Mechanism of the Guanine Nucleotide Exchange Reaction of Ras GTPase—Evidence for a GTP/GDP Displacement Model

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ABSTRACT: Ras GTPases function as binary switches in the signaling pathways controlling cell growth and differentiation by cycling between the inactive GDP-bound and the active GTP-bound states. They are activated through interaction with guanine nucleotide exchange factors (GEFs) that catalyze the exchange of bound GDP with cytosolic GTP. In a conventional scheme, the biochemical roles of GEFs are postulated as stimulating the release of the bound GDP and stabilizing a nucleotide-free transition state of Ras. Herein we have examined in detail the catalyzed GDP/GTP exchange reaction mechanism by a Ras specific GEF, GRF1. In the absence of free nucleotide, GRF1 could not efficiently stimulate GDP dissociation from Ras. The release of the Ras-bound GDP was dependent upon the concentration and the structure of the incoming nucleotide, in particular, the hydrophobicity of the β and γ phosphate groups, suggesting that the GTP binding step is a prerequisite for GDP dissociation, is the rate-limiting step in the GEF reaction, or both. Using a pair of fluorescent guanine nucleotides (*N*-methylanthraniloyl GDP and 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-GTP) as donor and acceptor probes, we were able to detect fluorescence resonance energy transfer between the incoming GTP and the departing GDP on Ras under controlled kinetic conditions, providing evidence that there may exist a novel intermediate of the GEF−Ras complex that transiently binds to two nucleotides simultaneously. Furthermore, we found that Ras was capable of binding pyrophosphate (PPi) with a dissociation constant of 26 μM and that PPi and GMP, but neither alone, synergistically potentiated the GRF1-stimulated GDP dissociation from Ras. These results strongly support a GEF reaction mechanism by which nucleotide exchange occurs on Ras through a direct GTP/GDP displacement model.

Ras GTPases play a pivotal role in the transduction of signals from membrane receptors to downstream intracellular effectors. They act as molecular switches, interconverting between the inactive GDP-bound state and the active GTP-bound state. The conversion of Ras-GDP to Ras-GTP following cell stimulation is mediated by direct interaction of Ras with a family of guanine nucleotide exchange factors (GEFs)[1] that include Sos1/Sos2 (*1*), RasGRP (*2*), smgGDS (*3*), GRF1/CDC25Mm (*4*), and GRF2 (*5*). The Sos proteins have been shown to activate Ras upon receptor tyrosine kinase stimulation (*1*). RasGRP is involved in coupling calcium signals to Ras activation (*2*). GRF1/Cdc25^{Mm}, a neuron-specific RasGEF, and the homologous GRF2 are able to activate Ras in response to the heterotrimeric G protein coupled receptors (GPCR) (*6*), Ca^{2+} (*5*, *7*), and cyclic AMP (*8*).

The Ras guanine nucleotide exchange mechanism catalyzed by the GEFs has been intensively studied (for review, see refs *9*−*11*). A number of previous studies of Sos1 and GRF1/Cdc25^{Mm} have suggested that the nucleotide-free state of Ras binds to the GEFs with a much higher affinity than either of the nucleotide-bound states. The GDP-dissociation step appeared to be rate-limiting in the GEF reaction (*12*, *13*). A substituted GEF reaction mechanism, analogous to that of the heterotrimeric G proteins activation by the GPCR and to the interaction of elongation factor Tu (EF-Tu) with EF-Ts (*14*), has been proposed such that GEF binding to Ras-GDP results in the transient formation of a ternary GEF−Ras-GDP complex, followed by the formation of a nucleotide free binary Ras-GEF complex. GTP loading to this binary complex would prompt the release of the GEF from Ras and the reaction product Ras-GTP (*15*). In such a manner, GEFs appear to exert their functions by stimulating the bound GDP dissociation from Ras and stabilizing a transient nucleotide-free state of Ras. This sequence of the GEF reaction, termed the substituted mechanism, has been suggested to apply to the interactions of other members of the Ras superfamily with their cognate GEFs. In keeping with this model, binary G protein·GEF complexes have been successfully crystallized under conditions in which guanine nucleotide (GDP or GTP) is absent and the Mg^{2+} cofactor of the small GTPases is depleted. The three-dimensional structures of Ras·Sos1 (*16*), EF-Ts·EF-Tu (*17*), Arf1·Sec7

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[1] Abbreviations: GEF, guanine nucleotide exchange factor; mant-GDP, *N*-methylanthraniloyl GDP; TNP-GTP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-GTP; FRET, fluorescence resonance energy transfer; PPi, pyrophosphate.

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(*18*), Rab·Mss4 (*19*), Rac1·Tiam1 (*20*), Cdc42·Dbs (*21*), Rho·Dbs (*22*) and Ran·RCC1 (*23*) have been made available to demonstrate how the GEFs interact with the respective small GTPases and stabilize a nucleotide-free transition state. Significantly, a recent structural study of the Arf1-GDP−Mg^{2+} in complex with Brefeldin A, an Arf inhibitor, and the Sec7 domain of ARNO and the Arf1-GDP-ARNO moved the field a step further by providing a detailed picture of the Arf-GDP interaction with the Sec7 domain (*24*).

Despite these extensive biochemical and structural studies, it remains a challenge to establish the exact sequence of events involved in the GEF reaction. A few questions remain unanswered regarding the role of GEFs in stabilizing a nucleotide-free intermediate during the exchange process. First, the binary nucleotide-free complex is stable only in the absence of nucleotides; thus it can only be generated in vitro under nonphysiological conditions (*16*−*23*). Considering the abundance of GTP in the cytosol (∼150 μM) (*25*), it has been suggested that the nucleotide-free state of the GTPases is virtually nonexistent in the cell (*11*). In fact, the binding affinity of GEF·Ras-GDP is much lower than that of GEF·Ras (nucleotide-depleted) (*16*, *26*). Second, the dissociation of GDP from the GTPases may not follow the substituted mechanism. As shown for the interactions of Ras-GDP−Cdc25 and Ran-GDP−RCC1, the affinity of GDP in the ternary complexes was reduced by several orders of magnitude from pM to μM and the rate of dissociation was increased by a similar magnitude (*27*). Bound GDP is therefore expected to be released upon GEF binding. Unfortunately, assays for measuring the GEF-stimulated GDP release were routinely performed in the presence of excess nucleotide and in higher than micromolar concentration of the small GTPases (*28*, *29*). In the presence of high concentrations of free nucleotide, the observed GDP dissociation rate represents an apparent nucleotide exchange rate rather than a first step reaction of the substituted enzyme mechanism. To our knowledge, RCC1, a Ran-specific GEF, and smgGDS, a promiscuous GEF for Ras and Rho proteins, are the only regulators reported to be able to displace GDP efficiently in the absence of excess nucleotide (*27*, *30*). Third, the substituted mechanism does not explain how incoming GTP can re-enter the empty nucleotide binding pocket of Ras and reverse the nucleotide release reaction, since Sos1, Cdc25, and possibly other GEFs work equally well in stimulating GDP or GTP dissociation (*27*, *28*). On the other hand, GDP depletion results in the collapse of the switch regions and the p-loop of Ras protein, preventing the further GTP loading (*15*, *31*). Fourth, it remains controversial whether the GDP dissociation step is rate-limiting as suggested by the substituted mechanism. Taken together these concerns justify a detailed analysis of the substituted model of the Ras GEF reaction.

In the present study, we provide evidence strongly supporting a displacement model in the GRF1-catalyzed GDP/GTP exchange reaction of Ras. We show that the GRF1-stimulated GDP dissociation from Ras is dependent on the binding properties of the incoming nucleotide. The GDP/GTP exchange reaction appears to proceed via a transitory quaternary complex that contains both incoming and leaving nucleotides: GRF1-(GTP−Ras-GDP), as demonstrated by the transient fluorescence resonance energy transfer between the fluorescent mantGDP donor and TNP-GTP acceptor bound to Ras. In addition, we provide evidence suggesting that the β,γ-phosphates of incoming GTP may serve as the attacking group to initiate the GEF reaction.

EXPERIMENTAL PROCEDURES

Materials. All nucleotides used were from Sigma, except GppNHp and GMP−PCP which were obtained from Boehringer Mannheim. Sodium pyrophosphate (PPi) was purchased from Sigma. *N*-methylanthraniloyl derivate of GDP (mantGDP) and 2′(or 3′)-O−(trinitrophenyl)guanosine 5′-triphosphate (TNP-GTP) were obtained from Molecular Probes, Inc. The radiolabeled [^{32}P]PPi and the radioactive nucleotides [^{3}H]GDP and [^{35}S]GTPγS were obtained from NEN Life Sciences Products, Inc.

Preparation of Ras and GRF1 Proteins. The Ras protein containing full length H−Ras (amino acids 1−188) was expressed in *Escherichia coli* as amino terminal $(His)_6$-tagged fusion by using the pET expression system (Novagen). The cDNA encoding the CDC25 homology domain at the C-terminus of rat RasGRF1 (amino acids 798−1244) (*32*, *33*) was provided by Dr. Larry A. Feig (Tufts University School of Medicine, Boston, MA), and was subcloned into pGEX-KG vector to express glutathione-S-transferase (GST) fusion protein**.** The N-terminal tagged proteins were purified by glutathione or Ni^{2+} agarose affinity chromatography. The GST moiety of the fusion was cleaved by thrombin digestion, followed by incubation with *p*-aminobenzamidine immobilized on agarose beads (Sigma) to remove the thrombin (*34*). The quality of the proteins used in all assays was judged by SDS-polyacrylamide gel electrophoresis. Protein concentration was determined by BCA protein assay reagent (Pierce).

Radioactive Filter Binding Assay. The filter-binding assays were performed essentially as described (*35*). Briefly, Ras protein was loaded at 25 °C for 30 min with [^{3}H]GDP in buffer A (50mM HEPES, pH7.6, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) supplemented with 10 mM EDTA. Following incubation $MgCl_2$ was added to a net concentration of 5 mM and the reaction mixture was incubated on ice for 10 min. The unbound [^{3}H]GDP was removed by rapid gel filtration. The dissociation reaction was carried out in buffer A by addition of GTP or other analogue with or without GRF1 at the indicated concentrations. At the indicated time intervals, aliquots of the mixtures were withdrawn and filtered through nitrocellulose filter disks (0.45 mm, Millipore) followed by washing twice with 10 mL cold stop solution. To access the PPi binding property, Ras-[^{32}P]PPi was prepared in a similar procedure using [^{32}P]PPi as probe. After complex formation, the dissociation of bound [^{32}P]PPi was followed by addition of GTP, GDP or GMP in the presence or absence of GRF1. For the equilibrium binding, Ras-GDP was incubated at 25 °C for 10 min in buffer A containing 10 mM EDTA with [^{32}P]PPi at different concentrations followed by adding $MgCl_2$ to overcome EDTA. Radioactivity retained was measured in a Packard 2000 liquid scintillation analyzer.

Fluorescence Measurements. The fluorescence measurements were done under similar conditions as described for the filter binding assays, except that Ras-mantGDP was used instead of Ras-[^{3}H]GDP. The fluorescence measurements were carried out using an LS 50B Luminescence Spectrometer (Perkin-Elmer). All equilibrium and kinetic measure-

ments were performed at 20 °C in a 0.5-ml stirred cuvette in a buffer containing 50 mM HEPES, pH7.6, 100 mM NaCl, and 1 mM DTT. MantGDP showed a maximal excitation at 360 and a maximal emission at 440 nm. The binding of mantGDP to Ras causes an ~4-fold enhancement in mant emission intensity (*28*). This allowed studying of the interaction of guanine nucleotide with GTPases by easily following the decrease of the mant-moiety fluorescence. The raw spectra were corrected for both the contributions of Raman scattering and unbound mantGDP by subtracting the spectra of the buffer containing ligands only. For the recording of emission spectra of mantGDP bound to Ras in the presence of TNP-GTP, the excitation wavelength was set at 320 nm to minimize the inner-filter effect of nucleotides. Ras-GTP and Ras-TNP-GTP were prepared as described above for Ras-[^{3}H]GDP and the unbound nucleotides were removed by rapid gel filtration as stated for Ras-[^{3}H]GDP. In addition, all the measurements made for fluorescence resonance energy transfer (FRET) were corrected for the inner-filter effect of the nucleotides using the following formula (*36*): $F_{Corrected} = (F_{Observed} - F_{Background}) \times 10^{0.5b(A_{\lambda ex}+A_{\lambda em})}$, with $F_{Corrected}$ being the corrected value of the fluorescence intensity, $F_{Observed}$ the measured fluorescence intensity, $F_{Background}$ the fluorescence of the sample without protein, b the path length of the cuvette (in cm), $A_{\lambda ex}$ and $A_{\lambda em}$ the absorbance of the sample at the excitation and emission wavelengths, respectively.

RESULTS

GRF1 Alone Is Ineffective at Stimulating Release of Ras-Bound GDP. According to the substituted mechanism, GRF1 should be able to stimulate GDP dissociation from Ras-GDP by itself by lowering the nucleotide binding affinity of Ras. We performed two distinct assays to follow the GDP dissociation kinetics of Ras during GRF1 stimulation. In the nitrocellulose membrane filter binding assay (Figure 1A), when free GTP (100 μM) was present, a catalytic amount of GRF1 (10 nM) was sufficient to stimulate the release of 90% of bound GDP from 100 nM Ras-[^{3}H]GDP within 5 min. These results are in agreement with the previously reported GRF1 catalytic function in the Ras-GEF reaction (*4*, *32*, *33*). However, in the absence of free GTP, the presence of up to a 100-fold excess of GRF1 (10 μM) over Ras-GDP (100 nM) only caused about 10% release of Ras bound [^{3}H]GDP over a period of 15 min. The concentration of the Ras-[^{3}H]GDP employed was 100 nM to minimize the possible rebinding of [^{3}H]GDP to Ras after its dissociation, since the GDP binding affinity in the ternary Cdc25^{Mm}•Ras-GDP complex was estimated to be in the submicromolar range (28).

To confirm the results of the membrane filtration assay, the nucleotide exchange reactions were carried out by using mantGDP, a fluorescent analogue of GDP, as a tracer to follow the GRF1-catalyzed GDP dissociation kinetics. As shown in Figure 1B, addition of either GRF1 (100 nM) or GTP (100 μM) alone to the Ras-mantGDP complex (100 nM) had a marginal effect on mantGDP release. But the presence of both GRF1 and GTP resulted in a drastic increase in the mantGDP dissociation rate so that over 90% mantGDP was released from Ras in 5 min. Similar to the filter binding results, no significant mantGDP release was observed upon addition of increasing concentrations of GRF1 (0.1 and 5 μM) in the absence of free GTP. The results of these two independent assays show that GRF1 alone is ineffective at stimulating the dissociation of Ras-bound GDP. The presence of free GTP in addition to GRF1 is required for the effective release of GDP from Ras.

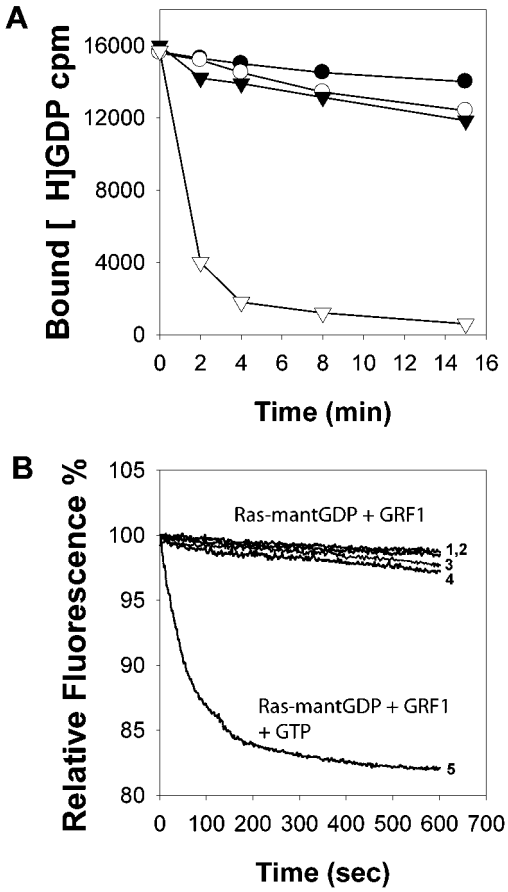

FIGURE 1: GRF1 alone is ineffective for stimulating GDP dissociation from Ras. (A) a filter binding analysis of [^{3}H]GDP release from Ras-[^{3}H]GDP. [^{3}H]GDP-loaded Ras (100 nM) was incubated at 20 °C in buffer A (●) or buffer A containing 1 μM GRF1 (○), 10 μM GRF1 (▼) or 1 μM GRF1 and 100 μM GTP (▽). At the designated times, aliquots of the reaction mixtures were withdrawn from each sample, and the amount of [^{3}H]GDP remained bound to Ras was determined by filter binding. (B) Fluorescence measurement of the mantGDP release from Ras-mantGDP. Ras-mantGDP (100 nM) was incubated at 20 °C in buffer A (trace 1) or supplemented with 0.2 μM GRF1 (trace 2), 5 μM GRF1 (trace 4), 100 μM GTP alone (trace 3), or 0.2 μM GRF1 plus 100 μM GTP (trace 5). The decrease in fluorescence at 440 nm due to dissociation of mantGDP was followed over time. Each graph represents the results of at least three independent experiments.

Dependence of the Nucleotide Exchange Rate on the Concentration and Structural Nature of Free Nucleotide. To examine the nucleotide dependence of the GRF1-catalyzed exchange reaction, we measured the rates of GDP release from Ras in the presence of different concentrations of GTP. By varying the GTP concentration between 0 and 500 μM, we observed that the [^{3}H]GDP release rate increased in correlation with the GTP concentration increase in the range of 0−10 μM (Figure 2A). Higher concentrations of GTP (100 and 500 μM) displayed a saturating effect in stimulating the GDP release by GRF1, which is consistent with the observations by Lenzen et al. (*28*) that addition of GTP from 10 μM to 10 mM did not affect the Cdc25^{Mm}-stimulated dissociation rate for Ras-bound mantGDP. Similar concen-

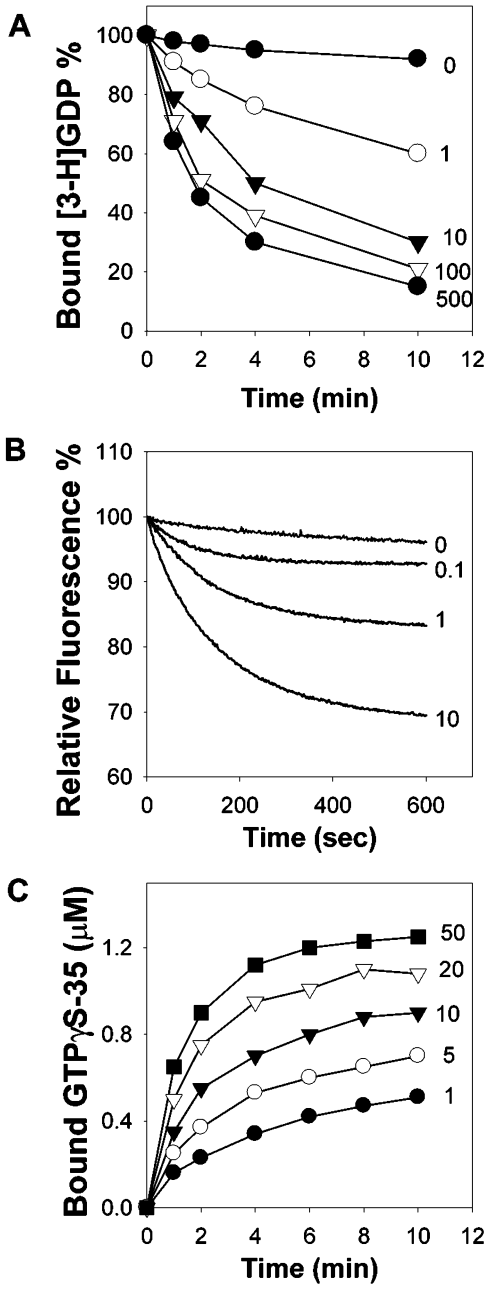

FIGURE 2: The GRF1-stimulated GDP release from Ras is dependent on the concentration of free GTP. (A) 100 nM Ras-[^{3}H]GDP was incubated with buffer A supplemented with 500 nM GRF and various concentrations of GTP. (B) fluorescence measurement of mantGDP release from Ras-mantGDP. Reaction conditions were similar to (A) except that the GTP concentrations were varied. C, nucleotide dose-dependence of [^{35}S]GTPγS binding to Ras. Free [^{35}S]GTPγS at the indicated concentrations (μM) were incubated with 1 μM Ras-GDP in the presence of 200 nM GRF1 in the exchange buffer.

tration dependence was also observed for the dissociation of mantGDP from Ras-mantGDP in the fluorescence-based assay (Figure 2B). In addition, we determined the rate of [^{35}S]GTPγS uptake by Ras-GDP catalyzed by GRF1 as a function of the concentration of [^{35}S]GTPγS. Similar to the GDP dissociation, the association rate of GTPγS also appeared to be concentration dependent (Figure 2C). Systemically increasing the concentrations of GTPγS from 1 to 50 μM led to increased GTPγS binding rates at fixed concentrations of GRF1 (200 nM) and Ras-GDP (1 μM).

It has been shown that structural modifications of guanine nucleotides can have strong effects on the binding affinities to the Ras GTPases (*30*, *37*). Given the observed GTP concentration-dependence of GDP-dissociation, we were interested to compare the efficiencies of GRF1-mediated GDP/GTP exchange in the presence of varying incoming nucleotides with different Ras binding affinities. A panel of nucleotide analogues with distinct Ras binding characteristics were chosen for kinetic analysis (Figure 3A). Using Ras-bound [^{3}H]GDP as a tracking signal, the dissociation reactions were studied by the addition of similar concentrations of GTP, TNP-GTP, GTPγS, mantGDP, GppNHp or GMPPCP together with GRF1. Figure 3B shows that [^{3}H]GDP was released most efficiently from Ras by the presence of GTP or TNP-GTP, to a lesser extent by GTPγS or mantGDP, and least efficiently by GppNHp or GMP−PCP. At 50 μM, TNP-GTP was indistinguishable from GTP in displacing Ras bound [^{3}H]GDP while GTPγS was about 1.5-fold less effective and GppNHp or GMPPCP was at least 4-fold less effective. Similar results were obtained in the mantGDP-based fluorescence assay when the kinetics of mantGDP dissociation was compared in the presence of 50 μM GTP, GTPγS or GMP−PCP (Figure 3C). The time courses appear to fit better into the equation $y = y_0 + a\cdot \exp(-bx)$ by assuming that the end point (y_0) for the three different nucleotide analogues is a fixed value, i.e., the same end point for different exchanging nucleotides. Based on the data in Figure 3C, y_0 is estimated to be 65%. Thus, the rate constants are derived as 0.017, 0.11 and 0.32 min^{-1} on the presence of 50 μM GMP−PCP, GTPγS and GTP, respectively. The differences in the GDP dissociation rates in the presence of various nucleotide analogues correlate well with the differences of the nucleotide binding affinities to Ras. Together, these data suggest that uploading of incoming nucleotide to Ras-GDP is a prerequisite and/or a rate-limiting step in GRF1-stimulated GDP release.

Fluorescence Resonance Energy Transfer between Ras-Bound mantGDP and TNP-GTP. Further understanding of the mechanism by which GRF1 accelerates GDP release from Ras requires a detailed characterization of potential intermediate(s) in the exchange reaction. The observation that the presence of GTP in the exchange buffer is a prerequisite and rate-limiting step for GDP dissociation raised the possibility that the GEF reaction may proceed via a transiently formed intermediate involving GTP. We designed a fluorescence resonance energy transfer (FRET) experiment to investigate the spatial relationship between the incoming and the departing nucleotides on Ras. The experiment strategy was to use two ribose-modified fluorescent derivatives of GDP and GTP, mantGDP and TNP-GTP (*38*), as the fluorescence donor and acceptor pair. As shown in Figure 4A, there is a substantial overlap between the emission spectrum of the Ras-bound mantGDP and the absorption spectrum of Ras-bound TNP-GTP that would give rise to a FRET between these two nucleotides if they are bound proximally on the Ras molecule. GTP, lacking the absorption spectral properties of TNP-GTP, was employed as a control. In addition, the GRF1 catalytic domain does not bind GDP or GTP, as demonstrated by radioactive filter-binding assays (Figure 4B). FRET was therefore determined under a well-controlled kinetic condition of the Ras exchange reaction,

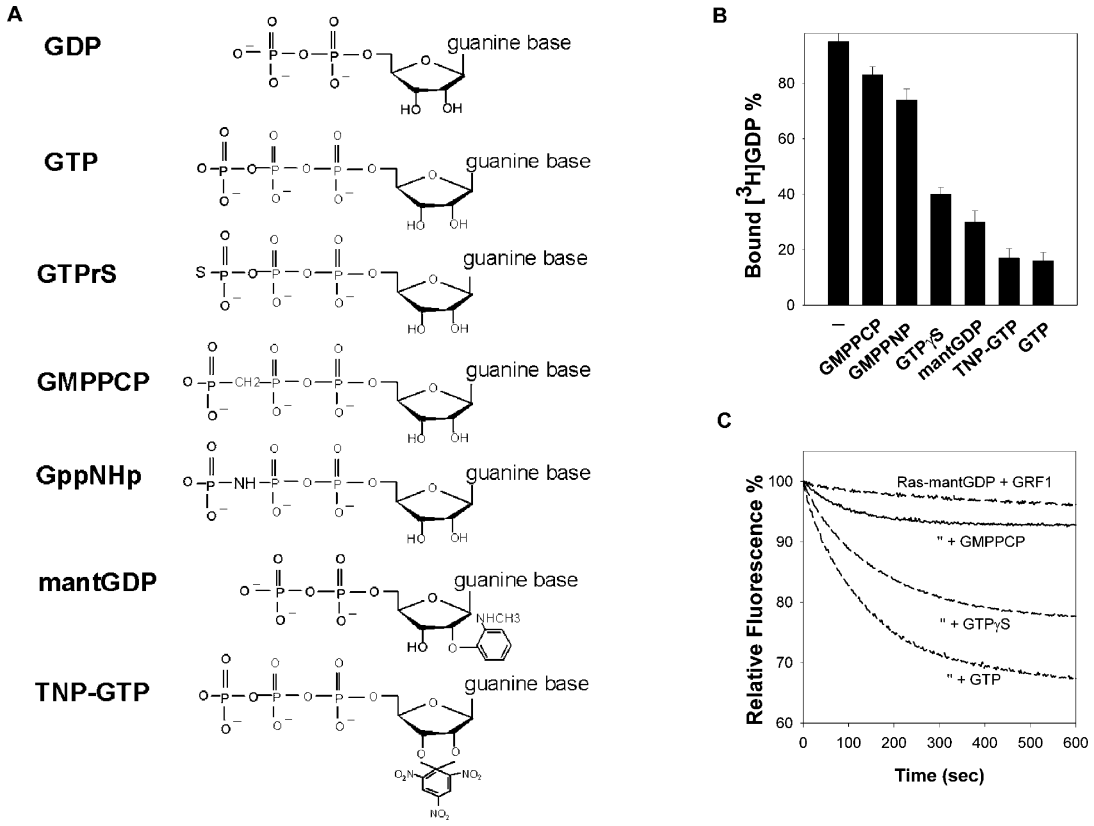

FIGURE 3: Effect of the structure of free guanine nucleotides on GRF1-catalyzed GDP dissociation. (A) Structure comparisons of a panel of guanine nucleotide analogues used in the studies. GMPPCP and GppNHp represent two non-hydrolysable GTP analogues with the replacement of the β,γ-bridging oxygen of GTP by a CH2 or NH group; GTPγS contains a thiolester group between the β and γ-phosphates; mantGDP and TNP-GTP are analogues with modification on the guanine ring or ribose moiety. (B) The dissociation of [^{3}H]GDP from Ras (100 nM) was assayed for 5 min in the presence of 200 nM GRF1 and the indicated nucleotide at 50 μM. (C) Time courses of mantGDP dissociation from Ras (100nM) in the presence or absence of 50 μM GTP, GTPγS, or GMPPCP were measured by monitoring mantGDP fluorescence changes. All data are representative of three independent experiments.

which as we have shown is highly sensitive to the free nucleotide concentration in the micromolar range.

Figure 4C shows the traces of the Ras-mantGDP fluorescence after mixing 100 nM Ras-mantGDP with 1 μM GTP or TNP-GTP in the presence or absence of 1 μM GRF1. The excitation was set at 320 nm to minimize the basal fluorescence of the acceptor, TNP-GTP. The intensity values at each time point were corrected for a potential inner-filter effect of the nucleotides as described under "Experimental Procedures". In the presence of GRF1, the interaction of GTP with Ras-mantGDP was characterized by a slow decay of mantGDP intensity, corresponding to the release of bound mantGDP. In contrast to GTP, TNP-GTP caused significantly more quenching of the Ras-mantGDP fluorescence under similar conditions (Figure 4C). When the loading/titration sequence of mantGDP and GTP/TNP-GTP was reversed (i.e. the free mantGDP (100 nM) was incubated with Ras-TNP-GTP or Ras-GTP (1 μM)), the resulting enhancement of mantGDP fluorescence with Ras-TNP-GTP, corresponding to the mantGDP loading, was significantly less than that observed with Ras-GTP (Figure 4D). The FRET is thus manifested as the fluorescence intensity difference between the two traces in Figure 4C (Ras-mantGDP + GTP or TNPGTP), or the two traces in Figure 4D (mantGDP + Ras-GTP or Ras-TNPGTP). We noticed that the FRET was strictly dependent on the concentrations of Ras-GDP, free nucleotide, and GRF1 in the reaction. The maximal FRET ($\sim$10%) was observed in a reaction mixture containing 100 nM Ras-mantGDP, 1.0 μM TNPGTP or GTP, and 1.0 μM GRF1. At the fixed concentrations of Ras-mantGDP (100 nM) and GRF1 (1.0 μM), increasing the free nucleotide (e.g. > 10 μM) could significantly accelerate the rate of mantGDP dissociation, making FRET detection difficult. In addition, the FRET is time-dependent and decreases as the reaction proceeds**.** After 2 h when the GEF reactions were completed, the FRET was undetectable.

One alternative explanation for the observed fluorescence differences between GTP and TNP-GTP exchanges for mantGDP is that these nucleotides might bind with a different affinity to Ras to confer different kinetics for exchanging mantGDP. To rule out this possibility, we performed the GTP or TNP-GTP assisted exchange assays by using [^{3}H]GDP in the reactions. Under similar conditions as employed for the FRET assays, the kinetics of TNP-GTP and GTP in displacing [^{3}H]GDP was essentially the same (Figure 3C and Figure 4D insert). In addition, the mantGDP based reactions for GTP and TNP-GTP achieved similar end points of fluorescence intensity. These results suggest a fluorescence energy transfer (FRET) between the two fluorescent probes mantGDP and TNP-GTP, but not mantGDP and GTP, resulting in a further decreased quantum yield of the bound mantGDP when exchanged for TNP-GTP.

Relationship of the γ-Phosphate Group and Guanine Ring Binding to Ras in the Exchange Intermediate. We next attempted to delineate a mechanism by which Ras could bind simultaneously to two nucleotides in a GEF reaction

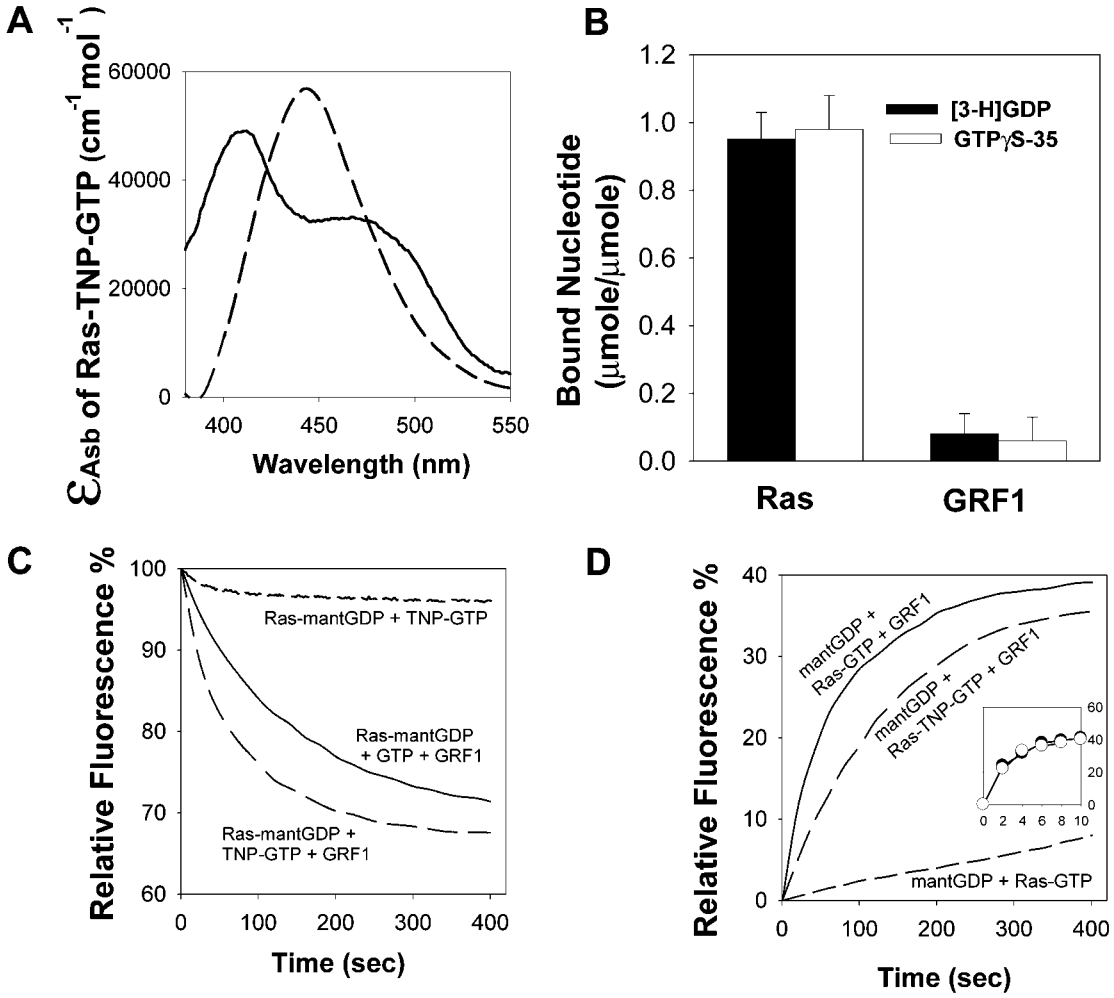

FIGURE 4: Fluorescence resonance energy transfer measurement between mantGDP and TNP-GTP bound to Ras during GRF-catalyzed nucleotide exchange. (A) The overlapping absorption spectrum of Ras-TNP-GTP (solid line) and emission spectrum of Ras-mantGDP (dashed line). Ras-TNP-GTP can act as the acceptor and Ras-mantGDP the donor in fluorescence energy transfer. The Ras-nucleotide complexes were prepared by exchange in the presence of excess nucleotide and subsequent removal of free nucleotide by gel filtration. (B) GRF1 catalytic domain does not bind guanine nucleotide. 1.0 μM GRF1 or nucleotide-depleted Ras (prepared as previously described (*49*)) was incubated at 25 °C with 10.0 μM [^{3}H]GDP or [^{35}S]GTP or [^{35}S]GTPγS in buffer A supplemented with 5 mM EDTA. After 60 min, $MgCl_2$ was added to overcome EDTA and aliquots were quantified for the bound radioactive nucleotides by filter binding. (C) The abilities of GTP and TNP-GTP (1 μM) in quenching the Ras-mantGDP (100 nM) fluorescence were compared when 1 μM GRF1 was present. The experiments were performed at 20 °C in buffer A. (D) Enhancement of the mantGDP fluorescence by Ras-GTP or Ras-TNP-GTP. 100 nM free mantGDP was incubated with 1 μM Ras preloaded with GTP or TNP-GTP in the presence or absence of 1 μM GRF1. The excitation wavelength was at 320 nm and the emission wavelength was 440 nm. Insert, time-courses of [^{3}H]GDP binding to Ras-GTP (●) or Ras-TNP-GTP (○) under the similar conditions as determined by the filter-binding assay (Bound [^{3}H]GDP % versus time (min)).

intermediate. Previous studies have concluded that the stoichiometry of Ras binding to guanine nucleotides is at 1:1 in a variety of stable structural complexes and that the binding affinity of guanine nucleotides arises from the direct interactions of the γ- and/or β-phosphate moiety and the guanine base of the nucleotide with specific Ras residues (*15*, *36*). We sought to test whether two subsites in the nucleotide-binding pocket of Ras could be involved in the binding of two nucleotides in a low affinity and transient fashion.

First, we used pyrophosphate (PPi) and GMP, representing the two parts of GTP or GDP, as ligands and explored their interactions with Ras. The equilibrium binding of PPi to Ras was assessed directly by a [^{32}P]PPi binding assay. Maximal loading of Ras with [^{32}P]PPi was observed in less than 10 min under saturating conditions (data not shown). As shown in Figure 5A, incubation of Ras-GDP with increasing concentrations of [^{32}P]PPi in the presence of GRF1 resulted in a stoichiometric incorporation of ^{32}PPi into Ras, yielding one PPi-binding site per Ras molecule at saturation. By assuming a simple one-step bimolecular association reaction, a nonlinear fit to the binding data yielded an equilibrium dissociation constant for the Ras-PPi interaction at 26 $\pm$ 2.9 μM. This value is comparable with a Kd of 29 μM previously determined for the Ras interaction with GMP (*39*). Interestingly, the PPi binding appeared to be facilitated by the GEF because omission of GRF1 from the reaction resulted in a drastic reduction in the binding activity (Figure 5A). In addition, Mg^{2+} was also required for the observed PPi binding. When [^{32}P]PPi was incubated with Ras in a binding buffer containing 1 mM EDTA in the absence of free Mg^{2+}, less than 0.2 mol of [^{32}P]PPi was found bound per mol of Ras in equilibrium conditions (Figure 5A).

Second, to ensure that the PPi binding site is located in the nucleotide-binding pocket of Ras, we examined the effect of free guanine nucleotides on PPi binding activity. Ras was

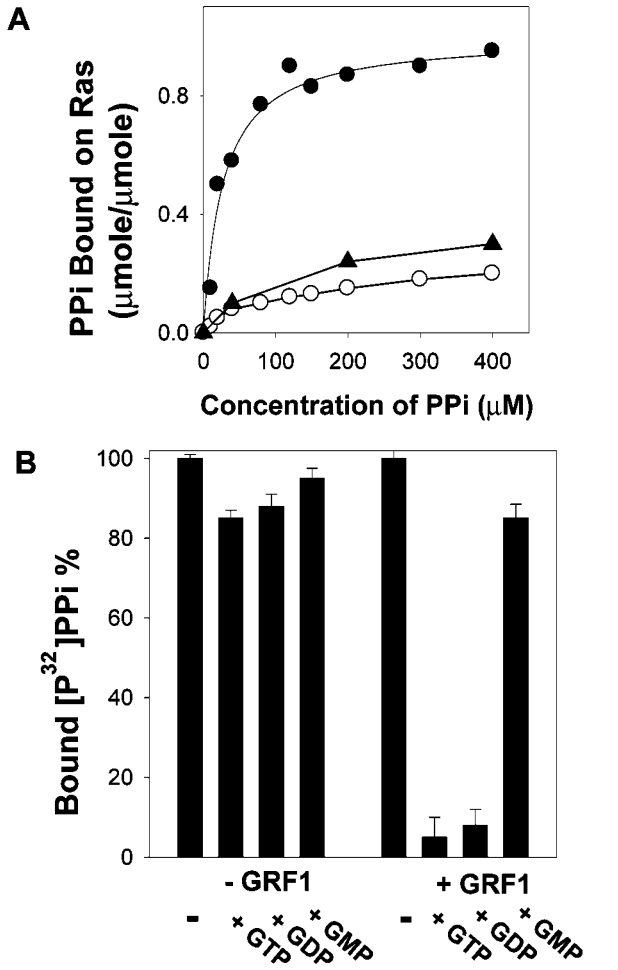

FIGURE 5: Independent binding of PPi and GMP to Ras. (A) Binding isotherm of [^{32}P]PPi to Ras. Ras-GDP (1.0 μM) was incubated at 25 °C with increasing concentrations of [^{32}P]PPi (up to 500 μM) in buffer A (○) or buffer A containing 5 μM GRF1 (●) or in a buffer containing 1 mM EDTA and 5 μM GRF1 in the absence of Mg^{2+} (▲). At equilibrium, the amount of [^{32}P]PPi bound to Ras was quantified by nitrocellulose filter binding. Data were fitted into a bimolecular binding equation yielding a dissociation constant of 26 μM for the Ras-PPi complex. Results are representative of three independent experiments. (B) nucleotide-induced release of [^{32}P]PPi from Ras preloaded with [^{32}P]PPi. The Ras-bound [^{32}P]PPi was determined at the 5-min time point in the absence (*left panel*) or presence (*right panel*) of 1 μM GRF1 in buffer A containing 500 μM GTP, GDP or GMP.

pre-loaded with [^{32}P]PPi as described under "Experimental Procedures" and its dissociation was traced upon the addition of 500 μM GTP, GDP or GMP. Figure 5B shows that in the absence of GRF1, none of these nucleotides significantly affected [^{32}P]PPi binding in a 5 min time point (left panel). In the presence of 5 μM GRF1, however, the Ras bound [^{32}P]PPi was completely replaced by GTP, mostly replaced by GDP, and not affected by GMP (right panel). These results suggest that the PPi-binding subsite overlaps with β- and/or γ-phosphate of GDP or GTP, accounting for the competitive displacement of PPi by GTP or GDP, whereas GMP appears to bind at a separate subsite that does not interfere with PPi binding.

Third, to further characterize the PPi binding site on Ras, we measured the dissociation of the Ras-bound [^{3}H]GDP after addition of PPi, GMP or their combination in the presence or absence of GRF1. In contrast to GTP, GMP or

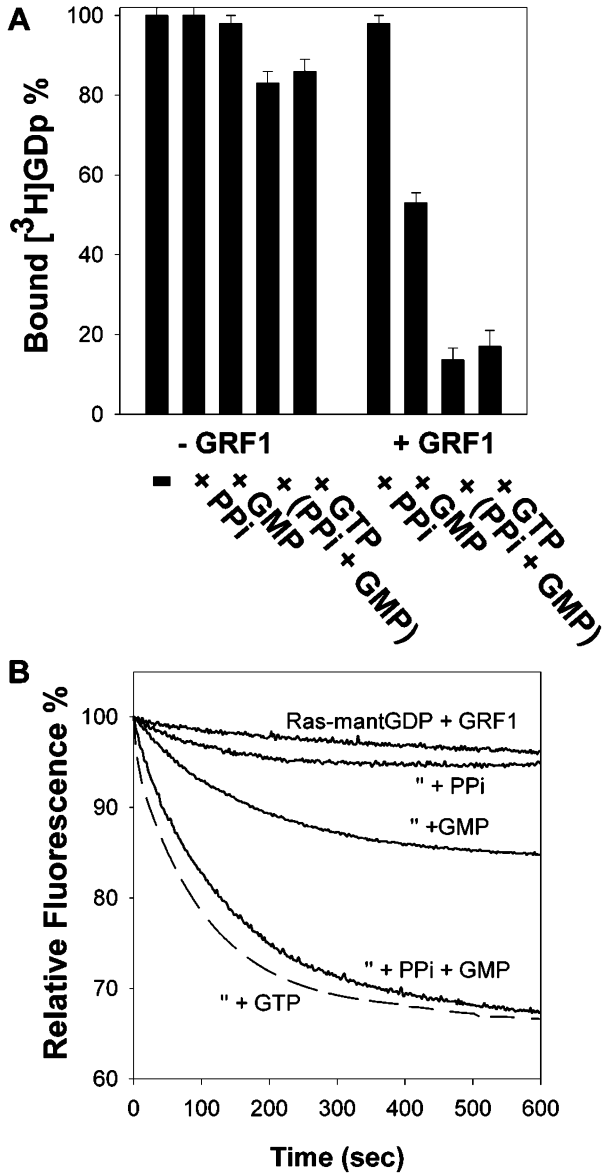

FIGURE 6: Synergistic effect of PPi and GMP in facilitating GRF1-stimulated GDP dissociation from Ras. (A) [^{3}H]GDP dissociation from 100 nM Ras-[^{3}H]GDP was measured in the absence or presence of 1 μM GRF1 in an exchange buffer containing 500 μM PPi, GMP, GTP, or their combinations. The reactions were terminated after 5 min. (B) Time courses of the mantGDP fluorescence change after rapidly mixing Ras-mantGDP (100 nM) with different combinations of 500 μM PPi, 500 μM GMP, or 500 μM GTP in the presence of 1 μM GRF1.

PPi alone failed to effectively induce the release of [^{3}H]GDP (Figure 6A). However, when GMP and PPi were added together to the reaction, the rate of [^{3}H]GDP release was significantly accelerated, suggesting GMP and PPi synergistically stimulate the release of the bound nucleotide. At 500 μM concentration, GMP together with PPi worked as potently as GTP in displacing [^{3}H]GDP from Ras. This synergistic effect was further confirmed by a fluorescence assay in which the mantGDP dissociation rate from Ras induced by GTP was found comparable to that induced by GMP together with PPi (Figure 6B). Similar to the filter binding results, in the mantGDP fluorescence assay the effect of PPi and GMP on GDP binding was dependent on the presence of GRF1. Taken together, these results further suggest that the phosphate moiety and guanine base of GTP

or GDP may occupy distinct subsites in the nucleotide-binding pocket of Ras, allowing transient formation of the dual nucleotide-bound intermediate.

DISCUSSION

In these studies, we demonstrate that (i) RasGRF1 itself does not efficiently stimulate GDP dissociation from Ras and the free GTP is required for efficient release of GDP; (ii) the GEF reaction kinetics is dependent on the concentration and structural nature/binding affinity of the incoming nucleotide, and the GTP-binding, rather than the GDP-dissociation, appears to be the rate-limiting step; (iii) in kinetically controlled conditions a fluorescence resonance energy transfer (FRET) occurs when the fluorescent analogue TNP-GTP exchanges for the mantGDP on Ras, suggesting the transient formation of the GRF1-[GDP−Ras-GTP] intermediate; (iv) when the nucleotide binding capability is studied, Ras is found to bind pyrophosphate (PPi) (mimicking the phosphate group of GTP) or GMP (mimicking the ribose moiety) with a Kd of ∼20−30 μM, providing a rationale for the accommodation of dual nucleotides in the nucleotide binding pocket of Ras. Although each line of evidence may be insufficient for the establishment of the displacement model, taken together the results provide strong support that the RasGRF1 catalyzed GTP/GDP exchange reaction of Ras may follow such a mechanism, i.e., proceeding via a quaternary complex containing both the leaving and the incoming nucleotides. The results also imply that the γ-phosphate moiety of incoming GTP may serve as the attacking group with the β-phosphate of GDP as the initial departing group (Figure 7C).

Ras GEFs catalyze a two-substrate reaction: Ras-GDP + GTP → Ras-GTP + GDP, which may follow either a substituted or a displacement mechanism (Figure 7A, B). The substituted mechanism, which is characterized by the formation of a stable nucleotide-free GEF−Ras intermediate and a nucleotide-independent GDP release, is widely accepted for the GEF reactions of the Ras superfamily of GTPases. As discussed, however, the binary G-protein (nucleotide-free)-GEF complexes can only be generated in vitro under nonphysiological conditions where the nucleotide is absent after the Mg^{2+} cofactor is removed. Our results clearly show that free GTP is required for the effective release of GDP from Ras catalyzed by GRF1. In light of its cellular abundance (100−200 μM), GTP may play an active role in replacing Ras bound GDP in cells. In this study, the concentration of Ras employed was 100 nM and GRF1 was varied from 10 nM to 10 μM, which appears to be a reasonable dose range mimicking the cellular conditions since the cellular Ras concentration could go up as much as μM or higher transiently at the action sites on the plasma or Golgi membrane (*40*, *41*). Although the lack of GDP dissociation in the absence of free GTP could be simply interpreted as the tight binding of GDP in Ras-GRF1 complex, the combined results suggest that a displacement reaction mechanism, proceeding via a transitory quaternary complex containing both the leaving and the incoming nucleotides, may be at work in the GRF1-catalyzed nucleotide exchange of Ras under physiologically relevant conditions.

Requirement for the Incoming Nucleotide for GEF-Catalyzed GDP Release from Ras-GDP. Our results from the [^{3}H]GDP filter-binding and mantGDP fluorescence assays demonstrate that neither GRF1 nor GTP alone can effectively stimulate the release of Ras-bound GDP. The presence of both GRF1 and GTP is required for the GDP dissociation (Figure 1). Similar observations have been made previously

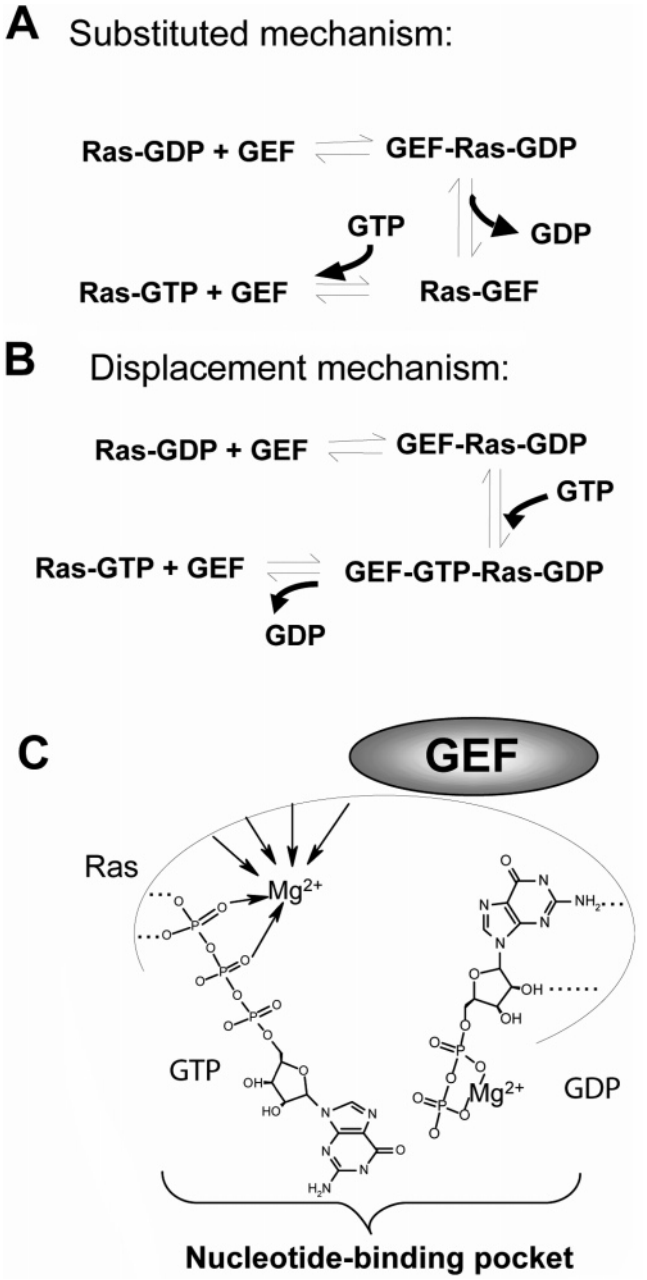

FIGURE 7: Comparison of the substituted mechanism with the nucleotide displacement model for GEF-catalyzed GDP/GTP exchange on Ras. (A) The scheme for the substituted exchange reaction mechanism. Ras-GDP interacts with GEF to form a ternary complex with Ras-GDP. The reaction intermediate is the nucleotide-depleted Ras-GEF complex and the rate-limiting step is the GDP dissociation. (B) The proposed nucleotide displacement mechanism. After the formation of the GEF−Ras-GDP ternary complex, GTP attacks by its phosphate moiety, yielding a quaternary intermediate GEF−(GDP−Ras-GTP). Thereafter the quaternary complex undergoes a conformation change, loosening the GDP binding to allow its dissociation. Displacement of GDP by GTP is accompanied by GEF release. The rate-limiting step is the GTP-binding. (C) Putative subsites in the nucleotide-binding pocket of Ras. Phosphate group (PPi) and GMP moiety binding sites constitute two independent subsites in the same nucleotide-binding pocket, rendering to Ras the ability to bind both GDP and GTP transiently.

with the eIF-2B stimulated eIF2 exchange (*42*, *43*) and the SopE catalyzed exchange on Cdc42 (*44*). In the case of eIF-2B, however, such a requirement may invoke a nucleotide-binding site on the exchange factor (*42*). We further demonstrate that the rate of GDP dissociation is dependent on the concentration of the incoming nucleotide, consistent with an earlier study of the GDP release mechanism from Ras2p stimulated by Cdc25p (*45*). It is noteworthy that the rate of GRF1-catalyzed GDP release increased significantly when the GTP concentration was increased from 0 to 10 μM, but did not increase further at higher concentrations (100 to 500 μM) (Figure 2A and 2B). The latter observation is consistent with Lenzen et al. (*28*), who reported that in the presence of excess of GTP (between 10 μM and 10 mM) there was little effect of the concentration of free nucleotide on the $Cdc25^{Mm}$-stimulated GDP dissociation rate from Ras. The nucleotide exchange of Ras is therefore influenced by the incoming GTP only in the low concentration range of 0−10 μM. These results suggest that, in cells, the rate of Ras activation may be dependent on the local concentration of GTP.

Rensland et al. (*31*) demonstrated that modification of the sugar residue of guanine nucleotides (e.g. mantGDP and TNP-GTP) is tolerated with little effect on the binding affinity of the nucleotides to Ras, whereas modification of the phosphate moiety, particularly altering the hydrophobicity of the β,γ-phosphate groups of the nucleotide (e.g. GTPγS, GppNHp and GMPPCP), leads to a significant loss in affinity. We show here that the rate of the GDP dissociation stimulated by GRF1 in the presence of the nucleotide analogues correlates well with the nucleotide binding affinities to the Ras GTPase; the higher the binding affinity of the incoming nucleotide, the higher GDP dissociation rate catalyzed by GRF1. The fact that two substrates (i.e. Ras-GDP and free GTP) are required for the GRF1-catalyzed exchange reaction suggests that the cellular GEF reaction favors the displacement mechanism rather than the substituted mechanism.

Possible Intermediate of the Guanine Nucleotide Exchange Reaction. The displacement mechanism is characterized by the formation of a transitional quaternary complex. We designed a FRET experiment using two fluorescent nucleotide analogues, mantGDP and TNP-GTP, as the donor−acceptor pair in an attempt to capture such an intermediate. The feasibility of this set of experiments relied to a large extent on the nucleotide concentration-dependence of the exchange rate, which allowed manipulation of the reaction kinetics for the detection of possible FRET. With nucleotide concentrations in the micromolar range, we were able to find a ratio of GRF1, Ras-mantGDP and TNP-GTP concentrations that permitted the detection of FRET between the Ras bound donor and acceptor nucleotide probes, which indicates a physical proximity between the probes during GRF1 catalysis. In addition to FRET, there are at least two other explanations for the observed fluorescence difference when TNPGTP or GTP was employed to exchange for Ras bound mantGDP. First, the two nucleotides might bind with different affinity to Ras to confer different kinetics for mantGDP dissociation. However, this possibility was ruled out by our data showing that TNPGTP and GTP display identical kinetics in displacing [^{3}H]GDP from Ras (Figure 3B and Figure 4D insert), which is consistent with the observation that modification of sugar residue of guanine nucleotide had no effect on the binding affinity to EF-Tu (37). Second, the apparent FRET could be also due to other sources such as nucleotide quality/concentration and collision quenching. Although this remains a possibility, we believe that it is not very likely because an opposite FRET response was observed when the loading/titration sequence of the GEF reaction was reversed, i.e., when free mantGDP was incubated with Ras-TNPGTP or Ras-GTP, the resulting enhancement of mantGDP fluorescence in the presence of Ras-TNPGTP that corresponded to mantGDP loading was significantly less than that observed in the presence of Ras-GTP (Figure 4D). In light of that the Ras protein could not dimerize under the experimental conditions (*46*) and the GRF1 catalytic domain does not bind nucleotide (Figure 4B), we interpret that the observed FRET of the Ras bound donor−acceptor nucleotides represents a transient intermediate, i.e., a quaternary GRF1-[GTP−Ras-GDP] complex. This finding is novel for Ras GTPase research since it presents the strongest evidence yet that Ras may undergo a displacement reaction mechanism in the GEF reaction, and that the reaction intermediate is a Ras-nucleotide (1:2) quaternary complex (as opposed to the nucleotide-free GEF binary complex of the substituted model).

Putative Binding Sites on Ras for Incoming and Departing Nucleotides. Guanine nucleotide binds to Ras mostly through the guanine base and phosphate groups (*47*). While guanine base is trapped in a hydrophobic pocket to be recognized by several interactions with the conserved residues of Phe28, the NKXD motif and the SAK motif of Ras, the β,γ-phosphate groups are bound by a number of important interactions that involve the P-loop, Thr35, and Gly60 of Ras. Another contributing factor to the high affinity binding is Mg^{2+} coordination, release of which could lead to a 500-fold reduction in the GDP or GTP binding affinity for Ras (*48*). Here we show that PPi, mimicking the phosphate group of GTP, can bind to Ras in a GEF-dependent manner with an affinity (Kd$\sim$26 μM) comparable to that of GMP (*39*) or ATP (*31*), providing a molecular basis for the observed dual nucleotide binding. An examination of the interplay of GMP, GDP or GTP with the PPi binding on Ras led us to conclude that the PPi-binding site overlaps with that of GDP or GTP but not GMP. Consistent with this conclusion, a combination of PPi and GMP was shown to be able to synergistically displace GDP from Ras. It appears that there may exist two subsites in the nucleotide binding pocket of Ras to allow the transient accommodation of the β/γ phosphate moiety of the incoming GTP and the guanine base of the departing GDP in the GRF1 involved intermediate (Figure 7C). We propose that the reaction involves three major steps. First, in agreement with the substituted mechanism, GRF1 forms a low-affinity, docking complex with Ras-GDP. Second, GTP associates with this ternary complex to form a quaternary intermediate complex where GDP remains bound tightly while GTP is bound loosely; subsequently, the complex undergoes a conformational change through isomerization, releasing GDP and allowing GTP to bind tightly. We also predict that the incoming GTP may attack Ras-GDP through its phosphate moiety first, whereas the departing GDP leaves with its guanine base last. Whether this is a generalized mechanism for other GEFs remains to be determined.

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