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Conformational States of ADP Ribosylation Factor 1 Complexed with Different Guanosine Triphosphates As Studied by ^{31}P NMR Spectroscopy

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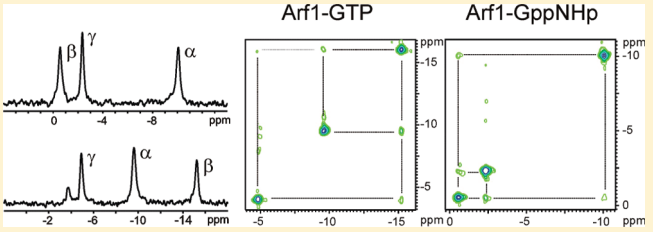
Supporting Information

ABSTRACT: Guanine nucleotide binding proteins (GNB-proteins) play an essential role in cellular signaling, acting as molecular switches, cycling between the inactive, GDP-bound form and the active, GTP-bound form. It has been shown that conformational equilibria also exist within the active form of GNB-proteins between conformational states with different functional properties. Here we present ^{31}P NMR data on ADP ribosylation factor 1 (Arf1), a GNB-protein involved in Golgi traffic, promoting the coating of secretory vesicles. To investigate conformational equilibria in active Arf1, the wild type and switch I mutants complexed with GTP and a variety of commonly used GTP analogues, namely, GppCH$_2$p, GppNHp, and GTPγS, were analyzed. To gain deeper insight into the conformational state of active Arf1, we titrated with Cu^{2+}-cyclen and GdmCl and formed the complex with the Sec7 domain of nucleotide exchange factor ARNO and an effector GAT domain. In contrast to the related proteins Ras, Ral, Cdc42, and Ran, from ^{31}P NMR spectroscopic view, Arf1 exists predominantly in a single conformation independent of the GTP analogue used. This state seems to correspond to the so-called state 2(T) conformation, according to Ras nomenclature, which is interacting with the effector domain. The exchange of the highly conserved threonine in position 48 with alanine led to a shift of the equilibrium toward a conformational state with typical properties obtained for state 1(T) in Ras, such as interaction with guanine nucleotide exchange factors, a lower affinity for nucleoside triphosphates, and greater sensitivity to chaotropic agents. In active Arf1(wt), the effector interacting conformation is strongly favored. These intrinsic conformational equilibria of active GNB-proteins could be a fine-tuning mechanism of regulation and thereby an interesting target for the modulation of protein activity.



The guanine nucleotide binding protein (GNB-protein) ADP ribosylation factor 1 (Arf1) acts as molecular switch cycling between an inactive GDP-bound "off" state and an active GTP-bound "on" state. Because vesicle coat proteins can interact with only the active form, Arf1 induces vesicle coating or uncoating by switching between the active and inactive conformation. Therefore, Arf1 is a key regulator of the Golgi apparatus and traffic from the Golgi.[1−3] Besides the regulation of vesicular trafficking and endo- and exocytosis, Arf1 directly activates phospholipase D.[4] It controls the activation of the phosphatidylinositole 3-kinase pathway and thereby contributes to breast cancer progression and migration.[5] Besides these tasks, Arf1 is also known as the downstream partner of Nef for CD4 lysosomal targeting.[6] CD4 is the primary receptor of the human (HIV) and simian (SIV) immunodeficiency viruses and is downregulated by the early protein Nef of these viruses.

The cycling between the active and inactive forms is regulated by proteins accelerating the exchange of the bound nucleotide [guanine nucleotide exchange factors (GEFs)] or the activation of GTP hydrolysis [GTPase-activating proteins (GAPs)].[2] The investigations presented here were performed on the variant [Δ17]Arf1.[7] It differs from the full-length protein because it lacks the first 17 N-terminal amino acids, which form an amphipathic helix. In vivo, this helix becomes post-translationally myristoylated and allows membrane attachment of Arf1 in the GTP-bound state.[8] With the truncated variant used here, in vitro investigations can be performed in the absence of lipids or detergents. This 18 kDa protein consists of 164 amino acids, building a six-stranded β-sheet with five α-helices. There are two switch regions (switch I, amino acids 42−52; switch II, amino acids 70−85), which show the most structural differences between the GDP- and GTP-bound forms.[9,10] The switch regions are linked by β-sheets called the interswitch. Bound GDP can be exchanged with GTP through the Sec7 domain of its activating protein, ARNO.[11,12] In contrast to most other GNB-proteins, for Arf1 no intrinsic GTPase activity is observed. Arf1 hydrolyzes bound GTP only in the presence of GTPase-activating proteins,[13] which themselves are positively regulated by the coatamer, but with a different sensitivity.[14−16]

It could be shown by ^{31}P NMR spectroscopy that the related protein Ras occurs in two conformational states (states 1 and 2) when it is complexed with the GTP analogues GppNHp and

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$GppCH_2p$[17,18] or the physiological ligand GTP.[19] To distinguish between conformational states in the GDP-bound form (D) in ambiguous cases, state 1(T) and state 2(T) are termed the active triphosphate nucleoside-bound complexes. Both conformational states are characterized by different functional properties. Ras variants existing in conformational state 1(T), like the partial loss-of-function mutant Ras(T35S), show a drastically reduced affinity for effectors,[18−21] but this conformation seems to be recognized by guanine nucleotide exchange factors (GEFs).[22] State 2(T) is the high-affinity state for effector interaction.[17−21] In the meantime, ^{31}P NMR spectroscopy was also applied to a variety of other members of the Ras superfamily such as M-Ras,[23,24] Ral,[25,26] Rap,[27] Cdc42,[28] and Ran[29] to investigate their conformational dynamics. It seems that the existence of different conformational states in active Ras-like GNB-proteins is a common property, but there are differences in the equilibrium constants. Therefore, these equilibria could be a fine-tuning mechanism of regulation of protein activity. Recent ^{31}P NMR experiments with [Δ17]Arf1(wt) complexed with GTP[30,31] indicated only one conformation, which corresponds to one single-resonance signal for each phosphate group of the nucleotide. To investigate if such dynamic properties are also found in Arf1 proteins, we tried to shift the equilibrium using different suitable methods: (i) complexation of Arf1 with different GTP analogues, (ii) formation of a complex between Arf1 and an effector domain, (iii) GdmCl treatment, (iv) replacement of the invariant threonine in switch I, (v) formation of a complex with the GEF ARNO-Sec7, and (vi) the use of paramagnetic Cu^{2+}-cyclen to probe the existence of conformational state 1.[32−34]

The objective of these investigations is to determine the conformational state in which Arf1 predominantly exists and if the replacement of GTP with commonly used analogues influences the conformational behavior and therefore also the functional properties of Arf1. Furthermore, the question of whether Arf1 exists in an equilibrium between different conformational states should be answered, which can be modulated to control the activation−inactivation cycle of Arf1 and consequently all the tasks like vesicle formation and transport. This would be of great interest in controlling metabolism.

EXPERIMENTAL PROCEDURES

Protein Purification. The wild type and T48 mutants of [Δ17]Arf1, a truncated form of Arf1, lacking the first 17 N-terminal residues, were expressed in *Escherichia coli* BL21-(DE3) cells using the pET11d expression vector. Purification was achieved with fast flow Q-Sepharose (Amersham Pharmacia) by applying a NaCl gradient (0 to 300 mM), followed by size exclusion chromatography using a G75 column in buffer, containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM DTE. The final purity of the protein was >95% as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). After purification, [Δ17]Arf1 is complexed to 60−70% with GDP and to 30−40% with GTP, as determined by C18 reverse phase liquid chromatography. To obtain uniformly loaded protein samples, we exchanged the nucleotide with GDP or GTP via incubation of [Δ17]Arf1 in the presence of 200 mM $(NH_4)_2SO_4$ and a 50-fold excess of GDP or GTP.[31] Exchange of the nucleotide with GTP analogues GppNHp, $GppCH_2p$, and GTPγS was done using alkaline phosphatase treatment in the presence of 200 mM $(NH_4)_2SO_4$ and a 2-fold excess of the GTP analogue. Free nucleotides were removed afterward by gel filtration. The [Δ17]Arf1 concentration as well as the success of the exchange was determined measuring the concentration of bound nucleotide by C18 reversed phase chromatography with a calibrated detector system.

The [Δ17]Arf1-T48 mutants were produced starting with the [Δ17]Arf1 pET11d plasmid by site-directed polymerase chain reaction mutagenesis. After transformation of the plasmid in competent *E. coli* BL21(DE3) cells, plasmids were isolated again; the correct single-mutation coding amino acid in position 48 was identified by DNA sequencing.

The Sec7 domain of nucleotide exchange factor ARNO was expressed in *E. coli* BL21(DE3) cells using the pET11d expression vector. Purification was conducted with a fast flow Q-Sepharose column (Amersham Pharmacia), applying a NaCl gradient (0 to 1 M), followed by size exclusion chromatography using a G75 column in buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, and 2 mM DTE. The final purity of the protein was >95%, as judged by SDS−PAGE. The ARNO-Sec7 protein concentration was determined with a calibrated Coomassie blue reagent and the absorbance at 280 nm using an extinction coefficient ε_{280} of 13075 M^{-1} cm^{-1}. The activity of the ARNO-Sec7 protein was proven, inducing fast exchange of Arf1·GDP with the GTP analogues.

The GAT domain of human GGA1 (residues 166−305) was expressed as a GST fusion protein in *E. coli* BL21(DE3)pLys cells using the pGEX-4-T1 vector. Purification was performed with a GSH column using Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM DTE as a buffer. Cleavage was performed using TEV (tobacco etch virus) protease. His-tagged TEV protease was removed afterward with Ni-NTA beads. The GAT domain could be concentrated to 0.4 mM in the buffer described above. The concentration was determined with a calibrated Coomassie blue reagent and by the absorbance using an extinction coefficient ε_{280} of 2980 M^{-1} cm^{-1}.

NMR Spectroscopy. Measurements were performed typically on 500 μL of a 1−2 mM [Δ17]Arf1 protein solution in buffer A [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 2 mM DTE containing 5% D_2O for the lock signal; 0.1 mM DSS was added to calibrate the spectra by indirect referencing]. ^{31}P NMR experiments were conducted with a Bruker Avance 500 spectrometer using a ^{31}P selective 10 mm probe and 8 or 10 mm Shigemi tubes at spectrometer frequencies of 500.1 MHz for protons and 202.4 MHz for ^{31}P. Measurements were performed using 70° pulses and a total repetition time of 7 s. Protons were decoupled during data acquisition by GARP sequence.[35] If not stated, the sample temperature was 278 K to detect slow conformational dynamics. During the processing procedure, spectra are exponentially filtered leading to an additional line broadening of 15 Hz.

^{31}P−^{31}P NOESY[36] spectra were recorded with a mixing time of 2 or 2.5 s and a total repetition time of 13 s. Protons were decoupled during t_1 evolution by a proton 180° pulse and during t_2 by a GARP sequence[35] with a B_1 field strength of 900 Hz.

^{31}P longitudinal relaxation times (T_1) were measured at 278 K with an inversion recovery sequence. A delay between two scans of 20 s was applied to ensure complete relaxation. The obtained signal integrals were fitted by a three-parameter fit to the equation

$$M_z(t) = M_0 + [M_z(0) - M_0]e^{-t/T_1} \quad (1)$$

where $M_z(t)$ is the z-magnetization (signal integral) at time t and M_0 the magnetization in thermal equilibrium. Protons were

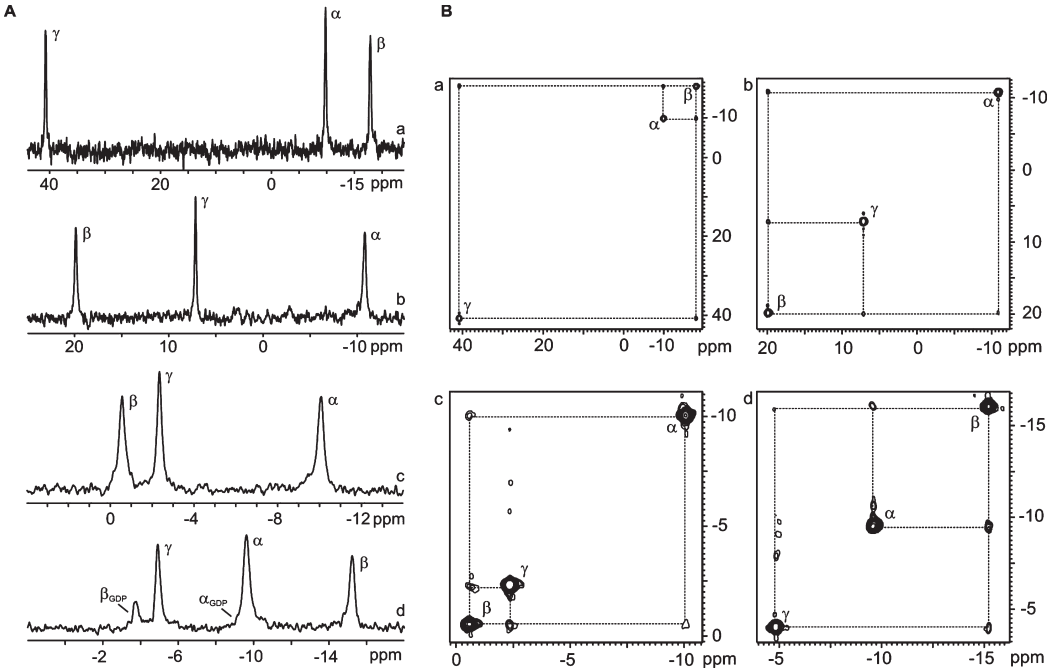

Figure 1. One-dimensional ^{31}P NMR spectra (A) and 2D ^{31}P−^{31}P NOESY spectra (B) of [Δ17]Arf1(wt) in complex with Mg^{2+}·GTPγS (a), Mg^{2+}·$GppCH_2p$ (b), Mg^{2+}·GppNHp (c), and Mg^{2+}·GTP (d). Measurements were taken on 1−1.5 mM [Δ17]Arf1(wt) in buffer A at 278 K. Spectra were recorded as described in Experimental Procedures. For the NOESY experiments with Mg^{2+}·GppNHp- and Mg^{2+}·GTP-bound Arf1 complexes, Arf1·$GppCH_2p$ complexes, and the GTPγS complex, 64, 96, and 128 t_1 increments were performed, respectively. Note that the scaling as well as spectral width is different for each spectrum.

decoupled during magnetization recovery and data acquisition by the GARP sequence. Experiments were conducted in a cyclic way to avoid instability effects of the sample or the spectrometer.

The ^{31}P saturation transfer experiments were performed at 278 K using a strength of the B_1 field of 18 Hz; 100 cycles of the experiments, each with eight scans, were summed to avoid instability effects of the sample or the spectrometer.

$$I_A(\tau) = \frac{I_A(0)}{\frac{1}{\tau_A} + \frac{1}{T_{1A}}}\left\{\frac{1}{\tau_A}\exp\left[-\tau\left(\frac{1}{\tau_A} + \frac{1}{T_{1A}}\right)\right] + \frac{1}{T_{1A}}\right\} \quad (2)$$

where $I_A(\tau)$ is the area of the nonsaturated signal after saturation time τ (at resonance B) and T_{1A} the longitudinal relaxation time of signal A (estimated from inversion recovery experiment). It should be mentioned that T_1 relaxation times of states 1a and state 1b could not be determined separately because of exchange. The exchange rate is $k_{BA} = 1/\tau_A$, with τ_A being the mean lifetime of the protein in state 1b.[37]

A Ξ value of 0.4048073561 reported by Maurer and Kalbitzer[38] was used, which corresponds to 85% external phosphoric acid contained in a spherical bulb. The temperature was controlled using ^{1}H line separation methanol in an external standard for calibration.[39]

RESULTS

Conformational States of Wild-Type [Δ17]Arf1 Bound to Different GTP Analogues. Stable GTP analogues are commonly used to demonstrate GNB-proteins in their active forms, e.g., for structure determination or in protein−protein interaction studies. From investigations of different members of the Ras superfamily, it is known that the nature of the bound nucleoside triphosphate influences the conformational dynamics and thereby the functional properties of GNB-proteins in their active forms.[17,19,25−29] Therefore, we have investigated wild-type [Δ17]Arf1 (Arf1 hereafter) bound to its natural ligand GTP, as well as analogues GppNHp, $GppCH_2p$, and GTPγS. ^{31}P NMR spectra were recorded on each Arf1(wt)·Mg^{2+}·nucleotide complex at various temperatures. In Figure 1A, the ^{31}P NMR spectra recorded at 278 K are presented. To verify the assignment of the phosphorus NMR signals, we performed two-dimensional (2D) ^{31}P−^{31}P NOESY experiments on each complex (Figure 1B). The resonance lines that correspond to the α-phosphate groups are rather unperturbed between free and protein-bound nucleotides with ^{31}P chemical shift values typically in the range of −10 to −12 ppm. In contrast, the chemical shifts of the β- and γ-phosphate resonance signals strongly depend on the degree of protonation within the complex, which is also influenced by Mg^{2+} coordination.[18,21] By 2D ^{31}P−^{31}P NOESY experiments, the resonances corresponding to the β-phosphorus can unambiguously be determined by the pattern of the cross-signals to both the α-phosphorus and the γ-phosphorus (Figure 1B). The obtained chemical shift values are listed in Table 1.

In contrast to the results obtained for Ras, Ral, or Ran protein[17,18,24,25,29] in all triphosphate nucleoside complexes, only one set of resonances is observed. For the GppNHp and GTPγS complexes of Arf1(wt), the temperature dependence of the line widths as well as the chemical shifts of the resonance signals was tested from 273 to 300 K. No indication of fast chemical exchange processes even at low temperatures could be found (data not shown). This indicates that Arf1(wt) with GTP bound, as well as when complexed with different GTP analogues, exists predominantly in a single conformation. If slow exchange conditions

Table 1. ^{31}P Chemical Shift Values and Line Widths of Different Guanine Nucleotides Bound to [Δ17]Arf1 and Its Thr48 Mutants[a]

	[Δ17]Arf1(wt)		[Δ17]Arf1(T48A)		[Δ17]Arf1(T48S)	
	δ (ppm)	$\nu_{1/2}$ (Hz)	δ (ppm)	$\nu_{1/2}$ (Hz)	δ (ppm)	$\nu_{1/2}$ (Hz)
GDP-Mg^{2+}	−9.74 (α)	56.8 (α)	−9.70 (α)	67.7 (α)	−9.71 (α)	51.9 (α)
	−3.71 (β)	55.0 (β)	−3.66 (β)	63.5 (β)	−3.67 (β)	53.4 (β)
GTP-Mg^{2+}	−9.69 (α)	34.8 (α)				
	−15.26 (β)	48.8 (β)				
	−4.93 (γ)	34.9 (γ)				
GppNHp-Mg^{2+}	−10.07 (α)	46.6 (α)	−10.23(α)	95.6 (α)	−10.04 (α)	76.9 (α)
	−0.57 (β)	40.5 (β)	−0.81 (β)	77.0 (β)	−0.68 (β)	68.1 (β)
	−2.35 (γ)	29.1 (γ)	−2.57 (γ)[b]	81.6 (γ)[b]	−2.57 (γ)	56.6 (γ)
			−3.08 (γ)[b]	81.6 (γ)[b]		
GTPγS-Mg^{2+}	−9.73 (α)	49.0 (α)	−9.87 (α)	93.9 (α)	−9.91 (α)	63.7 (α)
	−17.79 (β)	55.5 (β)	−18.07 (β)	78.1 (β)	−17.87 (β)	63.3 (β)
	40.75 (γ)	45.4 (γ)	38.99 (γ)	86.6 (γ)	39.53 (γ)	50.8 (γ)
			32.20 (γ)	86.6 (γ)		
$GppCH_2p$-Mg^{2+}	−10.76 (α)	53.9 (α)				
	19.90 (β)	34.3 (β)				
	7.20 (γ)	24.6 (γ)				

[a]Chemical shift values were determined by a fit of the Lorentzian function to the resonances. The total line width at half of the intensity is given. The error obtained by the fit for chemical shift values is less than ±0.1 ppm, and that in line width is less than ±2 Hz. The line broadening caused by exponential filtering of the data is subtracted. [b]Values obtained from the fit shown in Figure 3b.

relative to the time scale of the NMR experiment are assumed, one can give an upper limit for the equilibrium constant of a conformation with a small population of approximately 0.1, taking into account the signal-to-noise ratio. In contrast to the structure of the GDP complexes, the GppNHp-bound structure of Arf1 is rather similar to that of the active state of Ras. In the X-ray structure of the [Δ17]Arf(wt)·Mg^{2+}·GppNHp complex itself[10,40] or in the complex with the N-GAT domain of effector GGA1,[40] the invariant threonine residue of switch I (Thr48 in Arf1) is coordinated to the γ-phosphate via its main chain amide and contacts the Mg^{2+} ion, bound in the active center, by its side chain hydroxyl. This cannot serve as proof, but it indicates that Arf1 bound to GTP or the GTP analogues most likely exists in a conformation corresponding to state 2 in Ras.[17,20,21]

Formation of a Complex between Arf1 and the GAT Domain of Human GGA1. In terms of the sensitivity of ^{31}P NMR spectroscopy, the solubility of the GAT domain at ∼0.3 mM was rather low; thus, the measuring time for obtaining spectra with a satisfying signal-to-noise ratio was on the order of days. Figure 2 presents ^{31}P NMR spectra of the complexes between the GAT domain of the human GGA1 protein and Arf1(wt) bound to GppNHp (Figure 2A) or GTPγS (Figure 2B). Besides the line broadening of all three resonances by a factor of 2 due to the higher molecular weight and the corresponding change in the rotational correlation time, in both cases the γ-phosphate resonance shows a small downfield shift of 0.2 ppm in the GAT complex compared to that of free Arf1. Unfortunately, multistep titration experiments were not feasible so far, because of the low solubility of the GAT domain; therefore, ^{31}P chemical shift values as well as line width cannot be determined accurately. Although chemical shift changes do not directly reflect the extent of the structural rearrangement, one would expect only small structural changes because of effector binding in the proximity of the nucleotide binding site of Arf1.

Mutation of the Invariant Threonine in Switch I and Effect on the Conformational Equilibrium. Replacement of the crucial threonine in switch I with an alanine or serine should lead to the destabilization of state 2.[18−21,25,28] Therefore, we created the Arf1(T48S) mutant, still containing the side chain hydroxyl, and the corresponding Arf1(T48A) alanine mutant. These mutants seem to exhibit a drastically reduced affinity for GTP compared to GDP, which led to the fact that exchange of bound GDP to GTP was not feasible in a direct way (see also Figure S1 of the Supporting Information). Fortunately, the exchange with stable GTP analogues using alkaline phosphatase treatment was more successful. In Figure 3, ^{31}P NMR spectra of both mutants bound to Mg^{2+}·GppNHp or Mg^{2+}·GTPγS are depicted. For GppNHp-bound Arf1(T48S) (Figure 3A), 1.6-fold larger line widths and changes in chemical shift are shown. This is observed best for the chemical shifts of the β- and γ-phosphate resonance with Δδ values of 0.14 and 0.22 ppm, respectively, to higher field compared to signals observed for the wild-type protein (Table 1). The spectrum obtained for the Arf1(T48A)·Mg^{2+}·GppNHp complex (Figure 3A) shows clearly broadened resonances, which seems to split into two lines. Large line width and small chemical shift differences between the two states lead to an overlap of the resonances. From analytical size exclusion chromatography, NMR diffusion experiments, and differential light scattering (data not shown), there is no indication of dimerization of the mutant proteins, suggesting structural properties different from those of the wild-type protein. With an increase in temperature (Figure 3A, top spectrum), the γ-phosphate resonance of the alanine mutant results in only one homogeneous line, indicating fast exchange conditions between at least two conformational states at room temperature. In addition to the resonances corresponding to Arf1-bound GppNHp and GDP, sharp lines of free Mg^{2+}·GppNHp signals are found in the room-temperature spectrum. From the line width, there are no

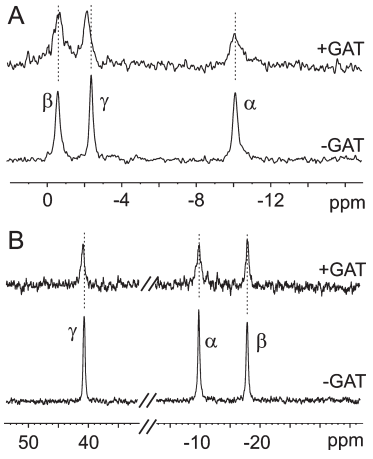

Figure 2. Formation of a complex between wild-type Arf1 and the GAT domain of human GGA1. (A) ^{31}P NMR spectra of the Arf1(wt)·Mg^{2+}·GppNHp complex in the absence (bottom) and presence of the GAT domain (top). (B) ^{31}P NMR spectra of the Arf1(wt)·Mg^{2+}·GTPγS complex in the absence (bottom) and presence of the GAT domain (top). Because the GAT domain was only soluble at concentrations up to 0.3 mM (in buffer A), active Arf1 nucleotide complexes were added at a lower concentration of 0.15 mM to ensure complex formation to a large extent (K_D value should be in the micromolar range[40]). Because of the low sensitivity and broad resonance signals found via ^{31}P NMR spectroscopy, 15000 FIDs were accumulated for spectra of the complex; i.e., the measuring time was 30 h for each spectrum. The temperature was adjusted to 274 K. The obtained ^{31}P chemical shift values for the Arf1−GAT complexes are −0.61 (β), −2.12 (γ), and −10.09 ppm (α) for the GppNHp complex (A, top spectrum) and 40.93 (γ), −9.78 (α), and −17.89 ppm (β) for the GTPγS complex (B, top spectrum).

indications of exchange processes. It is more likely that a fraction of nucleotide free Arf1 precipitated in the sample.

In Figure 3B, the γ-phosphate resonance of Arf1(T48A) in the GppNHp complex is given together with fitted lines from the Lorentzian function, corresponding to the γ-phosphate resonances with chemical shift values obtained for wild-type and Arf1(T48S) (Table 1). The expected exchange between the resonances obtained for Arf1(T48S) and Arf1(T48A) should result in additional line broadening; therefore, the increase in line width was allowed in the fitting procedure. Furthermore, the β-phosphate signal of bound GDP was taken into account using the experimentally determined parameters. From this simulation, an equilibrium constant between T48A and the corresponding T48S state was found to be 0.4 with a chemical shift of the additional resonance observed for Arf1(T48A) of −3.08 ppm. The corresponding free energy between the alanine and serine mutant conformations would then be −2.1 kJ/mol. The additional line broadening by exchange is 25 Hz and should give a direct estimation of the rate of exchange between the two states corresponding to the two Thr48 mutants.

In the GTPγS complex, Arf1(T48S) shows one signal for each phosphate, but with a resonance of the γ-phosphate that is shifted to higher field by 1.2 ppm compared to that of the wild-type spectrum. Arf1(T48A) further shows a splitting of the γ- and β-phosphate resonances (Figure 3C), which was obtained previously for the Ras state 1 mutants Ras(T35A) and Ras(T35S) assigned to states 1a and 1b.[21] Via performance of ^{31}P saturation transfer experiments with the irradiation frequency of state 1b (32.2 ppm), exchange is demonstrated and a rate k_{1a1b} of 2.5 ± 0.2 s^{-1} could be determined (Figure 3D). If the equilibrium constant K_{1a1b} of approximately 0.18 is taken into account, the total rate of exchange (k_{ex}) of 16 s^{-1} can be estimated at a temperature of 278 K. The corresponding difference in free enthalpy between the two substates (ΔG_{1a1b}) is 4.0 ± 0.5 kJ/mol. For wild-type Arf1, no saturation transfer could be detected, using an irradiation at the frequency corresponding to state 1b for 1.8 s. The serine mutant seems to take a position between the alanine mutant and wild-type Arf1. From chemical shift values as well as line broadening, it seems to be an intermediate between the more populated conformation of the T48A mutant and the wild-type state in GppNHp as well as in the GTPγS complexes. With the serine mutant, the effect of the analogously performed saturation transfer experiments was only in the limit of error. The obtained chemical shift values together with the line widths are listed in Table 1. As mentioned above, the T48 mutants seem to show a reduced affinity for triphosphate nucleosides compared to GDP. For a qualitative investigation, we incubated GTPγS-loaded Arf1(wt) and Arf1(T48A) with a 3-fold excess of GDP. In Figure S1 (Supporting Information), one can nicely see that in the case of the mutant the bound GTPγS is replaced with GDP but no bound GDP could be found for wild-type Arf1. These observations demonstrate that GDP has a higher affinity for the Arf1 mutant than the triphosphate nucleoside, probably accompanied by an expected increase in the nucleotide dissociation rate constant.[27]

Determination of Longitudinal Relaxation Rates of Wild-type and Mutant [Δ17]Arf1. The longitudinal relaxation time (T_1) of NMR-active nuclei can be used to probe differences in their chemical environment and coordination. Although the obtained values cannot be structurally interpreted in a simple way, differences in the environment of such nuclei can be detected and compared with the results obtained for mutants or other related GNB-proteins. In Table 2, the determined values for wild-type and mutant Arf1 are summarized. Whereas nucleotides or Mg^{2+}-nucleotide complexes show ^{31}P T_1 relaxation times in the range of 0.5 s, those obtained for protein-bound nucleotides vary typically in the range of 4−6 s.[18] ^{31}P T_1 relaxation times for wild-type Arf1 and Arf1(T48A) were determined by a standard inversion recovery experiment as described in Experimental Procedures. In the GDP-bound complex, significant differences are found. Despite the estimated error of up to 1 s (Table 2), a significantly longer T_1 relaxation time is obtained for the β-phosphate in the T48A mutant than for the wild type. In the GppNHp complexes, the largest differences could be obtained for the α-phosphate and especially the β-phosphate, which again are longer in the mutant complex. Analogous results were obtained previously for Ras protein.[18] For the GTPγS complexes, the largest difference is in the γ-phosphate (1.7 s). Here wild-type protein shows with a value of more than 6 s a significantly longer relaxation time compared to that of the mutant with a time of approximately 4.5 s (Table 2). It must be mentioned that for Arf1(T48A) the values are averaged values of the mixture of at least two different conformations, because the rate of exchange is much faster than T_1 relaxation. Although one cannot deduce from these results the detailed structural properties of the Arf1 variants, they indicate clearly differences in the interaction phosphate groups between wild-type Arf1 and Arf1(T48A).

Response of Arf1 to Guanidine Hydrochloride Treatment. Guanidine hydrochloride (GdmCl) is often used for protein unfolding or stability experiments. The treatment of the GNB-protein Ras·Mg^{2+}·GppNHp with small amounts of GdmCl shifts the conformational equilibrium toward state 1.[22] We added increasing

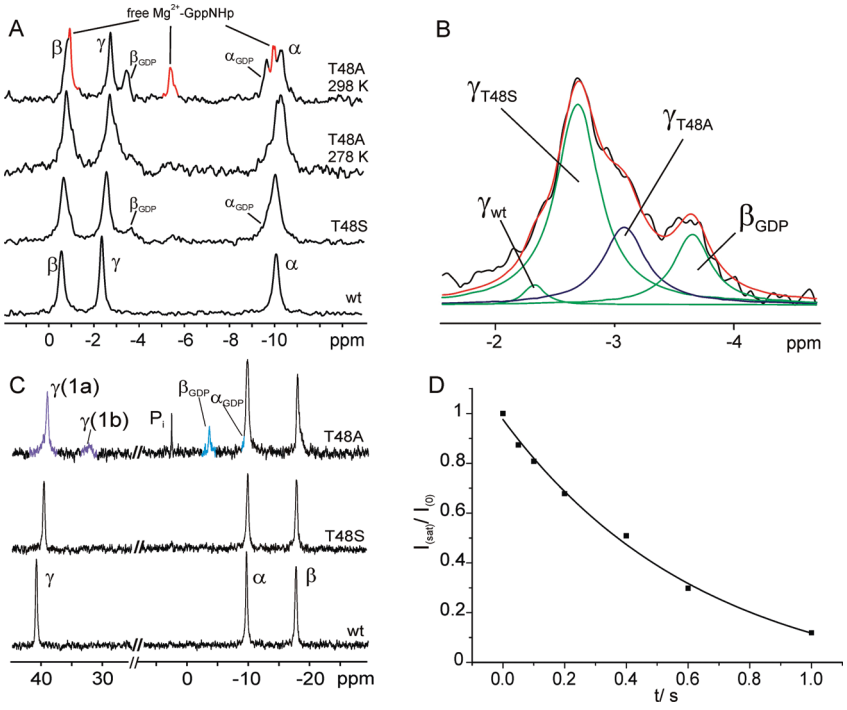

Figure 3. ^{31}P NMR spectra of 1−1.5 mM wild type and Thr48 mutants of [Δ17]Arf1 in buffer A. Obtained chemical shift values are listed in Table 1. (A) Spectra of Arf1 complexed with Mg^{2+}·GppNHp recorded at 278 K and Arf(T48A) (top) recorded at 298 K. The resonances arising at −0.9, −5.4, and −10.0 ppm correspond to the γ-, β-, and α-phosphates of free Mg^{2+}·GppNHp, respectively. (B) Line shape of the γ-phosphate resonance of the T48A mutant at 278 K with the fit of the resonances with the parameters corresponding to the states of the wild type and Arf1(T48S). The equilibrium constant between the states found for the alanine and serine mutants is 0.4. (C) Spectra of the Arf1·Mg^{2+}·GTPγS complexes at 278 K. (D) Results of the saturation transfer experiment with Arf1(T48A) with the irradiation frequency on state 1b. $I(\mathrm{sat})/I(0)$ is given vs saturation time. A value for k_{1a1b} of 2.5 s^{-1} is obtained. The fit equation is given in Experimental Procedures.

Table 2. ^{31}P T_1 Relaxation Times for Different [Δ17]Arf1·Mg^{2+}·Nucleotide Complexes[a]

	T_1 relaxation time of α-phosphate (s)		T_1 relaxation time of β-phosphate (s)		T_1 relaxation time of γ-phosphate (s)	
Arf1-bound nucleotide	wt	T48A	wt	T48A	wt	T48A
GDP-Mg^{2+}	4.8 ± 0.54	5.1 ± 0.64	6.9 ± 1.00	4.8 ± 0.58		
GTP-Mg^{2+}	4.3 ± 0.19		4.7 ± 0.34		4.9 ± 0.34	
GppNHp-Mg^{2+}	3.6 ± 0.18	5.7 ± 0.54	4.8 ± 0.23	5.9 ± 0.74	5.3 ± 0.26	5.3[b] ± 0.77
GTPγS-Mg^{2+}	3.9 ± 0.29	4.2 ± 0.24	4.2 ± 0.18	4.3 ± 0.60	6.2 ± 0.31	4.5[b] ± 0.66
$GppCH_2p$-Mg^{2+}	4.4 ± 0.45		6.3 ± 0.45		6.1 ± 0.12	

[a] Values were determined by the inversion recovery sequence as described in Experimental procedure. The error of the fit procedure of the data is given. [b] Values give an average over different states caused by exchange processes, which are faster than T_1 relaxation.

amounts of GdmCl to Arf1(wt), as well as to the T48A mutant (Figure 4). For wild-type Arf1, no indication of unfolding over conformational state 1(T) could be obtained. At a GdmCl concentration of 1.5 M, the equilibrium constant between bound and free nucleotides is 1. The Arf1(T48A)·Mg^{2+}·GppNHp complex is less stable. At a GdmCl concentration of 0.25 M, free nucleotide can be already observed by the resonances at −0.9, −5.4, and −10.0 ppm, which correspond to the γ-, β-, and α-phosphates of free Mg^{2+}·GppNHp, respectively (Figure 4). In the presence of 0.75 M GdmCl, the nucleotide is totally released. It is not clear if the equilibrium can be shifted by GdmCl treatment, because the unstable complex dissociates at very low concentrations of the chaotropic reagent and increasing amounts of nucleotide-free protein precipitated. The missing contact among Thr48, the metal ion, and probably the γ-phosphate leads to a drastic loss of stability of the protein·nucleotide complex.

Cu^{2+}-cyclen Binding for the Identification of Conformational States of Arf1. Zn^{2+}- or Cu^{2+}-cyclen complexes selectively bind to active Ras protein only if it exists in the conformation defined as state 1,[32−34] which is able to interact with GEFs[22] and shows a 2 order of magnitude decrease in affinity for effectors.[20,21] Very recently, we demonstrated that Cu^{2+}-cyclen can be used as probe to further prove the existence of conformational states in Ras-like proteins, such as Ras, Arf1, and Ran, leading to strong line broadening of the γ-phosphate resonance if the protein exists in conformational state 1,[34] because of paramagnetic relaxation enhancement (PRE). At a Cu^{2+}-cyclen:Arf1(wt) molar ratio of up to 16, effects on the chemical shifts, relative integrals, or line widths could not be obtained, suggesting that wild-type Arf1 exists in a conformation corresponding to state 2.[34] Equal results were obtained for

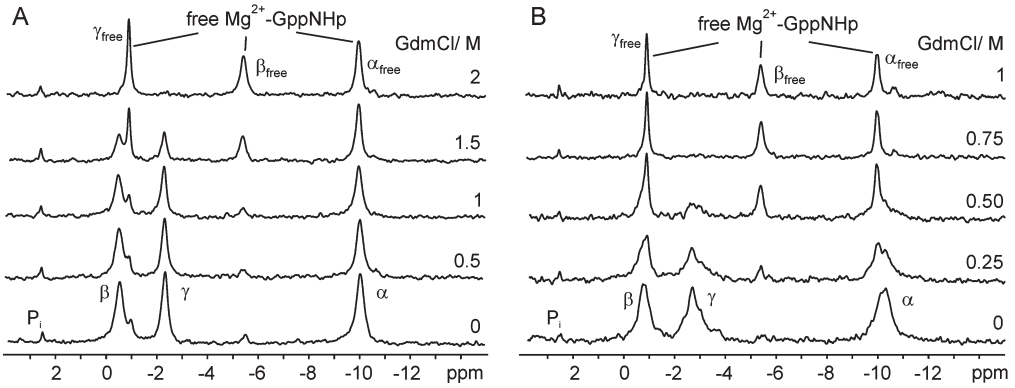

Figure 4. GdmCl titration of 1.6 mM [Δ17]Arf1(wt) (A) and 1.6 mM [Δ17]Arf1(T48A) (B), bound to Mg^{2+}·GppNHp. Measurements were performed in buffer A at 278 K. Highly concentrated GdmCl in the same buffer at the same pH was added. Measurements were started 30 min after mixing. The resonances arising at −0.9, −5.4, and −10.0 ppm correspond to the γ-, β-, and α-phosphates of free Mg^{2+}·GppNHp, respectively.

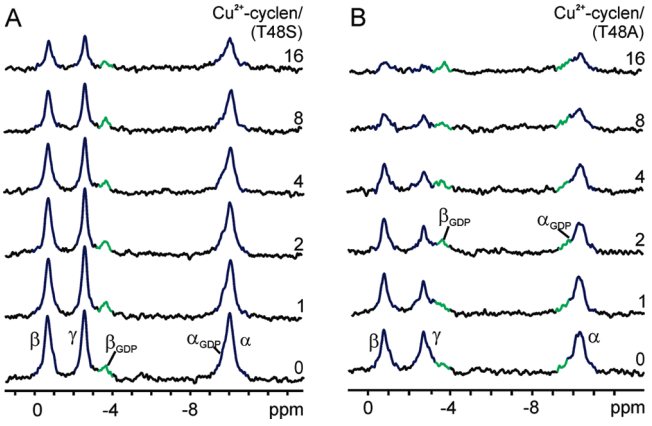

Figure 5. Titration of GppNHp-bound [Δ17]Arf1(T48S) (A) and the T48A mutant (B) with Cu^{2+}-cyclen. To initially 1.0−1.4 mM [Δ17]Arf1 in buffer A was added a 200 mM concentrated solution of Cu^{2+}-cyclen to the indicated Cu^{2+}-cyclen:Arf1 molar ratio. All measurements were performed at 278 K.

Arf1(wt) in complex with GTP or GTPγS, and again no effects are found in the corresponding ^{31}P NMR spectra (data not shown). Arf1(T48S) behaves like the wild type. No effects could be determined up to a molar excess of the metal cyclen of 16 (Figure 5A); i.e., the γ-phosphate group seems not to be accessible to the metal-bound cyclen in the conformational state of Arf1(T48S). In contrast, when Arf1(T48A) is titrated with Cu^{2+}-cyclen, the resonance of the γ-phosphate and at higher concentrations also the one corresponding to the β- and α-phosphates become strongly broadened, which indicates the binding of Cu^{2+}-cyclen near the γ-phosphate that shows the strongest PRE effect. Therefore, this mutant should at least partially exist in a conformation in which the γ-phosphate is accessible to the Cu^{2+}-cyclen complex (Figure 5B).

Formation of a Complex between ARNO-Sec7 and Arf1. Previously, it could be shown that guanine nucleotide exchange factor Sos (son-of-sevenless) binds to the conformation of state 1 of GppNHp-bound Ras protein.[22] Here we performed titration experiments between the Sec7 domain of the Arf1-activating regulator (ARNO) and wild-type Arf1 bound to Mg^{2+}·GppNHp and Mg^{2+}·GTPγS. Neither line broadening nor changes in ^{31}P chemical shifts could be observed (Figure 6A,B). With increasing concentrations of ARNO-Sec7, a small fraction of free nucleotide could be obtained in the ^{31}P NMR spectra that could reflect the nucleotide free Arf1·ARNO-Sec7 complex. We also recorded ^{1}H NMR spectra for both proteins and their mixture. Again no indications of a strong complex between Arf1(wt)·GppNHp and ARNO could be detected (Figure S2 of the Supporting Information). From line broadening of separated resonance lines, we could detect an increase in line width of 6% at an excess of 1 mM ARNO over Arf1·Mg^{2+}·GppNHp. Formation of an Arf1-ARNO complex should lead to an increase in the rotational correlation time by a factor of 2.4; thus, as a first approximation, one can estimate a fraction of the complex of approximately 5%.

Via the performance of analogous ^{31}P NMR experiments on the titration using the T48A mutant, the interaction can be clearly demonstrated, resulting in a strong line broadening of the resonances (Figure 6C,D). Although the resonances are not separated well, for the ^{31}P resonances corresponding to the phosphate groups of the bound nucleotide, a slight change in the equilibrium toward the high-field shifted signal typical for this mutant can be detected.

To investigate qualitatively the effect of the ARNO-Sec7 domain on active Arf1(wt) and Arf1(T48A), we incubated these complexes bound to GppNHp with a 2-fold excess of GTP in the absence and presence of a small fraction of ARNO-Sec7 protein (Figure S3 of the Supporting Information). By ^{31}P NMR spectroscopy, one can distinguish easily between the nucleotides themselves and their complexation state. In wild-type Arf1, the GTP analog was exchanged with GTP only in the presence of ARNO-Sec7 (Figure S3A of the Supporting Information). This result demonstrates that Arf1(wt)·GppNHp must interact with ARNO-Sec7, but as determined via line shape analysis of the spectra presented in Figure 6A,B, the fraction of the Arf-nucleotide·ARNO complex should be lower than 5% as estimated from the line width or signal-to-noise ratio. In the experiment with Arf(wt), in the presence of the GEF, added GTP replaces completely the corresponding analogue; i.e., GTP exhibits a higher affinity compared to the analogue. In the mutant, even the presence of a small amount of GDP, which was still in the sample, replaces both types of triphosphate nucleosides directly, without a GEF present (Figure S3B of the Supporting Information). Addition of ARNO-Sec7 protein shows here no further effects. That means GDP is more strongly bound to the Arf1 mutant than either triphosphate nucleoside (see also Figure S1 of the Supporting Information). The rate of dissociation of the bound

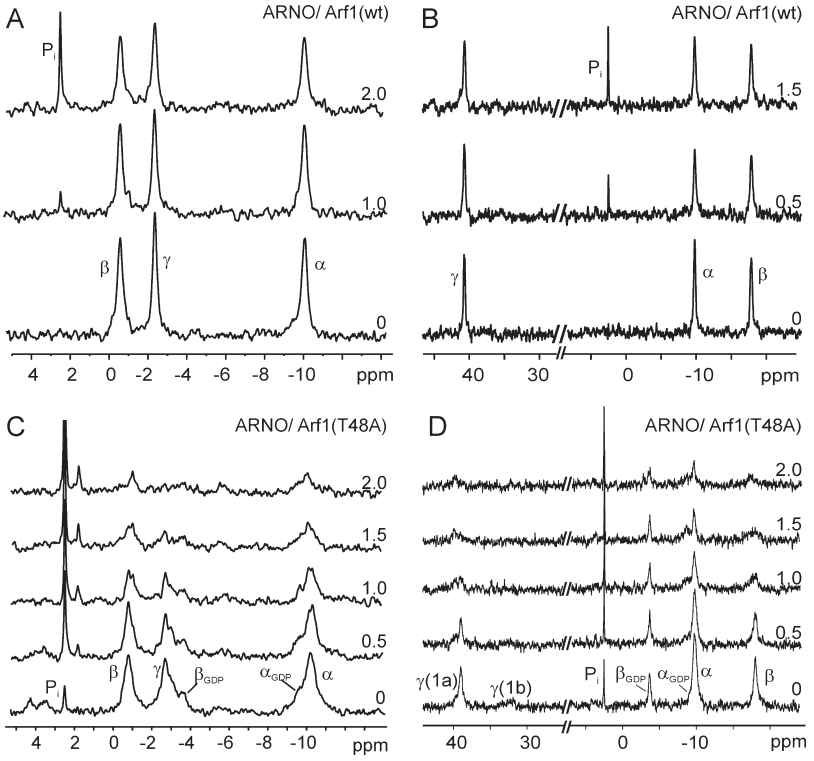

Figure 6. Results of ^{31}P NMR spectroscopy of the titration of [Δ17]Arf1·Mg^{2+}·GppNHp (A and C) or [Δ17]Arf1·Mg^{2+}·GTPγS (B and D) with ARNO-Sec7 in buffer A (see Experimental Procedures). Measurements were taken initially with 1.2 mM [Δ17]Arf1(wt)·Mg^{2+}·GppNHp (A) and 1.2 mM [Δ17]Arf1(wt)·Mg^{2+}·GTPγS (B), 1.3 mM [Δ17]Arf1(T48A)·Mg^{2+}·GppNHp (C), and 1.0 mM [Δ17]Arf1(T48A)·Mg^{2+}·GTPγS (D). ARNO-Sec7 protein was added until the indicated ARNO-Sec7:Arf1 molar ratio was reached. The temperature was 278 K.

nucleotide seems to be higher than that of the wild type, as one would expect with the loss of the contact between the nucleotide and/or Mg^{2+} ion and the crucial threonine residue of switch I. It must be mentioned that the recording times of the ^{31}P NMR spectra are on the order of hours.

DISCUSSION

Conformational State of Wild-Type Arf1. Conformational dynamics of proteins play an important role in the performance of their cellular tasks, allowing fast enzymatic reactions as well as proper protein−protein interaction. Guanine nucleotide-binding proteins must be able to adopt a variety of different conformations to fulfill their function as molecular switches. Besides the change between the inactive, GDP-bound and active, GTP-bound conformation, they have to interact with activating and inactivating regulators, i.e., the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs) as well as effector proteins, to induce the cellular response. For the Arf-related proteins of the Ras, Rho, and Ran family, the coexistence of two different conformational states (state 1 and state 2) has been shown, when they are bound to guanine nucleoside triphosphates.[17−29] The equilibrium constants (K_{12}) depend on the nature of the bound guanine nucleotide; therefore, modifications in the nucleotides such as replacement of the bridging oxygen between β- and γ-phosphorus lead to a shift of the equilibrium toward state 1(T) in Ras[18,19] or Ral.[25,26] State 1(T) of Ras, which is recognized by the catalytic site of the GEF Sos,[22] seems to be a conformation that lacks the coordination between the totally conserved threonine residue in switch I and the Mg^{2+} ion and most probably the γ-phosphate.[41] State 2(T) is the high-affinity state for effector binding, presenting the contacts mentioned above.[17−19] Previously, it was shown by ^{31}P NMR spectroscopy that Arf1(wt) bound to Mg^{2+}·GTP shows one resonance for each phosphate group over the tested temperature range, indicating a single conformational state.[30] Analogous results were obtained by Seidel and co-workers.[31] In this work, we tested the commonly used GTP analogues GppNHp, $GppCH_2p$, and GTPγS. All Arf1(wt)·nucleotide complexes show only single ^{31}P resonances for each phosphate, independent of the GTP analogue used. Taking into account the crystal structure of Arf1(wt)[10] (there the truncated variant [Δ17]Arf1 was used, too), as well as the structure obtained with mouse Arf1 in complex with an effector domain,[40] we find the conformation should correspond to state 2, according to our definition. State 2 is the conformation that is stabilized by effector interaction,[17−19] with the conserved threonine of switch I (Thr48 in Arf1 and Thr35 in Ras) coordinated to the γ-phosphate as well as to the Mg^{2+} ion. In contrast to the GDP complexes, the active conformation of Arf1(wt) is very similar to that of Ras(wt)·Mg^{2+}·GppNHp.[2,10,40] The crystal structure of Ras[42] represents state 2 only, although the protein should exist in an equilibrium between the two states in the crystal, too, as it was previously demonstrated by ^{31}P solid state NMR on Ras crystals.[43,44] Both switch regions in state 1 mutant T35S were found to be disordered or flexible in the crystal and therefore invisible in the crystal structure.[20] Very recent results published by Shima and co-workers using other crystallization conditions show two different forms of active Ras(T35S).[41] In both cases, Thr35 is not coordinated to the Mg^{2+} ion or the γ-phosphate. The difference

in the coordination pattern of the phosphate groups is the interaction of Gly60 from switch II with the γ-phosphate.

The truncated version of Arf1 ([Δ17]Arf1) used in this work allows investigations of Arf1 in its active state without membrane mimics present, e.g., interaction with GEFs. Therefore, in many previous structural investigations, that Arf1 variant was used. A very recent work determined the structure of membrane-anchored yeast Arf1.[45] In that work, the authors found the obtained NMR structure of myristoylated yeast Arf1-GTP bound to bicelles, especially in terms of its switch regions, to be quite similar to the crystal structure derived from mouse Arf1-GTP, which seems to represent the state 2 conformation interacting with the N-GAT domain of GGA.[40]

Mutation of the Conserved Threonine in Arf1. Mutation of the crucial threonine residue in switch I of Arf1 to alanine or serine should destabilize the conformation of state 2(T).[18−21,25,28] The ^{31}P NMR spectra of the corresponding GppNHp complexes show that both Arf1 mutants behave differently. Compared to the wild type, the mutant Arf1(T48S) shows small changes in the ^{31}P chemical shift values, especially in those corresponding to the β- and γ-phosphates, and a 1.6-fold broadening of the resonances. The latter observation is an indication of chemical exchange and/or exchange between the bound and free nucleotide. The alanine mutant shows larger changes in chemical shift values and broader lines with an inhomogeneous line shape. This indicates an overlay of at least two resonances in slow exchange relative to the time scale of the NMR experiment. The temperature-dependent behavior confirms this hypothesis. At room temperature, the line results in one sharp resonance (fast exchange conditions on the NMR time scale). Furthermore, NMR diffusion measurement, light scattering, and size exclusion chromatography did not give a hint of dimerization or aggregation of the protein as it could be shown for membrane-bound Arf1.[46]

The resonances are not separated well; thus, determination of exchange rates or equilibrium constants cannot be determined accurately from the spectra. As it was proven for Ras·nucleotide complexes (unpublished results), there is no advantage in performing the measurements at higher magnetic fields, because relaxation effects caused by the chemical shift anisotropy become dominant and lead to a further decrease in resolution. With respect to the temperature dependence of the line width, one would expect it to be on the millisecond time scale at room temperature, which is in good agreement with the results found for other GNB-proteins.[18,26] At low temperatures, the γ-phosphate line of the T48A mutant bound to GppNHp can be explained by three lines, corresponding to the state of the wild type (with a small amount), predominantly by the one obtained for the T48S mutant, and the one exclusively found for the alanine mutant. The resulting chemical shift value of the T48A state is −3.08 ppm with a ratio of 0.4 relative to the T48S-like state. The rate of exchange is 25 s^{-1}, as estimated from additional line broadening compared to the resonance obtained for the T48S mutant.

In complex with Mg^{2+}·GTPγS, only Arf1(T48A) shows a splitting of the γ-phosphate signal, corresponding to two conformers of state 1 called states 1a and 1b in analogy to the data published for Ras protein.[21] For Ras protein, these properties could be shown for both corresponding mutants, Ras(T35A) and Ras(T35S). This splitting seems to be possible only in the conformation of state 1(T), caused by different orientations of the sulfur in the complex.[21,34] The equilibrium constant (K_{1a1b}) is 0.18, and a rate of exchange (k_{ex}) of 16 s^{-1} at 278 K can be estimated. The latter is 1 order of magnitude lower compared to that determined for Ras(T35S)·Mg^{2+}·GTPγS by line shape analysis of the temperature-dependent spectra (approximately 200 s^{-1}).[21] This means that Arf1(T48A) at least partially exists in a conformational state corresponding to state 1, whereas wild-type Arf1 is predominantly in state 2. The serine mutant exists structurally in something between the two. It seems that this mutant exists in the more populated state detected in the equilibrium of the T48A mutant. Because the side chain hydroxyl is still present, the interaction with the Mg^{2+} ion should stabilize the conformation with switch I close to the γ-phosphate and the metal ion as it is found in the crystal structure.

Interaction with Cu^{2+}-cyclen, Arno-Sec7, and GAT. As an effector of Arf1, we expressed the GAT domain of human GGA1 protein. Although the GAT domain showed only a low solubility of 0.3 mM (which is rather low in terms of ^{31}P NMR sensitivity), we could record spectra of the complexes between the effector GAT and Arf1 bound to GppNHp as well as Arf1-GTPγS. In both cases, the ^{31}P resonances show line broadening due to complex formation. Further, the γ-phosphate signal shows a small change in chemical shift value of 0.2 ppm to lower field. These results indicate that Arf1(wt) exists in a conformation very similar to the effector binding state. These results confirm that Arf1 bound to guanosine triphosphates exists in a conformation corresponding to state 2(T), which is in accordance with crystal structures of Arf1(wt)·GppNHp[21] and its complex with a N-GAT domain.[40] In both structures, the switch regions are coordinated to the γ-phosphate.

As shown with the Arf1 mutants, the difference in chemical shift between the two states is smaller compared to those obtained for other GNB-proteins. Effector binding usually leads to minor chemical shift changes as well as to line broadening because of the increase in the molecular weight of the complex. These effects make it difficult to analyze the obtained results. Therefore, we used two other ligands to investigate the Arf1 variants in terms of their conformational states. Cu^{2+}- or Zn^{2+}-cyclen complexes are known to bind to the active site of Ras only if Ras is in state 1(T) of the protein.[32,33] We could demonstrate that Cu^{2+}-cyclen can be used to identify conformational states of Ras-like proteins using the effect of paramagnetic relaxation enhancement.[34] In that work, we could show that Arf1(wt)·GppNHp is insensitive to the paramagnetic probe, a further indication of the state 2(T) conformation. We have tested both Thr48 mutants in terms of these properties. Like the results obtained for the wild type, also Arf1(T48S) seems to be resistant to the Cu^{2+} probe in its active site. The resonances remain unperturbed. In contrast, all ^{31}P resonances of the Arf1(T48A) spectrum become strongly broadened in a distance-dependent manner, because of the paramagnetic properties of the copper(II) ion. The γ-phosphate resonance with a chemical shift value of −3.08 ppm is especially sensitive. At higher concentrations, the equilibrium seems to be shifted toward the latter mentioned state, because the magnitudes of the resonances typical for the serine mutant decrease. This result indicates that the interaction of Cu^{2+}-cyclen occurs in a manner similar to that found for Ras protein.[32−34]

Formation of a complex of GTP-bound Arf1 with the activator domain ARNO-Sec7 was assumed to stabilize the state 1(T) conformation of the protein, which resembles a more opened switch I region.[47,48] As shown previously by Kremer and co-workers,[30] there was no significant interaction between

Arf1(wt)·Mg^{2+}·GTP and ARNO-Sec7 detectable in the spectra. In this paper, we obtained similar results for Arf1(wt) bound to the different GTP analogues and ARNO-Sec7, but we could demonstrate that the Sec7 domain promotes nucleotide exchange also in active Arf1(wt); thus, interaction must happen. In principle, four scenarios are possible. (i) The Sec7 domain binds completely to the dominantly existing active state of Arf1. This would lead to an increase in the rotational correlation time by a factor of 2.4 and thus in first approximation to a line broadening of the ^{31}P resonances by a factor of 2.4. (ii) The Sec7 domain has a dissociation constant in the millimolar range for the dominant conformational state, and thus, even at the highest concentration of ARNO-Sec7 used, only a small fraction of Arf1 forms a complex with the Sec7 domain. The resulting line broadening could be thus smaller than 10%. (iii) The Sec7 domain interacts with a conformation with a low population (probably the one found for Arf1 mutant) that cannot be detected in the signal-to-noise level (as estimated 5−10%). Thus, a line broadening is not visible, and the intensity of the visible resonance line potentially could decrease slightly. (iv) The Sec7 domain has a higher affinity for the nucleotide-free state. This would lead to a dissociation of the nucleotide from the complex of Arf1 and ARNO. In this case, free nucleotide could be observed by NMR.

From our ^{31}P NMR data, we can exclude the first case for wild-type Arf1. Case (ii) would be possible, but we can estimate a maximal level of complex formation of 5%, taking into account an estimated error in line width determination (iii) or the signal-to-noise ratio (iii). With regard to case (iv), at high ARNO-Sec7 concentrations (2 mM), a small fraction of approximately 5−10% of free nucleotide can be observed, which is in agreement with the increase in line width within the ^{1}H NMR spectra due to the addition of ARNO-Sec7 to Arf1(wt).

In contrast, addition of ARNO-Sec7 to Arf(T48A) led to strong line broadening of the resonance signals, which can be due to the increase in the molecular weight of the complex, as well as broadening caused by exchange processes of bound and free nucleotide, which is found in the spectra. In both cases, these results clearly demonstrate the direct interaction of ARNO-Sec7 and T48A mutant to an large extent.

Comparison with Other GNB-Proteins. Intrinsic conformational equilibria seem to be a general property of GNP-proteins in the active GTP-bound form.[17−30] At a minimum, one would expect three different GTP-bound states from the regulation cycle, the interaction state(s) with effectors and two states interacting with the activating and inactivating regulators, the nucleotide exchange factors and the GTPase-activating proteins, respectively. For several GNB-proteins, two states could be distinguished by ^{31}P NMR spectroscopy because they lead to resonances with different chemical shift values. The rates of exchange are commonly slow on the NMR time scale at temperatures below 278 K and can therefore be resolved. In contrast to Ras, Ral, Cdc42, or Ran proteins,[17−21,23−30] in the Arf1 equilibrium the state 2(T) conformation, i.e., the effector interacting state, is energetically much more favored; thus, even when the protein is bound to GTP analogues, state 2 clearly predominates. In the case of Arf1, the GTP analogues seem to be good analogues for structural as well as biochemical investigations, in contrast to the results obtained for many other GNB-proteins, where the equilibria are shifted (and thereby the biochemical properties) when using GTP analogues instead of the natural ligand GTP. The truncated Arf1 variant used here allows investigations in the absence of membrane mimics. Therefore, the results cannot be directly transferred into in vivo conditions; nevertheless, one would expect the state 2 conformation also in the membrane-anchored form that very recently was also shown for yeast Arf1 anchored in a membrane mimic.[45] Because these different conformational states have different physiological properties, in terms of their affinity for effectors or regulators such as GEFs or GAPs, as well as their nucleotide binding kinetics[27] or intrinsic GTPase activity,[19] it seems that within the cellular network, fine-tuning of these molecular switches could be achieved by nature adjusting these equilibria. In terms of academic and medicinal research, targeting these equilibria is a promising approach to modulating signaling pathways.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures demonstrating intrinsic and catalyzed nucleotide exchange of wild-type and mutant [Δ17]Arf1 as well as ^{1}H NMR data for the titration of Arf1-GppNHp with ARNO-Sec7. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ARNO, Arf nucleotide binding site opener; Arf, ADP ribosylation factor; CD4, cluster of differentiation 4; COPI, coat protein I; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTE, 1,4-dithioerythritol; GdmCl, guanidine hydrochloride; GAP, GTPase-activating protein; GGA, Golgi-associated γ-adaptin homologous Arf-interacting; GEF, guanine nucleotide exchange factor; GppNHp, guanosine 5′-(β,γ-imido)triphosphate; $GppCH_2p$, guanosine 5′-(β,γ-methyleno)triphosphate; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Nef, negative factor; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser exchange spectroscopy; Ran, Ras-related nuclear protein; Ras, rat sarcoma.

■ REFERENCES

(1) D'Souza-Schorey, C., and Chavrier, P. (2006) ARF proteins: Roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* 7, 347–358.

(2) Gillingham, A. K., and Munro, S. (2007) The small G proteins of the Arf family and their regulators. *Annu. Rev. Cell Dev. Biol.* 23, 579–611.

(3) Hsu, V. W., Lee, Y. S., and Yang, J.-S. (2009) The evolving understanding of COPI vesicle formation. *Nat. Rev. Mol. Cell Biol. 10*, 360–364.

(4) Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Steinweis, P. C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell 75*, 1137–1144.

(5) Boulay, P., Cotton, M., Melancon, P., and Claing, A. (2008) ADP-ribosylation factor 1 controls the activation of the phosphatidylinositol 3-kinase pathway to regulate epidermal growth factor-dependent growth and migration of breast cancer cells. *J. Biol. Chem. 283*, 36425–36434.

(6) Fauré, J., Stalder, R., Borel, C., Sobo, K., Piguet, V., Demaurex, N., Gruenberg, J., and Trono, D. (2004) ARF1 Regulates Nef-Induced CD4-Degradation. *Curr. Biol. 14*, 1056–1064.

(7) Kahn, R. A., Randazzo, P., Serfini, T., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P., Orci, L., and Rothmann, J. E. (1992) The amino terminus of ADP-ribosylation factor (ARF) is a critical determinant of ARF activities and is a potent and specific inhibitor of protein transport. *J. Biol. Chem. 267*, 13039–13046.

(8) Randazzo, P. A., Terui, T., Sturch, S., Fales, H. M., Ferrige, A. G., and Kahn, R. A. (1995) The myristoylated amino terminus of ADP-ribosylation factor 1 is a phospholipid- and GTP-sensitive switch. *J. Biol. Chem. 270*, 14809–14815.

(9) Amor, J. C., Harrison, D. H., Kahn, R. A., and Ringe, D. (1994) Structure of the human ADP-ribosylation factor 1 complexed with GDP. *Nature 372*, 704–708.

(10) Goldberg, J. (1998) Structural basis for activation of ARF GTPase: Mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. *Cell 95*, 237–248.

(11) Chardin, P., Paris, S., Antonny, B., Robineau, S., Béraud-Dufour, S., Jackson, C. L., and Chabre, M. (1996) A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature 384*, 481–484.

(12) Cherfils, J., Menetrey, J., Mathieu, M., La Bras, G., Robineau, S., Béraud-Dufour, S., Antonny, B., and Chardin, P. (1998) Structure of the Sec7 domain of the Arf exchange factor ARNO. *Nature 392*, 101–105.

(13) Kahn, R. A., and Gilman, A. G. (1986) The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J. Biol. Chem. 261*, 7906–7911.

(14) Luo, R., Ha, V. L., Hayashi, R., and Randazzo, P. A. (2009) Arf GAP2 is positively regulated by coatomer and cargo. *Cell. Signalling 21*, 1169–1179.

(15) Weimer, C., Beck, R., Eckert, P., Reckmann, I., Moelleken, J., Brügger, B., and Wieland, F. (2008) Differential roles of ArfGAP1, ArfGAP2, and ArfGAP3 in COPI trafficking. *J. Cell Biol. 183*, 725–735.

(16) Beck, R., Adolf, F., Weimer, C., Bruegger, B., and Wieland, F. (2009) ArfGAP1 activity and COPI vesicle biogenesis. *Traffic 10*, 307–315.

(17) Geyer, M., Schweins, T., Herrmann, C., Prisner, T., Wittinghofer, A., and Kalbitzer, H. R. (1996) Conformational transitions in p21-(Ras) and in its complexes with the effector protein Raf-RBD and the GTPase activating protein GAP. *Biochemistry 35*, 10308–10320.

(18) Spoerner, M., Nuehs, A., Ganser, P., Herrmann, C., Wittinghofer, A., and Kalbitzer, H. R. (2005) Conformational states of Ras complexed with the GTP analogue GppNHp or $GppCH_2p$: Implications for the interaction with effector proteins. *Biochemistry 44*, 2225–2236.

(19) Spoerner, M., Hosza, C., Poetzl, J. A., Reiss, K., Ganser, P., Geyer, M., and Kalbitzer, H. R. (2010) Conformational states of human rat sarcoma (Ras) protein complexed with its natural ligand GTP and their role for effector interaction and GTP hydrolysis. *J. Biol. Chem. 285*, 39768–39778.

(20) Spoerner, M., Herrmann, C., Vetter, I. R., Kalbitzer, H. R., and Wittinghofer, A. (2001) Dynamic properties of the Ras switch I region and its importance for binding to effectors. *Proc. Natl. Acad. Sci. U.S.A. 98*, 4944–4949.

(21) Spoerner, M., Nuehs, A., Herrmann, C., Steiner, G., and Kalbitzer, H. R. (2007) Slow conformational dynamics of the guanine nucleotide-binding protein Ras complexed with the GTP analogue GTPγS. *FEBS J. 274*, 1419–1433.

(22) Kalbitzer, H. R., Spoerner, M., Hozsa, C., Ganser, P., and Kremer, W. (2009) A fundamental link between functional state and folding states of proteins. *J. Am. Chem. Soc. 131*, 16714–16719.

(23) Ye, M., Shima, F., Muraoka, S., Liao, J., Okamoto, H., Yamamoto, M., Tamura, A., Yagi, N., Ueki, T., and Kataoka, T. (2005) Crystal structure of M-Ras reveals a GTP-bound "off" state conformation of Ras family small GTPases. *J. Biol. Chem. 280*, 31267–31275.

(24) Matsumoto, K., Shima, F., Muraoka, S., Araki, M., Hu, L., Ijiri, Y., Hirai, R., Liao, J., Yoshioka, T., Kumasaka, T., Yamamoto, M., Tamura, A., and Kataoka, T. (2011) Critical roles of interactions among switch I-preceding residues and between switch II and its neighboring α-helix on conformational dynamics of the GTP-bound Ras family small GTPases. *J. Biol. Chem. 286*, 15403–15412.

(25) Fenwick, R. B., Prasannan, S., Campbell, L. J., Nietlispach, D., Evetts, K. A., Camonis, J., Mott, H. R., and Owen, D. (2009) Solution Structure and Dynamics of the Small GTPase RalB in Its Active Conformation: Significance for Effector Protein Binding. *Biochemistry 48*, 2192–2206.

(26) Fenwick, R. B., Campbell, L. J., Rajasekar, K., Prasannan, S., Nietlispach, D., Camonis, J., Owen, D., and Mott, H. R. (2010) The RalB-RLIP76 complex reveals a novel mode of ral-effector interaction. *Structure 18*, 985–995.

(27) Liao, J., Shima, F., Araki, M., Ye, M., Muraoka, S., Sugimoto, T., Kawamura, M., Yamamoto, N., Tamura, A., and Kataoka, T. (2008) Two conformational states of Ras GTPase exhibit differential GTP-binding kinetics. *Biochem. Biophys. Res. Commun. 369*, 327–332.

(28) Phillips, M. J., Calero, G., Chan, B., Ramachandran, S., and Cerione, R. A. (2008) Effector proteins exert an important influence on the signaling-active state of the small GTPase Cdc42. *J. Biol. Chem. 283*, 14153–14164.

(29) Geyer, M., Assheuer, R., Klebe, C., Kuhlmann, J., Becker, J., Wittinghofer, A., and Kalbitzer, H. R. (1999) Conformational states of the nuclear GTP-binding protein Ran and its complexes with the exchange factor RCC1 and the effector protein RanBP1. *Biochemistry 38*, 11250–11260.

(30) Kremer, W., Steiner, G., Béraud-Dufour, S., and Kalbitzer, H. R. (2004) Conformational states of the small G protein Arf-1 in complex with the guanine nucleotide exchange factor ARNO-Sec7. *J. Biol. Chem. 279*, 17004–17012.

(31) Seidel, R. D., III, Amor, J. C., Kahn, R. A., and Prestegard, J. H. (2004) Conformational changes in human Arf1 on nucleotide exchange and deletion of membrane-binding elements. *J. Biol. Chem. 279*, 48307–48318.

(32) Spoerner, M., Graf, T., König, B., and Kalbitzer, H. R. (2005) A novel mechanism for the modulation of the Ras-effector interaction by small molecules. *Biochem. Biophys. Res. Commun. 334*, 709–713.

(33) Rosnizeck, I. C., Graf, T., Spoerner, M., Tränkle, J., Filchtinski, D., Herrmann, C., Gremer, L., Vetter, I. R., Wittinghofer, A., König, B., and Kalbitzer, H. R. (2010) Stabilizing a weak binding state for effectors in the human Ras-protein by small compounds. *Angew. Chem., Int. Ed. 49*, 3830–3833.

(34) Meierhofer, T., Rosnizeck, I. C., Graf, T., Reiss, K., König, B., Kalbitzer, H. R., and Spoerner, M. (2011) Cu^{2+}-cyclen as probe to identify conformational states in guanine nucleotide binding proteins. *J. Am. Chem. Soc. 133*, 2048–2051.

(35) Shaka, A. J., Baker, P. B., and Freeman, R. (1985) Computer-optimized decoupling scheme for wideband applications and low-level operation. *J. Magn. Reson. 64*, 547–552.

(36) Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys. 71*, 4546–4553.

(37) Hausser, K. H., and Kalbitzer, H. R. (1991) *NMR in Medicine and Biology. Structure Determination, Tomography, In Vivo Spectroscopy*, Springer-Verlag, Heidelberg, Germany.

(38) Maurer, T., and Kalbitzer, H. R. (1996) Indirect referencing of ^{31}P and ^{19}F NMR Spectra. *J. Magn. Reson., Ser. B 113*, 177–178.

(39) Raiford, D. S., Fisk, C. L., and Becker, E. D. (1979) Calibration of methanol and ethylene glycol nuclear magnetic resonance thermometers. *Anal. Chem. 51*, 2050–2051.

(40) Shiba, T., Kawasaki, M., Takatsu, H., Nogi, T., Matsugaki, N., Igarashi, N., Suzuki, M., Kato, R., Nakayama, K., and Wakatsuki, S. (2003) Molecular mechanism of membrane recruitment of GGA by ARF in lysosomal protein transport. *Nat. Struct. Biol. 10*, 386–393.

(41) Shima, F., Ijiri, Y., Muraoka, S., Liao, J., Ye, M., Araki, M., Matsumoto, K., Yamamoto, N., Sugimoto, T., Yoshikawa, Y., Kumasaka, T., Yamamoto, M., Tamura, A., and Kataoka, T. (2010) Structural Basis for Conformational Dynamics of GTP-bound Ras Protein. *J. Biol. Chem. 285*, 22696–22705.

(42) Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. *Nature 341*, 209–214.

(43) Stumber, M., Geyer, M., Graf, R., Kalbitzer, H. R., Scheffzek, K., and Haeberlen, U. (2002) Observation of slow dynamic exchange processes in Ras protein crystals by ^{31}P solid state NMR spectroscopy. *J. Mol. Biol. 323*, 899–907.

(44) Iuga, A., Spoerner, M., Kalbitzer, H. R., and Brunner, E. (2004) Solid-state ^{31}P NMR spectroscopy of microcrystals of the Ras protein and its effector loop mutants: Comparison between crystalline and solution state. *J. Mol. Biol. 342*, 1033–1040.

(45) Liu, Y., Kahn, R. A., and Prestegard, J. H. (2010) Dynamic structure of membrane-anchored Arf*GTP. *Nat. Struct. Mol. Biol. 17*, 876–881.

(46) Beck, R., Zhe, S., Adolf, F., Rutz, C., Bassler, J., Wild, K., Sinning, I., Hurt, E., Brügger, B., Béthune, J., and Wieland, F. (2008) Membrane curvature induced by Arf1-GTP is essential for vesicle formation. *Proc. Natl. Acad. Sci. U.S.A. 105*, 11731–11736.

(47) Mossessova, E., Gulbis, J. M., and Goldberg, J. (1998) Structure of the guanine nucleotide exchange factor Sec7 domain of human ARNO and analysis of the interaction with ARF GTPase. *Cell 92*, 415–423.

(48) Béraud-Dufour, S., Robineau, S., Chardin, P., Paris, S., Chabre, M., Cherfils, J., and Antonny, B. (1998) A glutamic finger in the guanine nucleotide exchange factor ARNO displaces Mg_2F and the β-phosphate to destabilize GDP on ARF1. *EMBO J. 17*, 3651–3659.

Conformational States of the ADP Ribosylation Factor 1 Complexed with Different Guanosine-Triphosphates as Studied by ^{31}P NMR Spectroscopy

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Supporting information:

Figures S1 and S3:

^{31}P NMR titration experiments on Arf1in order to investigate nucleotide exchange. All experiments were performed in buffer A: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 2 mM DTE, containing 10% D_2O for lock signal, and 0.1 mM DSS allowing calibration of the spectra by indirect referencing.

Figure S2:

^{1}H NMR spectra on Arf1-GppNHp in absence and presence of ARNO-Sec7. Potential complex formation can be displayed by line broadening.

Further descriptions are found in the main article.

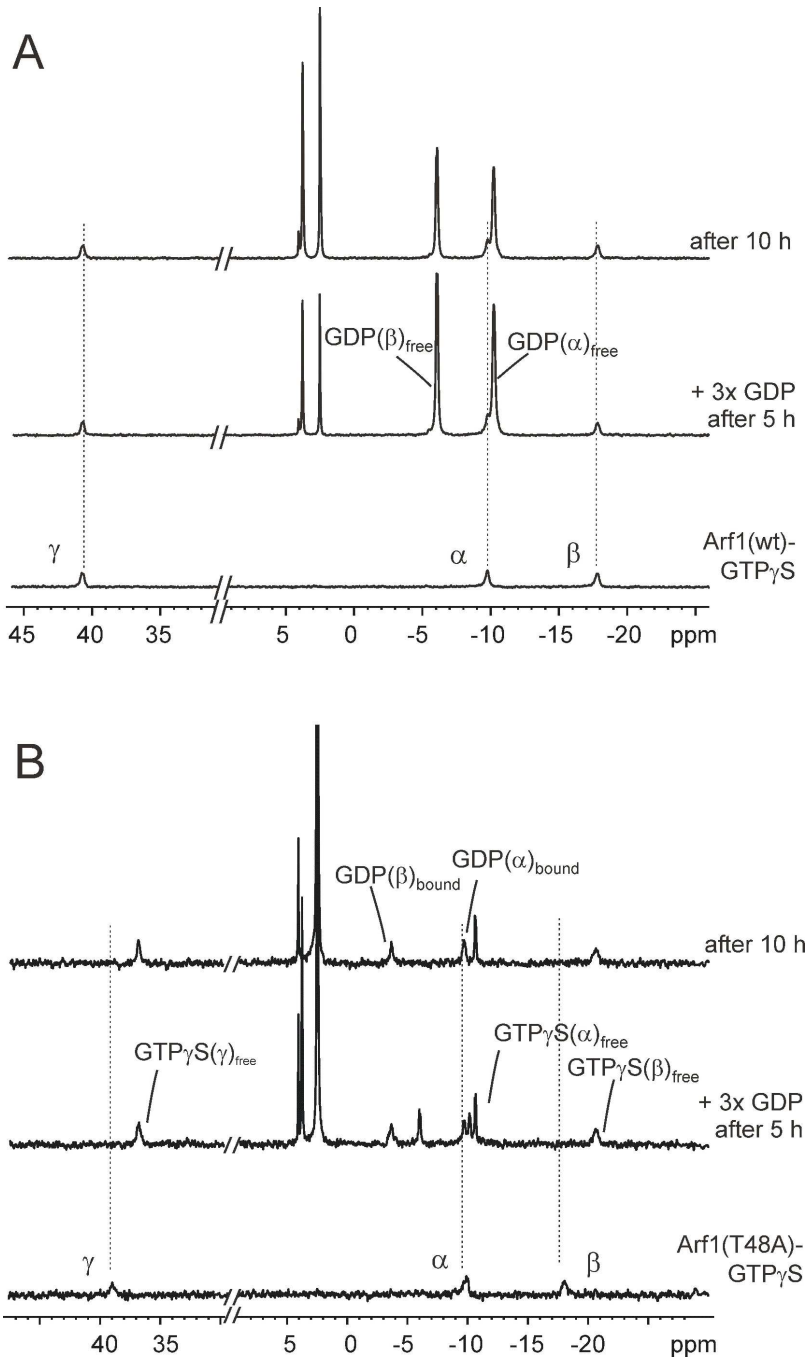

Figure S1:

^{31}P NMR investigation on the nucleotide exchange of wild-type (A) and T48A mutant (B) of [Δ17]Arf1. (A) To initially 1.5 mM [Δ17]Arf1(wt)-GTPγS (bottom spectrum) a 3-fold molar excess GDP was added (middle and upper spectrum). (B) To initially 1.1 mM [Δ17]Arf1(T48A)-GTPγS a 3-fold molar excess of GDP was added (middle and upper spectrum). For the middle spectra FIDs were summarized during the first 5 hours after adding the GDP, the upper spectra during further 5 hours. Temperature was 278 K.

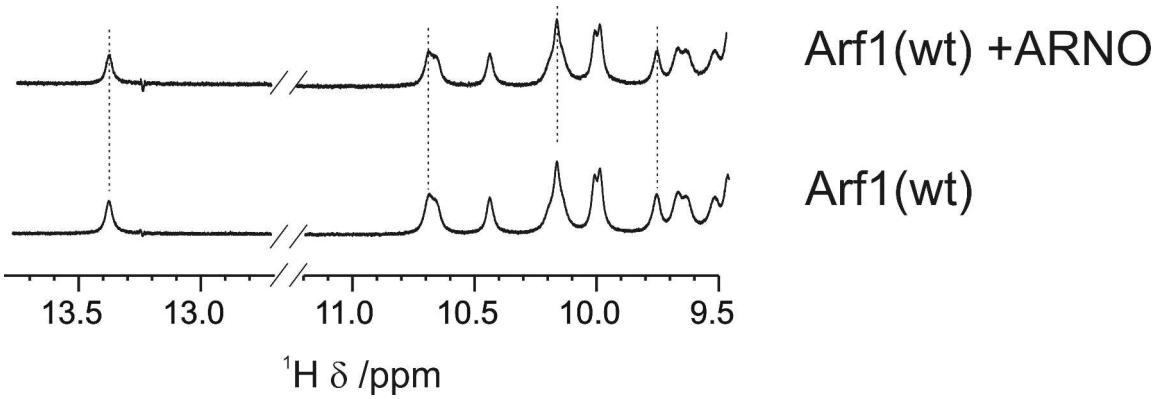

Figure S2:

Cutout of ^{1}H NMR spectra of 0.5 mM solution of Arf1(wt)-GppNHp in absence and presence of 1.5 mM ARNO-Sec7. Spectra were recorded at 600 MHz ^{1}H resonance frequency at 293K in 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM DTE.

Within this cutout of the spectrum, ARNO-Sec7 shows no resonances, therefore analysis of chemical shift perturbation and line-broadening effects can be performed in a simple way. As described in the main text, line broadening due to partial complex formation of 6% was obtained. Taking into account the molecular weight of the complex (2.4 fold increase of the rotatorial correlation time of the complex compared to free Arf1) one can estimate a fraction of Arf1-ARNO complex of 4.5%.

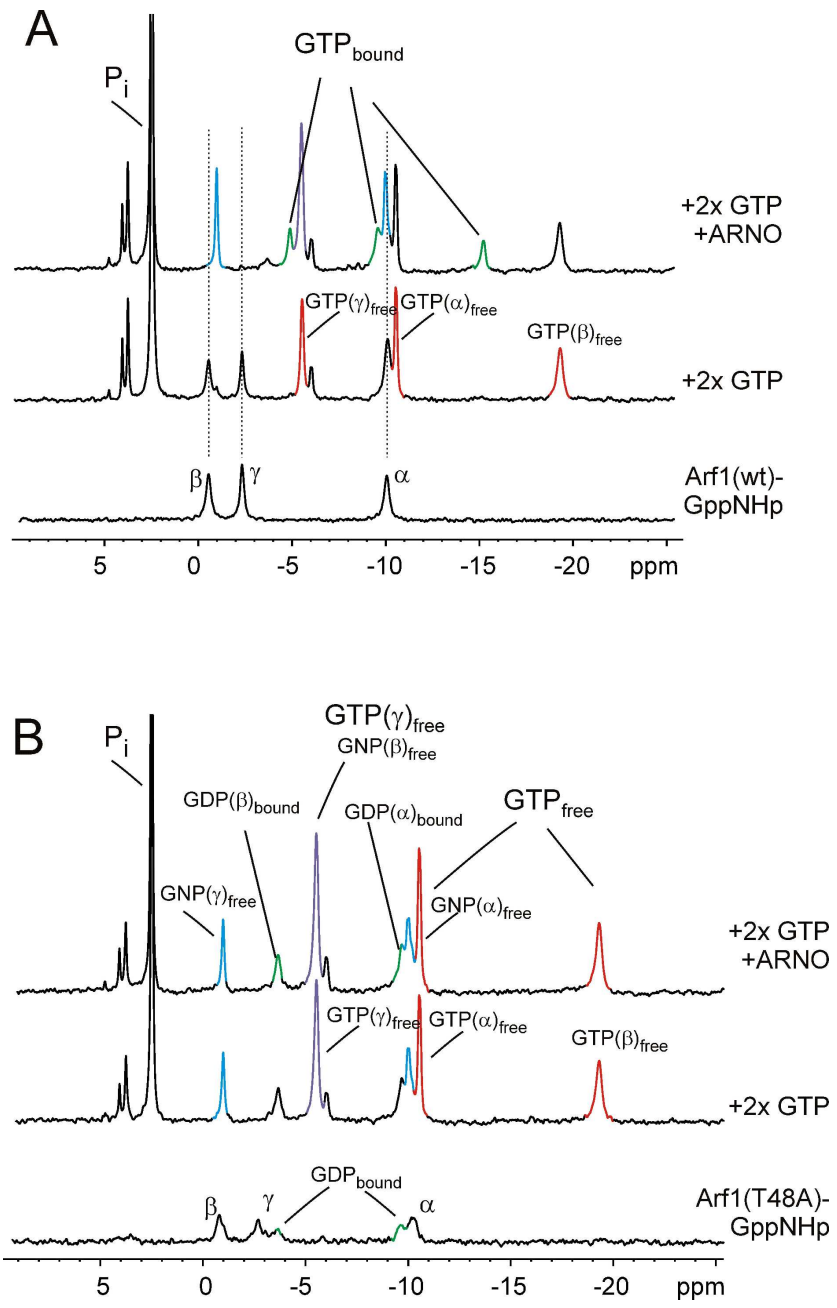

Figure S3:

^{31}P NMR investigation on the nucleotide exchange of wild-type (A) and T48A-mutant (B) of [Δ17]Arf1 in absence and presence of ARNO-Sec7. (A) To initially 1.6 mM [Δ17]Arf1(wt)-GppNHp a 2-fold excess GTP was added (middle spectrum). The sample of the upper spectrum contained further 0.16 mM ARNO-Sec7. (B) To initially 1.5 mM [Δ17]Arf1(T48A)-GppNHp a 2-fold excess GTP was added (middle spectrum). The sample of the upper spectrum contained further 0.15 mM ARNO-Sec7. FIDs were summarized over 5 hours after adding GTP or GTP and ARNO. Temperature was 278 K.