Mg$^{2+}$ inhibits GDP release from Rab5$^{\text{WT}}$ but not from Rab5$^{\text{S34N}}$, a mutant lacking Ser34 critical for Mg$^{2+}$ coordination in the nucleotide binding pocket. Thus, inhibition of GDP release is apparently exerted via coordination of Mg$^{2+}$ between Rab5 and GDP. Mg$^{2+}$ also induces conformational changes in Rab5$^{\text{WT}}$, demonstrated by increased tryptophan fluorescence intensity and a red shift in $\lambda_{\text{max}}$ for the GDP-bound protein. Mg$^{2+}$-induced fluorescence changes are not observed for Rab5$^{\text{S34N}}$. The correlation between Mg$^{2+}$ effects on nucleotide exchange and the fluorescence properties of Rab5 suggests that a conformation promoted through Mg$^{2+}$ coordination with Ser34 also contributes to inhibition of GDP release. The role of structural changes in GDP release was investigated using C- and N-terminal truncation mutants. Similar to Rab5$^{\text{WT}}$, Mg$^{2+}$ inhibits GDP release and alters the fluorescence of Rab5$^{1-198}$ but only partially inhibits release from Rab5$^{23-198}$ and fails to induce changes in the latter’s fluorescence properties. Since Rab5$^{23-198}$ maintains Ser34 necessary for Mg$^{2+}$ coordination, the lack of Mg$^{2+}$-induced fluorescence changes suggests a requirement for the N-terminal domain to promote a conformation blocking GDP release. A model for mechanisms of interaction between Ras-like proteins and their exchange factors is proposed.

Rab proteins are a family of Ras-like small molecular weight GTPases that are localized to distinct subcellular compartments and believed to regulate specific steps of intracellular membrane trafficking. The functional cycle of Rab proteins involves the delivery of the GDP-bound forms to the target membrane by a GDP dissociation inhibitor (GDI) (7-9), the exchange of GDP for GTP at membrane surface catalyzed by a guanine nucleotide exchange factor (GEF) (8,9) and the retrieval of the GDP-bound forms from the membrane by GDI after GTP hydrolysis and membrane fusion (7). Localized on plasma membrane, clathrin-coated vesicles, and early endosomes, Rab5 has been shown to play an important role in early events of endocytosis (4,5), although the exact mechanism of its function remains to be determined.

It is known that Mg$^{2+}$ is essential for GTPase function and structure. Crystallographic studies of several GTP-binding proteins reveal a single Mg$^{2+}$ in the guanine nucleotide binding pocket, coordinating between the protein and guanine nucleotide in both GDP- and GTP analog-bound conformations (10-15). Effects of Mg$^{2+}$ on guanine nucleotide binding, GTPase activity, and the structural integrity of GTP-binding proteins have been widely documented (16-32). A key observation is that Mg$^{2+}$ inhibits GDP release from Ras-like GTP-binding proteins and therefore prevents binding of GTP$\gamma$S (16-27). However, the exact mechanism for this inhibitory effect and its physiological significance remains unknown.

Important functional roles of the N-terminal domains of several Ras-like GTP-binding proteins also have been noted in studies of guanine nucleotide exchange (33-38). Myristoylation at the N terminus of ARF enhances its rate of GDP release (27), and N-terminal truncation of ARF results in loss of function by reducing its affinity for GDP and permitting GDP/GTP exchange in the absence of phospholipids (33). Moreover, deletion of the N terminus enables isolation of ARF in a nucleotide-free form (38). Finally, deletion of the N-terminal domain of Rab5 results in a loss of function (34-37) and interferes with the protein’s post-translational processing (37). These observations suggest that N-terminal domains of Ras-like GTP-binding proteins may participate in the regulation of guanine nucleotide exchange and represent crucial structural domains necessary for the function of the proteins.

We have investigated mechanisms through which Mg$^{2+}$ and the N-terminal domain of Rab5 participate in its regulation of GDP release. Physiologic concentrations of Mg$^{2+}$ block GDP release from Rab5$^{\text{WT}}$ but not from Rab5$^{\text{S34N}}$, a mutant lacking Ser34 critical for Mg$^{2+}$ coordination. Mg$^{2+}$ also alters the intrinsic tryptophan fluorescence properties of Rab5$^{\text{WT}}$ but not Rab5$^{\text{S34N}}$. While the structure and function of Rab5$^{1-198}$, a C-terminal truncation mutant, is influenced by Mg$^{2+}$ in the same fashion as Rab5$^{\text{WT}}$, an N- and C-terminal truncation mutant, Rab5$^{23-198}$, is resistant to the cation’s effects. Thus, inhibition of GDP release by Mg$^{2+}$ appears to be exerted via chemical constraints due to the cation’s coordination between GDP and Rab5, as well as conformational restraints involving the protein’s N-terminal domain that are induced by Mg$^{2+}$ coordination with Ser34 of Rab5. Based on the correlation between inhibition of GDP release and conformational changes promoted by Mg$^{2+}$, we propose that in vivo, Mg$^{2+}$ prevents GDP dissociation from Rab5 until a guanine nucleotide exchange factor acts to promote GDP/GTP exchange, perhaps through interactions with the GTP-binding protein’s N-terminal domain.

MATERIALS AND METHODS

Reagents—[35S]GTP$\gamma$S, [3H]GDP and [35S]methionine were purchased from DuPont NEN. Unlabeled guanine nucleotides, CHAPS, trypsin, and restriction enzymes for subcloning were from Boehringer Mannheim. Rabbit reticulocyte lysate was from Promega. Construction of Rab5 Mutants—Rab5$^{1-198}$ was amplified by polymerase chain reaction from wild-type Rab5 using the oligonucleotides 5’-CCCGGATCCATATGGCAGGAGCC-3’ and 5’-CCGGTTCATCAGTCCGAG-3’.
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The absence of any added Mg\(^{2+}\) was subcloned into pAGA between BamHI and Sall sites. Similarly, Rab5\(^{23–198}\) was amplified using oligonucleotides 5'-GAATTCTATTGCTGATCTCTTGGAGA-3' and 5'-GGTACCATTTATCCTCCTCT-3' as primers and subcloned into pAGA between EcoRI and Sall sites. The vectors containing Rab5\(^{23–198}\) and Rab5\(^{23–198}\) were digested with NdeI and Sall, and the excised fragments were also inserted into pT7.7 by directional subcloning. A point mutant Rab5\(^{534N}\) was constructed by the Kunkle method using oligonucleotide 5'-AAGCACTAGGCTTTTTTGCACAAGC-3' and also inserted into pT7.7. The construction of Rab5\(^{23–198}\) in pAGA and Rab5\(^{S34N}\) and Rab5\(^{W74F}\) in pT7 were described previously (37, 39). Sequences of all the Rab5 mutants were confirmed by the deoxy chain determination method of Sanger.

In Vitro Translation and Translation—Procedures for in vitro transcription and translation of nascent peptides in rabbit reticulocyte lysate have been described in detail (40). Briefly, 10 \(\mu\)l wild-type or mutant cDNAs was transformed into BL21(DE3) cells by electroporation. Cells were grown in TB in the presence of 50 \(\mu\)g/ml carbenicillin, and protein expression was induced by 0.8 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. Rab5 or mutant proteins present in the cell lysate were purified by ion-exchange chromatography using DEAE-cellulose (Whatman). Fractions containing Rab5 proteins were concentrated by vacuum dialysis, and the final protein concentration was determined by the method of Bradford. Samples were frozen at –80°C until use.

Measurement of \([^{3}H]\)GDP Dissociation Rates—Purified recombinant wild-type or mutant Rab5 protein was rapidly thawed and exchanged into buffer A (50 mM Hepes-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM dithiotreitol). Cells were grown in TB in the presence of 10 \(\mu\)Ci/ml \[^{3}H\]GTP. The polymerase chain reaction product was amplified using oligonucleotides 9' TGCTAGTACTTCTGGGA-3' and 9' TATCCTCCTCCTCTCTCTCTCTCTCT-3'. The polymerase chain reaction product was subcloned into pAGA between BamHI and Sall sites. Similarly, Rab5\(^{23–198}\) was amplified using oligonucleotides 5'-GAATTCTATTGCTGATCTCTTGGAGA-3' and 5'-GGTACCATTTATCCTCCTCTCTCTCT-3' as primers and subcloned into pAGA between EcoRI and Sall sites. Five \(\mu\)l wild-type or mutant Rab5 cDNA was transformed into BL21(DE3) cells by electroporation. Cells were grown in TB in the presence of 50 \(\mu\)g/ml carbenicillin, and protein expression was induced by 0.8 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. Rab5 or mutant proteins present in the cell lysate were purified by ion-exchange chromatography using DEAE-cellulose (Whatman). Fractions containing Rab5 proteins were concentrated by vacuum dialysis, and the final protein concentration was determined by the method of Bradford. Samples were frozen at –80°C until use.

Intrinsic Tryptophan Fluorescence Measurements—Proteins were thawed and exchanged into buffer A as described above. Wild-type or mutant Rab5 protein was rapidly thawed and exchanged into buffer A (50 mM Hepes-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM dithiotreitol) and translated in vitro in the presence of 106 cpm/\(\mu\)l \[^{3}H\]methionine (1200 Ci/mmol) at 30°C for 20 min, and expressed proteins were quantified by trichloroacetic acid precipitation.

Proteolysis of in Vitro Synthesized Peptides—Proteolysis of in vitro translation products was as described by Sanford et al. (37). Briefly, translated peptides were preincubated at 30°C for 45 min in aliquots containing 10 or 25 \(\mu\)M guanine nucleotide or 25 mM EDTA in 80 \(\mu\)l of buffer A (50 mM Hepes-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM dithiotreitol). After equilibration at room temperature, an aliquot of each sample was filtered to determine the amount of \[^{3}H\]GDP bound. Excess unlabeled GDP were incubated in parallel to measure non-specific binding. After equilibration at room temperature, an aliquot of each sample was filtered to determine the amount of \[^{3}H\]GDP bound. Excess unlabeled GDP were incubated in parallel to measure non-specific binding. After equilibration at room temperature, an aliquot of each sample was filtered at indicated time points, and the amount of \[^{3}H\]GDP bound was determined by scintillation counting. Specific binding was determined as difference in cpm measured in samples incubated with or without excess GDP. Shown is the mean of duplicate measurements plotted as a function of ln(B/B0) against time (B0 initial specific binding; B, specific binding at each time point). Identical results were obtained on three separate occasions.

RESULTS

Mg\(^{2+}\) Inhibits GDP release from Rab5\(^{WT}\) but Not from Rab5\(^{534N}\). From Table I, we estimate that micro- to millimolar levels of free Mg\(^{2+}\) strongly inhibit GDP release, whereas free Mg\(^{2+}\) in the submicromolar range does not.

To investigate the mechanism through which Mg\(^{2+}\) inhibits GDP release, the point mutant Rab5\(^{534N}\) was constructed by site-directed mutagenesis. Sed\(^{2+}\) of Rab5 is expected to coordinate with Mg\(^{2+}\) in the guanine nucleotide binding pocket in both GDP- and GDP-bound states based on crystallographic evidence obtained for other GTP-binding proteins (10–15). The

FIG. 1. Effects of Mg\(^{2+}\) on the rate of \[^{3}H\]GDP dissociation from Rab5\(^{WT}\) and Rab5\(^{534N}\). Purified Rab5\(^{WT}\) (panel A) and Rab5\(^{534N}\) (panel B) (5 \(\mu\)l) were incubated at 37°C for 30 min with 200 \(\mu\)M \[^{3}H\]GDP (specific activity, –2.2 \(\times\) 10\(^{4}\) cpm/\(\mu\)l). Control reactions containing the same amount of protein and \[^{3}H\]GDP, but with 20 mM unlabeled GDP were incubated in parallel to measure nonspecific binding. After equilibration at room temperature, an aliquot of each sample was filtered to determine the amount of \[^{3}H\]GDP bound. Excess unlabeled GDP (20 mM) with 0.5 mM (triangle) or without (square) Mg\(^{2+}\) were then added, and GDP dissociation was monitored at room temperature. An aliquot of each sample was filtered at indicated time points, and the amount of \[^{3}H\]GDP bound was determined by scintillation counting. Specific binding was determined as difference in cpm measured in samples incubated with or without excess GDP. Shown is the mean of duplicate measurements plotted as a function of ln(B/B0) against time (B0, initial specific binding; B, specific binding at each time point). Identical results were obtained on three separate occasions.

The assay completely blocks the release of GDP, while little effect is observed with 0.5 mM Mg\(^{2+}\). The values of \(k_{off}\) determined from the slopes of these lines are presented in Table I. The inhibitory effect is reflected in the markedly reduced \(k_{off}\) at [Mg\(^{2+}\)] > 1.5 mM (data not shown). Therefore, under these assay conditions (Table I), we estimate that micro- to millimolar levels of free Mg\(^{2+}\) strongly inhibit GDP release, whereas free Mg\(^{2+}\) in the submicromolar range does not.
cognate mutant of Ras at this position, RasS17A, displays an accelerated rate of GDP release that is insensitive to Mg$^{2+}$ (20). Fig. 1B demonstrates that Rab5$^{534N}$ also exhibits a rate of GDP dissociation greater than Rab5$^{WT}$, but more importantly, the release of GDP is no longer affected by Mg$^{2+}$. As summarized in Table I, this effect is reflected in the large difference in $k_{off}$ determined in the presence of high Mg$^{2+}$ (0.245 versus 0.006 min$^{-1}$ for Rab5$^{WT}$). Thus, it is likely that coordination of Mg$^{2+}$ with Ser$^{34}$ participates in a direct chemical restraint preventing dissociation of the nucleotide. However, it is also possible that Mg$^{2+}$ could promote structural rearrangements in Rab5 through interactions with Ser$^{34}$, resulting in a protein conformation that is unfavorable for GDP release. These two mechanisms are not mutually exclusive, and both may contribute to the observed inhibitory effect of Mg$^{2+}$.

Mg$^{2+}$ Alters the Intrinsic tryptophan Fluorescence of Rab5$^{WT}$, but Not of Rab5$^{534N}$. To examine whether protein structural changes are induced by Mg$^{2+}$, we investigated its effects on the intrinsic tryptophan fluorescence of Rab5; the latter provides a sensitive probe for local conformational changes in GTP-binding proteins (41–45). We previously characterized guanine nucleotide-dependent fluorescence changes in Rab5WT, but Not of Rab5S34N—

| Table I | Mg$^{2+}$ effect on $k_{off}$ for GDP release determined for Rab5 and its mutants |
|---------|--------------------------------------------------------------------------------------------------|
| $k_{off}$ for GDP release | No addition | 0.5 mM Mg$^{2+}$ | 5 mM Mg$^{2+}$ |
| Rab5$^{WT}$ | 0.120 | 0.114 | 0.006 |
| Rab5$^{534N}$ | 0.211 | 0.230 | 0.245 |
| Rab5$^{S34N}$ | 0.108 | 0.117 | 0.013 |
| Rab5$^{523-198}$ | 0.101 | 0.106 | 0.045 |

Estimated Mg$^{2+}$ concentration

| [Added] | 0 | 0.5 mM | 1.0 mM | 1.5 mM | 3.0 mM | 5.0 mM |
|---------|---|--------|--------|--------|--------|--------|
| [Free]  | 0 | 0.1 mM | 3.11 mM | 496.3 μM | 2.0 mM | 4.0 mM |

The increase in fluorescence intensity is accompanied by a slight red shift in $\lambda_{max}$. Further examination of Mg$^{2+}$ effects on the individual fluorescence properties of Rab5$^{W114F}$ and Rab5$^{W74F}$ indicates that each of the two tryptophans of Rab5 contributes to the observed fluorescence changes in Rab5$^{WT}$ (data not shown). Our results suggest that Mg$^{2+}$ induces conformational changes in GDP-bound Rab5 that place both of its tryptophans into a more polar environment. Since the inhibitory effect on GDP release and the induced structural changes in Rab5 occur within the same range of [Mg$^{2+}$], we postulate the increased fluorescence is due to an altered form of the protein that is unfavorable for GDP release. To test this hypothesis, we examined the influence of Mg$^{2+}$ on the fluorescence properties of Rab5$^{534N}$. In contrast to Rab5$^{WT}$, neither the fluorescence intensity (Fig. 3) nor $\lambda_{max}$ of Rab5$^{534N}$ (data not shown) is affected by high [Mg$^{2+}$]. This result supports the idea that protein conformation promoted by the coordination between Mg$^{2+}$ and Ser$^{34}$ plays an important role in the inhibition of GDP release.

Characterization of Rab5 Mutants by Protease Protection Assays—Several lines of evidence suggest potential roles for the N- and the C-terminal domains of small GTP-binding proteins in Mg$^{2+}$-induced conformational changes and the regulation of guanine nucleotide exchange. Ser$^{34}$ is located within the N terminus, thus it is easy to envision that coordination between Mg$^{2+}$ and Ser$^{34}$ could promote conformational rearrangements within this domain. Myristoylation at the N terminus and truncation of the N-terminal domain of ARF alter the protein's affinity for GDP and properties of guanine nucleotide exchange (33, 38). In addition, N-terminal truncation interferes with the function of both Rab5 and ARF (33–36) as well as post-translational processing of Rab5 (37). Finally, post-translational modification of Ras-like proteins at their C-terminal domains, including phosphorylation, isoprenylation, and carboxyl methylation, is important for GDP dissociation stimulator action (46, 47).

To evaluate the relative roles of the N and the C termini as potential regulators of guanine nucleotide exchange, three truncation mutants of Rab5 were characterized (Rab5$^{1-198}$, Rab5$^{23-198}$, and Rab5$^{23-215}$). Filter binding assays with [$^{3}H$]GDP and [$^{35}S$]GTPyS indicated that both Rab5$^{1-198}$ and...
of, but close to, position 198. The only practical assignment is Arg<sup>195</sup>. Trypsinization of the GDP-bound form of all the Rab5 molecules produces identical 14-kDa fragments, indicating that the cleavage sites must be downstream of position 23 and upstream of position 198. The fact that this peptide is heavily radiolabeled suggests that several methionine residues are present. Based on the size of this tryptic fragment, Lys<sup>70</sup> and Arg<sup>195</sup> are the most likely tryptic sites since cleavage at these residues would generate a 13.8-kDa fragment. Since the GTP<sup>S</sup>-protected fragments of Rab5<sup>WT</sup> and Rab5<sup>1–198</sup> migrate with a mass ~1.7 kDa larger than that of Rab5<sup>23–198</sup> on SDS gels, if there is an N-terminal tryptic site, it would be best positioned at Arg<sup>9</sup> or Arg<sup>8</sup>. However, because of the proximity of these residues to the N terminus, and given the vagaries of peptide mobilities on SDS gels, we cannot distinguish which Arg provides the tryptic cleavage site nor can we confirm that proteolysis at the extreme N terminus occurs. It should be noted that Steele-Mortimer and co-workers (35) have previously assigned Arg<sup>8</sup> as a tryptic site for Rab5 by N-terminal sequencing (35). A key prediction from the proposed tryptic map is that GTP<sup>S</sup>-y-mercaptobenzothiazole protects against proteolytic cleavage at Lys<sup>70</sup>, supporting the idea that the Rab5’s N-terminal domain undergoes conformational rearrangement upon GDP/GTP exchange.

In comparison with Rab5<sup>WT</sup>, Rab5<sup>S34N</sup> produces a distinct protease protection pattern (Fig. 6). In the absence of exogenously added guanine nucleotide, Rab5<sup>S34N</sup> is completely degraded by trypsin, unlike wild-type, which displays a 14-kDa fragment protected by endogenous levels of GDP. The accelerated rate of GDP release from the mutant (Fig. 1B) is most likely responsible for this effect, making Rab5<sup>S34N</sup> more susceptible to proteolysis. When saturating levels of GDP or GTP analogs are added, a 14-kDa fragment of Rab5<sup>S34N</sup> is protected.

The absence of the 20-kDa core peptide in the GTP analog-bound forms suggests that Rab5<sup>S34N</sup> resembles the GDP-bound conformation of wild-type, consistent with previous reports characterizing the cognate mutants of other Rab proteins (48–50). The failure of GTP<sup>S</sup> or Gpp(NH)p to protect the predicted Lys<sup>70</sup> cleavage site also suggests that Mg<sup>2+</sup> coordination with Ser<sup>24</sup> is necessary to induce conformational changes upon guanine nucleotide exchange.

**Characterization of Mg<sup>2+</sup> Effects on Rab5<sup>1–198</sup> and Rab23–198.** To further evaluate the structural role of the N-terminal domain of Rab5, the effects of Mg<sup>2+</sup> on GDP dissociation from Rab5<sup>1–198</sup> and Rab5<sup>23–198</sup> were compared. The data presented in Fig. 7A and summarized in Table I show that GDP release from Rab5<sup>1–198</sup> is markedly inhibited by 5 mM Mg<sup>2+</sup>, similar to the results obtained for Rab5<sup>WT</sup>. Thus, it is unlikely that the C-terminal domain of Rab5 participates in Mg<sup>2+</sup>-induced inhibition of GDP dissociation. In contrast, the ability to bind guanine nucleotides in solution, although Rab5<sup>23–198</sup> exhibits a reduced affinity for both nucleotides (data not shown). Previous studies demonstrated that Rab5<sup>23–215</sup> binds [3H]GDP (38). Further information on the tertiary structure of these proteins was obtained from protease protection assays (37). As shown in Fig. 4, in vitro synthesized [35S]-labeled Rab5<sup>WT</sup> migrates as a 27 kDa band on a urea/acrylamide gradient SDS gel. The synthetic protein binds endogenous GDP (40); limited digestion of this GDP-bound form with trypsin produces a single [35S]-labeled fragment of 14 kDa. Addition of 30 mM EDTA to chelate Mg<sup>2+</sup> essential for guanine nucleotide binding markedly reduces the amount of the latter tryptic peptide. However, incubation with GTP<sup>S</sup> prior to trypsinization results in protection of a [35S]-labeled 20-kDa peptide in addition to the 14-kDa fragment, representing a “core” structure of Rab5 in the GTP<sup>S</sup>-bound conformation. Fig. 4 further demonstrates that protease protection profiles for Rab5<sup>1–198</sup>, Rab5<sup>23–198</sup>, and Rab5<sup>3–215</sup> are all similar to Rab5<sup>WT</sup>, confirming that each of the truncation mutants binds guanine nucleotides.

By comparing the sizes of the GDP- and GTP<sup>S</sup>-y-mercaptobenzothiazole-protected fragments of Rab5<sup>WT</sup> and these truncation mutants, a tryptic cleavage map can be predicted as shown in Fig. 5. Our assignments are made on the following premises. Since both of the GTP<sup>S</sup>-y-mercaptobenzothiazole-protected fragments for Rab5<sup>23–198</sup> and Rab5<sup>23–215</sup> are identical and approximately equal in size to undigested Rab5<sup>WT</sup>, a C-terminal proteolysis site must exist upstream of Lys<sup>183</sup>; however, proteolytic cleavage at these sites would yield 18.3 kDa; and Rab5<sup>23–215</sup>, 14 kDa and 18.3 kDa. The lowest molecular weight species detected for Rab5<sup>23–198</sup> and Rab5<sup>23–215</sup> is not a tryptic peptide fragment since it appears in the (-) trypsin lanes.
of Mg$^{2+}$ to inhibit GDP release is partially lost when the N-terminal domain of Rab5 is truncated (Fig. 7B). Because all six coordinating ligands for Mg$^{2+}$ remain unperturbed in Rab5<sub>23–198</sub>, chemical constraint(s) due to its chelation within the guanine nucleotide binding pocket most likely account for the residual inhibitory action of the cation. The reduced inhibition of GDP release suggests, however, that the ability of Rab5<sub>23–198</sub> to undergo structural changes in response to Mg$^{2+}$-Ser<sub>34</sub> coordination could be impaired due to the absence of the N-terminal domain.

To test this possibility, the influence of Mg$^{2+}$ on the fluorescence properties of Rab5<sub>1–198</sub> and Rab5<sub>23–198</sub> was also compared (Fig. 8). Like Rab5<sub>WT</sub> the intrinsic fluorescence intensity of Rab5<sub>1–198</sub> is increased by Mg$^{2+}$. In contrast to Rab5<sub>WT</sub> but similar to Rab5<sub>S34N</sub>, the fluorescence properties of Rab5<sub>23–198</sub> do not change in response to increasing [Mg$^{2+}$]. Thus, in addition to chemical constraints exerted by Mg$^{2+}$ coordination within the guanine nucleotide binding pocket, the Mg$^{2+}$-Ser<sub>34</sub> link imposes structural restraints that involve the N-terminal domain and contribute to inhibition of GDP release.

**DISCUSSION**

Studies on crystallized Ras (10), EF-Tu (11), G<sub>a</sub><sup>i</sup> (12), ARF (13), and Ran (14) in their GDP-bound forms have revealed identical Mg$^{2+}$ coordination within the guanine nucleotide binding pocket. The cation interacts directly with the β-phosphate of GDP and a highly conserved Ser/Thr near the N terminus of these proteins; indirect interactions are mediated through four associated water molecules. In the GTP analog-bound forms of Ras (10), EF-Tu (1), G<sub>a</sub><sup>i</sup> (12), and G<sub>a</sub><sup>i</sup> (15), Mg$^{2+}$ coordination with two of these water molecules is replaced by a direct coordination with the γ-phosphate of the nucleotide and another highly conserved Thr. Inhibitory effects of Mg$^{2+}$ on GDP release have been documented for a wide variety of small GTP-binding proteins (16–27), and our results extend this general observation to include Rab5. Mutation of Ser<sub>34</sub> in Rab5 results in an increased rate of GDP release, which is no longer affected by Mg$^{2+}$, a phenomenon also observed for the cognate mutants of other small GTP-binding proteins, including Ras (20), Rab1 (48), Rab9 (49), and Rab3 (50).

In vivo, the release of GDP from Rab5, and most likely other small GTP-binding proteins, would be inhibited by intracellular free Mg$^{2+}$. Thus, a common mechanism to regulate guanine nucleotide exchange may be adopted by this family of proteins. In order to facilitate GDP/GTP exchange, a GEF must overcome the inhibitory restraints imposed by intracellular Mg$^{2+}$ levels. Through transient disruption of the Mg$^{2+}$-Ser/Thr coordination, GEF could be envisioned to induce structural changes such that the GTP-binding protein adopts a conformation similar to its Ser<sub>34</sub>Asn cognate mutant, thereby promoting GDP dissociation.

Several lines of evidence support this idea. Our fluorescence studies demonstrate that Rab5<sub>S34N</sub> adopts a Mg$^{2+}$-insensitive conformation that is distinct from the Mg$^{2+}$-sensitive conformation of Rab5<sub>WT</sub>. Although the strong dominant inhibitory effects of cognate mutants are generally attributed to their preferential affinity for GDP over GTP (6, 48, 49, 52–60), Wittinghofer and co-workers (20, 63) have argued that the distinct structures of the Ser<sub>34</sub>Asn mutations are most likely responsible for disruption of wild-type protein function through competition for GEF interactions. The fact that Ras<sup>D57A</sup> has an even greater affinity for GDP than Ras<sup>S17A</sup> but is not a suppressor of Ras function supports this idea (20). Moreover, Rab3A<sup>T17N</sup> (50), Ras<sup>S17N</sup> (51), and Ran<sup>T24N</sup> (52) have
It seems likely that Ser change by stabilizing Ras in a nucleotide-free state (53); thus, has been proposed that the yeast GEF Cdc25p promotes ex- corresponding GEs are thermodynamically more favorable. It been shown to have a much higher affinity for their respective GEs than wild-type, further suggesting that complexes formed between the Ser → Asn cognate mutants and their corresponding GEs are thermodynamically more favorable. It has been proposed that the yeast GEF Cdc25p promotes exchange by stabilizing Ras in a nucleotide-free state (53); thus, it seems likely that Ser → Asn cognate mutants of Ras-like factors are analogous to the nucleotide-free conformations of these proteins. The fact that exogenously added GDP and GTP analogs produce the same protease protection profile indicates that Rab5 must accommodate both nucleotides in its binding pocket but fails to undergo molecular rearrangements associated with guanine nucleotide exchange. This evidence supports the view that the mutant adopts a structure intermediate between GDP- and GTP-bound states. Ser34 is therefore predicted to be a critical residue involved in the conformational switch during guanine nucleotide exchange.

Our study demonstrates that Ser34-Ser34 coordination between GDP and Rab5 not only provides a chemical constraint, but also promotes conformational changes involving the N terminus, which impose additional structural restraints against GDP dissociation. The markedly reduced inhibition of GDP release by Mg2+ and the lack of Mg2+-induced fluorescence changes in Rab523–198 indicate that the N-terminal domain of Rab5 must play a key role in maintaining a conformation that blocks GDP release. It should be noted that the first 23 amino acids of Rab5 correspond to a rather small domain comprising only 4 residues in Ras based on pattern-induced multiple alignment analysis (37). As previously noted in a comparison with Ras (36), the N-terminal domain of Rab5 is one of five regions of this molecule predicted to impart functional specificity. Thus, although Ras517N (20) behaves in a fashion similar to Rab534N, whether Mg2+-induced N-terminal conformational changes contribute to inhibition of GDP release from Ras is uncertain. Comparison of Ras crystal structures in the GDP and GTP analog-bound states reveals no obvious conformational changes in its N-terminal domain (10), but this does not preclude the possibility of structural rearrangement during the guanine nucleotide exchange process. Since the N terminus of ARF also has been reported to participate in guanine nucleotide exchange regulation, it is possible that structural features of this domain may be shared among other Ras-like GTP-binding proteins. We speculate that GEF directly interacts with the N-terminal domain of Rab5 to interrupt Mg2+ coordination with Ser34, thereby promoting a transient conformation that facilitates GDP release. In support of this idea, truncation of the N-terminal domain abolishes the function of Rab5 (34, 35).

Since our data show that cellular levels of Mg2+ are sufficient to block GDP release, what is the role of the guanine nucleotide dissociation inhibitor GDI? Even though this factor was purified based on its ability to inhibit GDP dissociation (64, 65), under physiologic Mg2+ concentrations its relative activity is only marginal (65–67). Except for Rac (68, 69) and Rho (70), similar factors have yet to be identified for other Ras family members. This suggests that a GDI per se is not required to prevent GDP dissociation from small GTP-binding proteins since cellular Mg2+ levels are sufficient to provide this function. In fact, upon delivery of Rab proteins to membranes, exchange for GTP is not immediate after GDI dissociation (8, 9). Our study indicates that during this time period, Mg2+ most likely prevents GDP dissociation. Thus, we speculate that the true function of the Rab GDI may be one of a molecular escort protein or chaperone. In this capacity, GDI may stabilize the RabMg2+-GDP complex, blocking interaction sites for GEF until the appropriate membrane target is reached. This function is comparable with that of the βγ subunit complex of heterotrimeric G proteins, which also retards GDP release and targets α subunits to their appropriate membrane receptors. Indeed, our recent studies revealed structure-function similarities between Rab5 and the α subunits of GTP-binding proteins (39). However, GDP release from most Gα subunits is unaffected by Mg2+. In fact, the Gα subunits contain a unique α-helical domain absent from Ras-like GTP-binding proteins that may block GDP release (12, 13). The N-terminal domain of Rab5 may substitute, in part, for this structural element.

Fig. 8. Effects of Mg2+ on the fluorescence intensities of Rab5198 and Rab523–198. Effects of Mg2+ on the fluorescence properties of Rab5198 (filled square) and Rab523–198 (open square) were measured as described in Fig. 2. Normalized fluorescence intensities were plotted against added Mg2+ concentration as shown in Fig. 1. Similar results were obtained on at least three separate occasions.

Influence of Mg2+ on the Structure and Function of Rab5

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