Investigation of the GTP-Binding/GTPase Cycle of Cdc42Hs Using Fluorescence Spectroscopy†

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ABSTRACT: We have developed several high-resolution assays for the nucleotide state of a rho-subfamily low molecular weight GTP-binding protein, Cdc42Hs. The first involves the use of the fluorescent *N*-methylanthraniloyl derivative of GDP (mant-GDP). As has been shown for the ras protein, mant-dGDP fluorescence is significantly enhanced (~20%) upon binding to Cdc42Hs. It was further found that the binding of mant-nucleotides results in an efficient energy transfer between the single tryptophan residue of Cdc42Hs and the mant moiety. The exchange of mant-dGDP for GDP bound to Cdc42Hs, as read-out either by the enhancement of the mant fluorescence or by energy transfer, is inhibited by physiological (mM) Mg^{2+} concentrations and correlates exactly to the rate of [^{3}H]GDP exchange observed in filter-binding assays. Moreover, changes in the fluorescence of mant-dGDP are also sensitive to nucleotide dissociation induced by the dbl-oncogene product, a known nucleotide exchange factor for Cdc42Hs. A second fluorescence read-out for the nucleotide-bound state of Cdc42Hs involves the measurements of intrinsic fluorescence of a single tryptophan residue (W97) which is highly sensitive to whether GDP or GTP is bound in the nucleotide pocket. The hydrolysis of GTP to GDP by Cdc42Hs results in an ~30% enhancement of the protein fluorescence. The rate of this fluorescence change corresponds well to the rate of conversion of [γ-^{32}P]GTP to GDP plus [^{32}P]P_i as measured by filter-binding assays. This, taken together with the observation that a time-dependent enhancement of tryptophan fluorescence does not occur in the presence of a nonhydrolyzable GTP analog (GMP–PCP), suggests that the tryptophan enhancement that occurs subsequent to the binding of GTP is a direct outcome of GTP hydrolysis. Further support for this conclusion comes from the finding that the fluorescence enhancement is accelerated by the recombinant form of the recently cloned catalytic domain of Cdc42Hs–GAP (GTPase activating protein). Thus, changes in the tryptophan fluorescence of Cdc42Hs provide a real-time monitor for both the intrinsic- and GAP-stimulated GTPase activities.

GTP-binding proteins (G proteins, GTPases) have been shown to be key participants of many cellular signaling pathways, where they transduce incoming signals from cell-surface receptors to downstream effectors and ultimately mediate the regulation of a diversity of biological activities (Bourne et al., 1990, 1991; Kaziro et al., 1991). The key feature of all G proteins is their capacity to function as sensitive molecular switches. This property is the direct outcome of their ability to undergo a GTP-binding/GTPase cycle. Typically, the G protein is active in the GTP-bound form, mediating the regulation of a downstream effector in the signaling cascade, and is inactive in the GDP-bound form. Activation occurs by the exchange of bound GDP for GTP, and inactivation is the result of a GTPase activity which converts the bound GTP back to GDP.

Since the rates of the GDP–GTP exchange and GTPase reactions determine the overall fraction of activated G protein, they represent the key points of regulation in receptor/G protein-coupled signaling pathways. In the case of a heterotrimeric G protein, a cell surface receptor typically serves as an exchange factor that physically associates with the G protein and exchanges the bound GDP for GTP. In most cases, it is believed that the GTP hydrolytic event is an intrinsic property of the α subunit of the heterotrimeric G protein, although in some cases, the effector enzyme [i.e., PLCβ1 (Berstein et al., 1992) or the cyclic GMP phosphodiesterase (Arshavsky & Bownds, 1992)] was suggested to catalyze GTPase activity. The situation is somewhat more complicated in the superfamily of low M_w GTP-binding proteins since distinct proteins are responsible for stimulating GDP dissociation [guanine nucleotide exchange factors (GEFs)], inhibiting GDP dissociation (GDIs), and catalyzing GTPase activity [GTPase-activating proteins (GAPs)]. Activities which enhance or inhibit nucleotide exchange (GRFs and GDIs) as well as catalysts of the internal GTPase (GAPs) were shown to reside on separate proteins, which are transiently recruited to the active signaling machinery.

A good deal of effort has been devoted toward studying both structural and kinetic aspects of the interconversion of G proteins between their GDP-bound and GTP-bound states. X-ray diffraction analyses have provided three-dimensional structures for the α subunit of a heterotrimeric G protein, transducin, and for the ras protein, as well as for the more distantly related elongation factor, Tu (Pai et al., 1990; Kjeldgaard & Nyborg, 1992; Noel et al., 1993). Spectroscopic methods also have been developed for the direct monitoring of the GTP-binding/GTPase cycles of different G proteins. Changes in the intrinsic tryptophan fluorescence have provided kinetic information regarding the rhodopsin-stimulated GDP–GTP exchange and GTP hydrolytic reactions of transducin (Phillips & Cerione, 1988; Guy et al., 1990) as well as

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mechanistic information about the intrinsic GTP-binding and GTPase activities of related heterotrimeric G proteins, G_i and G_o (Higashijima et al., 1987a,b). The use of site-directed mutagenesis to prepare ras molecules that contain tryptophan residues has enabled investigators to directly monitor the GTP-binding and GTPase activities of ras (Antonny et al., 1991). In addition, studies using fluorescent guanine nucleotides (i.e., mant-nucleotides) have provided a significant amount of information regarding the mechanisms underlying GAP-stimulated GTPase activity in ras (Rensland et al., 1991; Moore et al., 1993).

In the present work, we have set out to develop spectroscopic read-outs to analyze the GTP-binding/GTPase cycle of Cdc42Hs, a member of the rho subgroup of the ras superfamily. Cdc42Hs is the human homolog of a *Saccharomyces cerevisiae* cell-division-cycle protein that is essential for bud-site assembly and suspected to play a role in cytoskeletal organization. A number of regulators of the GTP-binding and GTPase activities of Cdc42Hs have been identified, including a GEF [the dbl-oncogene product (Hart et al., 1991)], a GDI (Leonard et al., 1992), and a specific GAP [designated the Cdc42Hs–GAP (Barfod et al., 1993)]. The development of real-time fluorescence assays for different steps of the GTP-binding/GTPase cycle would facilitate obtaining mechanistic information concerning the mode of action of the different Cdc42Hs regulatory proteins. It is clear that the proteins that regulate the GTP-binding/GTPase cycle of ras [i.e., the GEF, son-of-sevenless (Egan et al., 1993), and the ras-GAP (Trahey & McCormick, 1987)] are markedly distinct from the regulators for Cdc42Hs. Thus, it may be that differences in the regulation of the GTP-binding/GTPase cycles of these proteins underlie differences in their biological functions. There also are likely to be differences in the intrinsic ability of Cdc42Hs (versus ras) to undergo GDP–GTP exchange since the former has the sequence TQID in place of the consensus sequence NKXD found in the guanine ring binding domain of ras and most other GTP-binding proteins. In addition, the rate of the intrinsic GTPase activity of Cdc42Hs is significantly faster (at least 30-fold) than that of ras. Thus, the development of spectroscopic read-outs for the individual steps of the GTP-binding/GTPase cycle of Cdc42Hs should ultimately allow for interesting comparisons with ras regarding the control points for the activation and deactivation of these G proteins.

MATERIALS AND METHODS

Proteins (1) Cdc42Hs. Escherichia coli strain RRIΔM15 (gift of A. Wittinghofer) was transformed with pGEX2T vector containing the Cdc42Hs gene originally cloned from a human placental library (Shinjo et al., 1990). A single colony of this transformant was grown in an overnight culture of 0.5 L of enriched LB media (42 mg/mL Bacto-Tryptone, 25 mg/mL yeast extract, and 12.5 mg/mL NaCl, supplemented with 0.1 M phosphate, pH 7.1, and 0.1 mg/mL ampicillin). This culture was used to seed 3.5 L of media, supplemented with 0.5 L of glycerol, in a high-density fermentor (Lab-Line Instruments, IL). After 8 h of growth at 37 °C, expression was induced with 200 μM isopropyl β-D-thiogalactoside for 2 h. Bacteria were harvested by centrifugation, washed once with buffer A (20 mM Tris-HCl, 50 mM EDTA, 1 mM DTT, and 1 mM sodium azide, pH 8.0), and frozen at −80 °C. The pellets were thawed in buffer A supplemented with 200 μM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, aprotonin, and benzamidine, and 1 mg/mL lysozyme. When lysis was complete, 40 mg of sodium deoxycholate and 10 mg of DNase I were added, and $MgCl_2$ was brought to 10 mM. The lysate was cleared by centrifugation, and the supernatant was incubated for 45 min at 4 °C with 20 mL of glutathione–agarose (Sigma) previously equilibrated with buffer A. The beads were then applied to a column and washed extensively with buffer B (20 mM Hepes, 5 mM $MgCl_2$, 1 mM sodium azide, pH 8.0). The fusion protein was eluted with buffer B supplemented with 10 mM glutathione. Protein-containing fractions were pooled, concentrated, and incubated for 2 h at 4 °C with 100 μg of bovine thrombin [purified from bovine plasma according to Ni et al. (1989)] to cleave the fusion protein. GST, thrombin, and any other minor impurities were removed by ion-exchange chromatography using a Q-Sepharose fast-flow (Pharmacia) column (2.5 × 40 cm) equilibrated with buffer B. The flow-through of this column contained highly pure Cdc42Hs (>95% as judged by SDS–PAGE). The protein was concentrated, dialyzed vs buffer B containing 40% glycerol, and stored in this buffer (typically at ~100 μM) at −20 °C.

(2) Cdc42Hs–GAP. Cloning and initial characterization of this protein were recently described (Barfod et al., 1993). The expression and purification of the recombinant GAP were performed generally as described above for Cdc42Hs, except that 2 L cultures were grown in a standard shaker rather then a fermentor, and the fusion protein was not cleaved with thrombin.

(3) dbl. Generation of the Glu-Glu epitope-tagged dbl in a recombinant baculovirus and expression of the construct in SF9 cells were identical to the described procedures for the expression of the rap1-GAP (Rubinfeld et al., 1991, 1992).

Chemicals. GDP and GTP were from Sigma. GMP–PCP and dGDP were from Boehringer Manheim. Mant-dGDP was synthesized from the parent nucleotide and *N*-methylisatoic acid (Molecular Probes, Eugene, OR) according to published procedure (Hiratsuka, 1983).

Fluorescence. All fluorescence measurements were taken with an SLM 8000C spectrofluorimeter in the photon-counting mode. Samples were continuously stirred and thermostated (25 °C unless otherwise stated) in either buffer C (20 mM Hepes, 50 mM NaCl, 5 mM $MgCl_2$, pH 7.5) or buffer D (20 mM Tris-HCl, 50 mM NaCl, 2.5 mM $MgCl_2$, pH 7.5). Spectral resolution, unless otherwise specified, was 8 and 16 nm for the excitation and emission paths, respectively.

Protein concentrations were determined using the Bradford assay (Bradford, 1976). Free Mg^{2+} concentrations were calculated from the total concentrations of $MgCl_2$ and EDTA using published association constants (Portzehl et al., 1964).

RESULTS AND DISCUSSION

Since the biological activity of virtually all GTP-binding proteins is conveyed by the GTP-bound form of the protein, the GTP-binding/GTPase cycle represents a key aspect of their regulation. It is the relative rates of the GDP–GTP exchange and the GTP hydrolytic reactions that determine the fraction of the GTP-binding protein in an activated (GTP-bound) conformation. In the work presented below, we describe fluorescence spectroscopic assays that provide real-time, high-resolution read-outs for the guanine nucleotide exchange and hydrolytic events occurring on the rho-subtype GTP-binding protein, Cdc42Hs, and the modulation of these activities by distinct regulatory proteins.

Guanine Nucleotide Exchange. Previously, it was shown that guanine nucleotides substituted at ribose hydroxyls with *N*-methylanthraniloyl (mant-) derivatives have fluorescent properties that are environmentally sensitive (Hiratsuka, 1983). Specifically, they were shown to exhibit marked increases in fluorescence intensity upon binding to GTP-binding proteins such as c-H-ras (John et al., 1990). We

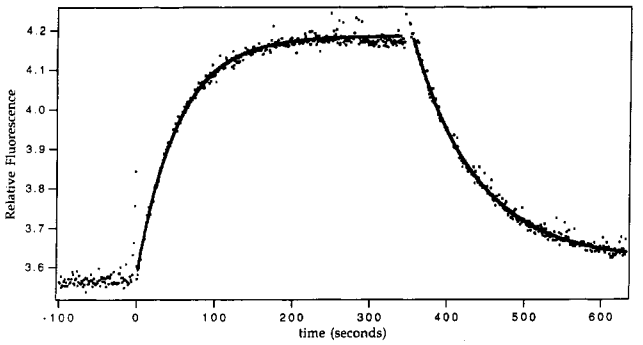

FIGURE 1: Time course of mant-dGDP fluorescence upon binding and release from Cdc42Hs. 0.19 μM mant-dGDP was incubated with 0.9 μM Cdc42Hs–GDP in buffer C. At t = 0 EDTA was added to 11.5 mM (free Mg^{2+} concentration 0.9 μM) to induce exchange onto the protein. At t = 350 s unlabeled GDP was added to 100 μM. The solid lines represent fits of the data to first-order kinetics for the fluorescence increase and decrease portions of the curves.

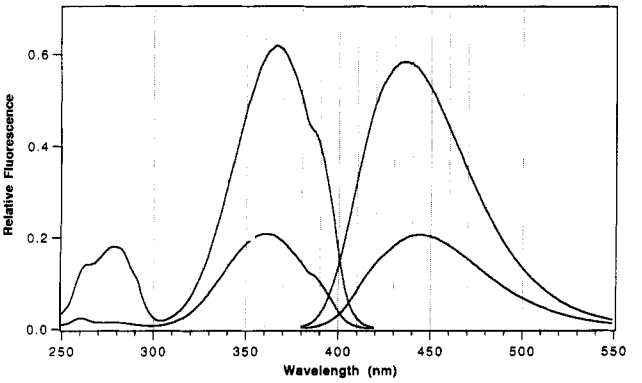

FIGURE 2: Fluorescence spectra of mant-GDP in solution and when bound to Cdc42Hs. 0.2 μM mant-GDP was incubated with 1.1 μM Cdc42Hs in buffer C, and excitation spectra (emission = 440 nm, left-hand curves) and emission spectra (excitation = 366 nm, right-hand curves) were recorded before and after addition of 15 mM EDTA (lower and upper spectra, respectively). Spectral resolution, 2 nm.

have found the same to be the case for Cdc42Hs. Figure 1 shows a time course for the binding of mant-dGDP to Cdc42Hs, as monitored by changes in the mant fluorescence (excitation = 360 nm, emission = 440 nm). The addition of mant-dGDP to Cdc42Hs, in the presence of EDTA which catalyzes the exchange of the fluorescent nucleotide for the GDP originally bound to Cdc42Hs (by removing excess Mg^{2+}), results in an ~20% increase in the mant fluorescence. On the basis of the known three-dimensional structure of ras (Pai et al., 1990), it has been proposed that Mg^{2+} regulates GDP dissociation from ras-like proteins because it is coordinated to both the guanine nucleotide and the protein. Consequently, the chelation of Mg^{2+} results in an increased rate of GDP dissociation (Hall & Self, 1986). The fluorescence change is rapid ($t_{1/2}$ = 20 s) and is completely reversible upon the addition of excess GDP or GTP (but not GMP), albeit at a slower rate ($t_{1/2}$ = 45 s). The rates of the fluorescence enhancement measured for mant-GDP, mant-GTP, and the nonhydrolyzable GTP analog, mant-GMP–PCP, are all similar (data not shown). These results are consistent with the interpretation that the enhancement of the mant fluorescence accompanying the addition of a mant-nucleotide to Cdc42Hs reflects the rate of dissociation of the (unlabeled) GDP that was originally bound to the GTP-binding protein. The fact that the decay in the mant-GDP fluorescence occurs with a slower time course than the initial enhancement indicates that the mant-guanine nucleotide dissociates from Cdc42Hs more slowly compared to unlabeled GDP. Figure 2 shows the excitation spectra (emission wavelength = 466 nm) and emission spectra (excitation wavelength = 360 nm) for mant-GDP free in solution (lower spectra) and bound to Cdc42Hs (upper spectra). The excitation spectra illustrate that there are significant enhancements in mant-GDP fluorescence, upon binding to Cdc42Hs, when exciting both at 280 nm and at 360 nm. The latter most likely reflects a change in the immediate environment of the mant moiety, following the interaction of mant-GDP with Cdc42Hs. This is further supported by the finding that the wavelength of the emission maximum is blue-shifted 10 nm (from 445 to 435 nm) when mant-GDP is bound to Cdc42Hs, consistent with the mant moiety being less accessible to solvent. The enhancement accompanying excitation at 280 nm, which is ~10-fold greater than the enhancement following excitation at 360 nm, most likely reflects resonance energy transfer between the single tryptophan residue on Cdc42Hs (W97) and the mant moiety.[1]

We have used the changes in mant-nucleotide fluorescence to examine further how Mg^{2+} influences the intrinsic guanine nucleotide exchange activity of Cdc42Hs. Figure 3A shows the time courses for GDP dissociation, as monitored by changes in the fluorescence of mant-dGDP following its exchange for GDP on Cdc42Hs, at different Mg^{2+} concentrations. Figure 3B presents a plot of the half-time for the dissociation of GDP as a function of $[Mg^{2+}]$. The data clearly illustrate that the half-time for GDP dissociation increases with increasing concentrations of Mg^{2+}. The titration profile (Figure 3B) is consistent with a single class of Mg^{2+} binding sites with an apparent dissociation constant of 7.5 μM, similar to what was shown for ras (John et al., 1993) and EF-Tu (Wittinghofer & Leberman, 1979). Thus, as has been suggested for the ras protein, Mg^{2+} binding to Cdc42Hs increases the affinity of the GTP-binding protein for guanine nucleotide by decreasing its off rate (Hall & Self, 1986).

It has been shown that distinct regulatory proteins [referred to as guanine nucleotide exchange factors (GEF)] counteract the effects of physiological levels of Mg^{2+} and stimulate GDP dissociation from ras-like proteins. The "son-of-sevenless" (SOS) proteins represent one group of GEFs for ras. In the case of Cdc42Hs, it has been suggested that the dbl-oncogene product serves as a GEF (Hart et al., 1991). Figure 4A presents the results of a fluorescence experiment where mant-dGDP and recombinant dbl were added to the Cdc42Hs–GDP complex, in the presence of 5 mM Mg^{2+}. While, in the absence of added dbl, there was essentially no change in the mant-dGDP fluorescence, in the presence of dbl an enhancement in the mant fluorescence was observed which was complete in 5–10 min. Either unlabeled GDP or GTP, in the presence of dbl, caused a decay in the fluorescence of mant-dGDP, due to the dbl-catalyzed exchange of GDP for the mant nucleotide, whereas GMP could not be exchanged for the bound mant-dGDP. Figure 4B shows that the time course for the dbl-catalyzed enhancement of the mant-dGDP fluorescence is essentially identical to the time course for dbl-catalyzed [^{3}H]GDP association. Thus, the changes in the mant fluorescence accompanying the exchange of mant-dGDP for (unlabeled) GDP provide a real-time monitor of this important regulatory event. Using this fluorescence assay for dbl-

[1] We have utilized this energy transfer to estimate the distance between the mant moiety and W97. Assuming $\kappa^2 = {}^2/_3$, and measuring the tryptophan quantum yield of fluorescence to be 0.08, we arrive at a donor–acceptor distance of 21 Å. This is in close agreement with the distance (25 Å) between the 3′ hydroxyl of the nucleotide and the Cα carbon of the analogous residue in ras (R97), as determined from the atomic coordinates generated from the X-ray crystallographic structure of the ras-p21 protein.

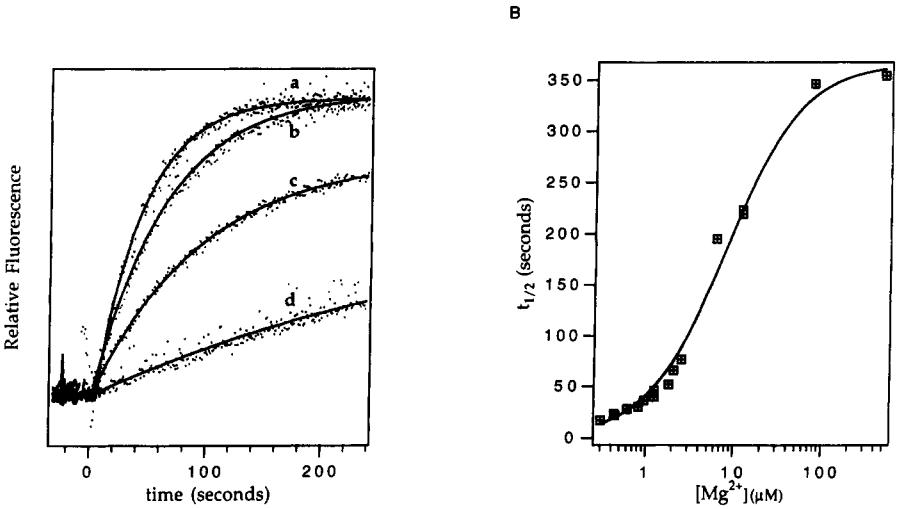

FIGURE 3: Effect of Mg^{2+} on rate of GDP dissociation from Cdc42Hs. (A) Cdc42Hs–GDP was incubated with mant-dGDP as in Figure 1, and at $t = 0$ EDTA was added to final concentrations of 15, 10, 8, and 5 mM (curves a–d, respectively). The solid lines through the data represent a fit to a single exponential process. (B) The extracted half-life values ($t_{1/2}$) for GDP dissociation are shown as a function of the calculated free Mg^{2+} concentration. The solid line through the data represents the best fit to a simple binding equilibrium, yielding an apparent dissociation constant of 7.6 μM.

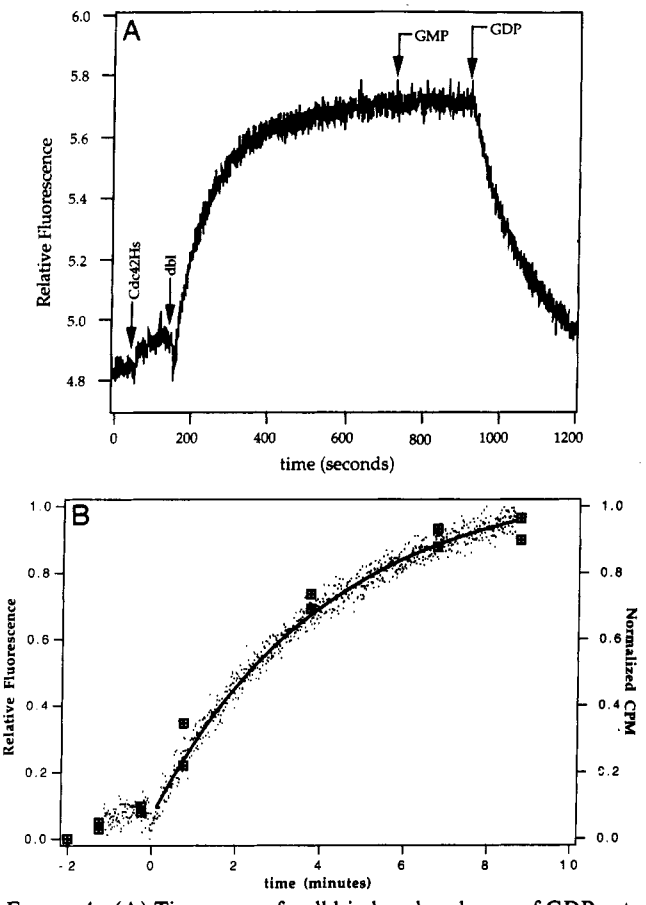

FIGURE 4: (A) Time course for dbl-induced exchange of GDP onto Cdc42Hs. 0.14 μM mant-dGDP was incubated in buffer C, and at the time indicated by arrows, Cdc42Hs–GDP and dbl were added to 2.6 μM and 60 nM, respectively, while monitoring mant fluorescence emission (excitation = 360 nm, emission = 440 nm). Following saturation, GMP and GDP were added at the indicated times to 100 μM. (B) Comparison of the time course for dbl-induced mant-GDP and [^{3}H]GDP exchange onto Cdc42Hs. Fluorescence measurement (dots) was done as in Figure 4 with 1.1 μM Cdc42Hs and 3 μM mant-dGDP. The solid line represents the best fit of the data to a single exponential process. For radioactive binding (boxes), 6 μM [^{3}H]GDP was substituted for mant-dGDP in an otherwise identical reaction mixture, and at the indicated times aliquots were filtered through nitrocellulose filters and counted as described (Hart et al., 1991).

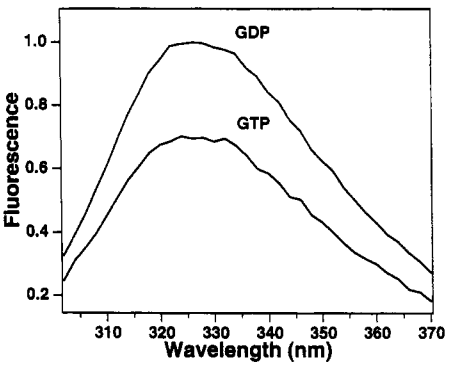

FIGURE 5: Tryptophan emission spectra for the GTP- and GDP-bound forms of Cdc42Hs. Cdc42Hs–GTP (10 μM) was prepared by mixing protein in buffer D with 33 μM GTP in the presence of 6.7 mM EDTA. Following a 5 min incubation, during which exchange was completed (see Figure 7), $MgCl_2$ was added to 2.5 mM to initiate hydrolysis, and two emission spectra (excitation = 295 nm) were collected and averaged. After an additional 30 min incubation (sufficient for complete hydrolysis, see below) two additional emission spectra were collected and averaged. Each data set was normalized to the fluorescence level of the Cdc42Hs–GDP complex. Spectral resolution 4 nm, scanning rate 4 nm/s.

catalyzed GDP dissociation, we have found that the dbl protein acts catalytically and stimulates GDP dissociation from multiple Cdc42Hs molecules (i.e., 1 dbl molecule can caytalyze GDP dissociation from at least 20 molecules of Cdc42Hs within 10 min).

GTP Hydrolysis. The GTP hydrolytic activity represents the deactivation step for most GTP-binding proteins. We also were interested in developing real-time fluorescence read-outs for this regulatory event; however, the fact that significant differences in the fluorescence enhancements accompanying the binding of mant-GDP and mant-GTP to Cdc42Hs were not consistently observed precluded the use of changes in mant fluorescence as an assay for Cdc42Hs GTPase activity. Since the tryptophan residues of heterotrimeric GTP-binding proteins (G proteins) have provided a sensitive monitor for G protein GTPase activity, and because Cdc42Hs (unlike ras) has a single tryptophan residue (at position 97), we examined whether changes in the tryptophan fluorescence might be correlated with the guanine nucleotide-bound state of Cdc42Hs.

Figure 5 compares the intrinsic tryptophan emission spectra for Cdc42Hs in the GDP- and GTP-bound states (excitation

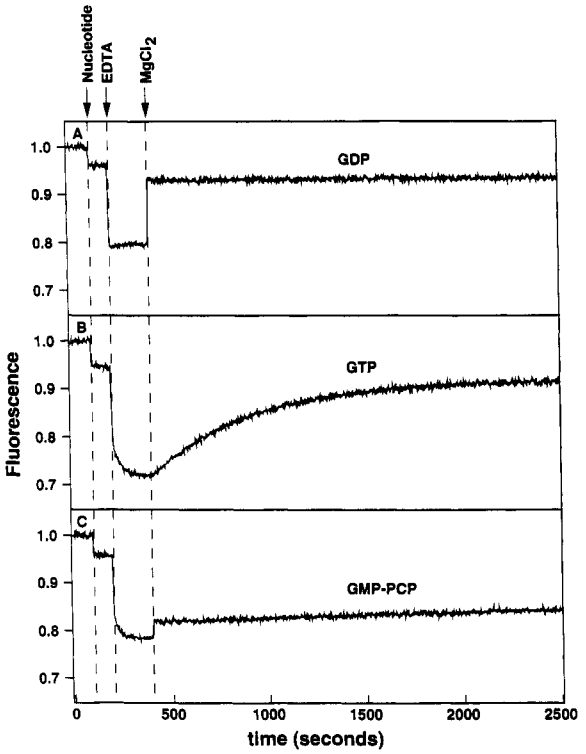

FIGURE 6: Tryptophan fluorescence of Cdc42Hs complexed with GDP, GTP, or GMP–PCP. In all experiments, 1 μM Cdc42Hs was incubated in buffer D and the time course of tryptophan fluorescence (excitation = 295 nm, emission = 330 nm) was monitored. At the indicated times, the solution was brought to 20 μM with the indicated nucleotide, and EDTA was added to 6.7 mM to induce exchange. At the time indicated by the third arrow, the $MgCl_2$ concentration was brought back to 2.5 mM to inhibit further exchange and, in the case of GTP, to initiate hydrolysis.

= 295 nm). On the basis of the differences in the intrinsic fluorescence of these two forms of Cdc42Hs, we would predict that the conversion of bound GTP to GDP, as an outcome of GTPase activity, would be accompanied by an ~40% enhancement in the tryptophan fluorescence. There were no detectable differences in the emission maxima for the GTP-bound and GDP-bound forms of Cdc42Hs (both occur at 328 nm), which indicated that there were not significant changes in the polarity of the microenvironment of the tryptophan residue. In addition, acrylamide quenching experiments yielded linear Stern–Volmer plots that were essentially identical for the GTP-bound and GDP-bound forms of Cdc42Hs, further suggesting that the tryptophan residue has similar accessibility to solvent in these two guanine nucleotide states (data not shown). Taken together, these results suggest that the side chain of W97 does not undergo a major environmental change when Cdc42Hs shifts between the GDP- and GTP-bound conformations. Nonetheless, the changes in the fluorescence intensity that accompany the conversion of Cdc42Hs between the different guanine nucleotide states provide a convenient read-out for GTPase activity. It should be noted that the directions of the fluorescence changes for the tryptophan residue of Cdc42Hs are opposite to those observed for the G protein α subunits; specifically, in the case of transducin or the G_i or G_o protein, an enhancement of the tryptophan fluorescence accompanies GTP binding, and thus GTPase activity is read-out by a fluorescence decay.

Figure 6 shows the time courses for the changes in the intrinsic tryptophan fluorescence of Cdc42Hs following the addition of GDP (panel A), GTP (panel B), and GMP–PCP (panel C). The addition of GDP, alone, to Cdc42Hs resulted in a slight quenching in the tryptophan fluorescence (< 5%);

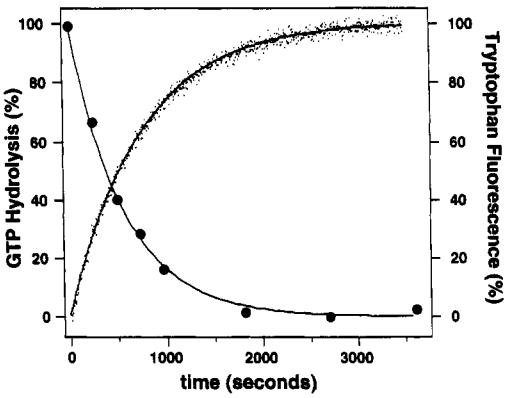

FIGURE 7: Time course for GTP hydrolysis by Cdc42Hs as measured by tryptophan fluorescence and by radioactive filter binding assay. The fluorescence change (dots) of a 0.87 μM solution of Cdc42Hs was monitored as in Figure 7, after it was allowed to exchange with 2.0 μM GTP, and then brought to a final $MgCl_2$ concentration of 9.1 mM. The filter-binding hydrolysis assay was carried out in an identical reaction mixture, except that the 2.0 μM GTP was supplemented with [α-^{32}P]GTP (final specific radioactivity 14,200 cpm/pmol). At the times indicated aliquots were removed, filtered, and counted for radioactivity as described in (Leonard et al., 1992).

this quenching was then accentuated when EDTA was added. The addition of millimolar concentrations of $MgCl_2$ then immediately restored some of the tryptophan fluorescence. These results indicate that W97 within a Cdc42Hs–GDP–Mg^{2+} complex has a significantly higher fluorescence quantum yield as compared to the Cdc42Hs–GDP species. The addition of GTP to Cdc42Hs also resulted in an immediate quenching of the tryptophan fluorescence that was accentuated by the addition of EDTA (Figure 6B). In this case, the extent of quenching was greater than that elicited by GDP, consistent with the results presented in Figure 5. However, the addition of $MgCl_2$ to the Cdc42Hs–GTP species did not result in an immediate restoration in the levels of tryptophan fluorescence, as was observed with the Cdc42Hs–GDP complex. Rather, a slow recovery of the tryptophan fluorescence, ultimately to a level essentially identical to that of the Cdc42Hs–GDP–Mg^{2+} complex, was observed. Given the relatively slow kinetics for the restoration of the tryptophan fluorescence, it seemed possible that this slow fluorescence change in fact reflected the GTP hydrolytic event. Consistent with this suggestion was the finding that when the same experiment was performed except that the nonhydrolyzable GTP analog, GMP–PCP, was substituted for GTP, the addition of $MgCl_2$ to the Cdc42Hs-GMP–PCP complex elicited a slight but immediate increase in the tryptophan fluorescence, and the fluorescence then remained constant over the period of several minutes. Moreover, as shown in Figure 7, the time course for [γ-^{32}P]-GTP hydrolysis, as measured by the release of [^{32}P]P_i, closely matched the time course for the change in fluorescence that accompanies the addition of Mg^{2+} to a Cdc42Hs–GTP complex. The best fits to these time courses yield rate constants of 0.0018 and 0.0013 s^{-1}, respectively.

Previous fluorescence studies using mant-nucleotides led to the suggestion that ras undergoes a conformational change that is the rate-limiting step for GTP hydrolysis (Rensland et al., 1991; Moore et al., 1993). Specifically, relatively slow changes in the fluorescence of mant-GTP and mant-GMP–PCP were observed when these nucleotides bound to ras, and based on the rate of these fluorescence changes it was concluded that they reflected a slow conformational change that precedes (and may be necessary for) GTP hydrolysis. Our fluorescence measurements do not reveal such a sequence of events for Cdc42Hs. Any changes that we observe in the fluorescence of the mant-nucleotides directly reflect guanine nucleotide

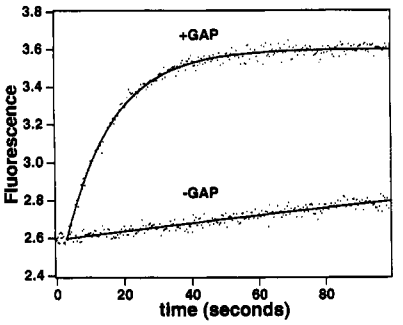

FIGURE 8: Acceleration of Cdc42Hs fluorescence change by Cdc42GAP. Cdc42Hs–GTP (2 μM) was prepared as described in Figure 7. Hydrolysis was initiated by bringing the $MgCl_2$ concentration to 2.5 mM, and tryptophan fluorescence was monitored as in Figure 7 in either the presence or absence of GST–Cdc42GAP (50 nM). The solid lines through the data represent the best fit to a single exponential process.

exchange while the change in tryptophan fluorescence that accompanies the conversion of the GTP-bound state of Cdc42Hs to the GDP-bound state appears to directly reflect the actual hydrolytic event (rather than reflecting a conformational change that precedes hydrolysis).

Figure 8 shows the time courses for the recovery of the tryptophan fluorescence following the addition of $MgCl_2$ to the Cdc42Hs–GTP species in the absence (lower time course) and in the presence (upper time course) of the recombinant Cdc42Hs–GAP. The Cdc42Hs–GAP strongly stimulates the rate of the fluorescence change, consistent with its ability to stimulate the GTP hydrolytic event. We have found that even when the ratio of GAP to Cdc42Hs–GTP is 1:40, there is a 30-fold stimulation in the rate of change of the tryptophan fluorescence.

SUMMARY

The results described in this study demonstrate that there are two fluorescence assays that now can be used to read-out directly the GTP-binding/GTPase cycle of the rho-subtype protein, Cdc42Hs. Changes in the fluorescence of mant-guanine nucleotides, that accompany their binding to Cdc42Hs, serve as a monitor for the guanine nucleotide exchange reaction, whereas changes in the quantum yield of a single tryptophan residue (W97) provides a direct read-out for the GTP hydrolytic event. The appropriate regulatory proteins, i.e., the Cdc42Hs–GEF (dbl) and the Cdc42Hs–GAP, stimulate the rates of the fluorescence changes that reflect guanine nucleotide exchange and GTPase activity. In the future, we intend to take advantage of the sensitivity provided by these fluorescence assays to compare the abilities of other members of the Cdc42Hs–GAP family (such as the bcr protein, the 85 kDa regulatory protein of the PI-3 kinase, and the *S. cerevisiae* Bem3 protein) and Cdc42Hs–GEF family (i.e., the *S. cerevisiae* cdc24 protein and the human vav oncogene product) to functionally couple to Cdc42Hs.

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