Investigation of the GTP-Binding/GTPase Cycle of Cdc42Hs Using Extrinsic Reporter Group Fluorescence†

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ABSTRACT: The overall goal of these studies was to examine the applicability of extrinsic reporter group fluorescence in monitoring the GTP-binding/GTPase cycle of a Ras-like GTP-binding protein. Toward this end, we have labeled the GTP-binding protein Cdc42Hs with the environmentally sensitive fluorophore succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate (sNBD) at a single reactive lysine residue. We find that the sNBD-labeled Cdc42Hs undergoes a fluorescence enhancement at 545 nm when Cdc42Hs exchanges bound GDP for GTP. This enhancement is then fully reversed upon GTP hydrolysis. The specific GTPase-activating protein for Cdc42Hs, the Cdc42Hs−GAP, strongly stimulates the rate of reversal of the fluorescence enhancement at 545 nm, consistent with its ability to fully catalyze the GTPase reaction of Cdc42Hs. Conversely, the specific guanine nucleotide exchange factor (GEF), Cdc24, strongly stimulates the fluorescence enhancement that accompanies GTP binding, consistent with its ability to stimulate the GDP−GTP exchange reaction on Cdc42Hs. Resonance energy transfer measurements yielded a distance of ∼32 Å for the sNBD moiety and the guanine nucleotide binding site occupied with either *N*-methylanthraniloyl- (Mant) dGDP or MantdGTP. Taken together, these results identify a conformationally sensitive reporter site on the Cdc42Hs molecule that is located some distance away from the guanine nucleotide binding site but nonetheless provides a highly sensitive monitor for GTP-binding, GTPase activity, and the interactions of key regulatory proteins.

The Ras-like low molecular weight GTP-binding proteins form a superfamily whose members are involved in a variety of biological pathways including the regulation of cell growth and differentiation, vesicular transport, and cytoskeletal organization. In all cases, these GTP-binding proteins appear to act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. This cycle is tightly regulated by distinct proteins; in particular, the exchange of GDP to GTP is stimulated by guanine nucleotide exchange factors (GEFs), and the hydrolysis of GTP back to GDP is catalyzed by GTPase-activating proteins (GAPs). In some cases, a third class of proteins participates in the regulation of the GTP-binding/GTPase cycle by inhibiting GDP dissociation (and thus has been designated GDP-dissociation inhibitors or GDIs) and GTP hydrolysis and stimulating the dissociation of the GTP-binding protein from membranes.

A major interest of our laboratory has been aimed at structure−function studies of the Cdc42Hs GTP-binding protein, which is the human homolog of the *Saccharomyces cerevisiae* cell division cycle protein (Cdc42Sc) (Johnson & Pringle, 1990; Shinjo et al., 1990). The Cdc42 protein appears to play an essential role in the establishment of cell polarity; in *S. cerevisiae* this is manifested by controlling the assembly of the bud site while in *Schizosaccharomyces pombe* it entails the regulation of both unidirectional and bidirectional cell growth (Miller & Johnson, 1994). Recent studies have implicated Cdc42 in the development of filopodia in mammalian cells (Kozma et al., 1995; Nobes & Hall, 1995) and in the stimulation of the nuclear MAP kinases, Jnk and p38 (Coso et al., 1995; Minden et al., 1995; Hill et al., 1995; Olson et al., 1995; Bagrodia et al., 1995). Several regulators of the GTP-binding/GTPase cycle of Cdc42Hs have now been identified. These include a GEF [the Dbl oncogene product (Hart et al., 1991); in yeast it is the Cdc24 gene product (Zheng et al., 1994)], a GDI (Leonard et al., 1992), and a specific GAP [designated the Cdc42Hs−GAP (Barfod et al., 1993)].

In order to better understand the molecular basis underlying the regulation of Cdc42Hs by these different proteins, we have set out to develop real-time fluorescence spectroscopic assays to directly monitor each step in the GTP-binding/GTPase cycle. Our initial approach was to take advantage of a single tryptophan residue on Cdc42Hs as an intrinsic fluorescence reporter group for monitoring GTP hydrolysis (Leonard et al., 1994). In addition, along the lines of earlier experiments performed with Ras (Antonny et al., 1991; Rensland et al., 1991; Moore et al., 1993), we have used the fluorescence of Mant-derivatized guanine nucleotides to monitor the binding of GDP and GTP. Although each of these methods has provided us with some kinetic information regarding GTP binding and hydrolysis, they both have disadvantages. For example, using the intrinsic tryptophan fluorescence of Cdc42Hs as a readout limits the concentration of target/regulatory proteins that can be added to the assay because of the significant background (tryptophan) fluorescence that they contribute. As a result, the tryptophan fluorescence readout would not be practical when monitoring changes in the guanine nucleotide-bound state of Cdc42Hs, where the amount of regulatory protein (e.g.,

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GEF) is similar to (and not sufficiently less than) the amount of Cdc42Hs.

In searching for an alternative approach that does not suffer from such disadvantages, we have examined the possible use of extrinsic reporter groups for monitoring the GTP-binding/GTPase cycle for Cdc42Hs. In the present study, we show that we can covalently attach an exogenous fluorescent probe, succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-hexanoate (sNBD), to Cdc42Hs with a stoichiometry of 1 probe molecule per GTP-binding protein. We further show that the sNBD-labeled Cdc42Hs serves as a sensitive extrinsic reporter for GTP binding, GTP hydrolysis, and the functional interactions between Cdc42Hs and its GEF and GAP regulators.

MATERIALS AND METHODS

Chemicals. GDP, GTP, and GTPγS were from Sigma. dGTP and dGDP were from Boehringer Mannheim. Mant-dGDP and MantdGTP were synthesized from the parent nucleotides and *N*-methylisatoic acid (Molecular Probes, Eugene, OR) according to the published procedure (Hiratsuka, 1983). Succinimidyl NBD was from Molecular Probes.

Proteins. Cdc42Hs and Cdc42Hs−GAP were expressed as GST fusion proteins in *Escherichia coli*. The expression and purification of these proteins have recently been described (Leonard et al., 1994). Analysis of the purified recombinant Cdc42Hs for bound guanine nucleotide, as described by Scherer et al. (1989), indicated that wild-type Cdc42Hs contains ∼1 mol of GDP/mol of protein and no detectable bound GTP. Cdc24 was expressed as a GST fusion protein in *Spodoptera frugiperda* (Sf9) insect cells, through infecting the cells with the appropriate recombinant baculovirus (Zheng et al., 1994).

Protein Modification. A 5 mL overnight culture of *E. coli* containing the expression plasmid for GST−Cdc42Hs was used to seed 2 L of superbroth (0.32 mg/mL bactotryptone, 0.02 mg/mL yeast extract, 0.005 mg/mL NaCl, pH 7.4). This was grown in a standard shaker at 37 °C. Protein expression was induced at an OD_{560nm} of ∼0.6 with 200 μM isopropyl β-D-thiogalactoside (IPTG) for 90 min. Cells were harvested by centrifugation, quick-frozen in liquid nitrogen, and stored at −80 °C. Pellets were thawed into buffer A (20 mM Tris, pH 8.0, 50 mM EDTA, 200 μM phenylmethanesulfonyl fluoride, and 10 μg/mL aprotinin and leupeptin) to which was added 1 mg/mL lysozyme. When lysis was complete, DNase was added to 0.05 mg/mL and $MgCl_2$ was adjusted to 2 mM. The lysate was cleared by centrifugation and the supernatant incubated with glutathione−agarose beads (Sigma) (∼300 μL of agarose/15 mL of lysate) for 15 min at 4 °C. This mixture was applied to a 2 mL column. The beads were first washed extensively with buffer B (20 mM Tris, pH 8.0, 1 mM EDTA, 2 μM GDP), followed by 10 mL of buffer C (20 mM HEPES, 50 mM K_2HPO_4, pH 8.7). A microstirbar was placed into the column, and the column was placed over a stirrer. A total of 10 μL of a 20 mM solution of succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate (sNBD) in DMF was added to the column. The reaction was carried out for 1 h at room temperature with continuous stirring. The column was then washed extensively with buffer B to remove free probe. sNBD−Cdc42Hs was released from the column by digesting with thrombin (Sigma). The column was first washed with 10 mL of buffer D (50 mM Tris, pH 8.3, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2 μM GDP), and 5 μL of a 1 unit/μL thrombin solution was added to the column. Digestion was carried out for 90 min at room temperature with continuous stirring. sNBD−Cdc42Hs, in the GDP-bound state, was eluted from the column by washing the column with 0.5 mL of buffer D. The stoichiometry of sNBD per Cdc42Hs was 1 ± 0.1, based on protein determination by the Cu^+/bicinchoninic acid complex (Pierce Chemicals, Inc.), using bovine serum albumin as a standard, and the absorbance of the sNBD moiety ($\epsilon_{max,488nm}$ = 22 000 M^{-1} cm^{-1}).

Isolation and Analysis of Fluorescently Labeled Peptides. Cdc42Hs that was specifically labeled at a reactive residue with lysine-selective reagents [sNBD or fluorescein isothiocyanate (FITC)] was reduced and *S*-carboxamidomethylated (Stone et al., 1989). The alkylation mixture was diluted 4-fold to a final buffer concentration of 2 M urea/0.1 M NH_4HCO_3. Chymotrypsin was added to this solution to maintain a Cdc42Hs to enzyme ratio of ∼25:1 (w/w), and the mixture was incubated at 37 °C for ∼20 h. The peptides generated by chymotrypsin treatment were separated by narrow-bore reverse-phase HPLC on a Hewlett-Packard 1090 HPLC equipped with a 1040 diode array detector, using a Vydac 2.1 mm × 150 mm C18 column. The gradient employed was a modification of that described by Stone et al. (1989), as outlined in Erickson et al. (1995). Peptide peaks were monitored for absorbance at 210 and 488 nm (i.e., the absorbance peak for sNBD or FITC). The fractions (1.5 mL) were collected manually and microsequenced by automated Edman degradation on an ABI Model 477A sequencer (Lane et al., 1991). Peptides that displayed absorbances at 214 and 488 nm were chosen for analysis; however, most of these were shown by mass spectral analysis to represent free label. One peak containing 488 nm absorbance was found to contain peptide based on mass spectral analysis. The chromatographic peak was sequenced and found to contain two predominant peptides. The first peptide sequence corresponded to residues 71−78 (SYPQTDVF) of Cdc42Hs and showed no indication that label was attached. The other peptide sequence was DLAVKYVEC (corresponding to residues 148−149 and 151−157 on Cdc42Hs). The third residue of this sequence should be lysine 150. This lysine was observed to have an anomalous retention time presumably due to the presence of the FITC reporter group attached to it. The size of an observed mass spectral peak at 1551.8 Da is the same as what one would calculate for the sequenced peptide with label (in this case FITC) attached (1553). Thus, on the basis of these analyses we conclude that the reactive residue on Cdc42Hs is lysine 150.

Fluorescence Spectroscopy. The fluorescence measurements were made using an SLM 8000c spectrofluorometer operated in the photon-counting mode. Samples were stirred continuously and thermostated at 30 °C in buffer E (20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$).

Calculations. The Förster distance R_0 is defined as the distance between donor and acceptor at which energy transfer efficiency is 50%. R_0 values for MantdGDP−Cdc42Hs/sNBD−Cdc42Hs and MantdGTP−Cdc42Hs/sNBD−Cdc42Hs donor/acceptor pairs were calculated from the equation:

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6}\ \text{Å}$$

where J is an integral reflecting the spectral overlap of donor

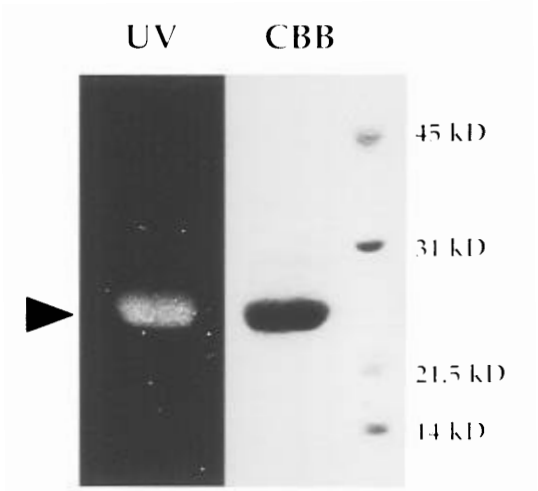

FIGURE 1: SDS−PAGE of sNBD−Cdc42Hs. sNBD−Cdc42Hs (10 μg), prepared as described in the Materials and Methods section, was electrophoresed on a 10% SDS−polyacrylamide gel. The gel was first photographed over a transilluminator to display the fluorescent band (left) and was subsequently Coomassie stained (right).

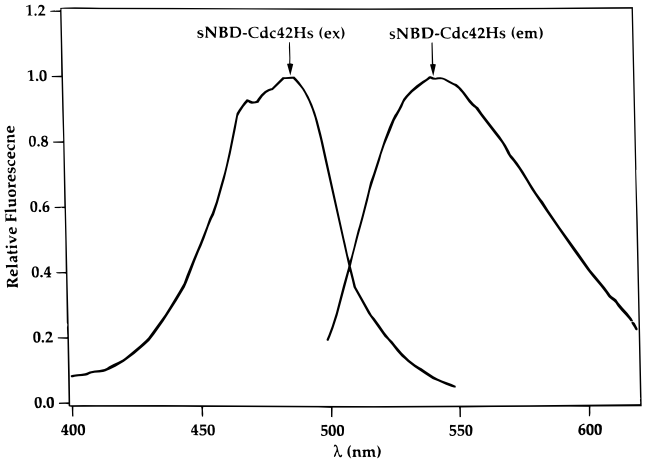

FIGURE 2: Normalized excitation and emission spectra for sNBD−Cdc42Hs. sNBD−Cdc42Hs (500 nM) was incubated in buffer E in the presence of 12.5 μM GDP and 6.7 mM EDTA. The excitation spectrum for sNBD−Cdc42Hs was obtained by recording emission at 545 nm. The emission spectrum of sNBD−Cdc42Hs was obtained by exciting the sample at 488 nm.

emission and acceptor absorption, κ^2 is a factor characterizing the relative orientation of donor and acceptor dipoles, and n is the refractive index of the medium. We used $^2/_3$ for κ^2, on the assumption that dipole orientations are random. To determine the error limits of κ^2, we determined the steady-state polarizations (p) of the labeled proteins. The average value of p for sNBD−Cdc42Hs was 0.192 ± 0.009 ($n = 5$), and the average value of p for MantdGDP−Cdc42Hs was 0.244 ± 0.006 ($n = 5$). These values correspond to an error in κ^2 of ∼10% (Hass et al., 1978). For n, we used 1.33, and we determined the Q_D values for MantdGDP or MantdGTP, bound to Cdc42Hs, to be 0.29. Using these quantities, we calculated an R_0 value of 33 ± 0.5 Å for the Mant guanine nucleotide/sNBD donor−acceptor pair. The efficiency of energy transfer between the sNBD moiety and the Mant nucleotides was measured under conditions where Cdc42Hs (1.5 μM) was in high excess over the Mant nucleotides (0.12 μM). To verify that the Mant nucleotides were fully bound under these conditions, we measured the enhancement of the Mant nucleotides [upon binding Cdc42Hs; see Leonard et al. (1994)] as a function of varying concentrations of Cdc42Hs (0.05−1 μM). These titrations yielded a saturable curve which when fit to a simple binding model yielded apparent K_d values of 1 nM for MantdGTP and 60 nM for MantdGDP. These values in fact underestimate the true affinities of the Mant nucleotides for Cdc42Hs, because unlabeled GDP was present with each addition of Cdc42Hs, resulting in competition by labeled and unlabeled GDP for the available Cdc42Hs pool.

RESULTS

Characterization of sNBD−Cdc42Hs. The primary aim of this study was to develop a fluorescent readout for the nucleotide state of Cdc42Hs using an environmentally sensitive fluorescence reporter group. Previously we found that Cdc42Hs could be fluorescently labeled using lysine-specific probes (C. Chang, unpublished data), and so we set out to attach the lysine-specific environmentally sensitive probe succinimidyl 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoate (sNBD) to Cdc42Hs. Figure 1 shows the purified recombinant Cdc42Hs protein that has been covalently labeled with sNBD (the fluorescence from the sNBD moiety is shown in the left panel and the protein staining is shown in the right panel). sNBD−Cdc42Hs has an excitation maximum at 488 nm and an emission maximum at 545 nm (Figure 2). The ability of sNBD−Cdc42Hs to bind and hydrolyze GTP was tested by monitoring guanine nucleotide-dependent changes in the tryptophan fluorescence of sNBD−Cdc42Hs. In a previous study, we have shown that changes in the fluorescence of the single tryptophan residue of Cdc42Hs (W97) provide a very sensitive monitor for its guanine nucleotide-bound state. Specifically, the GDP-bound state of Cdc42Hs has a 30% higher tryptophan fluorescence than the GTP-bound state (Leonard et al., 1994). Figure 3A shows a time course for changes in the tryptophan fluorescence of sNBD−Cdc42Hs, initially in the GDP-bound form, due to the binding and subsequent hydrolysis of GTP. The addition of GTP to the cuvette causes an ∼15% decrease in fluorescence, due to the absorbance of GTP at 295 nm. The addition of EDTA results in the chelation of Mg^{2+} at the nucleotide-binding site of Cdc42Hs, allowing for the exchange of GDP for GTP (Leonard et al., 1994). This is accompanied by a decrease in tryptophan fluorescence. The subsequent addition of $MgCl_2$ prevents further exchange of GDP for GTP and is followed by a slow gradual increase in tryptophan fluorescence as GTP complexed to the protein is hydrolyzed to GDP. The rates for both the exchange of GDP for GTP and the hydrolysis of GTP to GDP were indistinguishable from unlabeled Cdc42Hs (Figure 3B), indicating that fluorescent modification of Cdc42Hs does not interfere with nucleotide binding or hydrolysis.

sNBD Fluorescence of *sNBD−Cdc42Hs Is Sensitive to the Nucleotide State of Cdc42Hs*. Figure 4 shows the emission spectra for sNBD−Cdc42Hs in the GTP-, GDP-, and GTPγS-bound states. The fluorescence of sNBD−Cdc42Hs in the GTP-bound state is similar to that of the GTPγS-bound state. The fluorescence of the GTP-bound sNBD−Cdc42Hs (or GTPγS-bound sNBD−Cdc42Hs) is greater than the GDP-bound state by about 20%. This indicates that the binding of either GTP or GTPγS to sNBD−Cdc42Hs results in a conformational change in the vicinity of sNBD, giving rise to an enhancement of the sNBD fluorescence. The emission maxima for all three spectra are

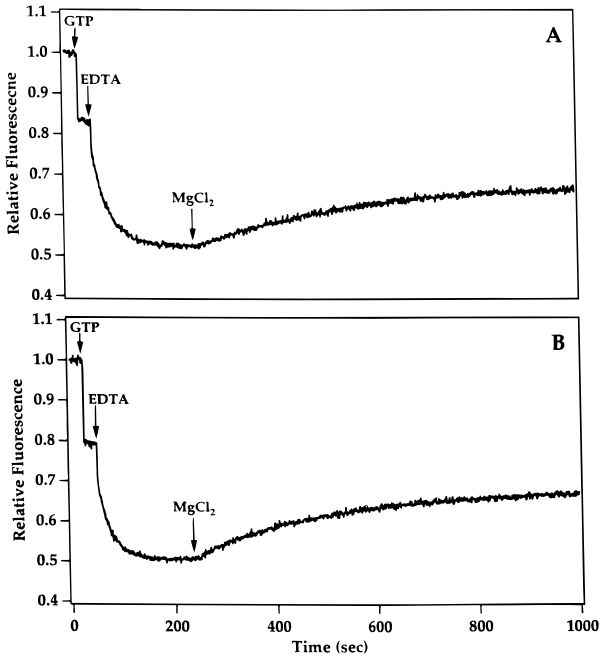

FIGURE 3: Tryptophan fluorescence of sNBD−Cdc42Hs and Cdc42Hs. sNBD−Cdc42Hs (A) or Cdc42Hs (B) (350 nM) was incubated in buffer E, and the time course of tryptophan fluorescence (excitation = 295 nm; emission = 330 nm) was monitored. At the indicated times, GTP was added to 12.5 μM, EDTA was added to 6.7 mM, and $MgCl_2$ was added so that the final $MgCl_2$ concentration was 2.0 mM.

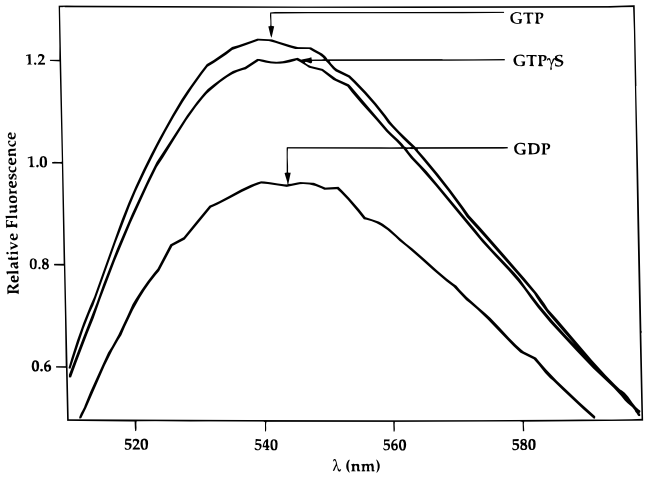

FIGURE 4: Normalized emission spectra of sNBD−Cdc42Hs in the GTP, GDP, or GTPγS states. sNBD−Cdc42Hs (350 nM) was incubated in buffer E with 6.7 mM EDTA and 12.5 μM GTP, GDP, or GTPγS. The emission spectra were obtained by exciting the samples at 488 nm.

about the same (∼545 nm). This suggests that the GTP-induced conformational change in the vicinity of the sNBD moiety does not involve a change in the hydrophobicity of the environment of sNBD but rather reflects a change in the degree to which the sNBD fluorescence is being quenched by neighboring residues.

Figure 5 shows the time courses for changes in sNBD fluorescence of sNBD−Cdc42Hs following the addition of GTP (Figure 5A), GDP (Figure 5B), or GTPγS (Figure 5C). As expected from the emission spectra in Figure 4, the binding of GTP to sNBD−Cdc42Hs results in an ∼20% increase in sNBD fluorescence. The subsequent addition of $MgCl_2$ to sNBD−Cdc42Hs(GTP) is followed by a gradual decrease in sNBD fluorescence. The rates of change of

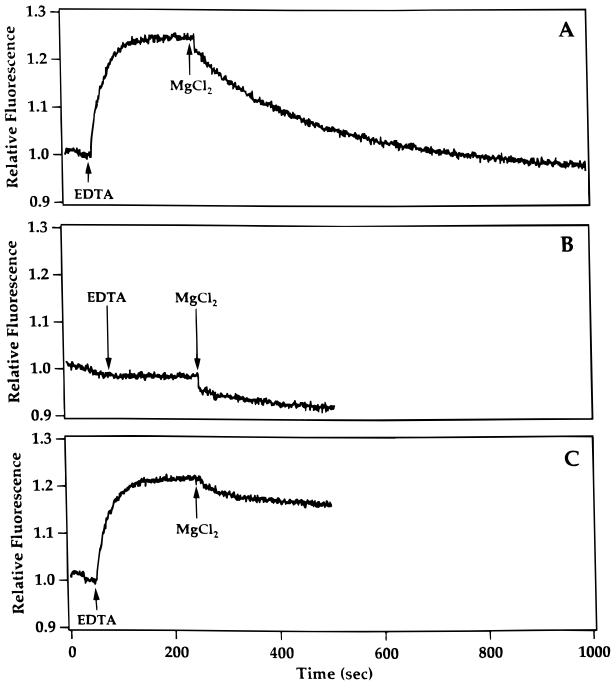

FIGURE 5: sNBD fluorescence of sNBD−Cdc42Hs. sNBD−Cdc42Hs (350 nM) was incubated in buffer E in the presence of 12.5 μM GTP (A), GDP (B), or GTPγS (C). The time course for sNBD fluorescence was monitored (excitation = 488 nm; emission = 545 nm), and at the indicated times, EDTA was added to 6.7 mM, and $MgCl_2$ was added so that the final $MgCl_2$ concentration was 2.0 mM.

sNBD fluorescence are very similar to the rates of change of tryptophan fluorescence in response to GTP binding and hydrolysis. This strongly suggests that the initial increase in sNBD fluorescence is due to GTP binding and the decrease in sNBD fluorescence upon the addition of $MgCl_2$ is due to the hydrolysis of GTP to GDP. The binding of GDP to sNBD−Cdc42Hs does not result in any significant changes in sNBD fluorescence (Figure 5B). A minor decrease is sometimes observed on the addition of $MgCl_2$; this appears to be due to an interaction between the sNBD moiety and $MgCl_2$ and is clearly distinguishable from the decrease in sNBD fluorescence due to the hydrolysis of GTP to GDP (compare panels A and B of Figure 5). The binding of the nonhydrolyzable analog of GTP, GTPγS, results in an increase in sNBD fluorescence similar to that caused by GTP (Figure 5C) but, as expected, does not show the significant reversal of the enhanced fluorescence that reflects GTP hydrolysis (i.e., Figure 5A).

sNBD−Cdc42Hs Interacts with Cdc42Hs GTPase-Activating Protein (Cdc42Hs−GAP) and the Guanine Nucleotide Exchange Factor (GEF). We next examined whether sNBD−Cdc42Hs was capable of functionally coupling to different regulators of the GTP-binding/GTPase cycle. Specifically, we examined the interactions of sNBD−Cdc42Hs with a specific guanine nucleotide exchange factor (GEF), the *S. cerevisiae* Cdc24 gene product, and with the Cdc42Hs−GTPase activating protein (Cdc42Hs−GAP). Figure 6A shows a time course monitoring changes in the sNBD fluorescence of sNBD−Cdc42 in response to the binding and hydrolysis of GTP in the presence and absence of Cdc42Hs−GAP. The measured rate for the intrinsic GTP hydrolysis (−GAP) for Cdc42Hs, as determined from the change in sNBD fluorescence, is 0.004 ± 0.0005 s^{-1}. The addition of Cdc42Hs−GAP then results in a rapid decrease

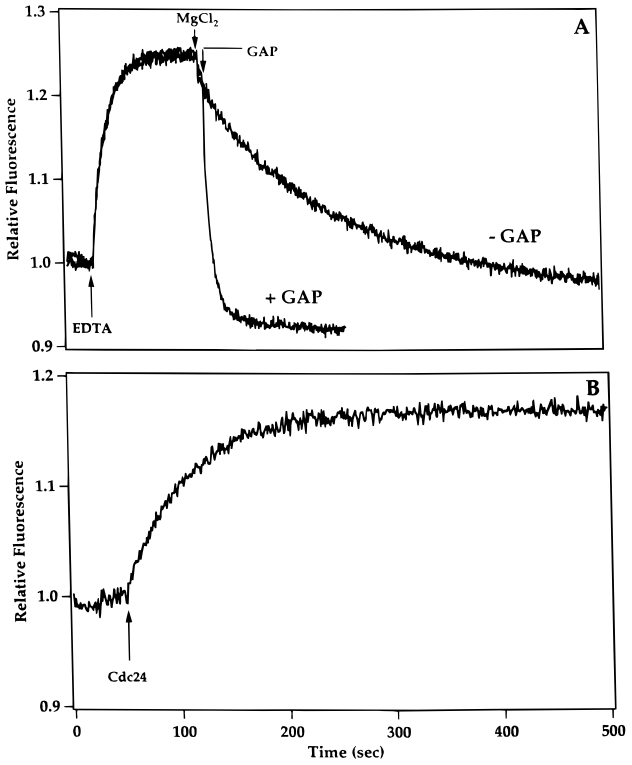

FIGURE 6: (A) Time course for Cdc42Hs−GAP accelerated hydrolysis of sNBD−Cdc42Hs. sNBD−Cdc42Hs (350 nM) was incubated in buffer E in the presence of 12.5 μM GTP. sNBD fluorescence was monitored (excitation = 488 nm, emission = 545 nm), and at the indicated times, EDTA was added to 6.7 mM, and $MgCl_2$ was added so that the final $MgCl_2$ concentration was 2.0 mM. GST−Cdc42Hs−GAP (25 nM) was added 10 s after the addition of $MgCl_2$. (B) Time course for Cdc24-induced exchange of GDP for GTP. sNBD−Cdc42Hs (350 nM) was incubated in buffer E in the presence of 12.5 μM GTP. sNBD fluorescence was monitored (excitation = 488 nm; emission = 545 nm), and at the indicated time, GST−Cdc24 was added to 62.5 nM.

in sNBD fluorescence, corresponding to a strong acceleration in the rate of hydrolysis of GTP to GDP. Figure 6B shows a time course monitoring the increase in sNBD fluorescence ($t_{1/2}$ ~10 s) that accompanies the Cdc24-catalyzed exchange of GDP for GTP on sNBD−Cdc42Hs. The rates for each of these fluorescence changes match those measured for GAP-stimulated GTP hydrolysis (Leonard et al., 1994) and Cdc24-catalyzed GDP−GTP exchange (Zheng et al., 1994) as determined in assays using [γ-^{32}P]GTP and [^{35}S]GTPγS, respectively. Taken together, these results demonstrate that the sNBD-labeled molecule is fully able to couple to GAPs and GEFs.

Resonance Energy Transfer between MantdGDP and sNBD−Cdc42Hs. To further characterize the nucleotide-induced conformational changes of Cdc42Hs, the distance between the nucleotide binding site of Cdc42Hs and the labeled lysine residue was determined using resonance energy transfer. Previously, it was shown that the binding of Mant-derivatized nucleotides to Cdc42Hs results in an increase in the Mant fluorescence (Leonard et al., 1994). Figure 7 shows the overlap between the emission spectrum of MantdGDP−Cdc42Hs and the excitation spectrum of sNBD−Cdc42Hs. There is significant overlap between the two spectra, thus indicating that sNBD would serve as an appropriate acceptor for the Mant moiety. Figure 8A shows a time course of the changes in the Mant fluorescence as MantdGDP binds to Cdc42Hs. The binding of MantdGDP to Cdc42Hs results

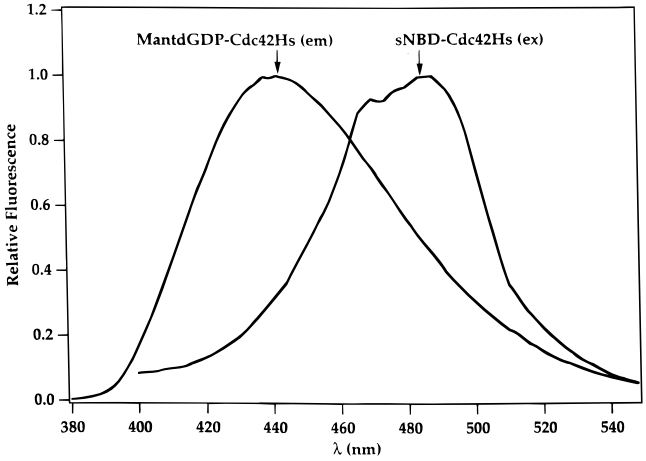

FIGURE 7: Normalized emission spectrum of MantdGDP−Cdc42 and excitation spectrum of sNBD−Cdc42Hs. The emission spectrum for MantdGDP−Cdc42Hs was obtained by first incubating 4 μM Cdc42Hs in buffer E in the presence of 120 nM MantdGDP and 6.7 mM EDTA. After the binding of MantdGDP to Cdc42Hs was complete, the emission spectrum was recorded by exciting the sample at 350 nm. The excitation spectrum for sNBD−Cdc42Hs was obtained as described in the legend for Figure 2.

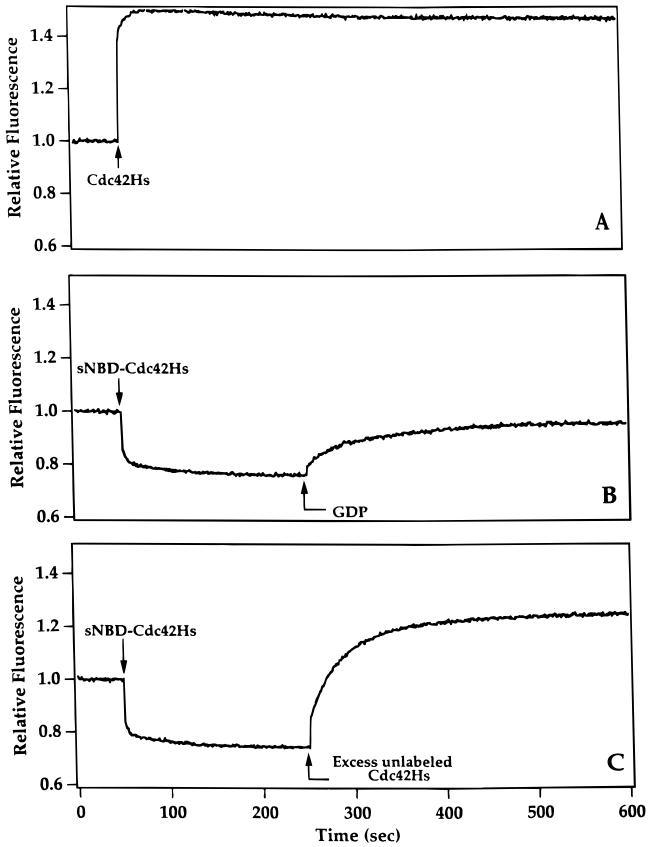

FIGURE 8: Time course for resonance energy transfer between MantdGDP and sNBD−Cdc42Hs. MantdGDP (120 nM) was incubated in buffer E in the presence of 6.7 mM EDTA. Mant fluorescence was monitored (excitation = 350 nm; emission = 440 nm), and at the indicated times, either 4 μM Cdc42Hs (A) or 350 nM sNBD−Cdc42Hs was added (B, C). Energy transfer was reversed by the addition of either 280 μM GDP (B) or 7.5 μM unlabeled Cdc42Hs (C).

in an ~50% increase in Mant fluorescence. On the other hand, the binding of MantdGDP to sNBD−Cdc42Hs results in a decrease in Mant fluorescence, suggesting that energy transfer has occurred between Mant and sNBD (Figure 8B,C). The decrease in Mant fluorescence can be reversed

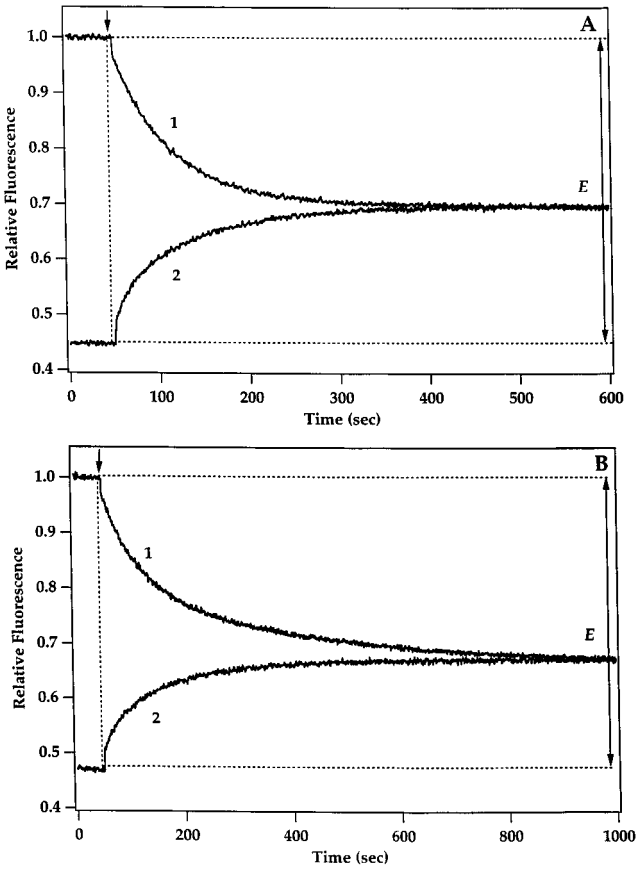

FIGURE 9: Determination of the efficiency of resonance energy transfer between MantdGDP−Cdc42Hs/sNBD−Cdc42Hs and MantdGTP−Cdc42Hs/sNBD−Cdc42Hs. MantdGDP (A) or MantdGTP (B) (120 nM) was incubated in buffer E with 6.7 mM EDTA and 4 μM Cdc42Hs (trace 1) or 1.5 μM sNBD−Cdc42Hs (trace 2). Mant fluorescence was monitored (excitation = 350 nm; emission = 440 nm), and the dissociation of Mant nucleotide was initiated (arrows) by the addition of 280 μM GDP.

by the addition of either excess unlabeled GDP (Figure 8B) or excess unlabeled Cdc42Hs (Figure 8C).

Distance Determination between the Nucleotide-Binding Site and the Labeled Lysine of Cdc42Hs in the GDP-Bound and GTP-Bound States. The efficiency of energy, *E*, was calculated from the relationship:

$$E = 1 - Q_{DA}/Q_D$$

where Q_D is the donor quantum yield in the absence of acceptor and Q_{DA} is the donor quantum yield in the presence of acceptor. The experiments carried out to determine the efficiencies of energy transfer for the MantdGDP−Cdc42Hs/sNBD−Cdc42Hs and MantdGTP−Cdc42Hs/sNBD−Cdc42Hs complexes are depicted in panels A and B of Figure 9, respectively. In this experiment, Mant nucleotide was bound to an excess of either Cdc42Hs or sNBD−Cdc42Hs (traces 1 and 2, respectively). The Mant nucleotide was then dissociated by the addition of unlabeled GDP (arrows). For each nucleotide state, traces 1 and 2 were normalized to the fluorescence of free Mant nucleotide. On the assumption that the quantum yield of Mant is proportional to its fluorescence, the initial fluorescence of the Mant nucleotide bound to Cdc42Hs was defined as Q_D, and the initial fluorescence of Mant nucleotide bound to sNBD−Cdc42Hs was defined as Q_{DA}. We calculated *E* values of 0.552 and 0.530 for MantdGDP−Cdc42Hs/sNBD−Cdc42 and MantdGTP−Cdc42Hs/sNBD−Cdc42, respectively. We then used the following relationship to calculate the distance between the Mant and sNBD probes:

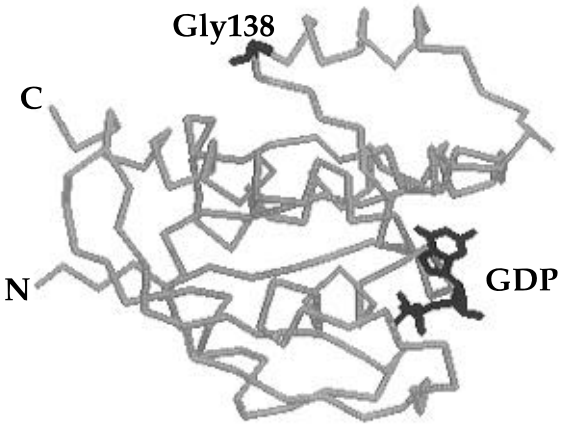

FIGURE 10: Three-dimensional model of H-ras in the GDP-bound state, displaying the spatial relationship between the nucleotide-binding site and Gly138. The model was created from the published coordinates (Tong et al., 1991) using the RasMac application.

$$E = (R_0/R)^6/[1 + (R_0/R)^6]$$

where R_0 is the Förster distance between MantdGDP−Cdc42Hs/sNBD−Cdc42 or MantdGTP−Cdc42Hs/sNBD−Cdc42 and *R* is the distance between the two probes. R_0 was calculated as described in the Materials and Methods section. On the basis of the values of R_0, we calculated a distance of 32 ± 0.5 Å between either MantdGDP or MantGTP and sNBD.

Sequence analysis of peptides incorporating the sNBD fluorescent moiety [using procedures that have been described previously (Erickson et al., 1995)] identified Lys150 as the most likely site of reaction (Harvard Microchemistry, data not shown), although we cannot absolutely rule out the possibility that other secondary reaction sites exist which were not detected by peptide sequence analysis. Careful sequence alignment of Cdc42Hs with H-ras indicated that Lys150 of Cdc42Hs is equivalent to Gly138 of H-ras (Sutcliffe et al., 1994). We used the published coordinates of the H-ras crystal structures to determine the distance between the C_α of Gly138 and the 3′-OH of the ribose ring for both the GDP and GTP states of H-ras (Tong et al., 1991; Pai et al., 1990) and found these distances to be 30.7 and 30.8 Å, respectively, which are consistent with our energy transfer measurements. The spatial relationship between the nucleotide-binding site and Gly138 on H-ras (GDP) is shown in Figure 10.

DISCUSSION

We have developed a fluorescent readout for the nucleotide state of Cdc42Hs using the environmentally sensitive fluorescent probe succinimidyl 6-[(7-nitrobenz-2-oxa,1,3-diazol-4-yl)amino]hexanoate (sNBD). The fluorescence of the sNBD moiety of sNBD−Cdc42Hs is sensitive to the nucleotide state, with the GTP-bound form of sNBD−Cdc42Hs having an ~20% greater quantum yield than the GDP-bound form. The time courses for GTP binding and hydrolysis of sNBD-Cdc42Hs are indistinguishable from unlabeled Cdc42Hs, as confirmed through monitoring both tryptophan and sNBD fluorescence. In addition, we have shown that

sNBD-Cdc42Hs is able to interact with regulators of the GTP-binding/GTPase cycle of Cdc42Hs, specifically the *S. cerevisiae* GEF of Cdc42 (the Cdc24 gene product), and Cdc42Hs−GAP. Although we have previously developed fluorescent readouts to monitor the GTP-binding/GTPase cycle using either the intrinsic tryptophan fluorescence of Cdc42Hs or fluorescent derivatives of nucleotides (Leonard et al., 1994), both of these methods have limitations. Using intrinsic tryptophan fluorescence as a readout for nucleotide state limits the concentrations of effector/regulatory proteins that can be added to the cuvette, as the tryptophan fluorescence of these proteins will mask changes in the tryptophan fluorescence of Cdc42Hs. Although this can be overcome by the use of fluorescent nucleotides, a disadvantage is that they cannot be efficiently used to monitor real-time changes in the nucleotide state of Cdc42Hs, such as the hydrolysis of GTP to GDP. Neither of these limitations is a problem when the sNBD-labeled Cdc42Hs is used. The sNBD fluorescence provides an extremely sensitive monitor for guanine nucleotide binding and can read out the functional interactions of a GEF or a GAP with Cdc42Hs, even when high concentrations of these regulators are used. We hope to be able to explore the possibility in more detail of using the sNBD fluorescence of sNBD−Cdc42Hs to directly monitor the binding of the putative Cdc42Hs target, the PAK serine/threonine kinase (Manser et al., 1994).

Our resonance energy transfer distance measurements indicate that the sNBD-reactive lysine is ∼32 Å away from the guanine nucleotide binding site. The main uncertainty regarding the measured distances between the reactive lysine and the nucleotide binding site on Cdc42Hs comes from the size of the succinimidyl NBD (and its extension via the side chain of the lysine molecule from the protein backbone) and the size of the *N*-methylanthraniloyl (Mant) moiety. These factors could certainly alter the measured distance by as much as 10 Å, although the exact extent depends on the relative orientation of the donor and acceptor moieties. Glycine 138 on the Ras molecule corresponds to lysine 150 on Cdc42Hs, and examination of the coordinates of the X-ray crystal structure for Ha-ras indicates that a distance of 31 Å separates Gly138 and the ribose hydroxyl moieties of bound guanine nucleotide. Thus, as well as can be determined at this point, the measured distance between sNBD and Mant guanine nucleotides bound to Cdc42Hs seems reasonable. Overall, these results suggest that the GTP-induced conformational changes in Cdc42Hs can impact on sites that are distant from the switch I and switch II domains and are located a significant distance from the guanine nucleotide binding domain. At present, we do not know the functional implications of this GTP-induced conformational change within the vicinity of Lys150. However, future studies will be directed toward determining whether Cdc42Hs regulatory proteins and/or target molecules that would be expected to respond to differences in the nucleotide-bound state of Cdc42Hs in fact bind in the vicinity of this region.

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