔC, consisting of amino acids 359 to 1160 (Fig. 5A, left). When expressed in Cos cells, GFP-LARGΔN/ΔC localizes to the cytoplasm (Fig. 5A, right), indicating that deletion of the N terminus prevents nuclear localization of the strongly nuclear GFP-LARGΔC. GFP-LARGΔN, in which only the N terminus of LARG has been removed, also localizes to the cytoplasm (Fig. 5A, right). It is interesting that GFP-LNPRGΔC, in which the N terminus of PRG has been replaced with the N terminus of LARG and the C terminus has been deleted, exhibits a strong nuclear localization (Fig. 5A, right), unlike the cytoplasmic/cortical actin localization of GFP-PRGΔC (Fig. 1). Thus, these results involving the deletion of the N terminus of LARG and swapping the N terminus of LARG into PRGΔC are consistent with the prediction that the N terminus of LARG carries a functional NLS.

To more precisely define the predicted N-terminal NLS of LARG, the positively charged lysines at positions 32 and 33 (Fig. 5B) were substituted with glutamines (2Q). The 2Q mutation was introduced into the background of GFP-LARG

![Fig. 3. Disruption of oligomerization induces nucleocytoplasmic shuttling of LARG. A, GFP-LARG FL-2IA localizes to the nucleus upon LMB treatment. Cos-7 cells transfected for 46 h with GFP-LARG FL, GFP-LARG FL-2IA, or GFP-arrestin3 and were incubated in the presence of 20 ng/ml LMB for the final 2 h. Cells were fixed and stained with DAPI and analyzed for the localization pattern of each mutant. Representative images of the dominant cellular distribution phenotype for each condition are shown. Bars, 10.2 μm. B, the nuclear fraction of GFP-LARG FL-2IA increases in the presence of LMB. Cellular distribution phenotypes were determined as listed: N = C, cells showing equal fluorescence intensities in the nucleus and cytoplasm; N > C, cells showing higher fluorescence intensity in the nucleus; and N < C, cells showing lower fluorescence intensity in the nucleus. Results are a percentage of cells with each phenotype and are the means ± S.E. from at least 100 cells analyzed in three independent experiments. Statistical differences between treated and nontreated cells for each phenotype are indicated by asterisks (two-tailed t test: **, P < 0.01; *, P < 0.05).](molpharm.aspetjournals.org)
FL-2IA and GFP-LARGΔC. GFP-LARG FL-2IA/2Q retains a cytoplasmic localization upon LMB treatment (Fig. 5C), in contrast to GFP-LARG FL-2IA, which shows a significant nuclear localization after LMB treatment (Fig. 3A). Moreover, the 2Q mutation prevents nuclear localization of GFP-LARGΔC; GFP-LARGΔC-2Q displays a cytoplasmic localization (Fig. 5C). It is noteworthy that these results demonstrate that lysines 32 and 33 are necessary for the nucleocytoplasmic shuttling of full-length monomeric LARG (i.e., GFP-LARG FL-2IA), suggesting that these lysines are critical components of a NLS.

Next, we investigated whether the N-terminal NLS of LARG could target an unrelated protein to the nucleus. The ChFP seemed as a good candidate for this purpose because it does not contain any NLSs or NESs. Because of its small size (approximately 28 kDa), which allows for passive diffusion into the nucleus, a significant portion of ChFP localizes to the nucleus (data not shown), making it difficult to visualize NLS-induced nuclear localization. To overcome this problem, we constructed an mCherry fluorescent fusion protein ChFP2x consisting of two repeats of the mCherry, which allowed for a stronger cytoplasmic content as detected in live cells expressing ChFP2x (Fig. 5D). ChFP2x-LARG(29–35) consisting of the mCherry fusion protein and the putative NLS of LARG showed a similar distribution to ChFP2x, although a control, ChFP2x-SV40NLS3x, which contains three repeats of the NLS of SV40, shows a completely nuclear distribution (Fig. 5D). Thus, we examined whether additional amino acids were necessary to define a functional NLS of LARG. When LARG amino acids 22 to 41 were fused to ChFP2x only a small increase in nuclear localization was observed (data not shown); however, a fusion protein of ChFP2x with amino acids 15–41 was sufficient to target the ChFP2x-LARG(15–41) to the nucleus (Fig. 5D).
acids 15 to 41 of LARG exhibited a nuclear distribution similar to that of ChFP2x–SV40NLS3x. Thus, the short basic stretch of amino acids 29 to 35 of LARG requires additional N-terminal basic residues in the linear sequence of LARG to target an unrelated protein into the nucleus. This may reflect the minimum number of residues required for the proper folding of the NLS and suggest that the functional NLS of LARG may conform to a bipartite nature. Taken together, the experiments shown in Fig. 5 demonstrate a functional NLS in the extreme N terminus of LARG.

**The C Terminus Is Required for Nuclear Export of Monomeric LARG.** Finally, we asked whether we could define regions of the protein that are involved in nuclear export of nucleocytoplasmic shuttling forms of LARG. The strong nuclear localization of GFP-LARGΔC compared with the much stronger cytoplasmic steady-state localization of GFP-LARG FL-2IA suggests that GFP-LARGΔC undergoes either no nuclear export or nuclear export that is slower than import. Indeed, consistent with little or no nuclear export, after photobleaching of the cytoplasm of cells with GFP-LARGΔC distributed in the cytoplasm and nucleus (similar to the image shown in Supplemental Fig. S3A), there was no significant recovery of fluorescence in the cytoplasm and/or loss of fluorescence in the nucleus over a time period of 1 h (data not shown).

To attempt to define further a role for the C terminus in nuclear export, we analyzed a series of C-terminal deletions. Cells expressing GFP-LARG FL, GFP-LARG(1–1490), GFP-LARG(1–1440), GFP-LARG(1–1340), GFP-LARG(1–1240), or GFP-LARGΔC were scored for the N < C, N = C, and N > C subcellular distribution phenotypes (Fig. 6A). A key feature of the distribution phenotypes for the truncation mutants is the strong presence of the N = C phenotype for GFP-LARG(1–1340), GFP-LARG(1–1440), and GFP-LARG(1–1490). Whereas the N > C phenotype, which predominates in GFP-LARG(1–1240) and GFP-LARGΔC, is consistent with a protein undergoing nuclear import but relatively slow nuclear export, the N = C phenotype is consistent with a protein undergoing nuclear export at a similar rate with nuclear import. Therefore, the strong presence of the N = C phenotype for GFP-LARG(1–1340), GFP-LARG(1–1440), and GFP-LARG(1–1490) suggests that these mutants are substantially exported out of the nucleus. Thus, we tested whether these deletion mutants were sensitive to LMB. Indeed, GFP-LARG(1–1340) shows an increased nuclear accumulation when cells are treated with LMB (Fig. 6B). Taken together, these results indicate that removal of the 384 C-terminal amino acids of LARG prevents nuclear export, but smaller C-terminal deletions recover some nuclear export function. Thus, the C terminus functions in mediating nuclear export of monomeric LARG. Analysis of the amino acid sequence of LARG for similarities with the leucine-rich CRM1-dependent nuclear export signal (ϕ-x_{n+1}ϕ-x_{n+2}ϕ-x_{n+3}ϕ-x_{n+4}; ϕ = L, I, V, F, M; x = any amino acid) (Kutay and Güttinger, 2005) reveals a potential NES between amino acids 1242 to 1268 in LARG. However, although fusion of amino acids 1242–1268 of LARG to the otherwise mostly nucleolar ChFP-LARG(15–41) led to an increase in cytoplasmic localization, extensive mutagenesis of leucine residues within amino acids 1242 to 1268 did not affect the localization of GFP-LARG-2IA (data not shown), indicating that these residues do not constitute an NES in the context of full-length monomeric LARG. The C terminus, therefore, may contribute an NES that is not apparent in the linear amino acid sequence of LARG but that becomes available in the structural folding of the protein. As an alternative, the C terminus could mediate nuclear export of monomeric LARG through interaction(s) with other NES-carrying protein(s).

**Discussion**

We have identified that a predicted coiled-coil sequence composed of amino acids 1507 to 1520 in the extreme C terminus mediates the oligomerization of LARG. We determined that disruption of the coiled-coil domain by replacing both isoleucine 1507 and isoleucine 1510 with alanines strongly impairs the ability of LARG to oligomerize (Fig. 2, A and B) and confers upon LARG a nucleocytoplasmic shuttling ability (Fig. 3). In addition, we establish that monomeric GFP-LARG FL-2IA is imported into the nucleus via an extreme N-terminal NLS. Furthermore, after nuclear targeting, monomeric LARG is rapidly exported out of the nucleus via the CRM1-dependent nuclear export pathway.

Our results demonstrate that the extreme C-terminal and N-terminal sequences of LARG, function as key regulators of its localization. Nuclear versus cytoplasmic localization of LARG is determined by a tightly regulated interplay between
the coiled-coil domain and a putative nuclear export sequence in the C terminus and the NLS in the extreme N terminus. Thus, the C-terminal coiled-coil domain of LARG, through its ability to oligomerize, functions as a cytoplasmic retention signal, and, although LARG carries a functional NLS in its N terminus, it can only undergo nucleocytoplasmic shuttling when its ability to oligomerize has been disrupted. The mostly monomeric GFP-LARG-2IA mutant localizes to the nucleus upon inhibition of nuclear export by LMB, whereas GFP-LARG FL remains cytoplasmic after LMB treatment (Fig. 3B). In addition, forced dimerization of GFP-LARGAC-FRB/GFP-LARGAC-FKBP through the rapamycin-inducible dimerization system inhibits nuclear localization of GFP-LARGAC (Fig. 4), consistent with a role for oligomerization in regulating nucleocytoplasmic shuttling of LARG. COILS predicts several other coiled-coil domains in the linear sequence of LARG; however, removal of a short C-terminal stretch of amino acids including only the coiled-coil domain between amino acids 1507 to 1520 is enough to disrupt the cytoplasmic retention of LARG. Thus, although it is possible that additional coiled-coil domains in the linear sequence of LARG may participate in its oligomerization, the C terminus coiled-coil domain represents the major determinant of oligomerization and cytoplasmic retention of LARG. Oligomerization may be a common mechanism for regulating nuclear transport of proteins. In addition to LARG, several proteins, including yeast Ste5, Smad4, and p53, have their nuclear transport regulated by oligomerization (Stommel et al., 1999; Watanabe et al., 2000; Wang and Elion, 2003).

We show that nuclear export of monomeric LARG, as revealed by the LMB sensitivity of GFP-LARG FL-2IA, occurs via the CRM1 export pathway. LMB treatment also increased the nuclear localization of GFP-LARG(1–1340) (Fig. 6B), GFP-LARG(1–1440), and GFP-LARG(1–1490) (data not shown). This, together with the distribution pattern of the series of truncation mutants in which N = C is a dominant phenotype for GFP-LARG(1–1340), GFP-LARG(1–1440), and GFP-LARG(1–1490) (Fig. 6A) indicate that the C terminus mediates nuclear export of monomeric LARG. Mutagenesis of putative NES in the linear sequence of LARG, however, had no effect on the localization of monomeric GFP-LARG FL-2IA. Thus, the C terminus could regulate nuclear export of monomeric LARG by mediating functionality of NES that were not apparent in the linear sequence of LARG and/or do not participate in protein-protein interactions or modifications that regulate the functionality of NES. Further studies will be needed to characterize the CRM1-dependent nuclear export mechanism of LARG.

The predicted N-terminal NLS (56PTDKKK56) of LARG is necessary for the nuclear import of monomeric LARG as revealed by the cytoplasmic retention of GFP-LARG FL-2IA or GFP-LARGAC mutants, in which lysines 32 and 33 have been replaced with glutamines (Fig. 5, B and C) or glutamic acids (data not shown). However, the stretch of amino acids that is sufficient for nuclear import requires additional N-terminal basic residues. As shown in Fig. 4D, fusion of LARG amino acids 29 to 35 or 21 to 35 to ChFP2 does not promote nuclear localization, but ChFP2-LARG(15–41) shows a mostly nuclear distribution. A role for flanking residues in mediating import receptor binding is a feature of several nuclear cargo proteins (Jans et al., 2000). As an alternative, when minimal stretch required for nuclear targeting contains two clusters of basic residues (amino acids 15–21 and 29–35), the functional NLS of LARG may be bipartite. It is interesting that the Net1 RhoGEF contains two N-terminal NLSs, whereas the Ect2 contains two NLSs located centrally in the linear sequence (Tatsumoto et al., 1999; Schmidt and Hall, 2002b). FRAP analysis (Supplemental Fig. S3A) for GFP-LARGC reveals that its nuclear import as monitored in live cells is slow. Consistent with a slow import, inhibition of nuclear export of GFP-LARG FL-2IA results in a drastic increase only in the N = C phenotype as opposed to a large increase in the N > C phenotype, which would indicate a faster nuclear import. LARG is a multidomain protein and as such it may participate in several protein interactions, which could function in keeping LARG out of the nucleus and in cytoplasmic compartments or at the PM, as indicated by the translocation of GFP-LARG FL or GFP-LARGC to the plasma membrane in the presence of α5QL (Fig. 1C).

It is well established that protein function is tightly regulated by temporal and spatial expression patterns. The ability of LARG to translocate to the nucleus may have evolved as a means to control its ability to activate its cytoplasmic targets such as the Rho GTPase, as has been previously suggested for the Net1 and Ect2 exchange factors (Tatsumoto et al., 1999; Schmidt and Hall, 2002b). A previous report showing higher activity of large C-terminal truncations of rgsRhoGEFs raised the possibility that monomeric forms could be more active than the oligomeric forms (Chikumi et al., 2004). Our results for LARG instead suggest that C-terminal-mediated oligomerization and regulation of activity are separable. Although the monomeric LARGC displays higher signaling activity than LARG FL (Supplemental Fig. S2), the monomeric LARG-2IA activates SRE-dependent gene transcription similarly to LARG FL (Fig. 2C). A recent study reported similar results for Lsc, the murine ortholog of p115RhoGEF (Eisenhaure et al., 2003). Thus, our results with LARG and those reported for Lsc are consistent with the C termini of rgsRhoGEFs having multiple regulatory functions. For LARG, the extreme C terminus mediates oligomerization. Other C-terminal regions may also be responsible for regulating activity, possibly via intramolecular inhibition or by mediating interaction with additional proteins.

In addition, the presence of LARG in the nucleus may indicate a nuclear-specific function of this protein. Recent work has revealed that the Rho-kinase Rho-associated coiled-coil containing protein kinase 2 localizes to the nucleus, in which it targets p300 for phosphorylation and regulates its acetyltransferase activity (Tanaka et al., 2006). In addition, Rho and additional Rho effectors such as phospholipase D have been detected in the nucleus through fractionation studies (Balboa and Insel, 1995; Baldassare et al., 1997). It is interesting that several RGS proteins have been identified to localize to the nucleus, and although their nuclear functions are still emerging, several lines of evidence suggest that they can directly or indirectly regulate gene transcription (Burchett, 2003). It is remarkable that the N terminus of LARG, which we have identified to carry a functional NLS, is absent from the MLL-LARG fusion protein (Kourlas et al., 2000; Popovic and Zeleznik-Le, 2005). It is tempting to speculate that in the x'-LARG fusion protein, the NLS, and DNA binding motifs of MLL (Li et al., 2005; Popovic and Zeleznik-Le, 2005) override the cytoplasmic retention signals of LARG and target MLL-LARG to the nucleus. In addition, as pro-
posed for many MLL-fusion partners (Li et al., 2005), LARG, through its ability to oligomerize, may also force oligomerization of MLL and lead to aberrant gene expression.

Numerous factors may function in disrupting oligomerization of LARG and inducing its nucleocyttoplasmic shuttling. Such factors may include signals from cell surface receptor, protein kinases and phosphatases, protein modifications such as ubiquitination, and interactions with proteins that may function as anchors for a particular cellular compartment. It will be important to determine the cellular mechanisms that regulate oligomerization of LARG and other Rho-GEFs.

Acknowledgments

We thank Dr. Peter Day for critical reading of the manuscript.

References


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