# Aluminium fluoride associates with the small guanine nucleotide binding proteins

Mohammad Reza Ahmadian[a], Rohit Mittal[a], Alan Hall[b], Alfred Wittinghofer[a,*]

[a] *Max-Planck-Institut für molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany*
[b] *MRC Laboratory for Molecular Cell Biology, University College London, London WC1E 6BT, UK*

Received 3 April 1997

**Abstract** $AlF_4^-$ **has long been known to associate with and activate the GDP-bound alpha subunits of heterotrimeric G-proteins. Recently the small guanine nucleotide binding protein Ras has also been shown to associate with** $AlF_4^-$ **in the presence of stoichiometric amounts of its GTPase activating protein (GAP). Here we present the isolation of a stable Ras·GDP·**$AlF_4^-$**·GAP ternary complex by gel filtration. In addition, we generalise the association of** $AlF_4^-$ **with the small GTP-binding proteins by demonstrating ternary complex formation for the Cdc42, Rap and Ran proteins in the presence of their respective GAP proteins.**



*Key words:* Aluminium fluoride; Ras; Cdc42; Rap; Ran

## 1. Introduction

Small guanine nucleotide binding proteins of the Ras superfamily are involved in a variety of cellular processes. They function as molecular switches and exist in an 'inactive' GDP-bound or an 'active' GTP-bound state. Their states of nucleotide occupancy are regulated by a variety of proteins including guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs)[1]. The GEFs promote the release of tightly bound GDP on the protein and thereby facilitate loading with cytosolic GTP. The GTP-binding proteins then return to the inactive state upon hydrolysis of the bound GTP to GDP by virtue of the GTPase reaction, which is usually very slow but can be accelerated by the action of the GAPs, in the case of Ras and Ras-GAPs by several orders of magnitude [2,3].

The superfamily of Ras-related GTP-binding proteins consists of several subfamilies such as the Ras, Rho, Rab, Ran and Arf subfamilies. While these have common sequence elements, and show the same overall three-dimensional fold as demonstrated by the structures of Ras [4,5], Arf [6], Ran [7] and Rab (P. Metcalf, unpublished data), the respective GEFs and GAPs are homologous only within and not across the subfamilies [8] and are expected to reveal different three-dimensional structures. Of the GAPs specific for Ras p120-GAP was the first to be isolated [9] and the next was neurofibromin (NF1) which is the product of the neurofibromatosis gene [10]. The three-dimensional structure of a catalytic fragment of the Ras-specific p120-GAP has recently been determined [11].

A number of GAPs specific for the members of the Rho subfamily (comprising the Rho, Rac and Cdc42 GTP-binding proteins) have been described. Of these Rho-GAP has been found to be active on several members of the Rho family but most active on Cdc42 [12]. A GAP for Ran has been cloned from human tissue [13] and has been shown to be the mammalian counterpart of RNA1, the yeast Ran-GAP. The *S. pombe* RNA1 protein has been shown to be active on human Ran [14]. Although Ras and Rap are from the same subfamily of Ras-related proteins, Rap-GAP has no sequence homology to Ras-GAP or to any of the other GAPs [15].

Recently we have shown by the use of fluorescence spectroscopic measurements that Ras-GAPs stabilize the binding of $AlF_4^-$ to Ras, which based on evidence from $G_\alpha$ proteins is believed to conformationally mimic the transition state of the GTPase reaction [16,17], and that stoichiometric amounts of the GAP are needed for this effect [18].

We have now isolated the stable complex between Ras·GDP, $AlF_4^-$ and NF1. Furthermore, by analysing the small GTP-binding proteins CDC42, Rap and Ran in the presence of their respective GAPs we demonstrate that the formation of quaternary G-protein·GDP·$AlF_4^-$·GAP complexes is a general principle applicable to other Ras-related GTP-binding proteins as well.

## 2. Materials and methods

### 2.1. Proteins

Recombinant H-Ras was prepared from *Escherichia coli* using a ptac-expression system as described [19]. Rap2B (expression plasmid kindly provided by Dr. Jean de Gunzburg) and CDC42 were expressed as GST-fusion proteins in *E. coli* and purified by glutathione-sepharose affinity chromatography. The protease thrombin was used (at a fusion protein:protease ratio of 50:1) to separate the GST and native G-proteins by cutting at a thrombin recognition site. Digestion was done in 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM DTE and 100 mM NaCl (HMDN buffer) supplemented with 2.5 mM $CaCl_2$, at 4°C for 3 h. Liberated GST subunits and undigested GST-fusion protein were removed by another round of glutathione sepharose chromatography. All G-proteins were dialysed against HMDN buffer before use in subsequent assays.

The catalytic domain of NF1, NF1–333, extending from amino acid E1198 to H1530, was isolated using the pLMM-expression system in *E. coli* as described previously [20]. Truncated Rap-GAP (amino acid residues 76–415) was prepared using the pLMM expression system in *E. coli* (Ahmadian et al., manuscript in preparation) and the 242-amino acid catalytic domain of Rho-GAP was prepared in *E. coli* as a GST-fusion protein and purified as described above for other GST-fusion proteins. Ran·GDP was a gift from Dr. Jörg Becker and Ran-GAP from Roman Hillig.

### 2.2. Gel filtration

Gel filtration (Superdex 75, 16/60, Pharmacia, Uppsala, Sweden) showing complex formation between Ras·GDP, $AlF_4^-$ and NF1-333 was performed in 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, and 5 mM DTE after incubating 150 μM Ras·GDP and 150 μM NF1-333 in the presence or absence of $AlF_4^-$ (10 mM NaF, 450 μM $AlCl_3$). The flow rate was maintained at 1.5 ml per min and 1 ml fractions were collected. Analytical gel filtration chromatography on CDC42 and Rho-GAP was performed over a Superdex G-75 column (10/30) using

*Corresponding author. Fax: (49) (231) 1206-230.
E-mail: alfred.wittinghofer@mpi-dortmund.mpg.de


*PII* S0014-5793(97)00422-5

HMDN buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM DTE, 100 mM NaCl). GDP-loaded CDC42 or the G12V mutant of CDC42 and the 242 amino acid C-terminal fragment of p50 Rho GAP [12] were used. 20 nmoles of each protein were incubated together for 15 min at room temperature in the presence or absence of $AlF_4^-$ in HMDN buffer in a total volume of 140 μl. Thereafter the sample was spun at 13000 rpm to remove any particulate impurities and loaded on the analytical gel filtration column. The flow rate was maintained at 1 ml per min and 250 μl fractions were collected. Peak fractions were visualised by 15% SDS-PAGE and subsequent staining by Coomassie Blue.

*2.3. GTPase assays*

G-proteins were loaded with radioactive nucleotide either by incubating nucleotide-free preparations [21] with sub-stoichiometric amounts of [γ-$^{32}$P]GTP at room temperature for 10 min or by the EDTA/$Mg^{2+}$ procedure [19]. Briefly, 10–20 pmoles of GTP-binding protein loaded with [γ-$^{32}$P]GTP and a 5 to 10 fold excess of the same protein loaded with GDP were mixed together and the extent of intrinsic and GAP-assisted GTP hydrolysis in the absence and presence of aluminium fluoride (30 μM $AlCl_3$ and 5 mM NaF) was monitored by quantifying radioactive phosphate release using the charcoal assay [22]. The reactions (total volume 50 μl) were started by addition of GAP (10 pmoles) or buffer to the samples, and after 6 min 40 μl of the reaction mixture were added to 360 μl of chilled charcoal slurry (5% Norit in 50 mM $NaH_2PO_4$, pH 3) and mixed well. This was then spun in a table top microfuge and 100 μl of the supernatant (containing the free $^{32}$P-labelled phosphate which cannot be absorbed by the charcoal) were added to scintillation fluid and counted. In a control reaction, the GAP stimulated GTPase reaction was done in the presence of $AlF_4^-$ (concentrations as above), and no inhibition was observed.

## 3. Results

*3.1. Isolation of the Ras·GDP·$AlF_4^-$·GAP complex*

In contrast to the α-subunits of heterotrimeric G-proteins [23,24], isolated Ras-related proteins in their GDP-bound form do not bind, and are not activated by aluminium fluoride complexes [25]. However, we have shown [18] that the addition of $AlF_4^-$ to Ras loaded with a fluorescent analogue of GDP induces a large fluorescence change indicative of $AlF_4^-$ binding – but only in the presence of the catalytic domains of the Ras-GAPs neurofibromin or p120-GAP. Stoichiometric amounts of the respective GAPs are required for this effect, which suggests that a 1:1:1 complex between Ras·GDP, Ras-GAP and $AlF_4^-$ is formed under these conditions. To examine this further, Ras·GDP was incubated with the catalytic domain of the Ras-GAP neurofibromin (NF1-333) in the presence or absence of $AlF_4^-$, and the mixtures analyzed by gel filtration. In the absence of $AlF_4^-$ the 36 kDa NF1-333 protein represented by the peak at 62 ml in Fig. 1A is well resolved from the 21 kDa Ras·GDP which elutes in the second peak (with a maximum at 74 ml). In the presence of $AlF_4^-$, however, the NF1-333 and Ras·GDP proteins are seen to elute in a single peak centred at 56 ml indicative of the formation of a Ras·GDP·$AlF_4^-$·NF1-333 complex. Analysis of the eluted fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) shows that the peak eluting in the presence of $AlF_4^-$ indeed contains a mixture of Ras and neurofibromin with an estimated 1:1 stoichiometry (Fig. 1B). SDS-PAGE analysis of control experiments done in the absence of $AlF_4^-$ shows that Ras and NF1-333 do not form a complex under these conditions and thus elute separately (Fig. 1C).

The gel filtration experiments thus constitute an independent readout monitoring complex formation of what we believe is a mimic of a transition state of the GAP-catalyzed GTPase reaction on Ras.

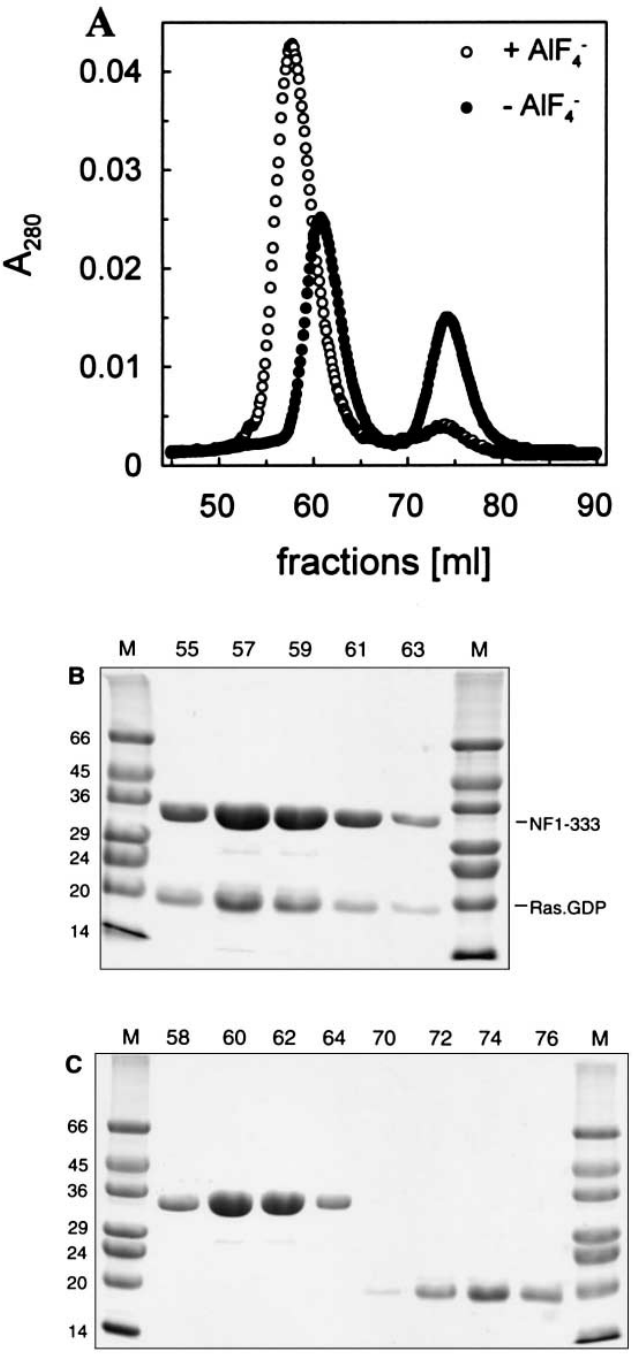


Fig. 1. Isolation of the quaternary complex Ras·GDP·$AlF_4^-$·NF1-333 by gel filtration. (A) Gel filtrations were performed in 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTE after incubating 150 μM Ras·GDP with 150 μM NF1-333 in the presence (○) and in the absence (●) of $AlF_4^-$ (10 mM NaF, 450 μM $AlCl_3$). Fractions from gel filtration with $AlF_4^-$ (B) and without $AlF_4^-$ (C) were analysed on a 15% SDS-polyacrylamide gel and visualized by Coomassie brilliant blue staining. Numbers indicate the fractions and M the molecular weight (in kDa).

*3.2. Ternary complex formation in the case of the Cdc42 GTP-binding protein*

Even though there are limited sequence and overall topological similarities between the low molecular weight GTPases (typified by Ras), there exist little or no sequence similarities between the corresponding GAP proteins. Hence we wanted

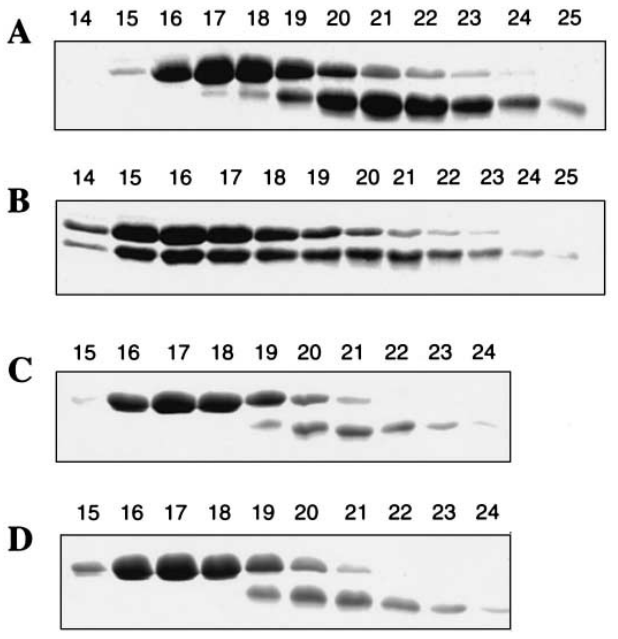


Fig. 2. Formation of the Cdc42·GDP·$AlF_4^-$·Rho-GAP complex with wild type Cdc42 but not with a GTPase negative mutant of Cdc42. The 21-kDa Cdc42·GDP protein and the 29-kDa catalytically active fragment from Rho-GAP (150 μM each) were incubated in the absence and presence of $AlF_4^-$ and the mixtures resolved over a Sephadex G-75 analytical gel filtration column. Peak fractions were analysed by SDS-PAGE and visualised by staining with Coomassie Blue. The numbers denote fraction numbers of the eluate from the gel filtration column. It is seen that whereas the Rho-GAP and Cdc42 proteins elute separately when incubated in the absence of $AlF_4^-$ (C), they are seen to coelute when incubated in the presence of $AlF_4^-$ (D). In the case of the GTPase negative Cdc42(G12V) mutant it is seen that Cdc42(G12V) is resolved from the Rho-GAP in the absence (A) and presence (B) of $AlF_4^-$.

to determine whether the binding of $AlF_4^-$ to GTP-binding proteins other than Ras could be induced by their respective GAPs.

With this intent we next examined the $AlF_4^-$ mediated ternary complex formation between the Rho subfamily GTP-binding protein Cdc42 and its GTPase activating protein Rho-GAP. Gel filtration analysis of the interaction revealed that Cdc42·GDP (21 kDa) and a catalytically active Rho-GAP fragment (29 kDa) [12] in the absence of $AlF_4^-$ are resolved on the gel filtration column (Fig. 2A). In the presence of $AlF_4^-$, however, they coelute (Fig. 2B) which demonstrates formation of the quaternary Cdc42·GDP·$AlF_4^-$·Rho-GAP complex. It has been shown that a mutation in the phosphate-binding loop (P-loop) of Cdc42 analogous to the oncogenic G12V mutation of Ras also reduces the intrinsic GTPase reaction which cannot be stimulated anymore by Rho-GAP [12]. Gel filtration experiments with Cdc42(G12V) showed that Cdc42(G12V)·GDP and Rho-GAP are seen not to coelute even in the presence of $AlF_4^-$ (Fig. 2C and D). Thus Cdc42(G12V) is unable to form a transition state analogue.

### 3.3. *Quaternary complex formation in the case of the Rap and Ran GTPases*

To test additional small GTP-binding protein/GAP combinations represented by the Ran/RNA1 and Rap2/Rap-GAP systems we developed another independent readout for ternary complex formation. The affinity of GAPs for the GDP-bound forms of the guanine nucleotide binding proteins is much lower than that for their GTP-bound forms [26]. Thus, at micromolar concentration, Ras·GDP, for example, should not inhibit the interaction of Ras·GTP with NF1. However, in the presence of $AlF_4^-$, the NF1-333 catalyzed hydrolysis should be inhibited by Ras·GDP owing to the formation of a Ras·GDP·$AlF_4^-$·NF1 complex that would in ef-

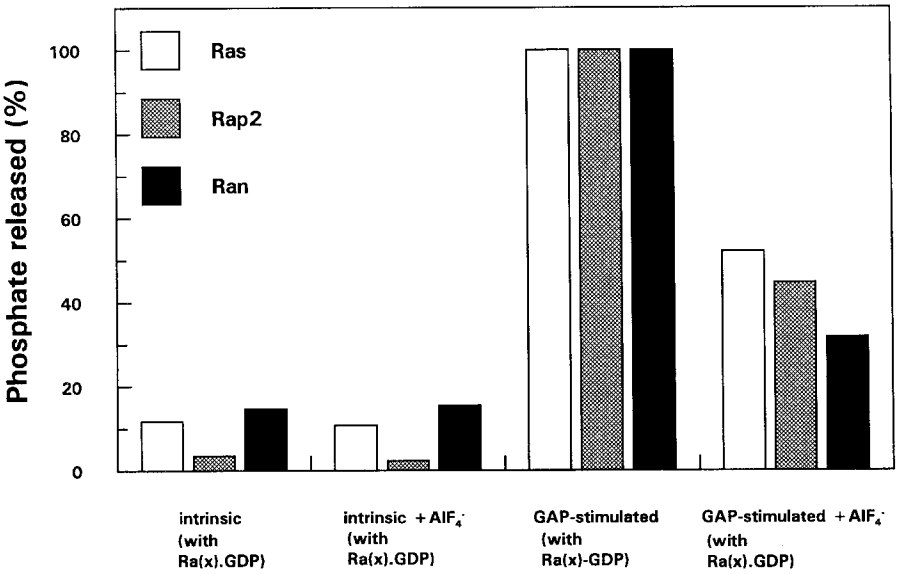


Fig. 3. Formation of the Ra(x)·GDP·$AlF_4^-$·GAP complex leads to inhibition of GAP-stimulated GTP hydrolysis on Ra(x)·GTP(γ-$^{32}$P). 300 nM Ra(x)·GTP(γ-$^{32}$P) was added to either 3.2 μM Ra(x)·GDP in the absence or presence of $AlF_4^-$ (to examine the effect of $AlF_4^-$ on intrinsic GTP hydrolysis), or to 3.2 μM Ra(x)·GDP preincubated with 200 nM of the respective GAP in the absence or presence of $AlF_4^-$ (to examine the effect of formation of the Ra(x)·GDP·$AlF_4^-$·GAP complex on GAP-stimulated GTP hydrolysis). Six minutes after mixing of the components release of $^{32}P_i$ was determined as described in the Section 2. Phosphate release under GAP-stimulated conditions for each Ra(x) protein (where Ra(x) represents Ras, Rap or Ran) was normalised to 100%.

fect titrate out NF1 from the hydrolysis reaction. This inhibition of GTP hydrolysis on Ras·GTP shown in Fig. 3 thus demonstrates Ras·GDP·$AlF_4^-$·GAP complex formation by yet another technique. The intrinsic GTPase reaction is seen not to be significantly affected by the presence of $AlF_4^-$. As controls we verified that $AlF_4^-$ alone did not inhibit the GAP-catalysed hydrolysis reaction, and neither did the oncogenic mutant RasG12V·GDP in the presence of $AlF_4^-$ (data not shown).

Using this GTPase inhibition assay we tested the available GAPs and GTP-binding protein combinations to determine whether their GAP-catalyzed GTPase reactions would also be inhibited by the GDP-bound form in the presence of $AlF_4^-$. Fig. 3 further shows that Rap as well as Ran show inhibition of the respective GAP-catalyzed reactions by GDP-bound Rap and Ran only in the presence of $AlF_4^-$ thus demonstrating formation of the corresponding quaternary complexes.

## 4. Discussion

We have shown here the association of $AlF_4^-$ with the GDP-bound form of various small G-proteins in the presence of their respective GAPs. As these apparently unrelated GAP molecules can induce the formation of quaternary transition state complexes in the presence of $AlF_4^-$ there may exist some unifying principles underlying the interaction of low molecular GTP-binding proteins with their GAPs. Even though the exact atomic details of the GTP hydrolysis reaction may be different in each case there may be reason to expect common topological elements recurring in the different systems.

For a full understanding of the details of the binding of the $AlF_4^-$ ion into the γ-phosphate binding site of the Ras-related proteins, more detailed structural studies on the complexes will be necessary. The isolation of such complexes by gel filtration in the case of the small GTP-binding proteins Ras and CDC42 is an important step towards their X-ray structure analysis. Such structures would, in turn, be a major step toward understanding the mechanism of GAP-mediated GTP hydrolysis on these proteins.

We have, furthermore, shown here that the binding of $AlF_4^-$ to GTP-binding proteins is not unique for Ras. The fact that we demonstrate it for Ran, Rap and Cdc42 and their respective GAPs, which have no sequence homology between each other, suggests that the GAP is generally necessary to stabilize the transition state of the GTPase reaction in Ras-related small GTP-binding proteins. As with any enzymatic mechanism, stabilisation of the transition state is believed to be responsible for increasing the rate of a chemical reaction, in the case of the GAP-stimulated GTPase reaction of Ras and Ran by several orders of magnitude [2,26,27]. It could also be that the mechanism for the increase in rate of GTP hydrolysis is similar for different GTP-binding proteins and their respective GAPs, such as using residues from GAP to neutralize negative charges in the transition state. Arginines in Ras-GAP, which have been implicated to be responsible for transition state stabilisation [18,28], are indeed conserved in all Ras-GAPs and are located around the active site in the structure of the catalytic domain of p120-GAP [11]. The GAPs for the other GTP-binding proteins such as Rho, Ran and Rap also contain totally conserved arginines. They may thus perform a similar function, as has been suggested for Rho-GAP [29], most likely however in a different structural context, since these GAPs are not expected to show the same structure as that found for Ras-GAP [11].

*Acknowledgements:* We thank Patricia Stege and Dorothee Vogt for expert technical help and Rita Schebaum for valuable secretarial assistance, J. de Gunzburg for the Rap2 plasmid, J. Becker for the Ran·GDP and R. Hillig for Ran-GAP from *S. pombe* (RNA1).

## References

[1] H.R. Bourne, D.A. Sanders, F. McCormick, Nature 349 (1991) 117–127.
[2] P. Gideon, J. John, M. Frech, A. Lautwein, R. Clark, J.E. Scheffler, A. Wittinghofer, Mol. Cell. Biol. 12 (1992) 2050–2056.
[3] J.F. Eccleston, K.J.M. Moore, L. Morgan, R.H. Skinner, P.N. Lowe, J. Biol. Chem. 268 (1993) 27012–27019.
[4] E.F. Pai, W. Kabsch, U. Krengel, K.C. Holmes, J. John, A. Wittinghofer, Nature 341 (1989) 209–214.
[5] M.V. Milburn, L. Tong, A.M. DeVos, A. Brünger, Z. Yamaizumi, S. Nishimura, S.-H. Kim, Science 247 (1990) 939–945.
[6] J.C. Amor, D.H. Harrison, R.A. Kahn, D. Ringe, Nature 372 (1994) 704–708.
[7] K. Scheffzek, C. Klebe, K. Fritz-Wolf, W. Kabsch, A. Wittinghofer, Nature 374 (1995) 378–381.
[8] M.S. Boguski, F. McCormick, Nature 366 (1993) 643–654.
[9] M. Trahey, F. McCormick, Science 238 (1987) 542–545.
[10] G.A. Martin, D. Viskochil, G. Bollag, P.C. McCabe, W.J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, R.M. Cawthon, M.A. Innis, F. McCormick, Cell 63 (1990) 843–849.
[11] K. Scheffzek, A. Lautwein, W. Kabsch, M.R. Ahmadian, A. Wittinghofer, Nature 384 (1996) 591–596.
[12] C.A. Lancaster, P.M. Taylor-Harris, A.J. Self, S. Brill, H.E. van Erp, A. Hall, J. Biol. Chem. 269 (1994) 1137–1142.
[13] F.R. Bischoff, H. Krebber, T. Kempf, I. Hermes, H. Ponstingl, Proc. Natl. Acad. Sci. USA 92 (1995) 1749–1753.
[14] J. Becker, F. Melchior, V. Gerke, F.R. Bischoff, H. Ponstingl, A. Wittinghofer, J. Biol. Chem. 270 (1995) 11860–11865.
[15] M. Hattori, N. Tsukamoto, M.S.A. Nur-E-Kamal, B. Rubinfeld, K. Iwai, H. Kubota, H. Maruta, N. Minato, Mol. Cell. Biol. 15 (1995) 552–560.
[16] J. Sondek, D.G. Lambright, J.P. Noel, H.E. Hamm, P.B. Sigler, Nature 372 (1994) 276–279.
[17] D.E. Coleman, A.M. Berghuis, E. Lee, M.E. Linder, A.G. Gilman, S.R. Sprang, Science 265 (1994) 1405–1412.
[18] R. Mittal, M.R. Ahmadian, R.S. Goody, A. Wittinghofer, Science 273 (1996) 115–117.
[19] J. Tucker, G. Sczakiel, J. Feuerstein, J. John, R.S. Goody, A. Wittinghofer, EMBO J. 5 (1986) 1351–1358.
[20] M.R. Ahmadian, L. Wiesmüller, A. Lautwein, F.R. Bischoff, A. Wittinghofer, J. Biol. Chem. 271 (1996) 16409–16415.
[21] J. John, M. Frech, J. Feuerstein, R.S. Goody, A. Wittinghofer, in: L. Bosch, B. Kraal, A. Parmeggiani (Eds.), The Guanine-Nucleotide Binding Proteins Plenum Press, New York, London, 1989, pp. 209–214.
[22] D.M. Berman, T.M. Wilkie, A.G. Gilman, Cell 86 (1996) 445–452.
[23] P.C. Sternweis, A.G. Gilman, Proc. Natl. Acad. Sci. USA 79 (1982) 4888–4891.
[24] J. Bigay, P. Deterre, C. Pfister, M. Chabre, EMBO J. 6 (1987) 2907–2913.
[25] R.A. Kahn, J. Biol. Chem. 266 (1991) 15595–15597.
[26] G.G. Brownbridge, P.N. Lowe, K.J.M. Moore, R.H. Skinners, M.R. Webb, J. Biol. Chem. 268 (1993) 10914–10919.
[27] C. Klebe, H. Prinz, A. Wittinghofer, R.S. Goody, Biochemistry 34 (1995) 12543–12552.
[28] T. Schweins, A. Wittinghofer, Curr. Biol. 4 (1994) 547–550.
[29] T. Barett, B. Xiao, E.J. Dodson, G. Dodson, S.B. Ludbrook, K. Nurmahomed, S.J. Gamblin, A. Musacchio, S.J. Smerdon, J.F. Eccleston, Nature 385 (1997) 458–461.