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Guanosine triphosphatase stimulation of oncogenic Ras mutants

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ABSTRACT **Interest in the guanosine triphosphatase (GTPase) reaction of Ras as a molecular drug target stems from the observation that, in a large number of human tumors, Ras is characteristically mutated at codons 12 or 61, more rarely 13. Impaired GTPase activity, even in the presence of GTPase activating proteins, has been found to be the biochemical reason behind the oncogenicity of most Gly12/Gln61 mutations, thus preventing Ras from being switched off. Therefore, these oncogenic Ras mutants remain constitutively activated and contribute to the neoplastic phenotype of tumor cells. Here, we show that the guanosine 5′-triphosphate (GTP) analogue diaminobenzophenone-phosphoroamidate-GTP (DABP-GTP) is hydrolyzed by wild-type Ras but more efficiently by frequently occurring oncogenic Ras mutants, to yield guanosine 5′-diphosphate-bound inactive Ras and DABP-P_i. The reaction is independent of the presence of Gln61 and is most dramatically enhanced with Gly12 mutants. Thus, the defective GTPase reaction of the oncogenic Ras mutants can be rescued by using DABP-GTP instead of GTP, arguing that the GTPase switch of Ras is not irreversibly damaged. An exocyclic aromatic amino group of DABP-GTP is critical for the reaction and bypasses the putative rate-limiting step of the intrinsic Ras GTPase reaction. The crystal structures of Ras-bound DABP-β,γ-imido-GTP show a disordered switch I and identify the Gly12/Gly13 region as the hydrophobic patch to accommodate the DABP-moiety. The biochemical and structural studies help to define the requirements for the design of anti-Ras drugs aimed at the blocked GTPase reaction.**

Tumor progression is an insidious and multistep process that is driven by various genetically defective factors (1). Among them is the proto-oncogene *ras* that participates in the early phase of tumor development (2). The guanine nucleotide-binding protein Ras controls a multitude of cellular signal transduction pathways regulating cell proliferation and differentiation in both normal and transformed cell types. Signal flow is controlled by the function of Ras as a molecular switch cycling between a guanosine 5′-diphosphate (GDP)-bound inactive and a guanosine 5′-triphosphate (GTP)-bound active state, which is regulated by guanine nucleotide exchange factors and guanosine triphosphatase (GTPase)-activating proteins (GAP) (3–5). The intrinsic GTPase reaction, which returns Ras·GTP to its inactive GDP-bound form, is rather slow but is accelerated on interaction with Ras-specific GAPs by orders of magnitude (6). The GTPase reaction is of great medical importance because ≈30% of human tumors contain a Ras protein with point mutations, mainly at codons 12 and 61, and more rarely at 13 (7). Ras-proteins with these somatic mutations have a defective GTPase activity and can no longer be switched off by GAPs. Therefore, they constitutively remain in their active state and consequently contribute to sustained cell proliferation and tumor formation (8–10). Ras oncogene activation is the most frequently occurring gain-of-function mutation detected in human tumors (7). This has invoked the idea that restoring the GTPase activity of oncogenic Ras might constitute a therapeutic concept against cancer. This vision has gained impact by the observation that Ras-specific GAPs participate directly in GTP-hydrolysis via an arginine residue and that violation of the steric/electronic requirements for transition state stabilization accounts for GAP insensitivity of oncogenic Ras mutants (11–13). Despite Ras being in the focus of drug discovery programs, no compound consistently increasing the GTPase activity of oncogenic Ras has been reported so far.

The GTP analogue 3,4-diaminobenzophenone-phosphonoamidate of GTP (DABP-GTP; Fig. 1) has been shown to be a substrate for Gsα and its GTPase-deficient mutant using crude erythrocyte membranes (14). Here, we have investigated the interaction of purified Ras oncoproteins (15) with DABP-GTP by using HPLC and fluorescence spectroscopy. We show that DABP-GTP is hydrolyzed by Ras, that the critical Gln61 is not required for efficient hydrolysis, and that the most common Gly12 mutations found in tumors dramatically enhance the rate of DABP-GTP hydrolysis. In addition, we have used x-ray crystallography to analyze DABP-GTP binding to Ras and its implications for drug development.

MATERIALS AND METHODS

Proteins and Nucleotides. Wild-type and mutant H-Ras proteins were prepared from *Escherichia coli* by using the ptac-expression system as described (15). The nucleotide-free and GTP-bound form of Ras proteins were prepared as described (16). The phosphonoamidate derivatives of GTP and guanosine 5′-β,γ-imidotriphosphate (GppNHp) were synthesized as described (14).

GTPase Assays. GTPase reaction rates of Ras-proteins have been determined by using different methods. (*i*) HPLC experiments have been performed to measure the GTP and DABP-GTP hydrolysis as described (17). Aliquots from a 140 μM Ras·GTP solution or a mixture of 150 μM nucleotide-free Ras and 140 μM DABP-GTP were taken at different time intervals, and the reaction was terminated by freezing in liquid

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Abbreviations: DABP, diaminobenzophenone; PDA phenylenediamine; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GppNHp, guanosine 5′-β,γ-imidotriphosphate; GTPase, guanosine triphosphatase; GAP, GTPase-activating proteins.
Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.rcsb.org [PDB ID codes 1clu (Pro12Ras) and 1rvd (Val12Ras)].
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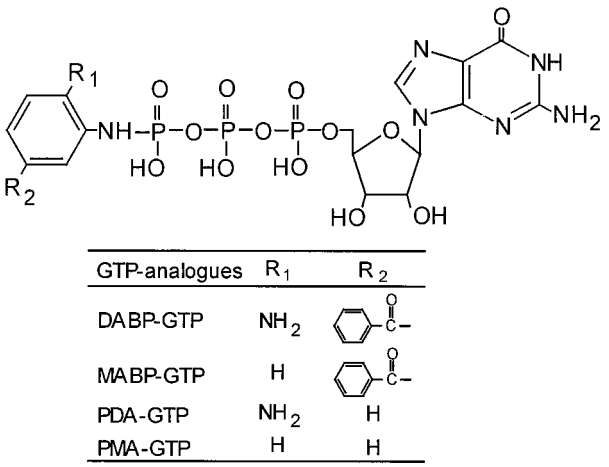
nitrogen. The samples were applied to a RP-18-HPLC column (250 × 4 mm), and isocratic acetonitril elution (7.5% for GTP analysis, 25% for DABP-GTP analysis) was performed at a flow rate of 1.8 ml/min. The HPLC solution contained 10 mM tetrabutylammonium bromide, 100 mM KP_i buffer (pH 6.5), and 2 mM NaN_3. (*ii*) GTP hydrolysis by slower-acting W32Ras-proteins was measured on a LS50B Perkin–Elmer spectrofluorometer using the tryptophane fluorescence of Y32W-mutant (excitation 295 nm, emission 350 nm) with 1 μM of the respective Ras·GTP as described (18). The measurements for faster DABP- or phenylene-diamine (PDA)–GTP hydrolysis were performed in an Applied Photophysics (Surrey, U.K.) SX16MV stopped-flow apparatus by rapidly mixing of 1.1 μM nucleotide-free Ras-proteins and 1 μM of the respective nucleotides. The tryptophane fluorescence of W32Ras was followed by using an excitation wavelength of 295 nm and a cut-off-filter (320 nm) in front of the emission monochromator. All of the reactions were followed at 30°C in 30 mM Tris·HCl (pH 7.5), 3 mM dithioerythritol, 5 mM KP_i buffer, and 5 mM $MgCl_2$ (standard buffer), unless indicated otherwise. Exponential fits to the data were done by using the program GRAFIT (Erithacus Software, Middlesex, U.K.).

Nucleotide Binding. Nucleotide binding experiments were performed as described (19). To determine the binding affinity of the nucleotide analogues, the double-modified non-hydrolyzable GTP analogues DABP-GppNHp and PDA-GppNHp were used.

Crystallization and Structure Determination. H-Ras (1–166) was purified (15) and bound GDP was exchanged for DABP-GppNHp as described by John *et al.* (16) and was crystallized in batch or with the sitting drop method, under conditions similar to those described by Scherer *et al.* (20), but requiring microseeding techniques to obtain diffraction quality crystals. Crystals belong to space group $P3_221$, with unit cell dimensions similar to those of the Ras·GppNHp crystals. Data collection of quartz capillary mounted crystals was performed as described by Pai *et al.* (21). Data processing was done with the program package XDS (22). The refined Ras·GppNHp-model was used as a starting model in alternate rounds of interactive model building (23) and refinement, following standard protocols in XPLOR (24). The presence of the mutations and of the nucleotide was verified in rounds of refinement omitting the respective component from the model. A model of DABP-GppNHp was constructed on the basis of the DABP-GTP coordinates, kindly provided by Sharona Elgavish and Boaz Shaanan. Data collection and structure refinement are summarized in *Results*. Structure visualization (Fig. 4) was done with the program MOLSCRIPT (25).

RESULTS AND DISCUSSIONS

DABP-GTP (Fig. 1) was bound to excess nucleotide-free Ras proteins (16), and the single turnover hydrolysis at 30°C was analyzed by HPLC (17). Although γ-phosphate esters or amides are normally resistant to hydrolysis by GTP-binding proteins, Ras-bound DABP-GTP is turned over with a rate of 0.18 min^{-1}, 10-fold faster than that of GTP (0.019 min^{-1}) (17) (Fig. 2*a*). This is different from Gα proteins, where both GTP and DABP-GTP have similar hydrolysis rates (14). The HPLC profile of the DABP-GTP hydrolysis reaction shows that the products are GDP and DABP-P_i (Fig. 2*b*), which seems to indicate that the reaction proceeds via nucleophilic attack on the γ-phosphate to yield inactive Ras-GDP. For convenience of kinetic analysis, the fluorescence-mutant W32Ras (18) was used to investigate the effect of various additional mutations on GTP hydrolysis in the (quasi-wild-type) W32Ras. The Y32W mutation has only a small effect on the kinetic properties of Ras (18), with W32Ras hydrolyzing DABP-GTP 2-fold faster than GTP (Fig. 2*c*). Control experiments with HPLC indicate that the fluorescence change indeed monitors



GTP-analogues	R_1	R_2
DABP-GTP	NH_2	O=C–
MABP-GTP	H	O=C–
PDA-GTP	NH_2	H
PMA-GTP	H	H

FIG. 1. Chemical structure of the GTP-phosphonoamidate derivatives. MABP, monoaminobenzophenone; PMA, phenylene-monoamine.

the hydrolysis reaction. The DABP-GTPase reaction is not stimulated by GAP (data not shown) as expected from the steric constraints revealed by the structure of the Ras:Ras-specific GAP complex (13).

DABP-GTP (Fig. 1) shows two possible functional groups that might be involved in stimulating catalysis when bound to Ras. It has been suggested for Gα (14) that the bulky aromatic group on DABP-GTP displaces the catalytic glutamine and that the aromatic amino group substitutes for its function. To understand which of the catalytic elements are required for the Ras DABP-GTPase, we have analyzed modified DABP-GTP variants (Fig. 1; ref. 14). The hydrolysis rate of PDA-GTP with W32Ras (Fig. 1; Table 1) is increased as compared with monoaminobenzophenone–GTP and phenylene-monoamine–GTP but is lower than DABP-GTP, suggesting that the benzoyl group does contribute to the rate acceleration. This is different from with Gα, where the benzoyl group has no effect on the cleavage rate (26). Hydrolysis resistance of monoaminobenzophenone–GTP and phenylene-monoamine–GTP underscores the critical role of the aromatic amino group as a catalytically functional group (Fig. 2*c*).

The equilibrium binding constant (K_D) of the β,γ-imido analogue of DABP-GTP to W32Ras, as derived from the ratio of the association and dissociation rate constants (16, 19), is 5.4 nM. This means that the overall affinity of the analogue is 22-fold lower compared with GppNHp (ref. 19; Table 2), still high enough to ensure complete saturation of Ras under all experimental conditions and to assume that the basic binding mode is similar to GTP. The K_D of PDA-GppNHp (Fig. 1) was found to be 1.3 nM (Table 2), intermediate between normal and DABP-modified GppNHp. Because DABP-GTP turned out to be a much better substrate for Ras than PDA-GTP, it was predominantly used in the study of Ras mutants.

The importance of Gln61 in GTP hydrolysis has been demonstrated for the GAP-accelerated reaction, where it is believed to stabilize the transition state by a double hydrogen bond involving both the transferred phosphate and the attacking nucleophile (13). It is assumed that Gln61 plays a similar role for the transition state of the intrinsic reaction, partly because Gln61 mutants have a markedly reduced intrinsic GTPase. HPLC analysis shows that the Q61A mutant hydrolyzes GTP very slowly (0.001 min^{-1}; Fig. 2*a*); its GTPase reaction is not stimulated by GAP, and it is expected to be oncogenic (9). However, hydrolysis with the GTP analogue DABP-GTP is strikingly faster (180-fold), the rate being identical to that of the wild type (0.18 min^{-1}; Fig. 2*a*; Table 3). Fluorescence measurements with the Gln61 mutants (in the

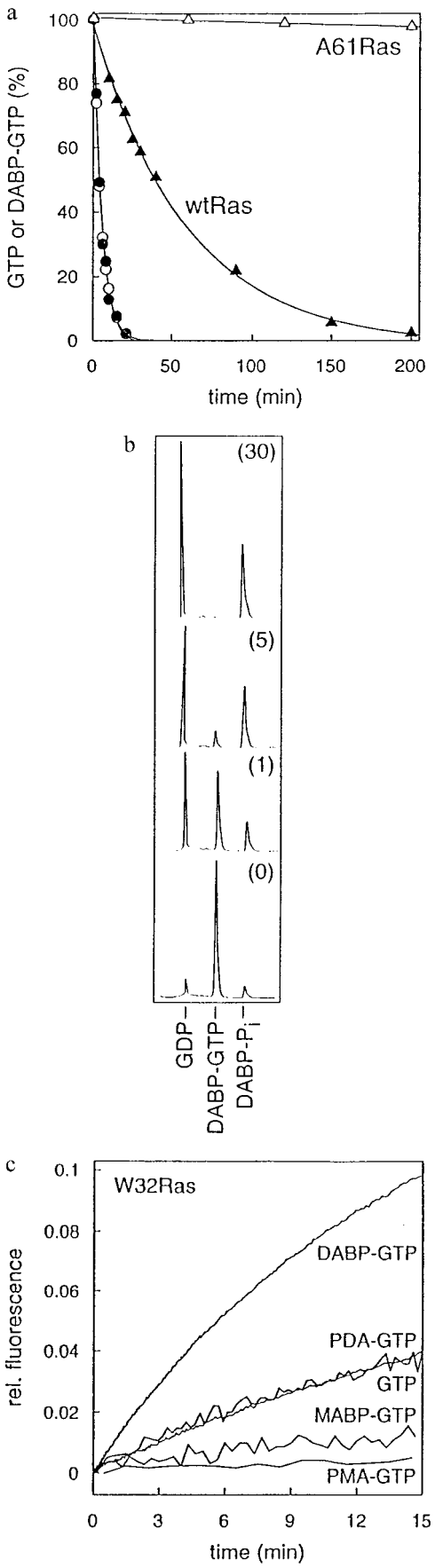


FIG. 2. HPLC and fluorescence measurements of the hydrolysis reaction of Ras-proteins loaded with either GTP or GTP-analogues. (*a*) Single turnover hydrolysis of 150 μM wild-type Ras (closed symbols) and A61Ras (open symbols) with 140 μM of either GTP (triangles) or DABP-GTP (circles) was measured in standard buffer at 30°C. Aliquots of the reaction mixture at the indicated time points were analyzed by HPLC as described in *Materials and Methods*. (*b*) HPLC analysis of DABP-GTPase reaction products by wild-type Ras after 0, 1, 5, and 30 min. Elution times of standards GDP (3.7 min), DABP-GTP (5.7 min), and DABP-P_i (7.3 min) are indicated. (*c*) Real time tryptophane fluorescence kinetics of the hydrolysis of GTP and various GTP-derivatives illustrated in Fig. 1 were followed by rapid-mixing of 1.1 μM nucleotide-free W32Ras with 1 μM of the respective GTP-analogues in standard buffer at 30°C by using stopped-flow apparatus. For calculation of the rate constants, the data were fitted to a single exponential.

Y32W background) Q61L and Q61N show 188- and 220-fold stimulation of the hydrolysis rates of DABP-GTP as compared with GTP (Table 1). The rates of DABP-GTP hydrolysis by Gln61/Y32W mutants are similar to those for W32Ras, indicating that the absence or presence of Gln61 has no significant effect on the DABP-GTPase reaction rate. γ-Phosphate esters of GTP are resistant to hydrolysis by Ras, possibly because of the displacement of the catalytic glutamine by the modification (14, 27, 28). DABP-GTP has a bulky aromatic group linked by an amide bond to the γ-phosphate and yet is hydrolyzed efficiently by wild-type Ras. Furthermore, in contrast to the GTPase reaction, the DABP-GTPase reaction does not require Gln61. The possibility that the rate acceleration observed is caused by the presence of the phosphoamide bond instead of a phosphate ester can be excluded from the observation that derivatives such as monoaminobenzophenone–GTP are resistant to hydrolysis by Ras.

Mutations of Gly12 of Ras are the most common oncogenic mutations in human tumors (7, 9), and codons for Arg, Asp, Val, Ala, Ser, or Cys have been identified (29). Therefore, it was of major importance to test whether the inability of these mutants to hydrolyze GTP could be bypassed by DABP-GTP. The intrinsic GTPase activity of the G12V mutant is 0.0024 min^{-1} (at 37°C) (30, 31), but the rate of DABP-GTP hydrolysis, as measured by HPLC, is 3.1 min^{-1}, a 720-fold increase as compared with the GTPase reaction of the mutant and 110-fold with respect to wild-type Ras. (Fig. 3*a*; Table 3). Similarly, the G12R mutant shows a 566-fold increase. To probe the influence of position 12 on DABP-GTP hydrolysis in more detail, several Gly12 mutations were introduced into W32Ras, and their GTPase and DABP-GTPase reactions were measured. The rate constant of DABP-GTP hydrolysis on V12Ras is 0.95 min^{-1}, consistent with the HPLC method. In general, the hydrolysis reactions of all of the Gly12-mutants tested are dramatically and selectively increased, as the DABP-GTPase rates of the mutants are much higher than both the GTPase and DABP-GTPase of wild-type Ras (or W32Ras). This increase is more pronounced by the more bulky side chains, as we observe a dramatic increase going from the methyl side chain of Ala12 to the isopropyl side chain of Val12 and a further small increase by Leu12/Ile12 (Table 1). Hydrophobicity of the side chain is not an absolute requirement, however, as a large effect also is observed for the G12D mutation, which is the most frequent mutation found in human tumors (29).

Mutants of Gly13 also have been observed in human tumors, albeit less frequently (32), and the HPLC and fluorescence analysis show the Ala13 and Arg13 mutants to hydrolyze DABP-GTP faster than the wild type (Fig. 3*a*), the more bulky side chain being more effective (Tables 1 and 3). Rescue of the catalytic properties of Ras is not only independent of the presence of Gln61 but is actually more efficient in the absence of the Gln61 side chain as shown by the experiments using the double mutants V12A61Ras and V12L61Ras (in W32Ras), which show ≈1,000-fold rate enhancement as compared with the GTP-hydrolysis (Table 1). Small differences between the

Table 1. Rate constants of the intrinsic GTP, DABP-, and PDA-GTP hydrolysis reaction by various oncogenic Ras-proteins in the Y32W background

Substrates/ Ras-proteins	GTP, min^{-1}	DABP-GTP, min^{-1}	DABP-GTP/ GTP	PDA-GTP, min^{-1}	PDA-GTP/ GTP
W32(wt)	0.039	0.07	1.8	0.039	1
P12	0.051	0.32	6.3	0.059	1.2
A12	0.028	0.33	12.0	0.053	1.9
D12	0.0011	0.15	136.4	0.049	44.5
V12	0.0017	0.95	559.0	0.106	20.8
L12	0.0013	0.93	715		
I12	0.0012	0.9	750		
R13	0.031	0.88	28.4		
A61	0.002	0.087	43.5		
L61	0.0006	0.11	188	0.056	15.6
N61	0.00035	0.077	220		
V12L61	0.00097	1.11	1144	—	
V12A61	0.00095	0.878	924.2	0.046	48.4

The rates have been determined by following the fluorescence increase of the single tryptophane residue of Ras(Y32W).

HPLC and the fluorescence data are observed, but the relative magnitude between the GTP and the DABP-GTP hydrolysis reactions is conserved between wild-type and W32Ras (Tables 1 and 3). Unlike wild-type Ras, some oncogenic mutations of Gly12 and Gln61 have a moderately increased PDA-GTPase rate as compared with the GTPase-rate, and the effect is most pronounced in the Val12-mutant (Table 1). In conclusion, it appears that the benzoyl group has some effect on the phosphoryl transfer reaction but that the most important contribution comes from the aromatic amine group. Following an earlier suggestion (14, 26) and from this study, it appeared plausible that the aromatic amino group of DABP-GTP would, at least partially, substitute for the loss of function of the carbonamide side chain of Gln61, which is involved in stabilization of the transition state but possibly also in increasing the nucleophilicity of the water molecule.

To obtain a three-dimensional view of DABP-GTP binding to Ras, we have determined the crystal structure of Val12Ras and of Pro12Ras in complex with DABP-GppNHp (21), at resolutions better than 2 Å (see Table 4). Although the overall structure of Ras is unaffected by the bound nucleotide, we found the effector loop (residues 30–37, switch I) and, less pronounced, the N-terminal part (residue 62–64) of switch II, with poor electron density indicating especially switch I to be disordered in the crystals, for no obvious reason. The hydroxyl group of Thr35, coordinating Mg^{2+} in all crystal structures of Ras bound to GTPs, is replaced by a water molecule in both mutants. The DABP moiety is accommodated close to a hydrophobic patch formed predominantly by Pro12/Val12-Gly13 (Fig. 4). Although the diamino-benzene moiety is close to residue 12, the benzoyl group has a higher than average B-factor, indicating higher flexibility. Comparisons with the structures of triphosphate-bound Val12Ras (33, 34) and Pro12Ras·GppNHp (35) shows Val12 in different conformations (rotamers) whereas the Pro12 conformation seems to be independent of the bound nucleotide. The exocyclic amino group of the DABP moiety is ≈4.5 Å from the γ-phosphate oxygen and thus could be involved in transition state stabilization. It is also in a position where it might activate a water molecule corresponding to Wat175 described for Ras·GppNHp, although a corresponding water molecule could not be found in either of the structures. Possible nucleophilic water molecules within reach of the γ-phosphate were detected in the Pro12Ras but not in the Val12Ras structure. Considering the importance of Thr35 and the switch I region for the intrinsic and GAP-mediated hydrolysis, the structure supports the conclusion that the mechanism of hydrolysis for DABP-GTP is different from that reported for GTP (13, 17). The rearrangement in the effector binding site seen in the crystal also prompted us to measure the binding of Ras·DABP-GTP to the effector RafRBD, which was, however, normal (data not shown).

Table 2. Association rate, dissociation rate, and dissociation equilibrium constant for the binding of various GTP-analogues to W32Ras

GTP-analogues	k_{on} M^{-1} s^{-1}	k_{off} s^{-1}	K_D nM
GppNHp	1.5×10^6	0.36×10^{-3}	0.24
DABP-GppNHp	1.5×10^6	8.1×10^{-3}	5.4
PDA-GppNHp	5.5×10^6	7.2×10^{-3}	1.3

For Ras-mediated GTP hydrolysis, the γ-phosphate of GTP has been proposed to act as the general base abstracting a proton from the nucleophilic water (17). It also was found, however, that this is not the rate-limiting step, as no solvent isotope effect was found for the overall reaction (36). To show whether proton transfer is taking place and is rate-limiting for the DABP-GTPase reaction, we measured the reaction in H_2O and deuterium oxide (D_2O). We found the same rate in either water or D_2O for the normal GTPase reaction of W32Ras, in line with earlier observations (36). However the DABP-GTPase reaction of Ras shows a strong isotope effect, the rate being reduced 2-fold in (D_2O) (Fig. 5). It is even further reduced for the oncogenic V12Ras (3-fold) (data not shown), in line with earlier observations in which solvent isotope effects for proton transfer from water of up to three have been found (37). Because the normal GTPase without any solvent isotope effect serves as a good internal control, major effects of D_2O on the enzyme structure can be excluded. In the analogue, the aromatic amine presumably substitutes for the catalytic glutamine, and this amino group is in a fixed position close enough to interact with a water molecule. Because the DABP-GTPase shows a strong solvent isotope effect and the GTPase reaction does not, we can conclude that the rate-limiting step of the two reactions is different.

The pKa of an aromatic amine such as aniline is 4.6, well in the range suitable to act as a general base. However, we have

Table 3. Rate constants of the intrinsic GTP, DABP-GTP hydrolysis reaction by various oncogenic Ras-proteins determined by HPLC

Substrates/ Ras-proteins	GTP, min^{-1}	DABP-GTP, min^{-1}	DABP-GTP/ GTP
Wild type	0.019	0.18	10
A12	0.0062		
V12	0.0043	3.1	720
R12	0.0053	3.0	566
A13	0.01	0.13	13
A61	0.001	0.18	180

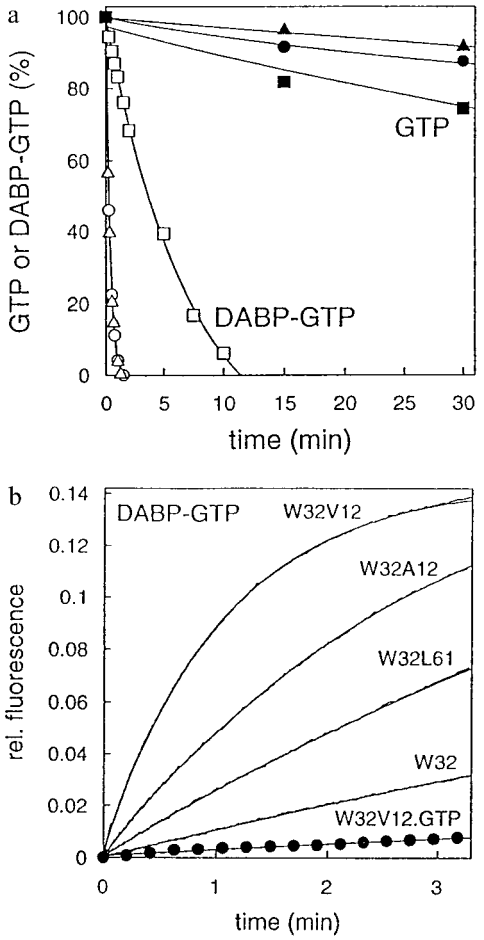


FIG. 3. HPLC and fluorescence measurements of the hydrolysis reaction of GTP and DABP-GTP by oncogenic Ras. (*a*) GTPase (full symbols) and DABP-GTPase (open symbols) of V12Ras (triangles), R12Ras (circles), and A13Ras (squares) were analyzed by HPLC as before. (*b*) Time course of tryptophane fluorescence of the DABP-GTPase reaction of oncogenic Ras mutants (as indicated) in W32-background was measured as before. As an internal control, the GTPase of W32V12Ras is shown. The data were analyzed by single exponentials.

shown that PDA-GTP, which does not have the electron-withdrawing parasubstituent of DABP-GTP, should have a higher pKa and thus should show a higher rate of hydrolysis on Ras. Because the effect found was actually in the opposite direction (Table 1), we suggest that a direct proton transfer from water to the aromatic amine does not take place and that other factors are contributing to the observed rate effects. One possible explanation could be the shielding of the attacking water molecule from the bulk solvent by the aromatic ring, thus increasing its nucleophilicity. Another explanation, as discussed before (26), could be that the aromatic amino group serves as a H-bond donor for the γ-phosphate to stabilize negative charges developing in the transition state similar to the role of Gln61 in the intrinsic and of Gln61 and an arginine from GAP in the GAP-accelerated GTPase reaction. Such an effect should be stronger in DABP-GTP, whose amino group is more acidic, in line with the experimental results.

CONCLUSIONS

It has become an attractive idea to develop anti-Ras drugs by supplying catalytically functional groups into the active site of oncogenic Ras to cure their primary biochemical defects. We have shown that the defective GTPase reaction of oncogenic

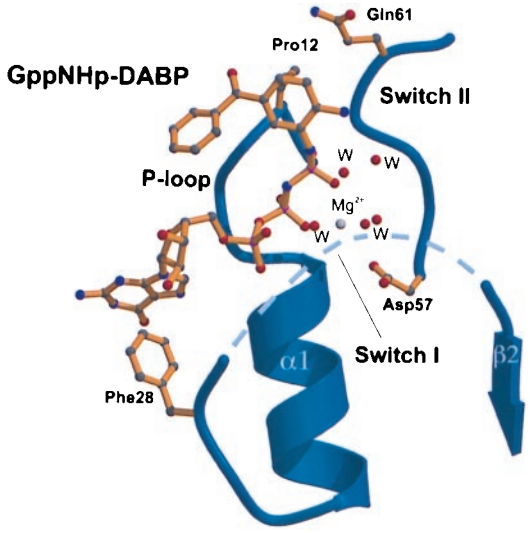


FIG. 4. Three-dimensional view of the active site of the Ras(G12P)·DABP-GppNHp complex showing the P-loop containing the G12P mutation, the visible part of switch I, part of switch II, the Mg^{2+} ion, and several water molecules, as well as DABP-GppNHp.

Ras-mutants can be rescued by using DABP-GTP instead of GTP. This surprising effect may be attributed to an optimal positioning of the catalytic amine of DABP-GTP, replacing the Gln61 and benefiting from the presence of a hydrophobic patch presented by residues in positions 12/13 to anchor the DABP-moiety.

The results show that oncogenic Ras mutants can be inactivated chemically and are not irreversibly damaged in their capability to act as molecular switches. In principle, it should therefore be possible to design or screen for chemical entities, not bound to GTP, that incorporate the relevant chemistry and at the same time bind with sufficient affinity into the active site of Ras. With the structures of the transition state complex of Ras and GAP (13) and of Ras·DABP-GppNHp available, it should be possible to design appropriate scaffolds containing the necessary chemistry indicated here and by the GAP mechanism (6, 12, 13) and to use combinatorial chemistry to find compounds specifically homing in into the active site of Ras. Such a compound would be a true inactivator, not inhibitor, of oncogenic Ras in human tumors. It would not be expected to interfere with the GTPase reaction of wild-type Ras because the latter is down-regulated much more efficiently

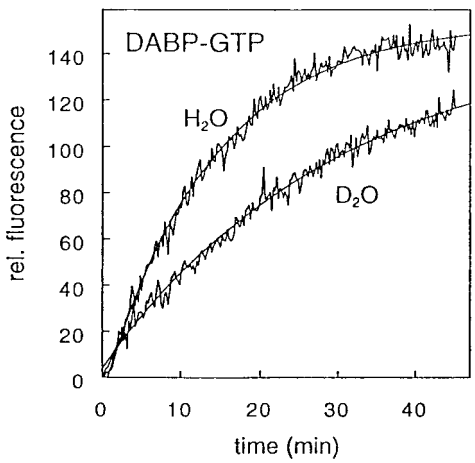


FIG. 5. Solvent isotope effect of the GTPase and DABP-GTPase reaction. W32Ras was incubated with DABP-GTP in either H_2O or D_2O as indicated. The reaction was monitored by the fluorescence increase as before. The data were analyzed by single exponentials.

Table 4. Summary of crystallographic analysis

	Pro12Ras	Val12Ras
Data Collection		
Resolution limit, Å	1.7	1.9
No. of observed reflections	151,581	83,084
No. of uniqe reflections	17,463	12,666
Completeness, %	99	98.7
R_{sym}, %*	8.4	9.0
Refinement		
Resolution range, Å	5–1.7	5–1.9
No. of reflections	16,413	11,593
R_{cryst}, %†	20.3	21.8
R_{free}, %‡	26.1	27.4
rms bond length, Å	0.006	0.008
rms bond angle, °	1.0	1.1

*$R_{sym} = \sum_h \sum_i |I_{hi} - I_h| / \sum_{hi}$, where I_{hi} is the scaled intensity of the ith symmetry-related observation of reflection h, and I_h is the mean value.

†$R_{cryst} = \sum_h |F_{oh} - F_{ch}| / \sum_h F_{oh}$, where F_{oh} and F_{ch} are the observed and calculated structure factor amplitudes for reflection h.

‡R-factor calculated for 10% of the randomly chosen reflections not included in the refinement.

by GAPs. It has been estimated that a 30- 50-fold stimulation of oncogenic mutants by a compound should be sufficient to serve as an anti-Ras drug. This estimation results from analysis of P12Ras that has an intrinsic GTPase activity 2- to 3-fold higher than that of the wild type but that is not stimulated by GAP (refs. 38 and 39 and this paper). The slight increase in GTP-hydrolysis rate is apparently the reason for P12Ras being nononcogenic (8). Considering that most oncogenic mutants of Ras have a 10- to 20-fold reduction of its intrinsic GTPase, it might be sufficient to stimulate their GTPase reaction up the level of the Pro12 mutant. The results presented here, with stimulations up to 1,000-fold, suggest that this should, in principle, be possible.

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