RGS14 Is a Centrosomal and Nuclear Cytoplasmic Shuttling Protein That Traffics to Promyelocytic Leukemia Nuclear Bodies Following Heat Shock*

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RGS14, a member of the regulator of G-protein signaling (RGS) protein family, possesses an N-terminal RGS domain, two Raf-like Ras-binding domains, and a GoLoco motif, which has GDP dissociation inhibitor activity. In this study we show that unique among the known mammalian RGS proteins, RGS14 localizes in centrosomes. Its first Ras-binding domain is sufficient to target RGS14 to centrosomes. RGS14 also shuttles between the cytoplasm and nucleus, and its nuclear export depends on the CRM-1 nuclear export receptor. Mutation of a nuclear export signal or treatment with leptomycin B causes nuclear accumulation of RGS14 and its association with promyelocytic leukemia protein nuclear bodies. Furthermore, a point mutant defective in nuclear export fails to target to centrosomes, suggesting that nuclear cytoplasmic shuttling is necessary for its proper localization. Mild heat stress, but not proteotoxic or transcription-linked stresses, re-localizes the RGS14 from the cytoplasm to promyelocytic leukemia nuclear bodies. Expression of RGS14, but not point mutants that disrupt the functional activity of its RGS domain or GoLoco motif, enhances the reporter gene activity. The multifunctional domains and the dynamic subcellular localization of RGS14 implicate it in a diverse set of cellular processes including centrosome and nuclear functions and stress-induced signaling pathways.

Many of the members of the regulator of G protein signaling (RGS) family of over 30 proteins regulate signaling pathways initiated by receptors that couple to heterotrimeric G proteins. They do so by enhancing the rate of GTP hydrolysis of Ga subunits, thus reducing the duration that the Ga subunits are GTP-bound (1–4), by antagonizing the interactions between activated Ga subunits and effectors (5, 6), by inhibiting the dissociation of guanine nucleotides from the Ga subunits (7). However, emerging evidence suggests that RGS proteins, particularly those that contain domains in addition to their signature RGS domain, have other cellular functions (for recent reviews see Refs. 8 and 9).

RGS14 is a GTPase-activating protein (GAP) for the Gα subfamily but not for the Gαs, Gαi, or G12/13α subfamily (10). It possesses an N-terminal RGS domain that is necessary and sufficient for its GAP activity and several other domains, including two Raf-like Ras-binding domains (RBD) and a GoLoco motif (7, 11). A region overlapping the first RBD domain and portion of the second RBD has been designated as a Rap-interaction domain (RID) because it mediated the interaction of RGS14 with GTP-bound forms of Rap1 and Rap2 but not Ras (12). The functional importance of this binding and whether the RBD domains might interact with other small GTPases is unknown. The GoLoco motif of RGS14 has GDP dissociation inhibitor activity (GDI) for Gα. Although its physiological significance remains enigmatic, the GoLoco motif does enhance the inhibitory effect of RGS14 on Gα signaling triggered through the muscarinic receptor (13). Three regions of RGS14 also show weak homologies to the DNA binding domains of high mobility group I and Y proteins (Alliance for Cellular Signaling RGS14 Molecular Page).

The expression of RGS14 occurs in many tissues, although the highest levels are in the caudate nucleus of the brain, spleen, and thymus (Gene Expression Atlas, expression.gnf.org). Among individual cell types and cell lines, high levels have been detected in lymphocytes, monocytes, dendritic cells, and lymphocyte cell lines (10). Most interestingly, the stimulation of human monocyte-derived dendritic cells with lipopolysaccharide and murine B cells with their cognate antigen results in a rapid reduction in RGS14 expression (14, 15). In contrast, the stimulation of human B cells via antigen receptor cross-linking or human T cells with the monoclonal antibody CD3 increases RGS14 expression (10). Protein kinase A phosphorylates rat RGS14 in vitro on serine 276 and threonine 494. Mimicking the phosphorylation of RGS14 at threonine 497 enhances its GDI activity (16). Underscoring the importance of RGS14 for cellular function, Rgs14−/− mouse embryos die prior to implantation (17).

In this study, we examine the intracellular localization of RGS14. We find that RGS14 is targeted to multiple intracellular locations, including centrosomes and promyelocytic leukemia protein (PML) nuclear bodies. RGS14 undergoes nuclear cytoplasmic shuttling that appears necessary for its centrosome targeting. We identify the regions in the RGS14 responsible for targeting to various intracellular locations, and we demonstrate a dynamic trafficking of RGS14 to the PML nuclear bodies in response to mild heat stress. Finally, we show that RGS14 may
directly or indirectly regulate gene transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Materials**—HeLa and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfection of the HeLa cells was performed using Superfect (Qiagen, Germantown, MD). The total amount of plasmid DNA for each transfection was always normalized with vector DNA. Leptomycin B and anti-FLAG monoclonal antibody were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). Anti-pericentrin and anti-PML antibodies were purchased from Covance (Princeton, NJ) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

**RGS14 Constructs and Site-directed Mutagenesis**—DNA fragments containing various regions of RGS14 protein were PCR-amplified by using gene-specific primers incorporating restriction sites (HindIII and BamHI) to facilitate their cloning into enhanced GFP vector. The isolated DNA fragments were then cloned into HindIII- and BamHI-digested pN1-enhanced GFP vector (Clontech) in-frame with C-terminal GFP following restriction enzyme digestion and agarose gel purification. All of the cloned RGS14 constructs were sequenced to confirm proper cloning, and protein lysates from HeLa cells transfected with the constructs were analyzed for proper expression of proteins by immunoblotting with anti-GFP antibody (Santa Cruz Biotechnology).

To introduce point mutations, Quick Clone mutagenesis kit (Stratagene, La Jolla, CA) was used as recommended by the manufacturer. Proper changes in the targeted amino acids were confirmed by DNA sequencing. To replace the GFP tag with the FLAG tag, regions of the RGS14 were PCR-amplified by using gene-specific primers incorporating restriction sites (HindIII and BamHI) and cloned into the pXFLAG-CMV10 vector (Sigma) as described above. The cloned RGS14 constructs were sequenced to confirm proper cloning.

**Antibodies and Immunocytochemistry**—To raise anti-RGS14 peptide antiserum, three peptide sequences of mouse RGS14 protein were chosen based on their high scores of antigenicity by using MacVector (Accelrys, San Diego, CA). The sequences of the mouse peptides are almost identical to those of human RGS14. The sequences of three peptides were as follows: peptide 1, "MPKPKHLLV" (human RGS14,1MPKPKHLLV59); peptide 2, "455SCRSQGSCP67" (human RGS14, 455SCRSQGSCP465); and peptide 3, "525PAQGPPSEQ544 (human RGS14, 525PAQGPPSEQ545). For each peptide, one cysteine residue was added to provide for easy labeling with fluorescein isothiocyanate and a krypton laser at 568 nm for Alexa568. Fluorochromes were excited using an argon laser at 488 nm for fluorescein isothiocyanate and a krypton laser at 568 nm for Alexa568. Hoechst 33342 was excited using an argon laser at 364 nm. Detector slits were configured to minimize any cross-talk between the channels. Differential interference contrast (DIC) images were collected simultaneously with the fluorescence images using the transmitted light detector. Images were processed using the Leica TCS-NT/SP software (version 1.65.587), Imaris 3.2 (Bitplane AG, Zurich Switzerland), and Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

**Reporter Assays**—Various DNA constructs directing the expression of wild type, a human short form, and three different mutants of RGS14 were transfected into HeLa cells along with pRL-TK vector or pCMV-luciferase construct. The pRL-TK vector contains herpes simplex virus thymidine kinase (HSV-TK) promoter and is designed to provide constitutive expression of Resilla luciferase in mammalian cells (Promega, Madison, WI). One day after transfection, cells were transfected into a passive lysis buffer (Promega), and the luciferase activities in each cell lysate were measured using Dual-Luciferase™ reporter assay kit (Promega). The reporter activities were normalized with transfection efficiency of GFP fusion constructs using flow cytometry.

**RESULTS**

**Dynamic Subcellular Localization of RGS14**—To examine intracellular localization of RGS14 and to map the region(s) responsible for its targeting to specific locales, GFP fusion constructs of RGS14 containing various deletions were generated and designated HC1 to HC11. The GenBank™ accession number of the mouse clone used in this study is U85055. RGS domain required for the GAP activity (amino acids 67–184), two Raf-like Ras binding domains (RBDs, amino acids 303–374 and 376–445), Rap-interacting domain (RID, amino acids 300–427), and GoLoco domain required for the GDI activity (Loco, amino acids 500–522) are indicated as filled boxes. The numbers indicate the length of corresponding RGS14 fragment. Lower-case letters on the right column indicate intracellular localization of each construct shown in B, localization of GFP fusion proteins. Confocal microscopy was performed with live cells expressing various GFP fusion constructs of RGS14. The constructs exhibited distinct intracellular localization ranging from exclusively cytoplasmic (b) to exclusively nuclear (e). Wild type RGS14 showed intense perinuclear dot-like staining (a). HC6, HC7, and HC9 also localized to the perinuclear dot-like structures (c). The vector GFP construct showed an evenly distributed GFP expression throughout the cell (f) as the HC8 construct did (d). The centrosomal and nuclear dot-like structures in this figure appear larger because they are overexposed to capture the much less prominent cytoplasmic or nucleoplasmic GFP staining. Confocal zoom factor of 1 was used to capture the images.

Fig. 1. Intracellular localization of RGS14. A, constructs. Mouse RGS14-GFP constructs containing various deletions were generated and designated HC1 to HC11. The GenBank™ accession number of the mouse clone used in this study is U85055. RGS domain required for the GAP activity (amino acids 67–184), two Raf-like Ras binding domains (RBDs, amino acids 303–374 and 376–445), Rap-interacting domain (RID, amino acids 300–427), and GoLoco domain required for the GDI activity (Loco, amino acids 500–522) are indicated as filled boxes. The numbers indicate the length of corresponding RGS14 fragment. Lower-case letters on the right column indicate intracellular localization of each construct shown in B, localization of GFP fusion proteins. Confocal microscopy was performed with live cells expressing various GFP fusion constructs of RGS14. The constructs exhibited distinct intracellular localization ranging from exclusively cytoplasmic (b) to exclusively nuclear (e). Wild type RGS14 showed intense perinuclear dot-like staining (a). HC6, HC7, and HC9 also localized to the perinuclear dot-like structures (c). The vector GFP construct showed an evenly distributed GFP expression throughout the cell (f) as the HC8 construct did (d). The centrosomal and nuclear dot-like structures in this figure appear larger because they are overexposed to capture the much less prominent cytoplasmic or nucleoplasmic GFP staining. Confocal zoom factor of 1 was used to capture the images.
prominent expression of RGS14-GFP (expressed from HC1) in the cytoplasm of the transfected cells. In addition, ~10% of the GFP-positive cells showed intense perinuclear dot-like staining suggestive of centrosome staining (Fig. 1A, a). The deletion of the N-terminal 61 or 185 amino acids (expressed from HC2 and HC3) did not change the intracellular location compared with that of full-length RGS14-GFP. The 185-amino acid N-terminal deletion, however, resulted in larger perinuclear dots than we had observed with the full-length RGS14. HC4 and HC5 expressed proteins that encompass the C-terminal 193 or 105 amino acids of RGS14, respectively, localized mainly in the cytoplasm and they lacked any perinuclear dot-like structures (Fig. 1B, b). The HC8 construct, which expresses only the first RBD domain, showed homogeneous GFP expression throughout the cell (Fig. 1B, d) but not in the nucleoli as seen with the GFP vector control (Fig. 1B, f). This truncation mutant also showed no perinuclear dot-like staining. The GFP fusion proteins expressed from the HC6, HC7, and HC9 constructs did localize to perinuclear dot-like structures, although in contrast to full-length RGS14 they had a more prominent nuclear expression (Fig. 1B, c). Most surprisingly, the RGS14-GFP fusion proteins lacking the C-terminal portion of RGS14 (HC10 and HC11) mainly localized within the nucleus. Some of the cells transfected with HC10 or HC11 exhibited intense staining of subnuclear dot-like structures in addition to the diffused nuclear staining. GFP expression from the HC10 or HC11 constructs was excluded from nucleoli. The intracellular localization of constructs described in this study is summarized. The length of each construct is shown in amino acids. Point mutations introduced are also indicated. + indicates predominant localization.

Next, we have confirmed that the perinuclear dot-like structures of RGS14-GFP staining are indeed centrosomes by colocalization with an endogenous centrosome protein, pericentrin (Fig. 2). Pericentrin is a pericentriolar matrix protein that likely functions as a scaffolding protein for proteins recruited to the centrosome (18). Hemagglutinin-tagged RGS14 also localizes to the centrosomes (data not shown), demonstrating that the localization of RGS14-GFP to the centrosomes is not an artifact caused by GFP tagging of RGS14. Colocalization between the putative centrosomal GFP staining of HC6 and HC9 and pericentrin (Fig. 2) showed that the region containing the first RBD was sufficient to target RGS14 into the centrosome region. However, the perinuclear centrosome dots of the HC6-expressed GFP fusion protein were generally much larger than those of wild type RGS14 (HC1) or HC9, suggesting other regions of RGS14 may be required for proper targeting of RGS14.

We then examined the staining pattern of endogenous RGS14 in adherent cells such as NIH3T3 and HeLa cells to determine whether the recruitment of RGS14 to the centrosomes was cell cycle-dependent. Compared with lymphoid cell lines such as HS-Sultan and Jurkat, these cells expressed much lower levels of RGS14 mRNA (data not shown). NIH3T3 and HeLa cells were immunostained with anti-centrin and anti-RGS14 antibodies, and DNA was stained with Hoechst 33342 to determine the cell cycle positions (Fig. 3). At the G1 phase of the cell cycle, the anti-centrin antibody clearly stained a pair of centrioles within one centrosome. Cells in G2 or mitotic phase contained two centrosomes that were located at opposite ends. Cells in the mitotic phase were also identified by...
the presence of condensed chromosomes as shown with the Hoechst 33342 staining. Cells in S phase were identified by having two closely located centrosomes. Anti-RGS14 peptide antibody stained the area immediately adjacent to the centrioles that were immunostained by anti-centrin antibody, suggesting that RGS14 is a pericentriolar matrix protein. It appeared that endogenous RGS14 was associated with the centrosomes throughout the cell cycle. Previously characterized anti-recombinant RGS14 protein antiserum (10) that was further purified by using protein A/G affinity column chromatography also stained centrosomes (data not shown). The same immunostaining pattern by two antibodies raised independently in addition to expression of GFP- or hemagglutinin-tagged RGS14 in the centrosomes clearly demonstrates that RGS14 is a centrosome protein.

The results from the expression of the truncation mutants of RGS14 suggested that RGS14 might be a nuclear cytoplasmic shuttling protein. Full-length RGS14-GFP expression rarely occurred in the nucleus, whereas the C-terminally truncated RGS14 protein (HC10) resided mainly in the nucleus. To examine this possibility, we treated cells that expressed RGS14 with the fungicide leptomycin B (LMB) (Fig. 4), which inhibits the export of nuclear proteins mediated by CRM-1 receptor. We treated transfected HeLa cells with LMB for 2 h at 37 °C, and we monitored the localization of the RGS14-GFP fusion protein or GFP. We observed an apparent nuclear accumulation of RGS14-GFP within 1 h of drug treatment (Fig. 4A). To confirm the nuclear localization of RGS14, we merged the RGS14-GFP image and an image of the same cells stained the viable DNA dye Hoechst 33342 (Fig. 4B). The control cells that were not treated with the LMB exhibited cytoplasmic and centrosomal expression of RGS14-GFP. The drug did not affect the intracellular localization of the GFP protein expressed from the vector control DNA. These results demonstrate that RGS14 is a nuclear cytoplasmic shuttling protein and that the nuclear export of RGS14 depends upon the CRM-1 receptor.

A Functional RGS Domain, GoLoco Motif, or the Two Putative PKA-mediated Phosphorylation Sites Are Not Required for Centrosome Localization or Nuclear Cytoplasmic Shuttling of RGS14—In addition to the deletion constructs, we generated and expressed a series of constructs containing a point mutation(s) that disrupts the function of the RGS or the GoLoco domain or the interaction with Rap GTPase (Fig. 5A). We have also designed and expressed various constructs that are defective in PKA-mediated phosphorylation or that are phosphorylation mimics (Fig. 5A). The 120-amino acid RGS domain contains a number of residues previously defined as critical for GAP activity. The HC12 construct expresses an RGS14 protein that fails to inhibit G protein-coupled receptor signaling because of the introduction of mutations in two critical residues in its RGS domain, Glu-92 and Asn-93, needed for GAP activity (10). Previously identified by mutagenesis studies and characterized by structural studies, the GoLoco motif is essential for RGS14 GDI activity (19, 20). The highly conserved (D/E)QR triad in the GoLoco motif makes direct contact with the GDP- and H9251- and H9252-phosphates. Alanine substitution of a critical glutamine

![Endogenous RGS14 localizes to the centrosomes throughout the cell cycle.](image-url)
residue within the core GoLoco peptide is known to abolish GDI activity. Two hydrophobic leucine residues within the core sequence are also required for the GDI activity. We generated two GDI-defective mutants, one substituted an alanine for the critical glutamine (HC13), and the other substituted two alanines for the two leucine residues (HC14). Two specific sites in rat RGS14 protein, Ser-276 and Thr-494, are identified as the sites for PKA-mediated phosphorylation (16). Ser-261 and Thr-497 of mouse RGS14 and Ser-260 and Thr-495 of human RGS14 were identified as the rat counterparts. Mutants containing a single amino acid change in the serine or threonine residue to alanine or containing both amino acid changes (HC17) were constructed. In addition, we generated mutants that mimic a constitutively phosphorylated protein by changing the serine or threonine residue to a glutamic acid or an aspartic acid (HC18–21). Finally, we made an RGS14 mutant that no longer interacts with the small GTPase, Rap (HC22-1), by substituting a leucine for Arg-336 (13). Because it remained unclear whether the RBD domain or two RBD domains were necessary for RGS14 activity, we also introduced an alanine substitution to His-409 of the 2nd RBD that is the equivalent of Arg-336 of the 1st RBD (HC22-2). Additionally, we generated a mutant carrying both R336L and H409A changes (HC23).

Not surprisingly, the subcellular localization of the mutant lacking GAP activity (HC12) was similar to that of wild type RGS14-GFP (Fig. 5B) as the localization of a GFP fusion protein that lacked the entire RGS domain had not been disturbed. In addition, the localization pattern of HC13 expressing the Q518A mutant defective in the GDI activity was indistinguishable from that of RGS14-GFP (Fig. 5B). Mutant RGS14 proteins defective in phosphorylation, those that mimicked constitutive phosphorylation, and the RID/RBD mutants (HC22-1, HC22-2, and HC23) all showed the same localization pattern as wild type (Fig. 5B), suggesting that phosphorylation or small GTPase-binding activity is not necessary for the intracellular trafficking of RGS14. The RGS14 mutant protein expressed from the HC23 construct colocalized with the endogenous pericentrin (Fig. 5C). In contrast, the HC14 construct that directed expression of the L506A/L507A mutant predominantly localized in the nucleus as demonstrated in the merged image of GFP and Hoechst 33342 DNA staining (Fig. 5D). The nuclear GFP staining of the HC14 construct was excluded from nucleoli as seen with the HC10 and HC11 constructs. Approximately 10% of the cells transfected with the HC14 construct showed the staining of intra-nuclear dot-like structures in the cells expressing the HC14 construct. None of the intranuclear dot-like GFP staining of the L506A/L507A mutant colocalized with endogenous pericentrin (Fig. 5D). This result suggests that the nuclear cytoplasmic shuttling may be necessary for the centrosome localization of RGS14. The intracellular localizations of the RGS14 point mutants are also summarized in Table I.

Close examination of the amino acid sequences of mouse and human RGS14 proteins (21, 22) reveals that RGS14 contains multiple putative nuclear localization signals (NLSs) that we have named as NLS1, NLS2, and NLS3 (Fig. 6A). The NLS consists of very short amino acid stretches rich in basic positively charged amino acid residues such as lysine and arginine (23). Another type of NLS is bipartite NLS comprising two basic clusters separated by 10–12 variable amino acids (23). RGS14 appears to contain both types of NLSs with the NLS2 of RGS14 being the bipartite. The most common type of NES is a leucine-rich type found in proteins such as p53 and BRACA1 (23) and consists of a short peptide sequence with closely spaced large hydrophobic amino acids, in particular leucine or isoleucine (Fig. 6B). This leucine-rich type NES is recognized and bound by the export receptor, CRM-1. The leucine-rich type NES is found in the region overlapping with the GoLoco motif of RGS14. The nuclear accumulation of the HC14-expressed protein generated to abolish the GDI activity also indicates that one of the two leucine residues likely participates in the nuclear export of RGS14.

The region of RGS14 spanning from amino acids 297 to 423 (HC9, Fig. 1) was sufficient for centrosome targeting. That region contained the putative NLS3 near its C terminus (Fig. 6A). Therefore, we determined whether the NLS3 played any role in centrosome localization of RGS14. We mutated all five positively charged residues (three arginine and two lysine residues) in the NLS3 of HC9 to alanine by site-directed mutagenesis. The construct was named as HC25 and tested for its ability to localize to the centrosomes. The intracellular expression pattern of HC25 was similar to that of HC9. Colocalization of HC25-GFP with the endogenous pericentrin was further confirmed by immunocytochemistry (Fig. 6C), suggesting that the putative NLS3 may not be necessary for centrosome targeting of the HC9 construct. Because the GFP protein itself could be targeted to the nucleus (Fig. 1B, f), we tested whether the truncated RGS14 protein expressed from the HC9 construct that was tagged not with GFP but with the FLAG epitope tag could localize in the centrosomes. The GFP of both HC9 and HC25 was replaced with the FLAG tag. The new constructs were named HC9-FLAG and HC25-FLAG and were subjected to immunocytochemistry by using anti-FLAG and

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**Fig. 4.** CRM-1 receptor-dependent nuclear export of RGS14. A, HeLa cells were transfected with either GFP vector or wild type mouse RGS14-GFP and then treated with leptomycin B at 37 °C for 2 h. The nuclear accumulation of RGS14 caused by leptomycin B treatment was observed using confocal microscopy. The merged images (DIC merge) of GFP and differential interference contrast (DIC) are also shown. C stands for the control cells that were not treated with LMB. B, the same experiment was repeated with the wild type human RGS14-GFP. Cells were also incubated with a viable DNA dye, Hoechst 33324 (Ho) to stain the nuclei. The merged image of GFP and Hoechst 33324 clearly demonstrates the nuclear accumulation of human RGS14-GFP in the cells treated with leptomycin B.

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anti-pericentrin antibodies. Most surprisingly, the FLAG-tagged proteins localized in the cytoplasm without exhibiting any characteristic centrosome staining (Fig. 6C).

Most interestingly, Ser-261 and Thr-497 of mouse RGS14 (Ser-260 and Thr-495 of human RGS14) that are phosphorylated by PKA are located closely to the bipartite NLS2 and NES, respectively. Post-translational modification such as phosphorylation of specific sequences proximal to the nuclear import/export signals is a common mechanism to regulate the nuclear-cytoplasmic shuttling (23). Therefore, we examined whether the phosphorylation mimic RGS14 proteins (HC18–21) would respond differently to the LMB treatment; however, all of them exhibited the same nuclear accumulation pattern as wild type RGS14 did (data not shown). This suggests that phosphorylation of these sites in RGS14 does not regulate the nuclear cytoplasmic shuttling of the RGS14 protein.

RGS14 Is Mainly Associated with PML Nuclear Bodies in the Absence of Nuclear Export and Is Likely to Play a Nuclear Function in Heat Stress-induced Cellular Response—In silico analysis of RGS14 expressed sequence tag data base suggests the presence of a spliced variant, human RGS14-Short (hRGS14-S, GenBank™ accession number AF037194). The hRGS14-S consists of 201 amino acids. The sequence of amino acids 1–198 of the hRGS14-S is exactly the same as that of amino acids 154–350 of human RGS14 (RGS14, GenBank™ accession number GI:15559454). The last three amino acids (VQT) of hRGS14-S originate from a readthrough into intron 9. The RGS14-S contains the C-terminal half of the RGS domain and a portion of the first RBD domain. Neither the GoLoco domain nor the NES is present in this variant. Because it lacks...
the NES, we have generated a GFP fusion construct of RGS14-S (HC24), and we tested whether it localized (Fig. 7A). We found that RGS14-S-GFP is expressed mainly in the nucleus as expected; however, ~10% of the transfected cells show intense staining of subnuclear dot-like structures. The merged image of GFP and Hoechst (Ho) demonstrates the nuclear localization of hRGS14-S-GFP. Differential interference contrast (DIC) image is also shown. Confocal zoom factor of 2 was used for capturing the images. B, colocalization of hRGS14-S-GFP with the endogenous PML protein. HeLa cells transfected with hRGS14-S-GFP was indirectly immunostained with anti-PML antibody followed by goat Alexa568-conjugated anti-mouse antibody. Confocal microscopy was then performed with the immunostained cells. Confocal zoom factor of 6 was used for capturing the images. The merged image of green GFP and red Alexa568 is shown on the right. C, colocalization of RGS14-LL/AA-GFP with the endogenous PML protein. HeLa cells transfected with RGS14-LL/AA-GFP was indirectly immunostained with anti-PML antibody, and confocal images were collected as described above. The merged image of green GFP and red Alexa568 is shown on the right.
activity-defective Q518A mutant (HC13) exhibited the same pattern of trafficking in response to various cellular stresses (data not shown), suggesting the GAP and GDI activities were not required for heat-shock induced re-localization of RGS14 to the PML nuclear bodies.

When PML nuclear body-associated proteins are recruited to the PML nuclear bodies, they can often activate or repress gene transcription (26, 27). We therefore examined the ability of RGS14 protein to alter the gene transcription. Wild type (HC1), the human short form (HC24), and three different mutants (HC12–14) containing various point mutations were introduced into HeLa cells along with a luciferase reporter construct under the control of either HSV-TK or the CMV promoter. Both HSV-TK and CMV promoters direct constitutive expression of Renilla luciferase in mammalian cells. We then compared the luciferase activities of the cell lysates expressing various RGS proteins to those of control vector-transfected cells (Fig. 8A). In four independent experiments performed in duplicate, the expression of wild type RGS14 resulted in a 2-fold enhancement (p < 0.01) of luciferase activity. The nuclear export-defective L506A/L507A mutant also exhibited an increase (p < 0.01) in the luciferase activity. The GAP activity-defective E92A/N93A mutant, GDI activity-defective Q518A mutant, or hRGS14-S containing no functional RGS, RID, or GoLoco did not have a statistically significant effect of reporter gene activity.

**DISCUSSION**

In the present study, we demonstrate that RGS14 is a centrosomal and nuclear cytoplasmic shuttling protein, which undergoes heat shock-induced trafficking to PML nuclear bodies. Our study is the first to report localization of an RGS family member in the centrosomes and PML nuclear bodies, although there are reports of localization of RGS proteins in diverse intracellular compartments including the Golgi, endosomes, and mitochondria (4, 28, 29). Heterotrimeric G proteins have been implicated in regulation of centrosome movement and mitotic spindle formation. In *C. elegans*, Gα is important in regulating migration of the centrosome around the nucleus and hence in orientating the mitotic spindle. Gα is required for asymmetric spindle positioning in the one-celled embryo (30). A recent study has demonstrated that RGS-7, a *Caenorhabditis elegans* RGS protein, controls spindle positioning via the Goα-related G proteins likely in a receptor-independent manner (31). RGS14 may also regulate centrosome function via its ability to regulate the Gα subunits. Here we focus on the role of the different domains of RGS14 in its targeting to centrosomes and to other intracellular compartments.

Analysis of the expression of the GFP proteins fused to various portions of RGS14 reveals that RBD is sufficient for centrosome localization. The HC6 construct expressing a GFP fusion protein with amino acids 244–380 of RGS14 is localized in centrosomes, although the size of centrosome dots of this construct is larger than those of wild type or HC9. The HC8 construct lacking the N-terminal 56 amino acids of HC6 can no longer be targeted to the centrosomes, suggesting that the amino acids 244–299 may be critical. However, the HC9 expressing a GFP fusion protein that contains amino acids 300–427 of RGS14 exhibits a localization pattern similar to that of wild type. Therefore, it appears that the presence of additional amino acids at either the N or C terminus of the first RBD domain is necessary. The additional amino acids may provide further structural support for stabilizing the RBD domain or for interacting with other proteins. It is also possible that amino acids 381–427 of RGS14 facilitate interaction with a centrosome scaffolding protein(s) to properly localize RGS14 within the centrosome once RGS14 is targeted to the centrosome region. The mutants likely defective in interaction with small GTPases (HC22-1, HC22-2, HC23) also exhibit an intracellular localization pattern similar to that of wild type, arguing against the need for small GTPases for the centrosome targeting of RGS14. Most interestingly, the putative NLS3 resides within the amino acids 297–423, suggesting that it may play a role in centrosome targeting of the HC9 construct. However, a mutant containing alanine substitutions replacing three arginine and two lysine residues in the NLS3 exhibits an intracellular localization pattern similar to that of HC9. Most surprisingly, when the GFP tag of HC9 and HC25 is replaced with the FLAG tag, the truncated RGS14 protein can no longer localize in the centrosomes. Perhaps proper protein folding of the truncated RGS14 critical for centrosome localization is achieved in the presence of GFP tag but not in that of the FLAG tag. An alternative explanation is that the lack of the NLSs in the truncated RGS14 can be compensated with the nuclear targeting signals in GFP, and the nuclear localization of RGS14 is essential for the centrosome targeting. Presently, it appears that a structural motif(s) within the 1st RBD domain is necessary for the centrosome targeting of RGS14.
In general, the RGS14 fusion constructs localized to the centrosomes in ~10% of transfected cells. The GFP fusions of centrosome proteins such as pericentrin, γ-tubulin, and centrin, when transiently transfected, localized to the centrosomes in only a fraction of transfected cells, although endogenous counterparts are constitutively associated with the centrosomes throughout the cell cycle. Upon establishing a stable cell line, for example, pericentrin-GFP becomes centrosome-associated in all the cells throughout the cell cycle. There is no clear explanation for this phenomenon. It may be because the turnover of endogenous centrosome proteins and/or incorporation of newly synthesized centrosome components is cell cycle-regulated. The immunostaining of endogenous RGS14 reveals that RGS14 is also associated with the centrosomes throughout the cell cycle. Overexpression or underexpression of centrosome proteins often elicits G1 cell cycle arrest that cannot be observed in p53-compromised cells such as HeLa and Jurkat (32). So we used the MCF-7 cell line to examine the cell cycle distribution of cells expressing wild type and various mutants of RGS14. Unfortunately the GFP protein itself caused G1 cell cycle arrest in only a fraction of transfected cells. The GFP fusions of RGS14 and RGS16 are located in their N terminus, a region implicated in plasma membrane targeting (33, 34). Our site-directed mutagenesis studies demonstrate no requirement for either the GAP activity or the GDI activity of RGS14 for nuclear cytoplasmic shuttling or heat stress-induced trafficking to the PML nuclear bodies. Also we find no evidence to support a role for PKA-mediated phosphorylation in the shuttling. However, we cannot rule out the possibility that these activities may regulate the kinetics of shuttling or nuclear targeting of RGS14. Only the L506A/L507A substitution results in a drastic change in RGS14 localization. However, the nuclear accumulation of L506A/L507A mutant is not likely due to the loss of its GDI activity because the Q518A substitution, which also abolishes GDI activity, does not show a similar effect. The continuous shuttling of RGS14 may allow a tighter regulation of RGS14 protein activity in the nucleus. Cellular stresses such as heat shock may change the balance between import and export of RGS14, perhaps altering the transcriptional activity of certain genes.

Most interestingly, the nuclear export-defective mutant of RGS14 does not localize in the centrosome, suggesting that cytoplasmic RGS14 needs to traffic through the nucleus to target to the centrosomes. Several centrosome proteins shuttle between the cytoplasm and nucleus (35). The treatment of HeLa cells with leptomycin B causes the accumulation of centrin and pericentrin in the nucleus similar to our finding with RGS14. It will be interesting to see whether nuclear export-defective centrin or pericentrin also cannot be targeted to the centrosomes. Together, these results suggest that the nuclear cytoplasmic shuttling may be necessary for the centrosome localization of certain proteins. The nuclear cytoplasmic shuttling may allow post-translational modification required for centrosome targeting to occur in the nucleus and/or provide a means to tightly control the trafficking of these proteins to the centrosomes.

Most surprisingly, recent evidence indicates that several RGS family members may have functional role in the nucleus. Some RGS proteins such as RGS2, RGS3T (a truncated variant of RGS3), and RGS12TS-S (a variant of RGS12) localize predominantly in the nucleus. RGS3T induces apoptosis, and RGS12TS-S is a nuclear matrix protein that may assist in the regulation of transcription and cell cycle progression (36, 37). Brain-specific RGS9-2 also localizes to the nucleus and may alter transcriptional activity (38). Many RGS proteins including RGS2, RGS3, RGS6, and RGS13 undergo stress-induced nuclear trafficking, implicating them in cellular stress-related signaling pathway (25).

RGS14 also appears to be involved in cellular stress-related signaling pathways and the regulation of gene transcription. It is plausible that RGS14 is recruited to the PML nuclear bodies in response to heat stress to assist in the regulation of transcription of certain genes. The nucleus is functionally compartmentalized, and PML nuclear bodies associate with sites of active transcription (39). Proteins that localize to the PML nuclear bodies are often recruited to these sites in response to cellular stresses, modulating the transcriptional activity of specific genes (26, 27). We were surprised to observe that mutations that lead to defective GAP or GDI activity affected the ability of RGS14 to enhance transcriptional activity because the involvement of heterotrimeric G proteins in nuclear targeting or functions has not been described. However, the nuclear export-defective L506A/L507A mutant that also lacks GDI activity still enhances promoter activity. This may be because the higher level of nuclear expression compensates for its lack of GDI activity. Perhaps the GDI function of RGS14 alters nuclear import/export rates (or rate of subnuclear targeting), but not promoter activity.

Most interestingly, we have identified several nuclear and transcriptional factors as well as a centrosome protein as two-hybrid partners of RGS14, although the veracity of these interactions in mammalian cells needs testing. Nevertheless, it is tempting to speculate that RGS14 may act as coactivator for certain genes and that this activity requires its proper localization. Further studies will be required to determine the mechanism of centrosome and nuclear/subnuclear targeting of RGS14, the precise role of RGS14 in centrosome function and transcriptional regulation, and whether the localization and regulation are achieved via heterotrimeric G proteins. Together, our study provides the basis for further investigations into the role of RGS14 in centrosome and nuclear function and stress-induced cellular responses.

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Subcellular Localization and Trafficking of RGS14

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RGS14 Is a Centrosomal and Nuclear Cytoplasmic Shuttling Protein That Traffics to Promyelocytic Leukemia Nuclear Bodies Following Heat Shock

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