Minireview

The interaction of Ras with GTPase-activating proteins

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Abstract Ras plays a major role as a molecular switch in many signal transduction pathways which lead to cell growth and differentiation. The GTPase reaction of Ras is of central importance in the function of the switch since it terminates Ras-effector interactions. GTPase-activating proteins (GAPs) accelerate the very slow intrinsic hydrolysis reaction of the GTP-bound Ras by several orders of magnitude and thereby act as presumably negative regulators of Ras action. The GTP hydrolysis of oncogenic mutants of Ras remains unaltered. In this review we discuss recent biochemical and structural findings relating to the mechanism of GAP action, which strengthen the hypothesis that GAP accelerates the actual cleavage step by stabilizing the transition state of the phosphoryl transfer reaction.

Key words: Ras; Ras-GTPase-activating protein; GTPase; Transition state

1. The players

Ras-related GTP-binding proteins 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 nucleotide occupancies are regulated by a variety of proteins including guanine-nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs) [1,2]. GEFs promote the release of tightly bound GDP on the GTP-binding protein and thereby achieve loading with GTP. The GTP-binding proteins return to the inactive state by virtue of the GTPase reaction, which is usually very slow but can be accelerated by the action of GAPs, in the case of the Ras/Ras-GAPs and Ran/Ran-GAP interactions by several orders of magnitude [3–5].

The superfamily of Ras-related GTP-binding proteins consists of several subfamilies such as the Ras, Rho/Rac, Rab, Arf and Ran subfamilies. These proteins have common sequence elements, and show the same overall three-dimensional fold as demonstrated by the structures of Ras [6,7], Rap [8], Arf [9,10], Ran [11], Rac1 [12] and Rab (P. Metcalf, unpublished data). The structure very closely resembles the G-domain in other GTP-binding proteins such as EF-Tu [13,14] and the heterotrimeric G proteins transducin and Giα1 [15,16]. In contrast to the GTP-binding proteins themselves, the GAPs specific for the various members of the Ras superfamily are not sequence related and contain a different set of invariant residues, although their structures look similar in that they are purely α-helical [17–19]. For the discussion of the GTPase reaction and how it might be accelerated by GAP, we will focus on Ras itself in the present review.

Five mammalian GAPs for Ras have been described. The first, p120GAP, is the prototype of this class of proteins and was the first one to be isolated [20–22]. Apart from being a regulator of Ras, its N-terminal domain contains a number of signalling modules such as SH2, SH3, PH, Calb domains and is believed to be a signal transduction molecule that may act independently of Ras [23,24]. The second Ras-specific GAP is neurofibromin (NF1), which is the product of the neurofibromatosis gene [25] and has also been shown to stimulate the GTPase of Ras [26–28]. This gene has been found to be frequently mutated in patients with the disease neurofibromatosis type I [29–31] but also, albeit less frequently, in solid tumors [32]. $GAP1^{m}$, a mammalian homologue of the Drosophila GAP1 gene [33], has been described, and a close homologue GAPIII [34], both of which contain, in addition to the GRD (GAP-related domain), C2 domains and a PH domain. Recently an inositol-4-phosphate (IP4) binding protein $GAP1^{IP4BP}$ has been purified, cloned, and found to contain a Ras-GAP catalytic domain. In contrast to the other GAP mentioned, which are specific for Ras, $GAP1^{IP4BP}$ stimulates the GTPase of both Ras and Rap [35].

2. The chemical mechanism

The mechanism by which GAPs accelerate the GTPase reaction of Ras has been a matter of considerable debate [36]. This is particularly relevant since oncogenic Ras mutants contribute to tumor formation in 25–30% of all cancer patients, and the molecular defect in these proteins is the inability of mutant Ras to hydrolyze GTP. Furthermore and more importantly, the GTPase reaction cannot be stimulated by GAP. Therefore in the case of Ras we have one of the most significant relations between a biochemical defect of a protein and a pathological disorder. This makes the elucidation of the chemical mechanism of the GTPase and of its catalysis by GAP even more intriguing.

Contrary to phosphoryl transfer in solution it is generally believed that in enzymes the reaction proceeds as an associative in-line transfer of the phosphate group with a pentavalent intermediate and an inversion of configuration of stereochemistry. For the intrinsic (non-GAP-catalyzed) GTPase of Ras inversion of configuration has been demonstrated using a derivative of GTP which contains a chiral γ-phosphate [37]. Furthermore it has been found that activation of the nucleophilic water is achieved by the γ-phosphate of GTP itself acting as a general (or specific) base [38,39]. Other interpretations of the available data favoring a dissociative mechanism have also been put forward [40]. Further insight into the mecha-

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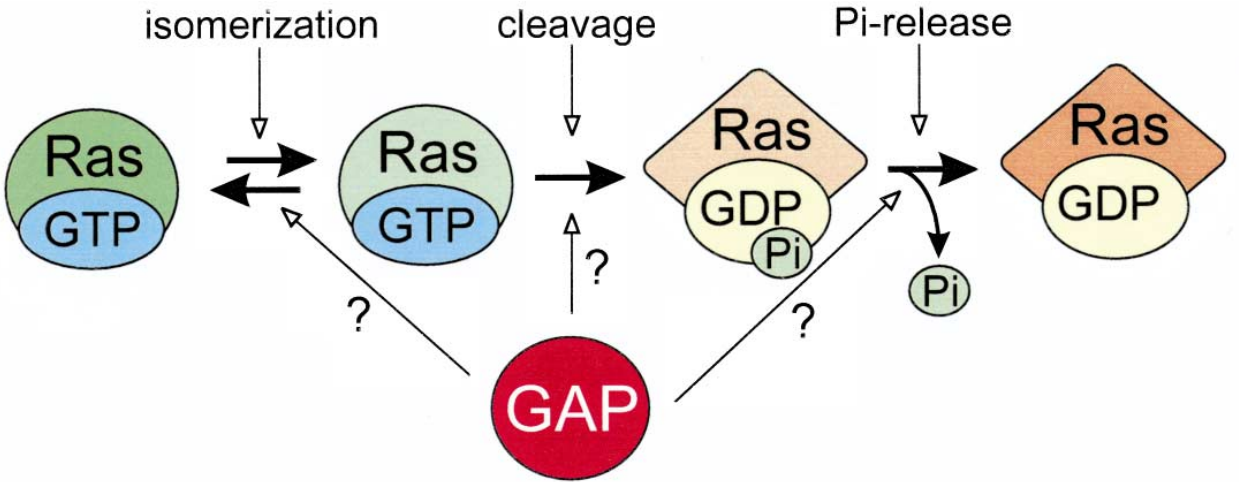

Fig. 1. Minimal scheme for the GTPase reaction of Ras with a hypothetical isomerization reaction preceding GTP hydrolysis. The binding of GTP is very fast and the affinity to GTP very high such that the protein is always saturated with nucleotide (GDP or GTP). GTP hydrolysis and release of inorganic phosphate have so far not been shown to be reversible reactions. Which of the individual reaction steps is rate-limiting and thus catalyzed by GAP is discussed in the text.

nism is expected from the structural analysis of the Ras/Ras-GAP complex, which is ongoing.

3. The biochemistry

So far only p120GAP and neurofibromin have been investigated in detail, and they can be distinguished with respect to their catalytic properties [3,26,41]. Under saturating conditions of p120GAP, the k_{cat} of the GTPase reaction of Ras is 19 s^{-1}, which means that the reaction is stimulated more than 10^5-fold, with a K_D of 9.7 μM for the GAP/Ras-GTP interaction [3]. The GAP activity of GAP-334, a fragment of GAP which contains the catalytic domain, is slightly lower and has a slightly higher K_D [4,42]. The catalytic fragment of neurofibromin, NF1–333, has a similar k_{cat} (5–10 s^{-1}) but a much higher affinity than GAP-334 (K_D of 0.1–0.3 μM) [4,41,42]. No apparent difference in GAP activity was found between the catalytic fragment and full-length neurofibromin [43,44]. Both Ras-GAPs work apparently equally well with K-, N- and H-ras [42]. The difference in affinity between GAP-334 and NF1–333 is due to a small difference in the association and a very large difference in the dissociation rate constants, and it is not entirely clear whether these differences reflect the different biological roles of the different GAPs [42].

Certain phospholipids and their breakdown products such as arachidonic acid were found to modulate GAP activity and the biological relevance of these observations is controversial [45,46]. The differential sensitivity of neurofibromin and p120GAP towards lipids has, however, been used to quantify the GAP activity of the respective proteins in crude cell extracts [43,47]. For both GAPs, the K_D for Ras•GTP is very sensitive towards salts with a doubling of K_D with the addition of 25 mM salts [4]. Interestingly, for NF1–333 the k_{cat} increases with increasing ionic strength, being 27 s^{-1} at 200 mM NaCl under saturating conditions of GAP [4]. The highest GTPase reaction rate can be measured by replacement of Mg^{2+} by Mn^{2+}, which increases both the intrinsic and the GAP-catalyzed reaction, the latter to a value of 155 s^{-1} [48].

4. GAP and the transition state

GAP catalyzes the overall reaction by several orders of magnitude, but which of the individual steps of the minimal scheme show in Fig. 1 is catalyzed has been at the center of the discussion. It has been argued that Ras itself is an efficient GTPase machine and that GAP favors the attainment of a conformation competent for a fast GTPase by catalyzing a rate-limiting isomerization reaction. Evidence for such an isomerization reaction in Ras has been presented by fluorescence spectroscopy whereby a fluorescent analogue of the non-hydrolyzable GTP analogue GppNHp showed a similar fluorescence change within a similar time range as the fluorescent GTP itself [49]. Since this was believed to represent a conformational change preceding GTP hydrolysis and was apparently rate-limiting, it was asked whether this fluorescent change is catalyzed by GAP in the same way as the chemical cleavage step. Conflicting evidence has been found to prove or disprove such a conclusion [42,50,51]. In another model for GAP action, the actual chemical cleavage step itself and not an isomerization preceding hydrolysis is influenced by GAP, the idea being that GAP contributes to creating and stabilizing the transition state of the reaction. In the most pronounced form of the model, the arginine-finger hypothesis, GAP supplies residues into the active site of Ras to favor GTP hydrolysis. This model has recently gained much support from biochemical studies involving aluminum fluoride complexes [52] and from the X-ray structure analysis of the catalytic domain of p120GAP [17]. It has also been shown that the release of P_i is not rate-limiting either in the intrinsic [53] or in the GAP-catalyzed reaction [54].

GTP hydrolysis by the α-subunit of heterotrimeric G proteins is usually a hundred times faster than hydrolysis by Ras [1,2]. Structural studies have shown that the active sites of transducin and Giα1 resemble very much those of Ras, with the conserved sequence elements of GTP-binding proteins involved in the binding of either the base, the ribose and the phosphates and Mg^{2+} ion. Compared to Ras-related proteins, the active site of Gα proteins contains an arginine that is invariant and is close to the phosphates in the triphosphate

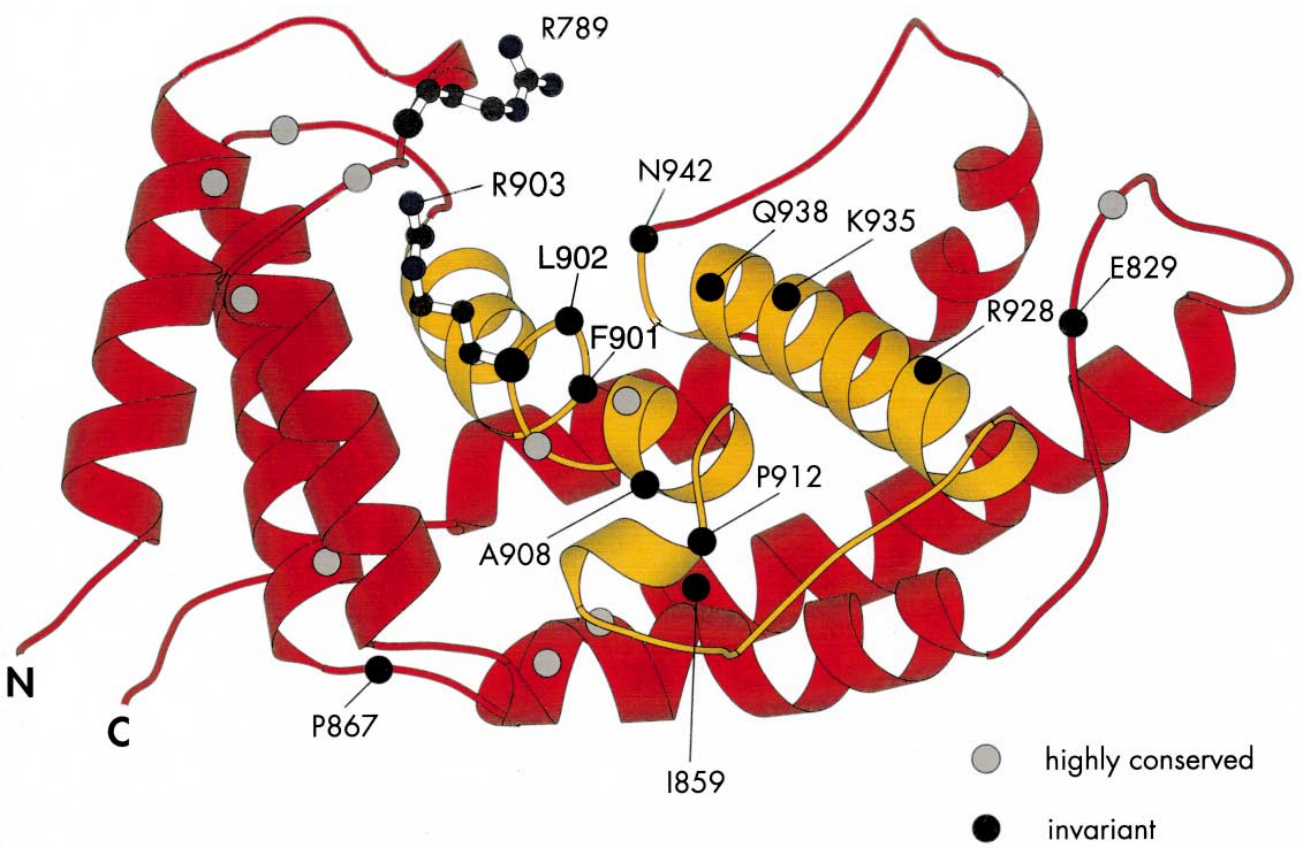

Fig. 2. Ribbon plot (drawn with MOLSCRIPT, see ref. [60]) of the structure of a catalytic fragment of p120GAP. It shows the position of the invariant and highly conserved residues as black and gray dots, respectively. The invariant arginines, one or both of which are believed to participate in the GTPase reaction, are shown as ball and stick models.

structures [15,16]. This arginine has been shown to be important for GTP hydrolysis since it is found mutated in oncogenic versions of Gα proteins with impaired GTPase and is the target of ADP-ribosylation by cholera toxin from *Vibrio cholera*, which reduces the GTPase reaction rate. All Gα proteins in the GDP-bound form, in contrast to Ras and other Ras-related proteins [52,55], bind AlF_4^- into the γ-phosphate binding pocket and thereby stimulate the actions of Gα proteins in their GTP-bound state. The structures of Gα proteins in the presence of GDP and AlF_4^- have shown that AlF_4^- mimics the transition state of the reaction by forming an octahedral arrangement, where the AlF_4^- forms the planar base and the oxygens from GDP (the leaving group) and the attacking water (the incoming group) occupy the apical positions of the octahedron [16,56]. This arrangement is believed to mimic the real pentavalent intermediate (or transition state) of the GTPase reaction. The transition state mimic is additionally stabilized by a glutamine, which in contrast to the arginine is conserved between Ras-related and Gα proteins.

As stated above, Ras and the Ras-related proteins do not bind AlF_4^-. Following suggestions [2,57] that Gα proteins have an in-built GAP, whereas Ras-related proteins have a *trans*-acting GAP, it was found that Ras•GDP also binds AlF_4^-, but only in the presence of stoichiometric amounts of Ras-GAP. In the absence of AlF_4^-, GAP does not bind to Ras-GDP [52]. Oncogenic mutants of Ras such as Ras(G12V), which binds to GAP but is unable to hydrolyze GTP, do not bind AlF_4^- in the presence of GAP. Since, in analogy to Gα proteins,AlF_4^- in the presence of GDP is believed to mimic the transition state, and since stoichiometric amounts are needed for this effect, it can be argued that the role of GAP is unlikely to be that of a catalyst that catalyzes a conformational change on Ras but rather that it participates in the reaction. This is further strengthened by the finding that a GAP with a mutation of the invariant arginine, analogous to R903 in Fig. 2, binds to Ras•GTP and forms the ground state complex, but does not catalyze GTP hydrolysis or form the transition state mimic [52,58].

5. The Ras-GAP structure

Originally, a 483-residue GAP-related domain (GRD) of neurofibromin had been described to contain full Ras-GAP activity [26,27]. Smaller fragments of 333–343 residues were also shown to possess full or almost full Ras-GAP activity [3,4]. The smallest fragment that is able to support full GTPase activating activity contains 230 residues from neurofibromin and can be stably expressed and purified as a recombinant protein [59]. The three-dimensional structure of a catalytic fragment of the Ras-specific p120GAP, GAP-334, has recently been determined [17]. The molecule is purely α-helical (Fig. 2) and consists of two domains one of which corresponds to the minimal fragment from neurofibromin and is called the catalytic domain. It contains residues from the three blocks of sequence homology defined earlier and all the invariant residues. Most of the invariant residues are centered around a shallow groove in the middle on the molecule and are presumably oriented to interact with GAP. To have a first look at the complex between Ras and Ras-GAP, it was attempted to dock the two proteins together using as guideline

the numerous reports on the biochemical features of this encounter. It was thus found that the two invariant arginines, one or both of which are involved in the chemical reaction step, are situated close to the γ-phosphate of Ras-bound GTP. This supports the notion that also from a structural view the participation of residues from GAP in the GTPase reaction is indeed very likely.

6. Conclusions

From both the structural and biochemical analysis of the Ras-GAP interaction it becomes more and more evident that residues from GAP participate in the GTPase reaction of Ras. Current data support the arginine-finger hypothesis whereby we expect arginines to be involved in the stabilization of the transition state. In both the associative and the dissociative mechanism of phosphoryl transfer there is a developing charge in the transition state that would need to be neutralized in order to speed up the reaction. In the case of the associative mechanism it would be on the γ-phosphate and in the dissociative case it would be on the leaving group, which is the β,γ-bridging oxygen. Thus it will be crucial to determine the X-ray structure of the Ras/Ras-GAP complex to see if indeed an arginine residue projects its side chain into the active site to interact with oxygens on GTP. It will also be interesting to see why oncogenic mutants of Ras are not stimulated by GAP. In the case of the Gln61 mutants this is because Gln itself is involved in stabilization of the transition state, in analogy to the Gα proteins [16,56]. For mutants of residue Gly12, for which there is no consistent explanation why any substitution at this position, even the minimal change to alanine, renders the protein resistant to stimulation by GAP. Finally one would hope that the structure of Ras/Ras-GAP complex allows one to design molecules that are able to increase the GTPase reaction of wild type and mutant Ras and thereby use the GTPase reaction as an attractive target for the development of anti-Ras drugs.

References

[1] Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) Nature 348, 125–132.
[2] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) Nature 349, 117–127.
[3] Gideon, P., John, J., Frech, M., Lautwein, A., Clark, R., Scheffler, J.E. and Wittinghofer, A. (1992) Mol Cell Biol 12, 2050–2056.
[4] Eccleston, J.F., Moore, K.J.M., Morgan, L., Skinner, R.H. and Lowe, P.N. (1993) J Biol Chem 268, 27012–27019.
[5] Klebe, C., Prinz, H., Wittinghofer, A. and Goody, R.S. (1995) Biochemistry 34, 1254–12552.
[6] Pai, E.F., Kabsch, W., Krengel, U., Holmes, K.C., John, J. and Wittinghofer, A. (1989) Nature 341, 209–214.
[7] Milburn, M.V., Tong, L., DeVos, A.M., Brünger, A., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1990) Science 247, 939–945.
[8] Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F. and Wittinghofer, A. (1995) Nature 375, 554–560.
[9] Amor, J.C., Harrison, D.H., Kahn, R.A. and Ringe, D. (1994) Nature 372, 704–708.
[10] Greasley, S.E., Jhoti, H., Teahan, C., Solari, R., Fensome, A., Thomas, G.M.H., Cockcroft, S. and Bax, B. (1995) Nature Struct Biol 2, 797–806.
[11] Scheffzek, K., Klebe, C., Fritz-Wolf, K., Kabsch, W. and Wittinghofer, A. (1995) Nature 374, 378–381.
[12] Hirschberg, M., Stockley, R.W., Dodson, G. and Webb, M.R. (1997) Nature Struct Biol 4, 147–152.
[13] LaCour, T.F.M., Nyborg, J., Thirup, S. and Clark, B.F.C. (1985) EMBO J 4, 2385–2388.
[14] Berchtold, H., Reshetnikova, L., Reiser, C.P.A., Schirmer, N.K., Sprinzl, M. and Hilgenfeld, R. (1993) Nature 365, 126–132.
[15] Noel, J.P., Hamm, H.E. and Sigler, P.B. (1993) Nature 366, 654–663.
[16] Coleman, D.E., Berghuis, A.M., Lee, E., Linder, M.E., Gilman, A.G. and Sprang, S.R. (1994) Science 265, 1405–1412.
[17] Scheffzek, K., Lautwein, A., Kabsch, W., Ahmadian, M.R. and Wittinghofer, A. (1996) Nature 384, 591–596.
[18] Musacchio, A., Cantley, L.C. and Harrison, S.C. (1996) Proc Natl Acad Sci USA 93, 14373–14378.
[19] Barrett, T., Xiao, B., Dodson, E.S., Dodson, G., Ludbrook, S.B., Nurmahomed, K., Gamblin, S.J., Musacchio, A., Smerdon, S.J. and Eccleston, S.F. (1997) Nature 385, 458–461.
[20] Trahey, M. and McCormick, F. (1987) Science 238, 542–545.
[21] Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G.A., Ladner, M., Long, C.M., Crosier, W.J., Watt, K., Koths, K. and McCormick, F. (1988) Science 242, 1697–1700.
[22] Vogel, U., Dixon, R.A.F., Schaber, M.D., Diehl, R.E., Marshall, M.S., Scolnick, E.M., Sigal, I. and Gibbs, J.B. (1988) Nature 335, 90–93.
[23] Martin, G.A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A.M. and McCormick, F. (1992) Science 255, 192–194.
[24] McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L.B. and Pawson, T. (1993) EMBO J 12, 3073–3081.
[25] Xu, G., O'Connel, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R. and Weiss, R. (1990) Cell 62, 599–608.
[26] Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connel, P., Cawthon, R.M., Innis, M.A. and McCormick, F. (1991) Cell 63, 843–849.
[27] Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R. and Tamanoi, F. (1991) Cell 63, 835–841.
[28] Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M. and Collins, F. (1990) Cell 63, 851–859.
[29] Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P. and White, R. (1990) Cell 62, 193–201.
[30] Wallace, M.R., Andersen, L.B., Saulino, A.M., Gregory, P.E., Glover, T.W. and Collins, F.S. (1991) Nature 353, 864–866.
[31] Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R. and O'Connell, P. (1990) Cell 62, 187–192.
[32] Li, Y., Bollag, G., Clark, R., Stevens, J., Conroy, L., Fults, D., Ward, K., Friedman, E., Samowitz, W., Robertson, M., Bradley, P., McCormick, F., White, R. and Cawthon, R. (1992) Cell 69, 275–281.
[33] Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Imai, Y., Kohsaka, S., Nakamura, S. and Hattori, S. (1994) Mol Cell Biol 14, 6879–6885.
[34] Baba, H., Fuss, B., Urano, J., Poullet, P., Watson, J.B., Tamanoi, F. and Macklin, W.B. (1995) J Neurosci Res 41, 846–858.
[35] Cullen, P.J., Hsuan, J.J., Truong, O., Letcher, A.J., Jackson, T.R., Dawson, A.P. and Irvine, R.F. (1995) Nature 376, 527–530.
[36] A. Wittinghofer, E.F. Pai, R.S. Goody, in: B.F. Dickey, L. Birnbauer (Eds.), GTPases in Biology. I. Handbook of Experimental Pharmacology, Vol. 108/I, Ch. 14. 1993:195–212.
[37] Feuerstein, J., Goody, R.S. and Webb, M.R. (1989) J Biol Chem 264, 6188–6190.
[38] Schweins, T. and Wittinghofer, A. (1994) Curr Biol 4, 547–550.
[39] Schweins, T., Geyer, M., Scheffzek, K., Warshel, A., Kalbitzer, H.R. and Wittinghofer, A. (1995) Nature Struct Biol 2, 36–44.
[40] Maegley, K.A., Admiraal, S.J. and Herschlag, D. (1996) Proc Natl Acad Sci USA 93, 8160–8166.
[41] Wiesmüller, L. and Wittinghofer, A. (1992) J Biol Chem 267, 10207–10210.
[42] Ahmadian MR, Hoffmann U, Goody RS, Wittinghofer A. Biochemistry 1997 (in press).

[43] Bollag, G., McCormick, F. and Clark, R. (1993) EMBO J 12, 1923–1927.
[44] Bollag, G. and McCormick, F. (1991) Nature 351, 576–579.
[45] Yu, Ch.-Li., Tsai, M.-H. and Stacey, D.W. (1990) Mol Cell Biol 10, 6683–6689.
[46] Serth, J., Lautwein, A., Frech, M., Wittinghofer, A. and Pingoud, A. (1991) EMBO J 10, 1325–1330.
[47] Gawler, D.J., Zhang, L.-J., Reedijk, M., Tung, P.S. and Moran, M.F. (1995) Oncogene 10, 817–825.
[48] Schweins, T., Scheffzek, K., Aßheuer, R. and Wittinghofer, A. (1997) J Mol Biol 266, 847–856.
[49] Neal, S.E., Eccleston, J.F., Hall, A. and Webb, M.R. (1988) J Biol Chem 263, 19718–19722.
[50] Moore, K.J.M., Webb, M.R. and Eccleston, J.F. (1993) Biochemistry 32, 7451–7459.
[51] Rensland, H., Lautwein, A., Wittinghofer, A. and Goody, R.S. (1991) Biochemistry 30, 11181–11185.
[52] Mittal, R., Ahmadian, M.R., Goody, R.S. and Wittinghofer, A. (1996) Science 273, 115–117.
[53] John J. Thesis, Ruprecht-Karls-Universität Heidelberg, 1990.
[54] Nixon, A.E., Brune, M., Lowe, P.N. and Webb, M.R. (1995) Biochemistry 34, 15592–15598.
[55] Kahn, A. (1991) J Biol Chem 266, 15595–15597.
[56] Sondek, J., Lambright, D.G., Noel, J.P., Hamm, H.E. and Sigler, P.B. (1994) Nature 372, 276–279.
[57] Markby, D.W., Onrust, R. and Bourne, H.R. (1993) Science 262, 1895–1901.
[58] Skinner, R.H., Bradley, S., Brown, A.L., Johnson, N.J.E., Rhodes, S., Stammers, D.K. and Lowe, P.N. (1991) J Biol Chem 266, 14163–14166.
[59] Ahmadian, M.R., Wiesmüller, L., Lautwein, A., Bischoff, F.R. and Wittinghofer, A. (1996) J Biol Chem 271, 16409–16415.
[60] P. Kraulis, J Appl Crystallogr 24 (1991) 946–950.