Articles

GTP Hydrolysis Mechanisms in ras p21 and in the ras–GAP Complex Studied by Fluorescence Measurements on Tryptophan Mutants†

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ABSTRACT: We have substituted leucine 56 or tyrosine 64 of p21 ras with a tryptophan. The intrinsic fluorescence of this tryptophan was used as an internal conformational probe for time-resolved biochemical studies of the ras protein. The slow intrinsic GTPase, GDP/GTP exchange induced by the SDC25 "exchange factor", and the fast GTP hydrolysis induced by GAP were studied. Tryptophan fluorescence of mutated ras is very sensitive to magnesium binding, GDP/GTP exchange, and GTP hydrolysis (changes in tyrosine fluorescence of wild-type ras are also observed but with a lower sensitivity). Nucleotide affinities, exchange kinetics, and intrinsic GTPase rates of the mutated ras could be measured by this method and were found to be close to those of wild-type ras. The SDC25 gene product enhances GDP/GTP exchange in both mutants. In both mutants, a slow fluorescence change follows the binding of GTPγS; its kinetics are close to those of the intrinsic GTPase, suggesting that a slow conformational change precedes the GTPase and is the rate-limiting step, as proposed by Neal et al. (1990) (*Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565). GAP interacts with both mutant ras proteins and accelerates the GTPase of (L56W)ras but not that of (Y64W)ras, suggesting a role for tyrosine 64 in GAP-induced GTP hydrolysis. However, GAP does not accelerate the slow conformational change following GTPγS binding in either of the mutated ras proteins. This suggests that the fast GAP-induced catalysis of GTP hydrolysis that is observed with (L56W)ras bypasses the slow conformational change associated with the intrinsic GTPase and therefore might proceed by a different mechanism.

The small guanine-nucleotide-binding proteins of the ras superfamily (Chardin, 1991; Hall, 1990a), like other proteins under the generic term of "G-proteins", switch back and forth between an "active" state, when the bound nucleotide is a GTP, and an inactive one, with bound GDP. Activation results from the exchange of the bound GDP for GTP and inactivation from the hydrolysis of the bound GTP into GDP + P_i. The precise targets of the active state of the ras proteins are not yet fully characterized, but the rate of hydrolysis of the bound GTP must be critical for the regulation of the effects of ras proteins on these targets. Isolated ras, in vitro, hydrolyzes its bound GTP slowly by a weak "intrinsic GTPase activity". A much faster hydrolysis rate of GTP in ras is induced by the interaction with GAP, a 115-kDa soluble "GTPase activating protein" that interacts with ras–GTP (Trahey & McCormick, 1987; Vogel et al., 1988). Oncogenic p21 ras proteins with substitutions in positions 12 or 61 have an impaired GTPase activity (Barbacid, 1987), and GAP has no effect on these oncogenic mutants (McCormick, 1989; Hall, 1990b).

The three-dimensional structure of the ras proteins in their GDP- and GTP-bound states has been determined at high resolution (Milburn et al., 1990; Pai et al., 1989, 1990). Active GTP-bound and inactive GDP-bound states of ras have different structures in two regions termed "switch domains": The switch I domain extends roughly from residue 30 to 37 and corresponds to an extended loop (L2) in the surface region; switch II spans residues 60–76, including another very flexible surface loop (L4) and an α-helical segment (H2) that partially unwinds. Time-resolved X-ray crystallographic studies have demonstrated that the local conformational changes correlate with the hydrolysis of the bound GTP (Schlichting et al., 1990). A physical probe of the structure of the protein in solution could therefore provide a very useful live monitor of GTP hydrolysis in ras and its simulation by GAP.

The fluorescence emissions of tyrosine and tryptophan are sensitive to their environment and may provide probes of the local conformation of proteins. Wild-type ras has no tryptophan but contains nine tyrosines, and their weak fluorescence changes slightly between the GDP-bound state and the GTP-bound state of ras. But given the large number of tyrosines spread over the full length of the polypeptide chain, this change is hard to interpret as one cannot unambiguously identify the sensitive tyrosine. Our approach was to introduce a single tryptophan at a selected position in ras, by site-directed mutagenesis, and to monitor the variations of its fluorescence emission upon nucleotide exchange or nucleotide hydrolysis.

The nucleotide-binding domains of ras proteins present significant sequence analogies with that of heterotrimeric signal-transducing G-proteins such as transducin or Gsα. These G-proteins contain two tryptophans, and the conformational change that occurs on GDP/GTP exchange correlates with a 60% increase of the tryptophan fluorescence emission (Higashijima et al., 1986), which decreases back upon the hydrolysis of GTP by the intrinsic GTPase. The sensitive tryptophan is probably one that is conserved in all the known Gα subunits and is located around the nucleotide site, at position 208 in transducin or 234 in Gsα (Bourne et al., 1988). By analogy, it seemed likely that the introduction in a ras

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protein of a single tryptophan at the equivalent locus might provide a sensitive conformational probe. Sequence alignments suggest that position 234 in Gsα corresponds to position 68 in ras. In the high-resolution structure of ras (Pai et al., 1990; Milburn et al., 1990), arginine 68 is located in helix H2, which is linked to the distal end of loop L4 and moves extensively between the GDP- and GPP-NH-P-bound conformations: this is the switch II domain, in the terminology of Milburn et al. (1990). Replacing an arginine by a tryptophan might, however, have perturbed the structure too much, and we looked for positions where the native structure was more likely to accept this bulky aromatic residue.

There is no tryptophan in p21 ras, but in the ras superfamily the rho and rab proteins all have two conserved tryptophans (Zahraoui et al., 1989); one of them is at position 56, in the internally located β-sheet segment that connects to the proximal end of loop L4. Preliminary fluorescence measurements on rab1 demonstrated that the fluorescence of this tryptophan decreases by 12% upon GDP/GTP exchange. This suggested an introduction of a tryptophan in ras at position 56 by mutating a leucine.

More sensitive loci for a sensor of conformation could be expected in segment 59–65 at the connection between loop L4 and helix H2, where the largest difference is seen between the GDP and GTP analogue complex structures. None of the residues in segment 62–65 interacts directly with the phosphate oxygens of the bound GTP, their mutation is therefore not expected to interfere with the mechanism of GTP hydrolysis. The aromatic residue tyrosine 64 (Y64) appeared as the best candidate to be mutated into a tryptophan.

In the switch I domain, segment 31–36 is displaced between the GDP- and GTP-bound conformations. In this segment, tyrosine 32 moves extensively and is probably responsible for the small nucleotide-dependent changes of tyrosine fluorescence emission that we detected in the wild-type ras protein. Mutating this tyrosine to tryptophan would probably give a good fluorescence probe, but this mutation might interfere with the switch mechanism of the ras protein or with GAP interaction. In the GTP-bound form, the hydroxyl of this tyrosine is not far from the γ-phosphate (Milburn et al., 1990), and the substitution of a phenylalanine for this tyrosine has been shown to interfere with the oncogenic power of the product of v-H*ras* (Stone et al., 1990).

Both ras mutants leucine 56 to tryptophan (L56W) and tyrosine 64 to tryptophan (Y64W) were genetically engineered and produced in *Escherichia coli*. In both mutant proteins, GDP/GTP exchange induced a large change of tryptophan intrinsic fluorescence, and the fluorescence of the GTP-bound state reversed slowly back to the level observed in the GDP-bound state, with a half-time close to that of the intrinsic GTPase of wild-type ras. This suggested that the mutations had not much altered the biochemical properties of the ras protein. Tryptophan fluorescence was used to monitor the kinetics of binding, dissociation, or hydrolysis of the guanine nucleotides under various conditions. We also studied the effect of GAP on the GTPase rate of the mutant proteins and that of an "exchange factor", the carboxy-terminal domain of the yeast SDC25 protein (Crechet et al., 1990).

It has been recently observed by Neal et al. (1990) that in wild-type ras a slow fluorescence change follows the binding of a fluorescently labeled GPP-NH-P. This change must reflect a slow conformational step that precedes the hydrolysis of GTP, since it is seen with a nonhydrolyzable analogue. The time constant of this fluorescence change is comparable to that observed for the hydrolysis of GTP, when fluorescently labeled GTP is used instead of GPP-NH-P. It has been hypothesized that this slow conformational change could be the rate-limiting step of the intrinsic GTPase. Then GAP should accelerate this conformational "pretransition" as it accelerates the GTPase. It was thus of interest to detect and analyze this conformation change, in our ras mutant proteins, through the evolution of the tryptophan fluorescence following the binding of GTPγS.

Materials and Methods

Site-Directed Mutagenesis and Purification of Recombinant Proteins.

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                                        P           M
 52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67
CTG TTG GAC ATC CTG GAT ACC GCC GGC CAG GAG GAG TAC AGC GCC ATG
 L   L   D   I   L   D   T   A   G   Q   E   E   Y   S   A   M
                 ---------- ----------

1 G TTG GAC ATC TGG GAT ACC GC    2 CAG GAG GAG TGG AGC GCC AT
                  W                               W
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The upper line represents the sequence of human c-Hras from amino acid 52 to 67; residues implicated in β- and γ-phosphate binding sites are underlined by dashes. Position 61, which is frequently mutated in human tumors, is indicated by "M" and position 59, which becomes a weak acceptor site for the γ-phosphate when Ala is substituted for a Thr, is indicated by "P".

The *Eco*RI–*Sal*I 0.7-kb fragment from ptac c-Hras (Tucker et al., 1986) was subcloned in M13mp19, and oligonucleotides 1 or 2 were used to introduce the CTG (Leu) to TGG (Trp) or the TAC (Tyr) to TGG (Trp) mutations at positions 56 or 64, respectively, by site-directed mutagenesis. The mutant M13 plaques were identified by hybridization with the ^{32}P-labeled mutagenesis oligonucleotide, the sequence was checked, the double-stranded replicative form of M13 was prepared, and the *Eco*RI–*Sal*I fragment was cloned into pPROK-1 (Clontech Lab., Inc.). Very high levels of soluble Hras protein were expressed in bacteria after IPTG induction. A 5-L fermentor was run at 35 °C, about 100 g of bacterial paste was collected, and the protein was purified from the soluble fraction essentially as described (Tucker et al., 1986; John et al., 1988). The purified proteins (>95% pure and including one bound GDP) were finally dialyzed against 10 mM Tris, 1 mM DTT, and 1 mM $MgCl_2$.

GAP and C-Terminal Truncated GAP (GAPette) Preparations. The GAP protein and its truncated version were a gift of A. Wittinghofer and F. McCormick. The GAP protein has a molecular mass of about 120 kDa, but it has been shown that the C-terminal third of the protein, of about 40 kDa, retains most of the activity on ras GTPase (Marshall et al., 1989). Both forms of GAP were used in this study: full-length GAP expressed in baculovirus-infected insect cells and purified to near homogeneity as described (Halenbeck et al., 1990) and the truncated version from methionine 714 to the end (1047), expressed in *E. coli* and purified (named "GAPette"). Both preparations were a gift of Matthias Frech and Alfred Wittinghofer.

SDC25 Exchange Factor Preparation. The SDC25 partially purified protein was a gift from J.-B. Crechet and A. Parmeggiani. The C-terminal part of the protein encoded by the SDC25 gene, a suppressor of CDC25 deficiency, has a ras exchange factor activity, this exchange activity is not restricted to yeast ras but is also efficient on mammalian ras. This SDC25 protein has been expressed in *E. coli* and partially purified (Crechet et al., 1990).

Fluorescence Measurements. Fluorescence measurements were performed on a Shimadzu RF 5000 fluorimeter, in a 10 × 10 mm cuvette at 37 °C, with stirring. For kinetics re-

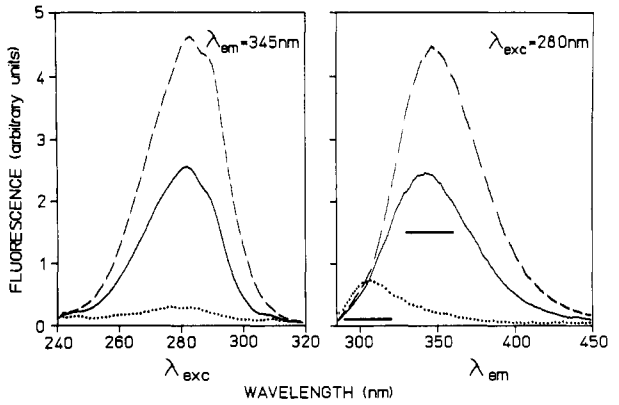

FIGURE 1: Fluorescence excitation and emission spectra of wild-type ras protein and of mutated (L56W)ras and (Y64W)ras proteins. (···) 5 μM ras–GDP, (—) (L56W)ras–GDP, or (– – –) (Y74W)ras–GDP in 10 mM HEPES, 1 mM $MgCl_2$, 1 mM DTT, pH 7.5, at 37 °C, with 5 μM GDP. The excitation wavelength was 280 nm for the emission spectra, and the emission wavelength was 345 nm for the excitation spectra. Optical bandwidths were 1.5 nm for excitation and emission. The horizontal lines in the emission spectra represent the emission bandwidths chosen for kinetic measurements of tyrosine or tryptophan fluorescence.

cording, the excitation wavelength was 280 nm (bandwidth 1.5 nm) and emission was at 345 nm (bandwidth 30 nm), unless otherwise stated.

Buffers and Salts. Standard buffer A included 50 mM HEPES, pH 7.5, 1 mM $MgCl_2$, and 1 mM, DTT. When indicated, the free magnesium concentration was reduced to about 1 μM (calculated 0.8 μM) by addition of 2 mM EDTA from a 0.5 M solution. The initial free magnesium concentration could be restored by injection of 2 mM $MgCl_2$ from a 1 M solution.

Guanine Nucleotides. GDP, GTP, and GTPγS were from Boehringer. As checked by chromatography, the GTPγS preparation typically contained 10% GDP and about 1% GTP; the GTP preparations contained about 5% GDP.

Results

In all our experiments, the ras proteins are initially in the "basal state", that is, with a GDP and a magnesium ion bound. This is achieved by purifying the protein in the presence of an excess stoichiometry of GDP and by maintaining 1 mM $MgCl_2$ in the medium.

Intrinsic Fluorescence Emission Spectra of Wild-Type ras Protein and Mutants (L56W)ras and (Y64W)ras. Wild-type ras fluorescence exhibits the characteristics of pure tyrosine fluorescence, which is maximal for an excitation at 275 nm and has an emission maximum at 305 nm (Figure 1). In (L56W)ras and (Y64W)ras, the fluorescence of the single tryptophan is much more intense than that of the nine (or eight) tyrosines. It might be noticed that the fluorescence emission of the mutant ras between 290 and 305 nm is smaller than that of wild-type ras, which contains the same tyrosines. This illustrates the energy transfer from tyrosine to tryptophan and the quenching of tyrosines emission by the added tryptophan. The fluorescence is maximal for an excitation at 280 nm, where both tryptophan and tyrosine absorb, but the emission maximum at 345 nm is characteristic of tryptophan. At 295-nm excitation, the tyrosines do not absorb anymore, and pure tryptophan fluorescence can be monitored on both ras mutants, with, however, lower emission yields than for the 280-nm excitation. One notices that tryptophan fluorescence is greater by about 80% for (Y64W)ras–GDP than for (L56W)ras–GDP, but this is not very informative as fluorescence yields are extremely dependent on the environment of the chromophore; this is precisely why tryptophan fluorescence changes can provide such sensitive probes of local conformational changes. We will see that if one compares the GTP-bound forms of the two mutants, the difference in fluorescence is now only 15%.

Tyrosine Fluorescence in Wild-Type ras Protein Is Sensitive to Magnesium and to the Bound Nucleotide. Tyrosine fluorescence emission on wild-type ras was monitored at 305 nm, (30-nm bandwidth), with excitation at 270 nm (1.5-nm bandwidth). The fluorescence emission of tyrosine is usally much less sensitive to conformation than that of tryptophan. The presence of nine tyrosines in wild-type ras, only a few of them being in or near the "switch" domains, is also expected to dilute the effect of a localized conformational change. However, the tyrosine fluorescence emission was found to be sensitive to the type of nucleotide bound into the site and also to the presence of magnesium (Figure 2a). Starting from ras–GDP in the presence of excess free GDP and 1 mM $MgCl_2$, an addition of 2 mM EDTA, which reduces the free magnesium concentration below 10^{-6} M, induces an instant 5% increase of the fluorescence, which remains stable thereafter, and fully reverses upon readdition of magnesium. If, instead of GDP, an excess of GTP is present in the medium, the exchange of the initially bound GDP for GTP during the magnesium-free period induces only a very small fluorescence increase after the initial step, but the fluorescence does not decrease back to the initial level when the magnesium is added back after completion of the exchange. Surprisingly, the exchange of GDP for GPTγS induces a much larger change of fluorescence than does GTP. The high fluorescence level with GTPγS bound is insensitive to magnesium, as is the fluorescence of the GTP-bound state.

Tryptophan Fluorescence of (L56W)ras and (Y64W)ras Is Sensitive to Magnesium and to the Bound Nucleotide. In (L56W)ras and (Y64W)ras proteins, the presence of magnesium also influences the tryptophan fluorescence. On excitation at 295 nm, in both mutants the removal of magnesium induces an instant change, by about 10%, of the fluorescence emission at 345 nm: a decrease for (Y64W)ras–GDP (Figure 2b) and an increase for (L56W)ras–GDP (Figure 2c). The fluorescence then remains stable at this new level if there is only GDP in the medium and will come back to the initial level when magnesium is added back. If the excitation is at 280 nm, where both tyrosine and tryptophan absorb, the effect of magnesium removal on fluorescence emission at 345 nm is reversed for (L56W)ras–GDP (Figure 2d). Thus, as discussed for wild-type ras in the preceding paragraph, the removal of magnesium must perturb some tyrosines whose fluorescence is here transferred to the tryptophan (see above, first paragraph of Results).

In the presence of GTP or GTPγS, after the initial fluorescence jump due to the removal of the bound magnesium, the tryptophan fluorescence at low magnesium concentration evolves within about a minute: it decreases by about 20% for (Y64W)ras (Figure 2b) and increases by about 10% for (L56W)ras, which shows the same evolution for the excitation wavelength of 295 nm (Figure 2c) or 280 nm (Figure 2d), suggesting that here the tryptophan alone is sensing a change, not the tyrosines. This evolution reflects the rapid exchange, at low magnesium concentration, of the initially bound GDP for GTP or GTPγS. The time course is not accelerated by higher concentrations of GTP or GTPγS, suggesting that the exchange is rate limited by the off-rate of the GDP. The amplitude of the fluorescence change depends on the relative concentrations of GTP or GTPγS versus that of residual GDP

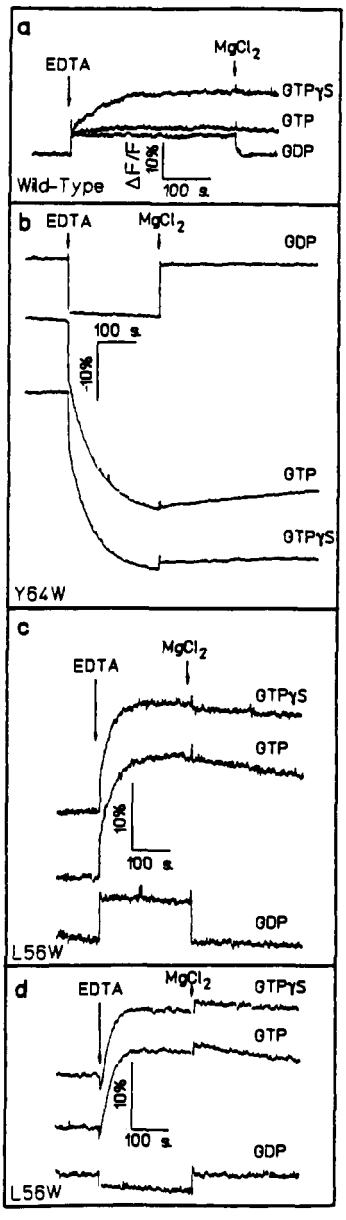

FIGURE 2: Intrinsic fluorescence changes correlated with the removal of magnesium, the fast nucleotide exchange in absence of magnesium, and the slow GTPase in the presence of magnesium, in wild-type ras protein (a), (Y64W)ras (b), or (L56W)ras (c,d). (a) Tyrosine fluorescence (λ_{ex} = 270 nm, λ_{em} = 305 nm) of wild-type ras–GDP. 1 μM protein was incubated in buffer A at 37 °C with 10 μM GDP, GTP, or GTPγS; after a 5-min incubation, EDTA is injected up to 2 mM (from a 0.5 M solution) to chelate the magnesium and allow a fast nucleotide exchange, and 6 min later 2 mM $MgCl_2$ was added (from a 1 M solution) to restore the initial free Mg^{2+} concentration. (b and c) Tryptophan fluorescence (λ_{ex} = 295 nm, λ_{em} = 345 nm) of (Y64W)ras–GDP (b) or (L56W)ras–GDP (c). 0.5 μM protein was incubated in buffer A with 5 μM GDP, GTP, or GTPγS; after 5 min, 2 mM EDTA was injected, and 4 min later 2 mM $MgCl_2$ was added. (d) Same experiment as in panel c, but with excitation at 280 nm.

in the solution, as the exchange leads to an equilibrium that depends on the relative affinities of the nucleotide site for GTP or GTPγS versus GDP. After the exchange is completed, the fluorescence level of ras remains stable, both with a bound GTP, which does not hydrolyze in ras in the absence of magnesium, and with a bound GTPγS. The readdition of magnesium at this stage induces only a very small fluorescence change that is not the reversal of the change that had been initially induced by the removal of magnesium from the corresponding ras–GDP complex. In the presence of magnesium, with excitation at 280 nm, the fluorescence emission levels are almost identical for the GTP- and GPTγS-bound forms and differ from that of the GDP-bound form by a 12% increase for the (L56W)ras and a 32% decrease for (Y64W)ras.

*Large Changes of Tryptophan Fluorescence Monitor the GTPase of (L56W)ras and (Y64W)*ras *Proteins.* With both mutated ras proteins, after completion of the GDP/GTP exchange at low magnesium concentration and readdition of millimolar magnesium, the fluorescence level of ras–GTP slowly reverses toward the level that had been initially observed for ras–GDP in the presence of magnesium: the fluorescence of (L56W)ras–GTP decreases (Figure 2c,d) and that of (Y64W)ras–GTP increases (Figure 2b). In both mutated ras proteins this evolution is complete within 2 h at 37 °C (Figure 3A,B); the time course of the fluorescence change is in the range of the time course of the GTPase in wild-type ras. This evolution must result from the hydrolysis of GTP in ras, which recovers its GDP-bound conformation. At this stage, with the excess nonhydrolyzed GTP still present in the solution, a second addition of EDTA will induce a new fast fluorescence increase for (L56W)ras (Figure 3C) or fluorescence decrease for (Y64W)ras (not shown), characteristic of a new cycle of GDP/GTP exchange.

Nucleotides Affinities, Exchange Kinetics, and Intrinsic GTPase Rate of the Mutated ras Proteins. The large fluorescence changes that correlate with nucleotide exchange at low magnesium concentration or with the GTPase in high magnesium concentration allowed us to follow the kinetics of these processes and to estimate the relative affinities and the kinetic constants of dissociation or hydrolysis of the nucleotides in the two mutants. The data are presented in Table I and compared to the values obtained by other methods for wild-type ras. The characteristics of (L56W)ras are very close to that of wild-type ras (John et al., 1988, 1989). Slightly lower intrinsic GTPase and nucleotide k_{off} rates are found for (Y64W)ras, whose characteristics are, however, closer to that of a normal ras than to that of any of the known oncogenic mutants.

Fluorescence Changes in ras–GTPγS Suggest That a Slow Conformation Change Occurs without Nucleotide Hydrolysis. The exchange of GDP for GTPγS, at low magnesium concentration, induces in both mutant ras proteins the same rapid fluorescence change as for GDP/GTP exchange (Figure 2). Then, after 1 mM free magnesium is added back to the newly formed ras–GTPγS, one observes for both mutants slow and partial reversals of the fluorescence levels toward that of the GDP states (Figure 3A,B). The time courses of these fluorescence changes are about the same as those observed upon the hydrolysis of GTP in ras–GTP. For (L56W)ras, the amplitude of the fluorescence change following GTPγS binding is about 40% of that observed with GTP, and for (Y64W)ras only 15%. Magnesium is required for these fluorescence changes to develop, as it is for the GTPase. Without magnesium, the ras–GTPγS fluorescences are as stable as that of ras–GDP: under the conditions of the experiment shown in Figure 3, they decayed at a constant rate of 2% per hour, due to photobleaching.

We checked that these changes were not due to the small contamination of 1% GTP in the GTPγS: doubling this proportion of GTP in the GTPγS did not increase significantly the amplitude of the fluorescence change. The evolution of fluorescence is not due to the hydrolysis of the bound GTPγS, which is very slow, does not saturate after 3 h, and was detectable over a much longer time scale (data not shown). A

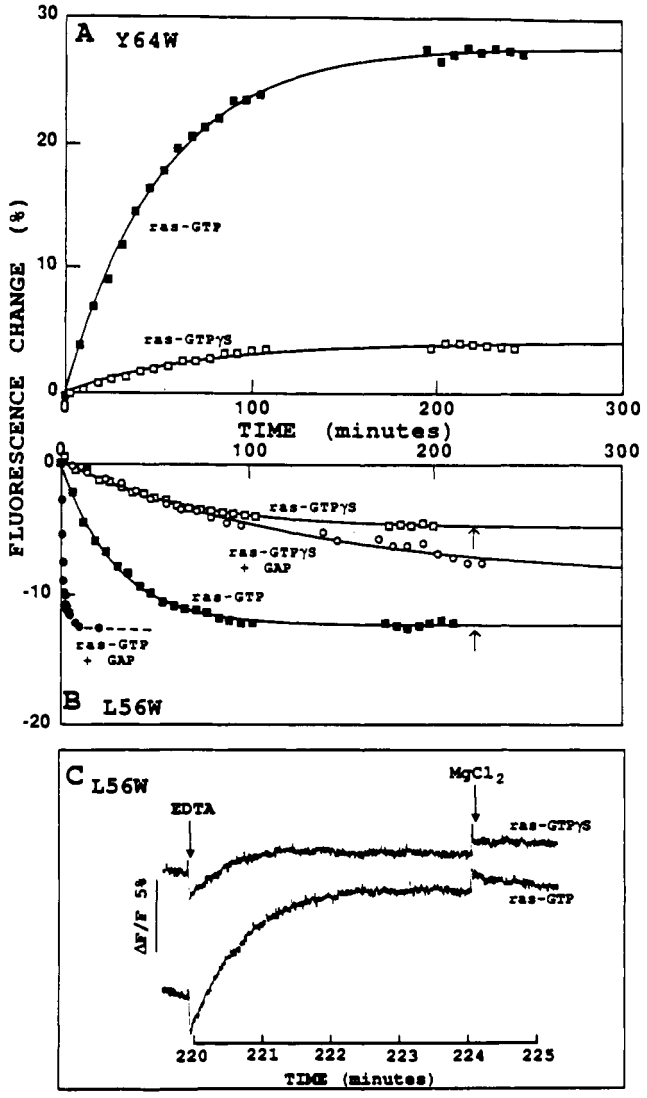

FIGURE 3: Changes of fluorescence that are correlated with GTP hydrolysis in ras–GTP and independent of nucleotide hydrolysis in ras–GTPγS. Three cuvettes containing 2-mL aliquots of a 2 μM solution of ras–GDP in buffer A were supplemented with 20 μM GTP in the first cuvette and 20 μM GTPγS in the two other cuvettes. In all three cuvettes, 2 mM EDTA was added. After incubation at 37 °C for 10 min to allow complete nucleotide exchange, 2 mM $MgCl_2$ was added to the cuvette with GTP and to one of the cuvettes with GTPγS, the third cuvette being kept as a control without magnesium. The three cuvettes were in a rotating holder in the fluorimeter, and the fluorescence measurements were stated at the time of magnesium addition, each cuvette staying in the UV beam for 2 min of every 6 min. The evolutions of the samples with GTP and magnesium (■) and the samples with GTPγS and magnesium (□) were corrected for the decays due to photobleaching that were monitored by the corresponding control samples without magnesium. (A) Evolution of the fluorescence of (Y64W)ras–GTP (■) and of (Y64W)ras–GTPγS (□) over 4 h following the binding of the nucleotides. (B) Evolution of the fluorescence of (L56W)ras–GTP (■, ●) and (L56W)ras–GTPγS (□, ○) in the absence (■, □), or presence (●, ○) of 25 nM GAP. (C) Fast evolution at low magnesium concentration of the fluorescence levels that were reached after 220 min in B (arrows).

careful analysis of the fluorescence spectra also demonstrated that the fluorescence emission spectrum of (L56W)ras–GTPγS evolves differently from that of (L56W)ras–GTP, i.e., the fluorescence spectrum of ras–GTPγS after the evolution is not similar to that of ras–GDP (data not shown).

These fluorescence changes reflect slow conformational changes that, in both mutant ras, follow the binding of a nonhydrolyzable analogue of GTP and thus must occur independently of GTP hydrolysis. They are probably the equivalent in the mutated ras of the conformational change previously detected with fluorescently labeled GPP-NH-P in wild-type ras, which was suggested to set a rate-limiting step for the intrinsic GTPase (Neal et al., 1990).

As magnesium was required to allow the evolution to proceed after the binding of GTPγS, we checked the effect of removing the magnesium after the evolution was complete: would this suppress the effect of the evolution and regenerate the initial state? The addition of EDTA, after the evolution of (L56W)ras–GTPγS was completed, induced an instant fluorescence change, revelative of the rapid release of magnesium. If magnesium was added back after only a few seconds, the fluorescence reverted to the level observed just before the addition of EDTA, not to the initial fluorescence level of ras–GTPγS (not shown). But if EDTA remained present for a sufficient time (Figure 3C, upper trace), the fluorescence level evolved rather rapidly toward the initial level observed for ras-GTPγS, as the bound GTPγS was exchanged for excess free GTPγS present in the solution. The kinetics of this evolution were significantly faster than that observed upon the same conditions for a ras–GTP sample that had evolved to ras–GDP and in which GDP was back-exchanged, at low magnesium concentration, with the excess GTP still present (Figure 3C, lower trace). Indeed, the exchange kinetics in the upper trace of Figure 3C is equal to that observed for a GTPγS/GDP exchange (see Table I). This added support to the interpretation that this fluorescence change is rate-limited by the k_{off} of a GTPγS and thus monitors the release and exchange of the initially bound and still nonhydrolyzed GTPγS for another (or the same) GTPγS. Upon the readdition of magnesium after the fast evolution in EDTA was completed, the fluorescence recovered the level it had in the initial ras–GTPγS conformation. Thus the regeneration of the initial conformation of ras–GTPγS is not induced by the removal of the bound magnesium but requires the transient release of GTPγS from the site.

Stimulation of GDP/GTP Exchange in ras by the Carboxyl-Terminal Domain of the SDC25 Gene Product. The carboxyl-terminal domain of the SDC25 yeast gene product accelerates the exchange of GDP for GTP in the RAS 2 protein of *Saccharomyces cerevisiae* and promotes the same effect on human ras (Crechet et al., 1990). The effect of a partially purified preparation (kindly provided by A. Permeggiani) of this exchange factor was tested on both our mutants by monitoring the fluorescence change correlated with the exchange of GDP for GTPγS in the presence of magnesium. Both mutants are sensitive to the exchange factor, which could lead to half-maximum exchange in about 30 min (Figure 4). Similar results were obtained for GDP/GTP exchange (data not shown), but the data evaluation is complicated by the hydrolysis of the bound GTP, which, in the presence of millimolar magnesium, is not negligible on this time scale.

Acceleration by GAP of the Hydrolysis of GTP in (L56W)ras and Not in (Y64W)ras. GAP is a 115-kDa protein that dramatically increases the rate of hydrolysis of GTP in wild-type ras (Trahey & McCormick, 1987). The GTPase activating capacity is localized in the carboxyl-terminal domain of the protein (Marshall et al., 1989) and is conserved in a 45-kDa fragment preparation (GAPette) that was kindly provided to us by A. Wittinghofer. The hydrolysis rate of GTP in a micromolar solution of (L56W)ras, as monitored by the decay of the tryptophan fluorescence, was considerably increased by nanomolar concentrations of GAP or of GAPette (Figure 5). In a buffer devoid of monovalent ions, at 37 °C, with 1 μM (L56W)ras–GTP and 10 nM GAP, the rate was

Table I: Nucleotide Affinities and Kinetic Constants of (L56W)ras and (Y64W)ras Compared to Wild-Type ras[a]

	$[MgCl_2] \leq 1$ μM					$[MgCl_2] \geq 1$ mM
	K_d GDP/ K_d GTP	K_d GDP/ K_d GTPγS	k_{off} GDP (s^{-1})	k_{off} GTP (s^{-1})	k_{off} GTPγS (s^{-1})	GTP hydrolysis (s^{-1})
(L56W)ras	2	1.1	2.3×10^{-2}	0.6×10^{-2}	3.7×10^{-2}	5.1×10^{-4}
(Y64W)ras	4.2	2.2	1.2×10^{-2}	0.3×10^{-2}	1.2×10^{-2}	3.3×10^{-4}
ras	1.9[b]	0.72[b]	1.8×10^{-2}[c]	0.9×10^{-2}[c]		4.67×10^{-4}[b]

[a] Measurements of intrinsic GTP hydrolysis activity of both mutants was performed as indicated in the legend to Figure 3. The ratio of dissociation constants (K_d) was measured in standard buffer with 500 nM (L56W)ras–GDP or (Y64W)ras–DGP and 5 μM GDP. The fluorescence of this solution was monitored after addition of the appropriate concentration of GTP or GTPγS and 2 mM EDTA. When equilibrium was reached, 2 mM $MgCl_2$ was added. The amplitude of the fluorescence change during the magnesium free phase is plotted as a function of the GTP/GDP or GTPγS/GDP ratio. The nucleotide ratio for which this amplitude is half-maximum gives the affinity ratio. The maximal rate of the GDP/GTP exchange for a high GTP/GDP or GTPγS/GDP ratio gives k_{off} GDP. GTP and GTPγS dissociation rates in absence of magnesium were measured by addition of 50 μM GDP to a 500 nM (L56W)ras or (Y64W)ras solution previously activated by 2.5 μM GTP or GTPγS. [b] From John et al. (1989). [c] Values calculated from data at 21 °C (John et al., 1988), assuming $Q_{10°C} = 2$.

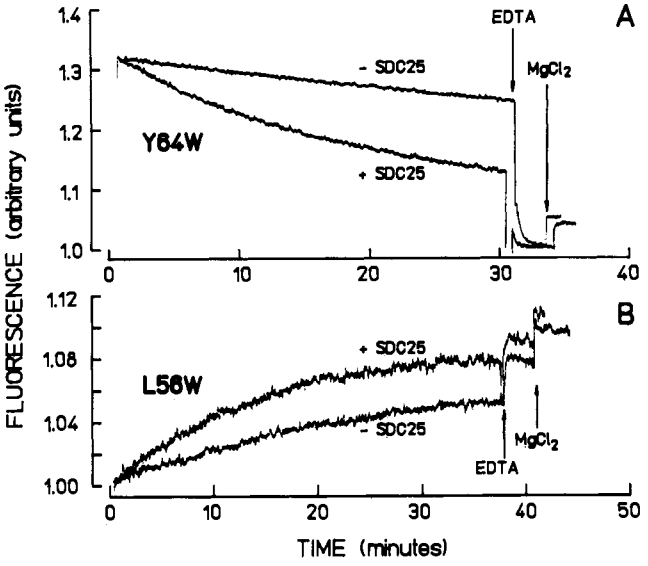

FIGURE 4: Effect of SDC25 C-terminal domain on the GDP/GTPγS exchange rate in (Y64W)ras (A) and (L56W)ras (B). The proteins (5 μM) were incubated at 37 °C in buffer A with 5 μM GDP and 8 μg/mL of the partially purified SDC25 C-terminal domain. The reaction was started by the addition of 50 μM GTPγS. GDP/GTPγS exchange is seen as an increase of tryptophan fluorescence in (L56W)ras and as a decrease in (Y64W)ras. In the presence of SDC25, the initial slopes, which monitor the rate of nucleotide exchange, are increased by factors of 4 and 2.3 for (Y64W)ras and (L56W)ras, respectively. After about 30 min, 2 mM EDTA was added in order to allow rapid saturation of the nucleotide exchange in the absence of magnesium, after which 2 mM $MgCl_2$ was added back. The final fluorescence level is identical in both samples after total exchange. The intrinsic fluorescence of the SDC25 preparation was not negligible (about 20% of the total fluorescence in these conditions), but extremely stable, and was substracted.

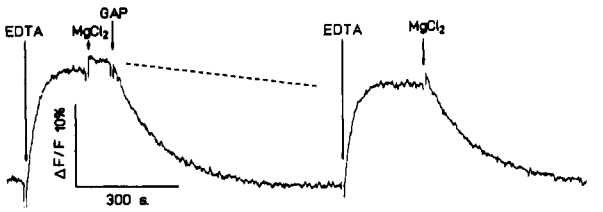

FIGURE 5: Acceleration by GAP of GTP hydrolysis in (L56W)ras. Tryptophan fluorescence recording of a 6 μM solution of (L56W)-ras–GDP protein incubated at 37 °C in buffer A with 60 μM GTP is shown. EDTA addition (up to 2 mM) allows fast GDP/GTP exchange, and later $MgCl_2$ addition (2 mM) activates the slow intrinsic GTPase. The addition of GAP (10 nM) considerably increases the GTPase rate, monitored by the decay of the tryptophan fluorescence (the dotted line shows the evolution in the absence of GAP). GAP is inactive in absence of magnesium, and further sequential additions of EDTA and $MgCl_2$ can trigger novel cycles of GDP/GTP exchange, followed by fast GAP-catalyzed GTP hydrolysis.

of 0.6 nM GTP hydrolyzed per second per 1 nM GAP; that is comparable to the rate observed under similar conditions for the catalysis of GTP hydrolysis in wild-type p21 ras by this GAP preparation (M. Frech, private communication). The catalytic activity of GAP or GAPette is reduced by a factor of about 2.5 when 120 mM KCl (that approximates the cytoplasmic concentration) is added to the buffer. This effect is not specific to K^+ cations or Cl^- anions, similar effects are observed with equivalent concentrations of NaCl or KNO_3.

No effect of GAP (or of GAPette) on the GTPase rate of the mutant (Y64W)ras protein was detectable by fluorescence. However, (Y64W)ras–GTPγS interacts with GAP and is able to compete with wild-type ras–GTP for GAP interaction: using exactly the assay described by Frech et al. (1990), we found (data not shown) that the affinity of (Y64W)ras–GTPγS for GAP is similar or slightly higher (2–3 μM) than the affinity of wild-type ras–GTPγS. The affinity of (L56W)ras–GTPγS for GAP is approximately 5 μM, the same as that of wild-type ras–GTPγS.

Kinetic Constants of the Interaction of GAP with (L56W)ras. The rate of GTP hydrolysis induced by 10 nM GAP in (L56W)ras was studied as a function of the concentration of ras. (L56W)ras–GDP was incubated at the appropriate concentration with a 10-fold excess of GTP in the presence of 2 mM EDTA for a time sufficient to allow total GDP/GTP exchange; 10 nM GAP were added, and GTP hydrolysis was initiated by the addition of 2 mM $MgCl_2$ (1 mM free Mg). The initial GTP hydrolysis rates were estimated from the slope of the fluorescence recording (see Figure 5) and were found to vary strictly linearly with the concentrations of ras, at least up to 10 μM (L56W)ras; higher concentrations were difficult to handle experimentally. This study gives for the apparent second-order rate constant of interaction of GAP with ras-GTP the value $k_{app}^+ = 6 \times 10^5\ M^{-1}\ s^{-1}$. The absence of saturation up to 10 μM ras suggests that the apparent dissociation constant of (L56W)ras–GTP from GAP is much larger than 10 μM and hence differs from that of (L56W)ras–GTPγS, which is on the order of 5 μM. This agrees with what has been observed previously on wild-type p21 ras by the classical method using ^{32}P-labeled GTP (Vogel et al., 1988). By competition experiments with excess cold ras–GTP to inhibit the rate of GAP-induced GTP hydrolysis in labeled ras–GTP, Vogel et al. (1988) obtained a value of 110 μM for the apparent dissociation constant of ras–GTP from GAP. On the other hand Frech et al. (1990) and Krengel et al. (1990), by competition with excess ras–GPP-NH-P obtained an apparent dissociation constant of 5–10 μM. Both experiments had been interpreted as measuring the K_d of the ras–GAP interaction. The apparent discrepancy was attributed to the different origins of the GAP preparations. To reinvestigate the origin of this discrepancy, we have measured, by monitoring the tryptophan fluorescence, the inhibition of the

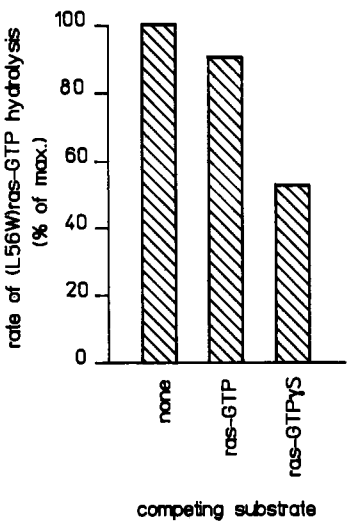

FIGURE 6: Inhibition of the interaction between GAP and (L56W)ras–GTP by competition with excess wild-type ras–GTP or ras–GTPγS. Shown are the initial rates of GTP hydrolysis promoted by 10 nM GAPette in 1 μM (L56W)ras–GTP with no competing substrate or supplemented with 10 μM wild-type ras–GTP or 10 μM wild-type ras–GTPγS, as monitored by tryptophane fluorescence. The ras preparations had been converted to the GTP or GTPγS form by preincubation with the required nucleotide at low magnesium concentration. A 1 mM free magnesium concentration was restored in all the samples by the addition of a suitable amount of 1 M $MgCl_2$ just before mixing them, and GAPette was added 1 min later. The fluorescence decay after the addition of GAPette monitors the GTPase of the (L56W)ras component only, since wild-type ras has no tryptophan.

GTPase induced by 10 nM GAP in 1 μM (L56W)ras–GTP, upon addition of wild-type ras–GTP or wild-type ras–GTPγS. We took advantage of the fact that wild-type ras is not detected in our tryptophan fluorescence assay. We found that the competition by 10 μM wild-type ras–GTPγS inhibits by more than 50% the GAP-induced GTPase in (L56W)ras–GTP but that the same amount of wild-type ras–GTP or ras–GDP inhibits it only by about 10% (Figure 6). Therefore, wild-type ras–GTPγS has a higher apparent affinity for GAP than wild-type ras–GTP.

Indeed, competition experiments do not give access to the catalytic constant k'_{cat} as defined in the reaction scheme

$$\text{GAP} + \text{ras–GTP} \underset{k_1^-}{\overset{k_1^+}{\rightleftharpoons}} \text{GAP–ras–GTP} \qquad K_d$$

$$\text{GAP–ras–GTP} \xrightarrow{k_{cat}} \text{GAP–ras–GDP} \underset{k_2^-}{\overset{k_2^+}{\rightleftharpoons}} \text{GAP} + \text{ras–GDP} \qquad k'_{cat}$$

The competitions by ras–GPP-NH-P or ras–GTPγS, which are not hydrolyzed and therefore not affected by k_{cat}, measure K_d, which we find to be on the order of 10 μM, in agreement with Frech et al. (1990). The competition by ras–GTP, which is affected by k_{cat}, measures K_m. The weak effect of 10 μM ras–GTP implies that $K_m \gg 10$ μM and is compatible with the K_m value of 110 μM that can be deduced from the measurement of Vogel et al. (1988).

At low concentrations, the reaction rate is linear with respect to the concentration of ras–GTP: $v_i = k_{app}^+[GAP][ras–GTP]$, with $k_{app}^+ = k'_{cat}/K_m$. $K_m \gg k_d$ implies $k'_{cat} \gg k_1^-$, hence $K_m = k'_{cat}/k_1^+$ and $v_i = k_1^+[GAP][ras–GTP]$. Therefore, the proportionality constant of initial GTP hydrolysis rate versus ras concentration gives the true second-order kinetic constant of the interaction of ras–GTP with GAP: $k'_{cat}/K_m = k_1^+ = 6.10^5\ M^{-1}\ s^{-1}$. With $K_d = 10$ μM, this gives $k_1^- = 6\ s^{-1}$ and $k'_{cat} \gg 6\ s^{-1}$. For the intrinsic GTPase of ras, $k_{cat} = 4.7\ 10^{-4}\ s^{-1}$ (Table I). Thus GAP increases the rate of GTP hydrolysis in ras by a factor of more than 10^4.

GAP Does Not Accelerate the Slow Conformational Change in (L56W)ras–GTPγS. The competition experiments discussed above suggest that GAP binds to (L56W)ras–GTPγS, with a true affinity comparable to that for the GTP-bound form. The binding of GAP to (L56W)ras–GTP considerably increases the GTPase rate, as detected through the tryptophan fluorescence change. If the fluorescence change observed after the binding of GTPγS in (L56W)ras monitored a conformational change that rate limits the GTPase mechanism, one would expect that GAP should also accelerate this phenomenon. But the addition of 25 nM GAP, a concentration that considerably accelerates the rate of GTP hydrolysis in (L56W)ras–GTP, did not significantly modify the kinetics of the fluorescence change of the GTPγS-bound form (Figure 3B): over the first hour, the evolution in the presence of GAP is the same as that of (L56W)ras–GTP alone. A slower but nonsaturable additional component becomes detectable later. We think (however without a definitive proof) that this very slow component reflects an increased hydrolysis of GTPγS in the ras–GAP complexes. But the surprising result remains that the initial evolution of fluorescence that monitors the slow conformational change of the GTPγS-bound form of (L56W)ras was not modified by a concentration of GAP that accelerated the rate of GTP hydrolysis in the same protein by several orders of magnitude. This observation may have important implications for the GTPase mechanism, as discussed below.

Discussion

In wild-type ras protein that is devoid of tryptophan, tyrosine fluorescence changes are seen upon the binding of magnesium to the GDP complex, but not when GTP or GTPγS is bound. In the triphosphate conformation, tyrosine fluorescence is sensitive to the replacement of an oxygen by a sulfur on the γ-phosphate. We suggest that among the nine tyrosine residues of the wild-type protein, Y_{32} is mainly responsible for these fluorescence changes. Y_{32} is located in loop L_2, which undergoes a large conformational change upon GDP/GTP exchange. In the structure of ras–GDP (Milburn et al., 1990), the hydroxyl of Y_{32} is not far from the magnesium ion, but in ras-GPP-CH_2-P Y_{32} has rotated extensively around the backbone of loop L_2 and its hydroxyl is close to the β- and γ-phosphates, which screen it from the magnesium. This might be correlated with the observations that tyrosine fluorescence is sensitive to magnesium in the GDP state only and, in the triphosphate conformation, it is sensitive to the substitution of a sulfur for an oxygen on the γ-phosphate. The main features of the active conformation must be conserved in ras–GTPγS, but Y_{32} could be affected by the presence of a modified γ-phosphate group. We noticed that the conformation of Y_{32} is different in the structure of ras–GPP-NH-P (Pai et al., 1990) from that in a ras–GPP-CH_2-P crystal (Milburn et al., 1990). In the ras–GPP-NH-P crystals, Y_{32} is in contact with the neighbor protein in the crystal lattice and is much closer (2.8 Å) to an oxygen of the GTP γ-phosphate of this neighbor than to any phosphate oxygen of its own GTP. Thus, this conformation of Y_{32} is probably distorted by the interactions between the close-packed proteins in the crystal and may differ from that in free ras.

The two ras mutants with a tryptophan at position 56 or 64 provide much more sensitive fluorescence probes of the conformational changes related to the exchange or hydrolysis of the bound nucleotide. The mutation of L_{56} that is located

rather internally in the protein structure, and does not interact directly with the bound nucleotide, does not modify the biochemical constants of binding, exchange, and hydrolysis of the nucleotide in ras. It also preserves the functional interactions of ras with the exchange protein derived from the SDC25 gene product and with the GAP protein. The fluorescence changes of the tryptophan introduced at position 56 are due both to direct perturbations of this tryptophan, which are probably small, and to perturbations of surrounding tyrosines, which can transfer their excitation to W_{56}. Y_{32} is again the main candidate since its distance and orientation with respect to L_{56} in wild-type ras change considerably between the GDP and GTP complexes' conformations.

The mutation of Y_{64} is not totally harmless, it modifies the nucleotide binding, exchange and hydrolysis constants a little, although Y_{64} is not interacting directly with the nucleotide (Pai et al., 1990). But Y_{64} does not seem really critical for the binding of the nucleotides in ras and for the mechanism of catalysis of intrinsic GTP hydrolysis. The changes in fluorescence of the tryptophan introduced at position 64 are very large and seem not to be influenced by energy transfer from tyrosines, as they are identical for excitations at 280 and 295 nm. The mutated W_{64} is in loop L4, exposed on the protein surface. The mutation suppresses the action of GAP, suggesting that the switch II domain that includes loop L_4 also takes part in the interaction with GAP and is essential for GAP-induced GTP hydrolysis, as already suggested by Srivastava et al. (1989).

The sensitivity of mutated (L56W)ras to GAP opened the use of the time-resolved fluorescence method to the kinetic analysis of the ras–GAP interaction. The specific monitoring of GTP hydrolysis in the tryptophan-tagged mutant by this technique, in the presence of a large excess of wild-type ras, allowed competition studies, which helped point out the origin of discrepancies between the apparent affinities of GAP for ras–GTP (Vogel et al., 1988) and for ras–GPP-NH-P (Frech et al., 1990; Krengel et al., 1990). The k_{cat} for the hydrolysis of GTP in ras–GTP–GAP complexes is very high, much higher than the k_1^- rate of dissociation in ras–GTP + GAP. The fast GTP hydrolysis and the quick subsequent dissociation of the ras–GDP–GAP complex in ras–GDP + GAP reduce the apparent affinity of GAP for ras–GTP. The true affinity of GAP for ras–GTP is probably on the order of 10 μM, as measured with ras–GTPγS, but is never observable. The high values of k_{cat} and low affinity of GAP for ras–GDP (or high value of k_2^-) ensure the unidirectionality of the switch.

Neither of the mutations in positions 56 and 64 suppressed the nucleotide-exchange enhancement induced by the yeast SDC25 gene product. The interface with this exchange factor must then not include the switch II domain of ras. It will be of interest to test the effect of the mammalian ras exchange factors that have been recently described (Huang et al., 1990; Downward et al., 1990; Wolfman & Macara, 1990).

The slow change of conformation of the ras–nucleoside triphosphate complex that was recently detected in wild-type ras complexed with a fluorescent analogue of GPP-NH-P (Neal et al., 1990) has been observed here directly through an evolution of the fluorescence of the tryptophan in both (L56W)ras and (Y64W)ras, following the binding of GTPγS. The structural change must therefore concern the entire switch II domain, that is, loop L4 and helix H2. The corresponding variations of tyrosine fluorescence could not be assessed in wild-type ras, due to the too weak tyrosine fluorescence, and we cannot determine whether the conformation of the switch 1 domain (loop L2) is also concerned. The presence of the magnesium ion in the GTP site is required for the conformational change to develop, but the magnesium can be removed afterward without disturbing the new structure. Thus the structure modification must not concern residues D_{33} and T_{35} in loop L2 that interact with the magnesium (Pai et al., 1990).

The conformational change of the ras–GTPγS complex having approximately the same time course as that of the hydrolysis of GTP in the ras–GTP complex, it has been suggested (Neal et al., 1990) that this transition would be an obligatory conformational step of the catalytic mechanism of GTP hydrolysis and that its slow time course rate-limits the intrinsic GTPase of ras. But our observation that concentrations of GAP that considerably accelerate the GTPase rate of (L56W)ras–GTP do not modify the "pretransition" observed with the GTPγS complex of the same mutant suggests that the action of GAP is not to accelerate the conformational step in ras that is presumed to rate-limit the intrinsic GTPase. The fast hydrolysis of GTP in the ras–GAP complex must then bypass this slow conformational change. Either this conformational change is not really a step of the intrinsic GTPase mechanism or the mechanism of the fast hydrolysis of GTP in the ras–GAP complex proceeds through a catalytic pathway different from that responsible for the slow hydrolysis of GTP in isolated p21 ras. Thus, the intrinsic GTPase might be a slow futile cycle of not much functional significance, while the catalytic mechanism of the very fast hydrolysis of GTP in the ras–GAP complex might be different.

In conclusion, we describe here two tryptophan mutants that allow time-resolved studies of conformational changes occurring in p21ras. This enabled us to study GDP/GTP exchange and GTP hydrolysis and to compare our results with the results obtained by other methods. One of the mutants, (L56W)ras, behaves as normal ras in every respect. GDP release in vitro is extremely slow (>1 h) and not compatible with a physiological function, suggesting that, in vivo, GDP release is promoted by an "exchange factor". We also studied the effect of GAP on GTP hydrolysis and found that GAP accelerates the hydrolysis rate by a factor of at least 10^4. However, GAP does not accelerate the slow conformational change that seems to rate-limit the GTP hydrolysis in isolated ras–GTP. Furthermore, there is no strict correlation between an impairment of GAP-induced GTP hydrolysis and a low intrinsic GTP hydrolysis rate. For instance, the Y64W substitution has very little effect on intrinsic GTP hydrolysis, does not decrease the affinity of ras–GTP for GAP, but inhibits the acceleration by GAP of the GTP hydrolysis rate in ras–GTP. These results suggest that the mechanisms of GTP hydrolysis in isolated ras or in the ras–GAP complex might be different.

Taken together, these results also strongly suggest that, in vivo and on a physiological time scale, GTP hydrolysis in ras strictly requires the interaction with GAP. By analogy with elongation factors, we hypothesize that GTP hydrolysis monitors the proper interaction of ras–GTP with GAP or the proper formation of a complex between ras–GTP, GAP, and a third component, the identity of which is still unknown. The (L56W)ras mutant will enable time-resolved studies of the effects of NF1, another protein that induces a fast GTP hydrolysis and shares sequence homologies with GAP (Martin et al., 1990). It will also be possible to test the effects of various compounds such as phosphatidic acid or eicosanoids on the catalytic properties of GAP (Han et al., 1991). Furthermore, it will be interesting to study the mechanisms of GDP release promoted by exchange factors (Wolfman &

Macara, 1990) as soon as these are purified to homogeneity. Since wild-type rho and rab proteins already possess a tryptophan in position 56, it is likely that similar tryptophan fluorescence measurements will turn out to be very useful for time-resolved studies of their biochemical properties.

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Registry No. GTPase, 9059-32-9; GTP, 86-01-1; GDP, 146-91-8; Mg, 7439-95-4; Tyr, 60-18-4.

REFERENCES

Barbacid, M. (1987) *Annu. Rev. Biochem. 56*, 779–827.

Bourne, H. R., Master, S. B., Miller, R. T., Sullivan, K. A., & Heideman, V. Y. (1988) *Cold Spring Harbor Symp. Quant. Biol. 53*, 221–227.

Chardin. P. (1991) *Cancer Cells 3*, 117–126.

Crechet, J.-B., Poullet, P., Mistou, M. Y., Parmeggiani, A., Camonis, J., Boy-Marcotte, E., Damak, F., & Jacquet, M. (1990) *Science 248*, 866–868.

Downward, J., Riehl, R., Wu, L., & Weinberg, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5998–6002.

Frech, M., John, J., Pizon, V., Chardin, P., Tavitian, A., Clark, R., McCormick, F., & Wittinghofer, A. (1990) *Science 249*, 169–171.

Halenbeck, R., Crosier, W. J., Clark, R., McCormick, F., & Koths, K. (1990) *J. Biol. Chem. 265*, 21922–21928.

Hall, A. (1990a) *Science 249*, 635–640.

Hall, A. (1990b) *Cell 61*, 921–923.

Han, J. W., McCormick, F., & Macara, I. G. (1991) *Science 252*, 576–579.

Higashijima, T., Ferguson, K. M., Sternweis, P. C., Ross, T. M., & Gilman, A. G. (1987) *J. Biol. Chem. 262*, 752–756.

Huang, Y. K., Kung, H. F., & Kamata, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8008–8012.

John, J., Frech, M., & Wittinghofer, A. (1988) *J. Biol. Chem. 263*, 11792–11799.

John, J., Schlichting, I., Schlitz, E., Rösch, P., & Wittinghofer, A. (1989) *J. Biol. Chem. 264*, 13086–13092.

Krengel, U., Schlichting, I., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E., & Wittinghofer, A. (1990) *Cell 62*, 539–548.

Marshall, M., Hill, W., Ng, A., Vogel, U., Schaber, M., Scolnick, E., Dixon, R., Sigal, I., & Gibbs, J. (1989) *EMBO J. 8*, 1105–1110.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'-Connell, P., Cawthon, R. M., Innis, M. A., & McCormick, F. (1990) *Cell 63*, 843–849.

Mc Cormick, F. (1989) *Cell 56*, 5–8.

Milburn, M. V., Tong, L., deVos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., & Kim, S. H. (1990) *Science 247*, 939–945.

Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565.

Pai, E. F., Kabsch, W., Krengel, U., Holmes, K., John, J., & Wittinghofer, A. (1989) *Nature 341*, 209–214.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351–2359.

Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990) *Nature 345*, 309–315.

Srivastava, S., Di-Donato, A., & Lacal, J. C. (1989) *Mol. Cell. Biol. 9*, 1779–1783.

Stone, J., Vass, W., Wilumsen, B., & Lowy, D. (1988) *Mol. Cell. Biol. 8*, 3565–3569.

Trahey, M., & McCormick, F. (1987) *Science 238*, 542–545.

Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986) *EMBO J. 5*, 1351–1358.

Vogel, U., Dixon, R., Schaber, M., Diehl, R., Marshall, M., Scolnick, M., Sigal, I., & Gibbs, J. (1988) *Nature 335*, 90–93.

Wolfman, A., & Macara, I. G. (1990) *Science 248*, 67–69.

Zahraoui, A., Touchot, N., Chardin, P., & Tavitian, A. (1989) *J. Biol. Chem. 264*, 12394–12401.