RGS6 is a member of a subfamily of mammalian RGS proteins that possess DEP (disheveled, Egl-10, pleckstrin) and GGL (G protein γ subunit-like) domains in addition to the hallmark RGS domain. RGS proteins negatively regulate heterotrimeric G protein signaling by virtue of the GTPase-activating protein activity of their RGS domains. RGS6 exists in multiple splice forms with a long (6L) or short (6S) N terminus, a complete or incomplete GGL domain, in combination with various C-terminal domains. Green fluorescent protein-tagged RGS6L and RGS6S forms exhibit predominantly cytoplasmic and nuclear patterns of distribution in COS-7 cells, respectively, and traffic from these sites to nucleoli in response to stress signaling. We undertook a yeast two-hybrid screen for nuclear RGS6-binding proteins and here identify DMAP1 as an RGS6-interacting protein. DMAP1 is a component of the Dnmt1 complex involved in recombination of newly replicated genes. The domains of interaction were mapped to the N-terminal region of the GGL domain of RGS6, a region distinct from its Gβ5 binding region, and the C-terminal domain of DMAP1. Gβ5 and DMAP1 did not compete for each other’s interaction with RGS6. Co-immunoprecipitation studies in COS-7 cells showed that RGS6L and RGS6S, but not RGS6LA258–293 deletion mutant lacking a DMAP1-binding module, co-immunoprecipitated DMAP1 as well as Dnmt1 in a DMAP1-dependent manner. A recombinant GGL domain of RGS6 precipitated endogenous DMAP1 and Dnmt1 in neuroblastoma cell lysates and endogenous DMAP1 co-immunoprecipitated with RGS6L from mouse brain. Co-expression of DMAP1 with RGS6L promoted nuclear migration of RGS6L and its co-localization with DMAP1, a response not observed with RGS6LA258–293. RGS6 inhibited the transcriptional repressor activity of DMAP1. RGS6 is the first member of the RGS protein family shown to interact with proteins involved in transcriptional regulation.

RGS1 proteins were discovered as negative regulators of heterotrimeric G protein signaling in Saccharomyces cerevisiae

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RGS6 Interacts with DMAP1 and DNMT1 and Inhibits DMAP1 Transcriptional Repressor Activity*
RGS6 Interaction with DMAP1 and Dnmt1

RGS proteins in the nucleus, we employed yeast two-hybrid analysis to identify proteins that interact with RGS6, one of the RGS proteins we found to exhibit constitutive or signaling-induced trafficking to and within the nucleus. Here we report a high affinity interaction between RGS6 and DMAP1, a protein identified and named based upon its interaction with Dnmt1 (17). DMAP1 has transcriptional repressor activity, and Dnmt1 is a multihit GTPase that is a critical domain structure and gene silencing as well as methyltransferase-independent transcriptional repression (17–21). Our studies demonstrate interaction of RGS6 and DMAP1 in yeast and mammalian cells and define the structural determinants required for this interaction. We define a new structural motif within the GGL domain of RGS6, identified originally as a binding motif for Gβγ (22), that mediates its high affinity interaction with DMAP1, and we show that RGS6 interacts with Dnmt1 in a DMAP1-dependent manner. Binding of DMAP1 and Gβγ to RGS6 is not mutually exclusive. RGS6 and DMAP1 were shown to interact in the context of whole cells, and this interaction was dependent upon the identified DMAP1-binding module of RGS6. RGS6, but not the DMAP1-interacting proteins TSG101 and Dnmt1, inhibited the functional activity of DMAP1 as a transcriptional repressor, demonstrating that the interaction between RGS6 and DMAP1 is functionally relevant. RGS6 is the first member of the RGS protein family shown to interact with proteins involved in transcriptional regulation. These results provide new evidence linking RGS proteins to proteins involved in regulating gene expression.

EXPERIMENTAL PROCEDURES

Materials—pEGFP vector, pGBK7, pACT2, pretransformed brain MATCHMAKER library, pM vector, and anti-GFP monoclonal antibody were purchased from Clontech. pCR2.1 TOPO TA cloning vector, pTrcHis2 TOPO TA cloning vector, and anti-GFP polyclonal antibody were from Invitrogen, and pCMV vector was from Stratagene. Antibody against human brain cDNA library cloned into pACT2 vector. First, amplified cDNAs were cloned in the T/A cloning vector pCR2.1 and confirmed by sequencing.

PCR Amplification/Preparation of EGFP-RGS6, FLAG-DMAP1, and FLAG-TSG101 Constructs—All of the human RGS6 protein constructs described originally as (8). Cells were used in experiments ~40 h following transfection.

Yeast Two-hybrid Screening and β-Galactosidase Assays—Full-length RGS6L and RGS6S, various truncation mutants of RGS6L, and truncation mutants of DMAP1 were PCR-amplified using gene-specific primers incorporating restriction sites to facilitate their cloning into pGBK7 or pACT2 vector. First, amplified cDNAs were cloned in LRCl2.1 (Invitrogen). Then restriction enzyme digestion and agarose gel purification of the cloned cDNAs was performed. RGS6 cDNAs were ligated to pGBK7 vector, and DMAP1 cDNAs were ligated to pACT2 vector. Constructs cloned into the pGBK7 vector were fused to an N-terminal Gal4 DBD, and constructs cloned into the pACT2 vector were fused to a N-terminal Gal4 DNA activating domain.

For yeast screening a human brain cDNA library cloned into pACT2 vector. The yeast strain AH109 was transformed with pGBK7-RGS6S and mated with the yeast strain Y187 already transformed with human brain cDNA library. An estimated 10⁷ diploid clones were screened. Yeast clones containing interacting proteins were identified by growth on selective media lacking leucine, tryptophan, histidine, and adenine and confirmed by β-galactosidase activity.

RGS6L and its truncation mutants fused with Gal4 DBD were co-transformed with different DMAP1 truncation mutants fused with Gal4 activating domain into AH109 cells. The activity of the reporter gene β-galactosidase was then assessed by liquid culture assay using o-nitrophenyl β-D-galactopyranoside as a substrate.

Fluorescence and Immunofluorescence—Transfected cells were rinsed three times with DPBS before fixation for fluorescence or immunofluorescence studies. For visualization of GFP-tagged RGS proteins, cells were fixed by treatment with 4% paraformaldehyde for 20 min at room temperature. For immunodetection of FLAG epitope, cells were fixed by treatment with 4% paraformaldehyde for 20 min at room temperature followed by permeabilization with DPBS containing 0.1% Triton X-100, 0.1% Nonidet P-40 for 10 min at room temperature. Cells were incubated with anti-FLAG M2 antibody (~1 μg/ml) in DPBS containing 5% bovine serum albumin for 1 h at room temperature. Cells then were rinsed three times with DPBS, incubated with Cy5-conjugated secondary antibodies (~1 μg/ml) in DPBS for 1 h at room temperature, and washed three times with DPBS. Finally, cells were air-dried and mounted using Vecta Shield mounting solution. Images shown are representative of a minimum of 400 cells derived from four or more separate transfections.

Co-immunoprecipitations—For co-immunoprecipitation studies, COS-7 cells were co-transformed with GFP-tagged forms of RGS6 proteins and FLAG-tagged DMAP1, with or without Gβγ, and grown for 48 h in 10-cm tissue culture dishes. Cells were harvested by lysis with 200 μl of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 1% Nonidet P-40, 6 mM MgCl₂, and 10 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 16,000 × g for 1 min at 4 °C. Resulting supernatants were incuitated with anti-GFP and protein A-conjugated agarose overnight at 4 °C. Precipitates were collected by centrifugation, washed three times in RIPA buffer, suspended in Laemmli sample buffer, and boiled for 3 min. Proteins were subjected to SDS-PAGE and immunoblotting with anti-GFP antibody and anti-FLAG-HRP. For co-immunoprecipitation studies on RGS6 and Dnmt1, COS-7 cells were co-transformed with GFP-tagged forms of RGS6 proteins and His₄-tagged Dnmt1 with or without FLAG-tagged DMAP1 and grown for 48 h in 10 cm of tissue culture. The cell lysate was processed and subjected to immunoprecipitation by using anti-GFP antibody as described above. Proteins were subjected to SDS-PAGE and immunoblotting with anti-GFP antibody and anti-polyhistidine antibody.

For co-immunoprecipitation studies in mouse brain, an adult mouse brain was homogenized in 1 ml of ice-cold RIPA buffer and centrifuged at 23,000 × g for 20 min at 4 °C. Anti-RGS6L-conjugated agarose (70 μl) was added to the resulting supernatant. After incubation overnight at 4 °C, agarose beads were collected by centrifugation, washed three times with 1 ml of RIPA buffer, and then resuspended in 50 μl of sample buffer. Precipitates were subjected to SDS-PAGE and immunoblotting with anti-GFP and anti-RGS6L antibodies.

Preparation of Recombinant GGL Domain Protein and in Vitro Pull-down Assays—A cDNA encoding amino acids 258–319 of RGS6L, encompassing the GGL domain and 10 amino acids C-terminal to the GGL domain, was PCR-amplified and cloned into pTrcHis2 TOPO TA vector. The DNA methyltransferase tag from pM vector was fused to the C-terminal protein sequences that were subject to SDS-PAGE and immunoblotting with anti-GFP and anti-RGS6L antibodies.

Preparation of Recombinant GGL Domain Protein and in Vitro Pull-down Assays—A cDNA encoding amino acids 258–319 of RGS6L, encompassing the GGL domain and 10 amino acids C-terminal to the GGL domain, was PCR-amplified and cloned into pTrcHis2 TOPO TA vector. The DNA methyltransferase tag from pM vector was fused to the C-terminal protein sequences that were subject to SDS-PAGE and immunoblotting with anti-GFP and anti-RGS6L antibodies.
by centrifugation at 5,000 × g for 20 min, and the resulting pellets were resuspended in 20 ml of lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 25 mg of lysozyme) and incubated on ice for 30 min. After brief sonication, supernatants were collected by centrifugation at 10,000 × g for 20 min and loaded onto a column filled with 2 ml of Ni-NTA slurry. The column was washed with 10 ml of wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole), and the Ni-NTA/protein mixture was collected and an aliquot subjected to SDS-PAGE to check for protein purity.

For in vitro pull-down assay, a T150 flask of neuroblastoma SH-SY5Y cells (~10⁷ cells) was lysed in 2 ml of ice-cold RIPA buffer and subjected to pull down by mixing with either empty Ni-NTA beads or RGS6 GGL domain-bound Ni-NTA beads, both of which were pre-incubated in RIPA buffer. The beads were washed and incubated in RIPA buffer. The beads were washed for 4 h at 4°C, collected, washed three times with RIPA buffer, and then resuspended in sample buffer. Samples were heated briefly and subjected to SDS-PAGE and immunoblotting with anti-DMAP1 antibody and anti-Dnmt1 antibody.

Luciferase Assay—Luciferase reporter gene construct containing five repeats of the Gal4-binding element and the herpesvirus thymidine kinase (TK) promoter was PCR-amplified and cloned in the pGL3 basic vector (Promega). Gal4 DBD fusion constructs of DMAP1 were cloned into the pM vector. NIH3T3 cells were transfected with various DNA constructs using LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol. Luciferase activity in transfected cells was determined with a luciferase assay kit (Promega) 40 h following transfection. Measurements were performed as described under "Experimental Procedures."

RESULTS

Identification of DMAP1 as an RGS6-binding Protein—We used yeast two-hybrid analysis to identify proteins that interact with RGS6, a form of RGS6 that exhibits a nuclear pattern of localization (15). A human brain library was screened with RGS6, and a strongly interacting clone, encoding the C-terminal 292 amino acids of DMAP1, was identified and recovered by bacterial transformation. Full-length DMAP1 then was amplified by PCR from a human brain cDNA library, and its sequence was verified by comparison with that of DMAP1 identified in yeast two-hybrid screens as a Dnmt1-associated protein (17). Although the tissue-specific pattern of expression was verified by showing that constructs encoding the GGL domain alone (258–319) or the GGL domain or construct 258–319 or the GGL domain interacted with DMAP1, although this region of DMAP1 is not unique to RGS6 and does not require sequences N-terminal to the GGL domain. However, further truncation to remove the GGL domain caused a loss of interaction with DMAP1, suggesting its involvement in interaction between RGS6 proteins and DMAP1. This finding was confirmed by showing that RGS6 alone and an RGS6 mutant truncated before the GGL domain interacted with the C-terminal DMAP1 protein as strongly as RGS6. These results suggest that the site of interaction between RGS6 proteins and this region of DMAP1 is not unique to RGS6 and does not require sequences N-terminal to the GGL domain. However, further truncation to remove the GGL domain caused a loss of interaction with DMAP1, suggesting its involvement in interaction between RGS6 proteins and DMAP1. This finding was confirmed by showing that constructs encoding the GGL domain and 10 additional C-terminal amino acids (258–319) or the GGL domain alone (258–309) interacted with the DMAP1 protein as strongly as that of full-length RGS6 or RGS6L. The interaction site within the GGL domain was further defined, by analysis of other truncation mutants, to the N-terminal 36 amino acids of the GGL domain (258–293). Most interesting, RGS6(GGL) splice forms possess only the first 28 N-terminal amino acids of the GGL domain. Thus, it was interesting to compare the interaction of this domain (258–285) to that of the identified DMAP1-interacting region within the GGL domain. As shown the truncated GGL domain present in RGS6(GGL) splice forms interacted with the DMAP1 protein, although this interaction was weaker than that observed with the complete GGL domain or construct 258–293. These findings suggest that RGS6(GGL) splice forms may interact with DMAP1, although this interaction may not be as strong as that of splice forms with complete GGL domains. These studies identify the site of interaction of RGS6 proteins with the DMAP1 protein to a specific sequence module present in the GGL domain and that sequences N- or C-terminal to this region are not required.

RGS6-DMAP1 Minimal Interaction Requirements—We initiated our studies by determining the structural sequences involved in interaction between RGS6 and DMAP1 in yeast. We examined interaction of RGS6L, RGS6S, and various RGS6 truncation mutants with the C-terminal region of DMAP1 identified as an RGS6S-binding region in our two-hybrid screen and DMAP1 truncation mutants of this region. RGS6 and DMAP1 constructs were used as bait and target proteins, respectively, and the relative strength of interaction between these proteins was assessed by β-galactosidase assays. p53 interaction with T antigen served as a positive control, and interaction of vector controls for the identified RGS6- and DMAP1-interacting regions were included as negative controls.

Fig. 1 illustrates the constructs examined and the results obtained. As shown, both RGS6L and an RGS6 mutant truncated before the GGL domain interacted with the C-terminal DMAP1 protein as strongly as RGS6S. These results suggest that the site of interaction between RGS6 proteins and this region of DMAP1 is not unique to RGS6 and does not require sequences N-terminal to the GGL domain. However, further truncation to remove the GGL domain caused a loss of interaction with DMAP1, suggesting its involvement in interaction between RGS6 proteins and DMAP1. This finding was confirmed by showing that constructs encoding the GGL domain and 10 additional C-terminal amino acids (258–319) or the GGL domain alone (258–309) interacted with the DMAP1 protein as strongly as that of full-length RGS6 or RGS6L. The interaction site within the GGL domain was further defined, by analysis of other truncation mutants, to the N-terminal 36 amino acids of the GGL domain (258–293). Most interesting, RGS6(GGL) splice forms possess only the first 28 N-terminal amino acids of the GGL domain. Thus, it was interesting to compare the interaction of this domain (258–285) to that of the identified DMAP1-interacting region within the GGL domain. As shown the truncated GGL domain present in RGS6(GGL) splice forms interacted with the DMAP1 protein, although this interaction was weaker than that observed with the complete GGL domain or construct 258–293. These findings suggest that RGS6(GGL) splice forms may interact with DMAP1, although this interaction may not be as strong as that of splice forms with complete GGL domains. These studies identify the site of interaction of RGS6 proteins with the DMAP1 protein to a specific sequence module present in the GGL domain and that sequences N- or C-terminal to this region are not required.

Fig. 1. Mapping the interaction domains between RGS6 and DMAP1. Full-length RGS6L, RGS6S, or partial domains of these proteins were evaluated in the yeast two-hybrid system for interaction with different C-terminal regions of DMAP1. Two negative controls (pGBK7 vector and DMAP1 and pACT2 and GGL domain of RGS6) and one positive control (p53 and T antigen) were included. Interactions between protein constructs were quantitated by measurements of β-galactosidase activity. Values represent means ± S.D. of four independent experiments. Preparation of constructs, yeast two-hybrid analysis, and β-galactosidase measurements were performed as described under "Experimental Procedures."
**Fig. 2.** Co-immunoprecipitation of RGS6 and DMAP1 and Dnmt1 in COS-7 cells. **Top panel,** immunoblots (IB) showing co-precipitation of DMAP1 with RGS6L, RGS6L–GGL, RGS6S, and RGS6S–GGL forms of RGS6 but not RGS6LΔ258–293. RGS6 proteins in cell lysates were immunoprecipitated (IP) with anti-GFP antibody, and immunoprecipitates were subjected to immunoblotting with anti-FLAG or anti-GFP. COS-7 cells were co-transfected with FLAG-DMAP1 and GFP fusions of RGS6 splice forms or the RGS6 deletion mutant. **Bottom panel,** immunoblots showing co-precipitation of Dnmt1 with RGS6L but not RGS6LΔ258–293 in cells co-expressing FLAG-DMAP1. COS-7 cells were co-transfected with His6-Dnmt1 and GFP fusions of RGS6L or RGS6LΔ258–293, with and without FLAG-DMAP1. RGS6 proteins in cell lysates were immunoprecipitated (IP) with anti-GFP antibody and immunoprecipitates were subjected to immunoblotting (IB) with anti-FLAG or anti-GFP. Transfections, immunoprecipitations, and immunoblotting were performed as described under “Experimental Procedures.”

and do not affect GGL domain interactions with this protein.

The region between amino acids 212 and 422 of DMAP1 contains a putative coiled coil region that was implicated in its interaction with Dnmt1 (17). Fig. 1 shows that this region of Dnmt1 showed strong interaction with a construct of RGS6 encompassing its GGL domain. These results identify the putative coiled coil region of DMAP1 as the RGS6-interacting domain.

RGS6 Interaction with DMAP1 and Dnmt1 in COS-7 Cells—It seemed essential to determine whether the interaction we observed between RGS6 and DMAP1 in yeast occurred in mammalian cells and whether this interaction was mediated by the identified region within the GGL domain of RGS6. Thus, we performed co-immunoprecipitation assays in COS-7 cells transfected with RGS6 proteins in cell lysates were immunoprecipitated (IP) with anti-GFP antibody and immunoprecipitates were subjected to immunoblotting (IB) with anti-FLAG or anti-GFP. Transfections, immunoprecipitations, and immunoblotting were performed as described under “Experimental Procedures.”

Our finding that RGS6 proteins interact with DMAP1 raised questions of whether RGS6 interacts with the DMAP1-Dnmt1 transcriptional complex or if binding of RGS6 to the coiled coil region of DMAP1 prevents DMAP1 interactions with Dnmt1. To address these issues, we determined whether RGS6 proteins associate with Dnmt1 and the dependence of such an interaction on the presence of DMAP1. Therefore, we performed co-immunoprecipitation assays in COS-7 cells transfected with and without FLAG-tagged DMAP1, with GFP-tagged RGS6L, or the RGS6L deletion mutant (RGS6LΔ258–293) that does not interact with DMAP1. Co-immunoprecipitation of Dnmt1 by RGS6L or the RGS6L mutant was examined by subjecting cell lysates to immunoprecipitation with anti-GFP antibodies followed by immunoblotting with anti-GFP or anti-FLAG. Anti-GFP immunoblotting was used to show the efficacy of the precipitation reaction. The **top panel** of Fig. 2 shows the result of these experiments. As shown, DMAP1 efficiently co-precipitated with RGS6L and RGS6S and their –GGL splice forms, the latter proteins possessing only the N-terminal 28 amino acids (258–285) of the GGL domain but being otherwise identical to their RGS6L and RGS6S counterparts. We examined co-immunoprecipitation of DMAP1 by RGS6 proteins, by subjecting cell lysates to immunoprecipitation with anti-GFP antibodies followed by immunoblotting with anti-GFP or anti-FLAG. Anti-GFP immunoblotting was used to show the efficacy of the precipitation reaction. The **top panel** of Fig. 2 shows the result of these experiments. As shown, DMAP1 efficiently co-precipitated with RGS6L and RGS6S and their –GGL splice forms of these proteins. However, no co-precipitation was observed with the RGS6L deletion mutant (RGS6LΔ258–293). These differences were not due to differences in the level of expression of RGS6 proteins or of DMAP1 or in the efficiency of their immunoprecipitation. These results demonstrate that DMAP1 interacts with RGS6 proteins in mammalian cells and confirm our yeast two-hybrid analysis identifying the N-terminal region of the GGL domain as the domain mediating this interaction. The lack of interaction of the RGS6L deletion mutant with DMAP1 further excludes the N-terminal region of RGS6L as well as sequences outside of the deleted region, including the RGD, as being involved in this interaction. Most interesting, the slight reduction in efficiency of co-precipitation of DMAP1 by RGS6(–GGL) and coiled coil region of DMAP1 as the RGS6-interacting domain.

**Gβ5 and DMAP1 Interactions with RGS6 Are Not Mutually Exclusive**—Our findings suggest different specificity requirements for DMAP1 and Gβ5 binding to the GGL domain of RGS6. The present results show that DMAP1 interaction with RGS6 requires sequences in the N-terminal region of the GGL domain and that this interaction occurs with –GGL splice forms of RGS6. Our previous study showed that –GGL splice
forms of RGS6 do not interact with Gβ5 (15). However, the finding that these two proteins bind to different regions of the GGL domain does not rule out their mutually exclusive binding. Therefore, we examined the ability of various RGS6 splice forms to co-precipitate co-expressed DMAP1 and Gβ5. Fig. 3A shows the results of these studies. As shown, Gβ5 efficiently co-precipitated with RGS6L and RGS6S but not their -GGL splice forms, whereas DMAP1 co-precipitated efficiently with all RGS6 splice forms. Thus, Gβ5 and DMAP1 exhibit the same patterns of interaction with RGS6 when expressed individually or together, demonstrating that their interactions with the GGL domain of RGS6 are not mutually exclusive.

**GGL Domain Pull-down of Endogenous DMAP1 and Dnmt1**—As a further test of our evidence for RGS6 interaction with the DMAP1-Dnmt1 complex by sequence modules in its GGL domain, we examined whether the GGL domain of RGS6 could be used to pull down native DMAP1-Dnmt1 complexes in cells. We discovered that no studies had examined the existence of a native complex (i.e., endogenous proteins) between Dnmt1 and DMAP1, although Dnmt1 had been shown to form a native complex with Rb, HDAC1, and E2F1 previously (19). We prepared and purified a recombinant His6 fusion protein encompassing the GGL domain of RGS6 and 10 additional C-terminal amino acids shown to exhibit strong interaction with DMAP1 in our yeast two-hybrid studies (Fig. 1). We examined whether the RGS6 GGL domain protein bound to Ni-NTA beads could pull down DMAP1-Dnmt1 complexes from human neuroblastoma SH-SY5Y cell lysates, where we identified endogenous expression of both DMAP1 and Dnmt1. Fig. 3B shows that the RGS6 GGL domain protein bound to Ni-NTA beads, but not Ni-NTA beads alone, efficiently precipitated both DMAP1 and Dnmt1 from SH-SY5Y cell lysates. These results confirm our findings in yeast and COS-7 cells demonstrating that the GGL domain of RGS6 is a DMAP1-binding module and represents the site of interaction of DMAP1 with RGS6. The observed co-precipitation of DMAP1 and Dnmt1 by the RGS6 GGL domain fusion protein shows that DMAP1 binding to the RGS6 GGL domain and to Dnmt1 is not mutually exclusive and provides the first evidence of a native complex between DMAP1 and Dnmt1.

**RGS6L and DMAP1 Interaction in Mouse Brain**—Previously we showed that our antibody to RGS6L detected native RGS6 in mouse brain by immunoblotting and immunohistochemistry (15). Although ectopically expressed RGS6L is primarily a cytoplasmic protein, we demonstrated its nuclear localization in response to heat or proteotoxic stress and during co-expression with Gβ5, and interestingly, we found evidence of some nuclear localization of RGS6L immunoreactivity in mouse brain (15, 16). Therefore, we undertook studies to see whether we could identify native DMAP1 in naive mouse brain and whether it showed any association with RGS6L. The upper panel of Fig. 4A shows the presence of DMAP1 immunoreactivity in mouse brain, and the lower panel shows that DMAP1 co-precipitated with RGS6L using anti-RGS6L agarose. These results provide the first evidence for interaction of endogenous RGS6L and DMAP1 in a naive tissue.

**Co-localization of RGS6 and DMAP1**—Rountree et al. (17) demonstrated co-localization of DMAP1 and Dnmt1 within the nucleus upon their combined expression, consistent with evidence that DMAP1 is part of the Dnmt1 repressive transcription complex. Recently we studied the subcellular distribution of RGS6 splice forms. RGS6L and RGS6L–(GGL) localize in the cytoplasm, RGS6S localizes both in the cytoplasm and nucleus, and RGS6S–(GGL) is found predominantly in the nucleus (15). These findings showed that the long N-terminal and GGL domain of RGS6 splice forms function as cytoplasmic retention sequences. We also reported recently that both RGS6L and RGS6S proteins undergo trafficking to and within the nucleus in response to activation of stress signaling (16) and that co-expression of the GGL domain-binding protein Gβ5 with RGS6 proteins promoted RGS6 transport to the nucleus (15). Thus, RGS6 splice forms are localized in the nucleus, in a position to interact with DMAP1-Dnmt1 complexes, constitutively or in response to stress signaling or protein interactions.

In view of our evidence demonstrating high affinity interactions between RGS6 proteins and DMAP1 in both yeast and mammalian cells, we performed experiments to see whether we could detect any evidence of co-localization of RGS6 and DMAP1. Therefore, we examined the subcellular localization patterns of RGS6L and RGS6S, and their –GGL splice forms, and the RGS6L deletion mutant (RGS6LΔ258–293) that does not interact with DMAP1 during their individual and/or combined expression with DMAP1 in COS-7 cells. The subcellular patterns of distribution of individually expressed GFP-tagged forms of RGS6L, RGS6S–(GGL), and DMAP1 and of co-expressed GFP-tagged forms of RGS6 and FLAG-DMAP1 are shown in Fig. 5, A and B, respectively. When expressed alone, DMAP1 is localized in the nucleus, and RGS6L and RGS6S–(GGL) are localized in the cytoplasm (Fig. 5A). Co-expression of these RGS6L splice forms with DMAP1 resulted
in co-localization of these proteins in the nucleus (RGS6L) or nucleoli (RGS6L(−GGL)). Thus, co-expression of DMAP1 with RGS6L splice forms promotes migration of RGS6L proteins from the cytoplasm to the nucleus or nucleoli and their co-localization with DMAP1 at these sites. In contrast, the RGS6L deletion mutant RGS6LΔ258–293 lacking the DMAP1-interacting domain did not undergo nuclear migration from the cytoplasm or co-localize with DMAP1 during its co-expression with DMAP1. DMAP1 was co-localized also with both forms of RGS6S during their combined expression. These results show that RGS6 proteins exhibit co-localization with DMAP1 during their expression in mammalian cells, in keeping with our evidence for direct interactions between these proteins. Moreover, the observed lack of nuclear migration of RGS6LΔ258–293 or its co-localization with DMAP1 shows that DMAP1 interaction with the N-terminal region of the GGL domain of RGS6 is required for its co-localization with RGS6. This latter finding confirms our yeast two-hybrid analysis and co-precipitation analysis in COS-7 cells, identifying this sequence module of RGS6 as the DMAP1-interacting domain, and shows the importance of this region in the interaction of these proteins in the context of a whole cell.

**RGS6 Inhibits DMAP1 Transcriptional Repressor Activity**—DMAP1 possesses transcriptional repressor activity that is independent of HDACs (17). This activity of DMAP1, and that of Dnmt1, is believed to contribute to transcriptional repression at sites of DNA replication. In view of our findings that RGS6 proteins interact with and co-localize with DMAP1, it seemed essential to examine possible effects of this interaction on the transcriptional repressor activity of DMAP1. For these experiments, we studied the effects of RGS6 on transcriptional repression by DMAP1 by using the Gal4 TK promoter-driven luciferase reporter construct that was used to document the transcriptional repressor activity of DMAP1 (17). Therefore, NIH3T3 cells were transfected with a Gal4 DBD-DMAP1 fusion protein alone or together with increasing amounts of GFP-RGS6S, GFP-RGS6S(−GGL), or EGFP, the latter serving as a negative control. Cells transfected with a Gal4 DBD represent non-repressed basal TK transcriptional activity. Fig. 6A shows that DMAP1 inhibits TK promoter-driven transcription ~75% when targeted to the promoter site of this construct. Co-transfection of cells with RGS6S or RGS6S(−GGL) inhibited transcriptional repression by DMAP1 on the TK promoter in a manner dependent upon the RGS6S cDNA transfected into cells. The transcriptional repressor activity of DMAP1 was unaffected by co-transfection with EGFP. These results demonstrate that RGS6S and RGS6S(−GGL), two proteins shown to interact with and co-localize with DMAP1, negatively mod-

![Image](http://www.jbc.org/)

**FIG. 4.** A, precipitation of endogenous DMAP1 and Dnmt1 from SH-SY5Y lysates with a recombinant RGS6 GGL domain protein. Recombinant His fusion of the GGL domain of RGS6 bound to Ni-NTA beads and Ni-NTA beads alone were tested for their ability to precipitate native DMAP1 and Dnmt1 from SH-SY5Y cell lysates. Proteins precipitated by the GGL domain bound Ni-NTA beads and Ni-NTA beads were subjected to immunoblotting (IB) with anti-DMAP1 and anti-Dnmt1. Preparation of the recombinant RGS6 GGL domain protein and the in vitro pull-down assay were performed as described under “Experimental Procedures.” B, co-immunoprecipitation of endogenous DMAP1 and RGS6L from mouse brain homogenate. Top panel, immunoblots showing anti-DMAP1 antibody recognized ectopically expressed DMAP1 as well as endogenous DMAP1 in mouse brain homogenate. Bottom panel, immunoblots showing DMAP1 co-immunoprecipitated with RGS6L. Mouse brain homogenate was subjected to immunoprecipitation with anti-RGS6L-agarose and immunoblotting with anti-DMAP1 and anti-Dnmt1. Immunoprecipitations and immunoblotting were performed as described under “Experimental Procedures.”
ulate the transcriptional repressor activity of DMAP1. Interestingly, we routinely observed that RGS6S is slightly more potent than RGS6S(-GGL) in inhibiting the transcriptional repressor activity of DMAP1, possibly reflecting its stronger interaction with DMAP1 (Figs. 1 and 2).

The transcriptional regulatory function of RGS6 on DMAP1 does not appear to be a consequence of protein overexpression or from a nonspecific effect due to its interaction with DMAP1. Dnmt1 and TSG101 are the only other known DMAP1-binding partners and have been shown to interact with DMAP1 in both yeast and mammalian cells (17). Therefore, we examined whether co-expression of Dnmt1 or TSG101 modulated DMAP1 transcriptional repression as observed during RGS6 expression. NIH3T3 cells were transfected with a Gal4 DBD-DMAP1 fusion protein alone or together with increasing amounts of RGS6S, Dnmt1, or TSG101 along the Gal4 TK promoter-driven luciferase reporter construct. Fig. 6B shows that Dnmt1 and TSG101, in contrast to RGS6S, failed to inhibit DMAP1-mediated transcriptional repression. Fig. 6C shows that Dnmt1 and TSG101 were expressed at levels comparable with those of RGS6S, RGS6S(-GGL), and GFP alone when expressed individually or in combination with DMAP1. Together, these results provide the first insight into the possible functional relevance of interaction of RGS6 with the Dnmt-DMAP1 complex and the first evidence for transcriptional regulatory actions of RGS6.

**DISCUSSION**

This study demonstrates specific interaction between RGS6 and DMAP1 and further delineates the structural determinants for this interaction on both proteins. The N-terminal region of the GGL domain of RGS6 and the C-terminal putative coiled coil region of DMAP1 are required for their high affinity interaction. DMAP1 interaction with RGS6 and Dnmt1, the originally identified DMAP1-interacting protein (17), is not mutually exclusive. Indeed, we found that RGS6L co-precipitates both DMAP1 and Dnmt1 when both proteins are expressed in cells and that its interaction with Dnmt1 is DMAP1-dependent. Precipitation of a native DMAP1-Dnmt1 complex from neuroblastoma cells with a recombinant GGL domain protein of RGS6 further showed the ability of the DMAP1-binding module of RGS6 to complex with a native DMAP1-Dnmt1 complex. Evidence for the existence of an RGS6L-DMAP1 complex in native tissue was provided by co-precipitation studies in mouse brain lysates. RGS6 and DMAP1 were shown to interact in the context of whole cells, and our studies showed that this interaction was dependent upon the identified DMAP1-binding module of RGS6. This binding module is distinct from that required for Gβδ binding, and co-precipitation experiments revealed that DMAP1 and Gβδ binding to RGS6 is not mutually exclusive. We showed that co-expression of DMAP1 with RGS6L proteins promoted trafficking of RGS6L proteins from the cytoplasm to the nucleus or nucleoli and co-localization with DMAP1 at these sites, a response that was not observed with a deletion mutant of RGS6L lacking the DMAP1-interaction site. Although less dramatic, due to their usual localization alone, a Gal4 DBD of DMAP1 alone, a Gal4 DBD of DMAP1 co-transfected with increasing amounts of GFP-tagged RGS6S or Dnmt1 or TSG101, along with the TK luciferase reporter construct. C, immunoblot showing expression levels of GFP, GFP-tagged RGS6S, and RGS6S-GGL, Dnmt1, and TSG101 in NIH3T3 cells transfected with increasing amounts of cDNAs encoding these proteins alone and in combination with DMAP1 cDNA. Data are expressed as luciferase activity relative to that observed following expression of the Gal4 DBD alone. Data are normalized for transfection efficiency by co-transfected β-galactosidase and represent means ± S.D. of three independent experiments. Transfection, luciferase expression, and immunoblotting were performed as described under "Experimental Procedures."
in the nucleus, RGS6L splice forms and DMAP1 also exhibited co-localization in cells. Particularly noteworthy is our finding that RGS6 inhibited the functional activity of DMAP1 as a transcriptional repressor. These findings show that the interaction between RGS6 and DMAP1 is functionally relevant and provide new evidence for transcriptional regulatory effects of RGS6 proteins on the DMAP1-Dnmt1 complex.

RGS6 is the first RGS protein shown to interact with proteins involved in transcriptional regulation. In 2000, we speculated that some RGS proteins might have a role in the nucleus distinct from their G protein regulatory actions when we reported that some RGS proteins are nuclear proteins or nucleocytoplasmic shuttle proteins (8). Subsequently, we reported that a splice form of RGS12 was a nuclear matrix protein that inhibited DNA and nascent RNA synthesis upon its expression in cells and possessed direct transcriptional inhibitory activity when targeted to a minimal TATA promoter (13, 14). Our recent findings that some splice forms of RGS6 are nuclear proteins (15) and that both nuclear and cytoplasmic splice forms of RGS6 undergo trafficking to the nucleus and nucleolus in response to stress signaling (16) raised the possibility that RGS6 may have G protein-independent functions in the nucleus. Indeed, G proteins are not believed to be present in the nucleus, and the present results demonstrate that interaction of RGS6 proteins with DMAP1 does not require its G protein-interacting RGS domain. Our results show clearly that interaction of RGS6 with DMAP1 requires a small N-terminal region of its GGL domain, although it remains a possibility that other structural regions, including the RGS domain, are involved in its ability to inhibit DMAP1-mediated transcriptional repression.

The present findings define a new structural motif within the GGL domain of RGS6 that mediates its high affinity interaction with DMAP1. The GGL domain was identified originally as a Gβγ-interacting domain, and amino acids in the C-terminal region of the GGL domain (Asp-297 and Thr-309) are required for its binding to Gβγ (22, 23). Our findings indicate that that the region of the GGL domain required for binding to DMAP1 is distinct from that required for binding to Gβγ. First, the DMAP1-interacting region requires a sequence module in the extreme N-terminal region of the GGL domain (258–293) and not C-terminal sequences involved in Gβγ binding. Second, we found that −GGL splice forms of RGS6 interact with DMAP1, although our previous and present studies showed that these proteins do not interact with Gβγ (15). RGS6(−GGL) splice forms lack all GGL domain sequences C-terminal to amino acid 284. As noted earlier, we did observe that −GGL splice forms of RGS6 interact slightly less well than their GGL-containing counterparts, indicating that the 8 C-terminal amino acids in the DMAP1-interacting domain (258–293) play some role in this interaction. Finally, we showed that DMAP1 and Gβγ do not compete for binding to RGS6, suggesting distinct binding sites for these proteins within the GGL domain of RGS6 and the lack of steric hindrance for each other’s binding to these sites. It is noteworthy that the region of the GGL domain identified here to represent the DMAP1-interacting domain has predicted secondary structural elements that include a coiled coil region (257–275), an α helix (257–279), and a part of a second α helix (281–297), whereas regions C-terminal to these sequences lack such secondary structure (23). It is interesting to speculate that these structural features, particularly the coiled coil region, provide the underlying basis for interaction of this sequence module with the coiled coil region of DMAP1 shown here to interact with RGS6.

Recently we showed the presence of both cytoplasmic and nuclear localized RGS6L immunoreactivity in mouse brain (15). We hypothesized that these patterns of localization could represent free RGS6L (cytoplasmic) and RGS6L complexed with Gβγ (nuclear), based upon our finding that Gβγ expression promoted nuclear localization of RGS6L. Our present findings raise the possibility that DMAP1 might also play a role in the nuclear localization of RGS6L.

The precise role of DMAP1 in regulation of gene expression in mammalian cells is not yet known. However, the finding that it possesses transcriptional repressor activity and forms a complex with the multifunctional protein Dnmt1 suggests that it may have important roles in gene regulation on its own or by virtue of association with Dnmt1. Indeed, the present study provides evidence that DMAP1 and Dnmt1 exist as a constitutive native complex in neuroblastoma cells. Dnmt1 is an essential DNA methyltransferase involved in maintaining the hemimethylated state of newly synthesized DNA at replication foci and in inheritance of DNA methylation patterns (17, 18, 24). In addition, Dnmt1 has intrinsic transcriptional repressor activity and recruits transcriptional co-repressor proteins, like DMAP1 (17, 19, 25). The N-terminal domain of Dnmt1 interacts with DMAP1, proliferating cell nuclear antigen, HDAC1/2, Rb, and several methyl-CpG-binding proteins (17, 19, 25). Dnmt1 functions as a co-repressor of Rb on E2F-driven gene transcription independently of its methyltransferase activity (19). The Dnmt1-DMAP1 complex is proposed to be involved in maintaining sparse methylation and transient gene silencing at early S phase and tight transcriptional repression of heterochromatin replicated in late S phase (17). Both genome-wide hypomethylation and region-specific hypermethylation (of CpG islands in promoters) are important in the formation and development of cancers (21). DNA hypermethylation, which often produces gene silencing, is associated with inactivation of many pathways involved in the cancer process, such as DNA repair, cell cycle regulation, and apoptosis (26). Promoter hypermethylation is found in essentially all human neoplasms and is the most characterized epigenetic change occurring in tumors. Dnmt1 is both necessary and sufficient to maintain aberrant CpG island methylation in human cancer cells, and overexpression of Dnmt1 in cells can promote cellular transformation (27, 28). Global hypomethylation induces chromosomal instability, loss of imprinting, and activation of intragenomic elements that may disrupt normal genes. Therefore, it is particularly noteworthy that mice harboring a hypomorphic allele of Dnmt1 alone or in a background of mutant p53 and Nf1 genes exhibit genomic DNA hypomethylation that promotes tumorigenesis (29, 30). How DMAP1 affects this role of Dnmt1 in maintaining normal versus cancerous cell phenotypes is not yet clear.

The present results provide new evidence linking RGS proteins to proteins involved in gene regulation. The finding that RGS6 forms high affinity complex with and functionally regulates DMAP1 suggests that this protein might be under regulatory control by RGS6. The ability of RGS6 to interact indirectly with Dnmt1 also raises intriguing questions regarding its functional role in the multifunctional activities of this important gene regulatory protein. The results presented here, including identification of the DMAP1-binding domain in RGS6, will facilitate studies to unravel the role of members of the RGS6 protein family in nuclear function.

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