Fluorescence Assays of Cdc42 Interactions with Target/Effector Proteins†

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ABSTRACT: The goal of these studies was to examine the interactions between the GTP-binding protein Cdc42 and its target/effectors by fluorescence spectroscopy. We have inserted fluorescent reporter groups at two distinct sites on Cdc42: *N*-methylanthraniloyl- (Mant-) derivatized nucleotides were complexed to the nucleotide-binding site of Cdc42, while a fluorescent succinimidyl ester was covalently attached to lysine 150. These two sites are separated by about 30 Å on the Cdc42 molecule. Thus, the attachment of reporter groups to these sites enables the effects of target/effector binding to be viewed over a significant portion of the GTP-binding protein surface. We have taken advantage of fluorescence changes occurring at both sites to compare the interactions of activated Cdc42 with the limit binding domains from the following target/effectors: the serine/threonine kinase PAK, the tyrosine kinase ACK-2, and the RasGAP-related protein IQGAP. In addition, a unique lysine residue on the Cdc42-binding domain of ACK-2 (GBD-ACK) was covalently modified with a fluorescent succinimidyl ester. The distances separating this reactive lysine from the nucleotide binding site and lysine 150 of Cdc42 were determined by fluorescence resonance energy transfer and yielded a picture for Cdc42/GBD-ACK interactions that is consistent with recent NMR structural determinations for Cdc42/effector complexes. The changes in reporter group fluorescence at the reactive lysine of GBD-ACK, which were induced by the binding of activated Cdc42, were also examined. Overall, the results of these studies suggest not only that Cdc42 can induce conformational changes within an effector but also that in a reciprocal fashion the target/effectors induce conformational changes that span a significant distance on the GTP-binding protein.

The Ras-like low molecular weight GTP-binding proteins form a superfamily whose members are involved in a variety of biological pathways, which include the regulation of cell growth and differentiation, vesicular transport, and cytoskeletal organization (*1*). A major interest of our laboratory has been directed toward studying the molecular regulation and biochemical activities of one such GTP-binding protein, Cdc42, which is a member of the Rho subfamily of the Ras superfamily. Other members of this subfamily include the Rho (A, B, C, D, and G) and Rac (1 and 2) proteins (*2*). In mammalian cells, these proteins have been implicated in several diverse pathways, including the organization of the actin cytoskeleton (*3*, *4*), the activation of nuclear (stress-regulated) MAP kinases (*5*−*8*), and cell cycle progression (*9*). Overall, a number of lines of evidence now argue that the Rho subfamily proteins play critical roles in the maintenance of proper cell growth and development.

Cdc42 participates in these pathways in the capacity of a molecular switch. It is active in the GTP-bound state and inactive in the GDP-bound state. When Cdc42 is active, it is able to bind specific target/effector proteins, thereby initiating the signal transduction pathways mediated by these effector molecules. A number of effectors have been identified for Cdc42. These include the p21-activated kinases (PAKs) (*10*−*13*), the Cdc42-activated tyrosine kinases ACK 1 and 2 (*14*, *15*), IQGAP (*16*, *17*), and phosphatidylinositol-3-kinase (PI 3-kinase) (*18*). In many cases, the specific domains that these effectors use to interact with Cdc42 have been identified. For example, the domain through which the PAKs and ACKs interact with Cdc42 contains a 12−16-residue consensus sequence known as the Cdc42- and Rac-interactive binding (CRIB) motif (*19*). IQGAP interacts with Cdc42 through a domain that shares sequence similarity to the GTPase-activating protein (GAP) for Ras, while the PI 3-kinase uses a domain that is found in members of the Rho-GAP family.

In this work, we have begun the development of fluorescence assays to study the interactions between Cdc42 and its target/effectors at high resolution and in real time. We are now able to insert fluorescent reporter groups at two distinct sites on Cdc42. Specifically, a fluorescent reporter group [*N*-methylanthraniloyl- (Mant-) derivatized guanine nucleotides] can be used to label the nucleotide binding site of Cdc42 (*20*) and a fluorescent succinimidyl ester can be covalently attached to lysine 150 of Cdc42 (*21*, *22*). We have estimated that the distance separating these two sites (as measured by fluorescence resonance energy transfer) is about 30 Å (*21*), which is in reasonably good agreement with the distances obtained from the recently solved NMR and X-ray crystallographic structures of Cdc42 (*23*, *24*).

Attachment of a fluorescent reporter group at a specific site on Cdc42 should make it possible to read out conformational changes that occur within the vicinity of the reporter

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group upon the binding of effector molecules. In a previous study, we demonstrated that the interactions between the GTPase-binding domain of PAK (designated GBD-PAK) and Cdc42 caused an increase in Mant fluorescence (*25*). In this study, we have examined the fluorescence changes at both the nucleotide binding site and lysine 150 of Cdc42, as a result of the binding of different effector molecules, including the GBD-PAK, as well as the GTPase-binding domains from ACK-2 (GBD-ACK) and IQGAP (GBD-IQ). In addition, we have covalently modified the single lysine residue (lysine 481) on GBD-ACK with a fluorescent reporter group. This has allowed us to examine whether the binding of Cdc42 also induces conformational changes within this effector molecule.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Cdc42 and the GTPase-binding domains of ACK (GBD-ACK) and PAK (GBD-PAK) were expressed as GST fusion proteins. The expression and purification of these proteins were carried out by standard procedures and the GST was then released by thrombin treatment (*21*). The recombinant GTPase-binding domain of IQGAP (GBD-IQ) was a gift from Dr. Matthew J. Hart (Onyx Pharmaceuticals). The protein was expressed with a Glu-Glu (EEEEYMPME) tag and then purified with an anti-Glu-Glu resin (Covance; Berkeley, CA).

Covalent Modification of Proteins with Fluorescent Probes. The preparation of Cdc42 covalently modified with the fluorescent probe sNBD has been previously described (*21*). In this study, Cdc42 was covalently modified with either sNBD or (diethylamino)coumarin (DEAC), and GBD-ACK was covalently modified with sNBD by using an identical procedure. The stoichiometry of fluorescent probe per protein molecule was ~1, based on protein determination by the Cu^+/bicinchoninic acid complex (Pierce Chemicals, Inc.) with bovine serum albumin as a standard, and the absorbance of the fluorescent moiety ($\epsilon_{max,463nm} = 22\,000\ M^{-1}\ cm^{-1}$ for sNBD and $\epsilon_{max,432nm} = 57\,000\ M^{-1}\ cm^{-1}$ for DEAC). For Cdc42, we had previously identified lysine 150 as the site of modification by the fluorescent succinimidyl ester (*23*). In the case of GBD-ACK, this domain has a unique lysine residue (lysine 481), which serves as the site of modification by the fluorescent succinimidyl ester.

N-Methylanthraniloyl Deoxyguanine Nucleotides. MantdGDP and MantdGTP were synthesized by the procedure described by Hiratsuka (26).

Fluorescence Spectroscopy. The fluorescence measurements were made on an SLM 8000c spectrofluorometer operated in the photon-counting mode. Samples were stirred continuously and thermostated at 25 °C in buffer FM (20 mM Tris, pH 8.0, 50 mM NaCl, and 2 mM $MgCl_2$).

Calculations. In this study, fluorescence resonance energy transfer measurements were made with two different donor/acceptor pairs: Mant/sNBD and DEAC/sNBD. The Förster distance R_0 (in angstroms) for each donor/acceptor pair was calculated from the equation:

$$R_0 = (9.79 \times 10^3)(Jk^2Q_Dn^{-4})^{1/6} \quad (1)$$

where J is an integral reflecting the spectral overlap of donor emission and acceptor absorption, k^2 is a factor characterizing the relative orientation of donor and acceptor dipoles, Q_D is the quantum yield of the donor probe, and n is the refractive index of the medium. We used a value of 1.33 for n. Q_D was determined by comparing the donor fluorescence to a sodium fluorescein standard and yielded values of 0.123 and 0.077 for GBD-ACK complexed to DEAC-Cdc42-GTP and Cdc42-MantdGTP, respectively. A value of $^2/_3$ for k^2 was used with the assumption that dipole orientations were random. To determine the error limits of our distance measurements as a result of making this assumption, the steady-state polarizations (p) of the labeled proteins were measured. In all cases, p values less than 0.2 were obtained, corresponding to an error in the distance between the donor and acceptor probes of about 10% (*27*). R_0 values of 28.2 and 34 Å were calculated for the Mant/sNBD and DEAC/sNBD donor/acceptor pairs, respectively.

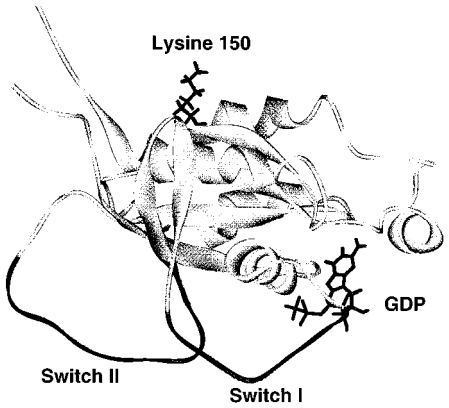

FIGURE 1: Structure of Cdc42-GDP: NMR solution structure of Cdc42-GDP (residues 1−182) showing the positions of the nucleotide binding site (GDP), lysine 150, and the switch I and switch II loops. The structure was drawn with the WebLab ViewerLite application (Molecular Simulations, Inc).

Calculation of Dissociation Constants. The K_d values for the binding of Cdc42 to the effectors GBD-ACK, GBD-PAK, and GBD-IQ were calculated from

$$F = F_i + F_f\{[(K_d + L_T + R_T) - [(K_d + L_T + R_T)^2 - 4R_TL_T)^{1/2}]/2R_T\} \quad (2)$$

where F is the change in fluorescence over initial fluorescence ($-\Delta F/F_0$), F_i is the initial value for ($-\Delta F/F_0$), F_f is the final value of ($-\Delta F/F_0$), L_T is the total concentration for either GBD-ACK, GBD-PAK, or GBD-IQ, and R_T is the total concentration of Cdc42. Fits were generated by IgorPro wavemetrics software.

RESULTS

Binding of Effectors to Cdc42 Results in Fluorescence Changes at both the Nucleotide-Binding Site and Lysine 150 of Cdc42. In previous studies, we have shown that *N*-methylanthraniloyl- (Mant-) derivatized guanine nucleotides can be used as fluorescent reporter groups for the nucleotide-binding site of Cdc42 (*20*). We have also been able to covalently attach a fluorescent reporter group to lysine 150 of Cdc42, using the succinimidyl esters of either sNBD or DEAC. The juxtaposition of these two sites on Cdc42 is depicted in Figure 1. In this study, we examined whether the binding of effector molecules to Cdc42 caused changes in the reporter group fluorescence at one or both of these sites. The limit Cdc42-binding domains for ACK-2 (GBD-

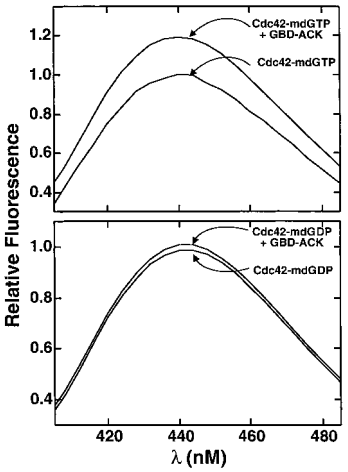

FIGURE 2: Mant fluorescence of Cdc42-MantdGTP is sensitive to GBD-ACK binding. (Upper panel) MantdGTP (30 nM) was incubated in buffer FM with EDTA (6.7 mM) and Cdc42 (200 nM), in either the presence or absence of GBD-ACK (600 nM). The emission spectra were obtained by exciting the sample at 350 nm. (Lower panel) Identical to the upper panel except that MantdGDP was used instead of MantdGTP.

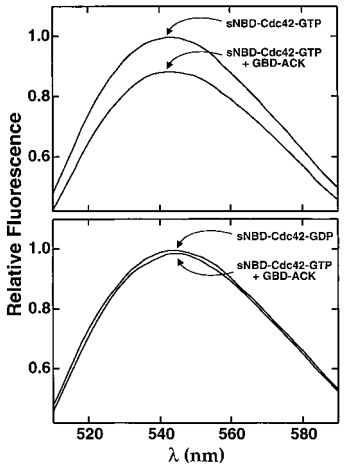

FIGURE 3: sNBD fluorescence of sNBD-Cdc42-GTP is sensitive to GBD-ACK binding. (Upper panel) sNBD-Cdc42 (150 nM) was incubated in buffer FM with EDTA (6.7 mM) and GTP (12.5 μM), in either the presence or absence of GBD-ACK (600 nM). The emission spectra were obtained by exciting the sample at 488 nm. (Lower panel) Identical to the upper panel except that GDP was used instead of GTP.

ACK) (residues 444−507), PAK (GBD-PAK) (residues 63−135), and IQGAP (GBD-IQ) (residues 864−1657) were compared. The binding domains of ACK and PAK share a common sequence, designated the CRIB motif, while the GBD domain of IQGAP shares sequence similarity with the GTPase-activating protein for Ras. Thus, we might expect IQGAP to bind Cdc42 at a site distinct from that used by CRIB motif-containing proteins such as PAK and ACK and that this might be reflected by different effects on reporter group fluorescence.

Figure 2 (upper panel) shows that the addition of GBD-ACK to Cdc42 bound to MantdGTP resulted in an approximately 20% increase in the Mant fluorescence. The fluorescence enhancement was specific for Cdc42 in the GTP-bound state, as the addition of similar amounts of GBD-ACK to Cdc42-MantdGDP did not result in any significant fluorescence changes (Figure 2, lower panel). The binding of ACK to activated Cdc42 also altered the local environment surrounding lysine 150 (which is ∼30 Å away from the nucleotide-binding site), as evidenced by an approximately 12% decrease in the fluorescence of the sNBD reporter group attached to this lysine (Figure 3, upper panel). As expected, the addition of GBD-ACK to sNBD-Cdc42-GDP did not result in any significant fluorescence changes (Figure 3, lower panel). For both Cdc42-MantdGTP and sNBD-Cdc42-GTP, these fluorescence changes were blocked when excess unlabeled Cdc42-GTP was added prior to the addition of GBD-ACK (data not shown).

Given that the binding of GBD-ACK to Cdc42 affected reporter group fluorescence in the vicinity of both the nucleotide binding site and lysine 150, we next examined whether GBD-PAK and GBD-IQ induced similar fluorescence changes upon binding to Cdc42. As shown in Figure 4, upper panel, GBD-ACK and GBD-PAK had almost identical effects on Mant fluorescence (an ∼20% increase) (traces 2 and 3, respectively), while GBD-IQ caused a 35% increase in Mant fluorescence (trace 4). The binding of both GBD-PAK and GBD-ACK caused decreases in the reporter group fluorescence of the sNBD-Cdc42-GTP species, with

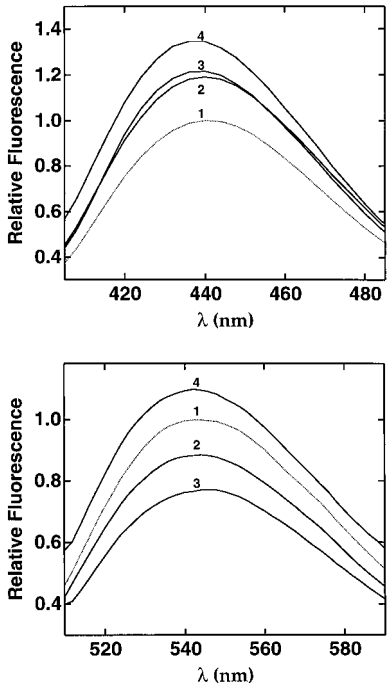

FIGURE 4: Mant and sNBD fluorescence of Cdc42 is differentially sensitive to the binding of different effector molecules. (Upper panel) MantdGTP (30 nM) was incubated in buffer FM with EDTA (6.7 mM) and Cdc42 (200 nM), either in the absence of effector molecules (1) (dotted line) or in the presence of GBD-ACK (600 nM) (2), GBD-PAK (500 nM) (3), or GBD-IQ (500 nM) (4). The emission spectra were obtained by exciting the sample at 350 nm. (Lower panel) sNBD-Cdc42 (150 nM) was incubated in buffer FM with EDTA (6.7 mM) and GTP (12.5 μM), in either the presence or absence of effectors as described for the upper panel. The emission spectra were obtained by exciting the sample at 488 nm.

the magnitude of the fluorescence change being greater upon the binding of PAK compared to ACK (20% versus 12%, respectively) (Figure 4, lower panel, traces 3 and 2, respectively). On the other hand, GBD-IQ caused an ∼5% increase in fluorescence (Figure 4, bottom panel, trace 4). Taken together, these results are consistent with the suggestion that PAK and ACK bind in a manner that causes similar

Table 1: Determination of the Dissociation Constants (K_ds) for the Binding of Effector Molecules to Cdc42[a]

	K_d (nM)	
effector	mdGTP	sNBD
GBD-ACK	38	30
GBD-PAK	50	20
GBD-IQ	28	ND

[a] To determine the K_d values for the binding of effector molecules to Cdc42-MantdGTP, MantdGTP (30 nM) was incubated in buffer FM with EDTA (6.7 mM) and Cdc42 (200 nM), and the Mant fluorescence was monitored (excitation = 350 nm, emission = 440 nm). Aliquots of effector molecules (either GBD-ACK, GBD-PAK, or GBD-IQ) were added so as to titrate the change in fluorescence. For each effector molecule, the change in fluorescence was plotted versus the effector concentration, and the points were fit to estimate the dissociation constant (K_d) of the effector for Cdc42, as described in the Experimental Procedures section. To determine the K_d values for the binding of effector molecules to sNBD-Cdc42, the sNBD-Cdc42 (100 nM) was incubated in buffer FM with EDTA (6.7 mM) and sNBD fluorescence was monitored (excitation = 488 nm, emission = 545 nm). Aliquots of effector molecules (either GBD-ACK, GBD-PAK, or GBD-IQ) were added so as to titrate the change in fluorescence. For each effector molecule, the change in fluorescence was plotted versus the effector concentration, and the points were fit to estimate the dissociation constant (K_d) of the effector for Cdc42, as described in the Experimental Procedures section.

conformational changes within Cdc42, whereas the binding of IQGAP results in distinct changes.

We titrated the fluorescence changes resulting from the interaction of these effectors with Cdc42, to estimate the K_d values for the different binding interactions (Table 1). In all cases, we obtained apparent K_d values ranging from 20 to 50 nM, indicating that although the different effectors appeared to induce distinct conformational changes within Cdc42, they exhibited similar binding affinities for the activated GTP-binding protein.

Use of a Fluorescence Reporter Group as a Probe for Cdc42 Binding to an Effector Molecule. GBD-ACK was chosen for these studies because it contains a unique lysine residue (lysine 481) that can be covalently modified by a fluorescent succinimidyl ester without loss of its binding interaction with Cdc42 (T. Nomanbhoy, unpublished results). In addition, an NMR structure for the Cdc42/GBD-ACK complex has recently been obtained (*28*), thus providing for some interesting comparisons between this structure and our resonance energy transfer mapping experiments. We modified lysine 481 on GBD-ACK with sNBD, as this would serve as an energy transfer acceptor for Mant derivatives at the nucleotide binding site of Cdc42. In addition, we also labeled lysine 150 of Cdc42 with the fluorescent reporter group (diethylamino)coumarin (DEAC). The emission spectrum of DEAC overlaps well with the excitation spectrum of sNBD and serves as a suitable energy transfer donor to the sNBD-labeled GBD-ACK. The overlap between the donor emission spectrum (Mant or DEAC) and the acceptor excitation spectrum (sNBD) is depicted in Figure 5.

The results of the fluorescence energy transfer experiments between Cdc42-MantdGTP and sNBD-GBD-ACK and between DEAC-Cdc42-GTP and sNBD-GBD-ACK are depicted in Figures 6 and 7, respectively. As described in Figure 2, the binding of GBD-ACK to Cdc42-MantdGTP resulted in an ~20% increase in Mant fluorescence. The addition of sNBD-GBD-ACK to Cdc42-MantdGTP, however, caused a

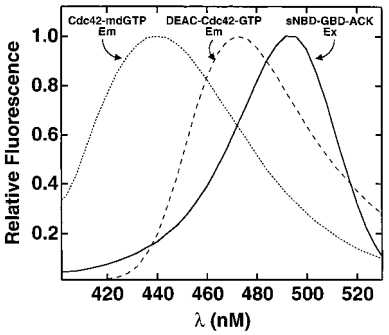

FIGURE 5: Overlap between the emission spectra of Cdc42-MantdGTP and DEAC-Cdc42-GTP and the excitation spectrum of sNBD-GBD-ACK. The emission spectrum of Cdc42-MantdGTP was obtained by incubating MantdGTP (30 nM) in buffer FM with EDTA (6.7 mM), Cdc42 (200 nM), and GBD-ACK (500 nM), and the sample was excited at 350 nm (dotted line). The emission spectrum of DEAC-Cdc42-GTP was obtained by incubating DEAC-Cdc42 (150 nM) in buffer FM with EDTA (6.7 mM), GTP (12.5 μM), and GBD-ACK (500 nM), and the sample was excited at 410 nm (dashed line). The excitation spectrum of sNBD-GBD-ACK was obtained by incubating sNBD-GBD-ACK (80 nM) in buffer FM in the presence of EDTA (6.7 mM), GTP (12.5 μM), and Cdc42 (600 nM), and fluorescence was recorded at 530 nm (solid line).

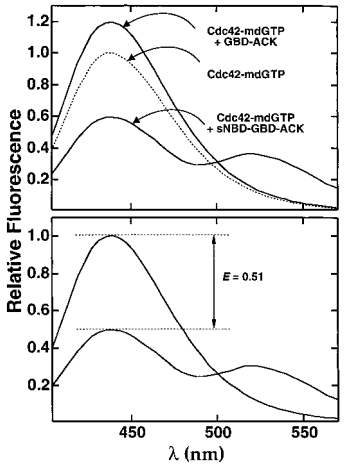

FIGURE 6: Fluorescence energy transfer between Cdc42-MantdGTP and sNBD-GBD-ACK. (Upper panel) MantdGTP (30 nM) was incubated in buffer FM with EDTA (6.7 mM) and Cdc42 (200 nM), either in the absence of effector (dotted line) or in the presence of GBD-ACK (600 nM) or sNBD-GBD-ACK (600 nM). The emission spectra were obtained by exciting the samples at 350 nm. (Lower panel) The spectrum obtained for Cdc42-MantdGTP in the presence of GBD-ACK and the spectrum obtained for Cdc42-MantdGTP in the presence of sNBD-GBD-ACK were normalized with respect to each other so that the emission maximum for Cdc42-MantGTP in the presence of GBD-ACK was 1. This allowed for the determination of the efficiency of energy transfer.

40% decrease in Mant fluorescence (Figure 6, upper panel). This fluorescence decrease was blocked by the presence of either excess unlabeled GBD-ACK or unlabeled Cdc42-GTP (data not shown), indicating that the fluorescence changes reflected a specific interaction between Cdc42-MantdGTP and sNBD-GBD-ACK. The efficiency of energy transfer, E, was calculated from

$$E = 1 - Q_{DA}/Q_D \quad (3)$$

where Q_D and Q_{DA} are the donor quantum yields in the absence and presence of acceptor, respectively. We measured an efficiency of energy transfer of 51% between the Mant

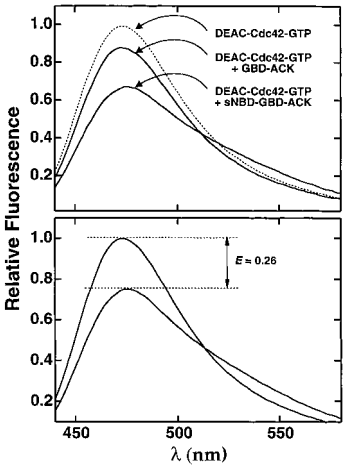

FIGURE 7: Fluorescence energy transfer between DEAC-Cdc42-GTP and sNBD-GBD-ACK. (Upper panel) DEAC-Cdc42 (150 nM) was incubated in buffer FM with EDTA (6.7 mM) and GTP (12.5 μM), either in the absence of added effector (dotted line) or in the presence of GBD-ACK (600 nM) or sNBD-GBD-ACK (600 nM). The emission spectra were obtained by exciting the samples at 410 nm. (Lower panel) The spectrum obtained for DEAC-Cdc42-GTP in the presence of GBD-ACK, and the spectrum obtained for DEAC-Cdc42-GTP in the presence of sNBD-GBD-ACK were normalized with respect to each other so that the emission maximum for DEAC-Cdc42-GTP in the presence of GBD-ACK was 1. This allowed for the determination of the efficiency of energy transfer.

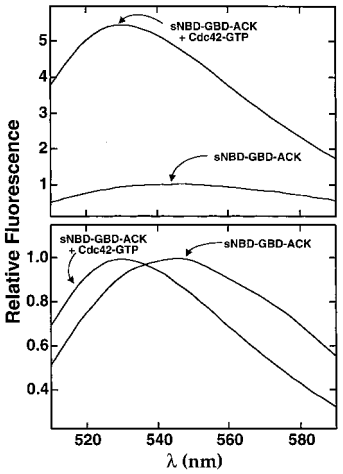

FIGURE 8: sNBD fluorescence of sNBD-GBD-ACK is sensitive to Cdc42-GTP binding. (Upper panel) sNBD-GBD-ACK (80 nM) was incubated in buffer FM with EDTA (6.7 mM) and GTP (12.5 μM), in either the absence or presence of Cdc42 (600 nM). (Lower panel) The spectra obtained in the upper panel were normalized so as to show the shift in the emission maxima between the spectra in the absence and presence of Cdc42-GTP.

moiety on Cdc42 and the sNBD group attached to GBD-ACK (Figure 6, lower panel). We then used the following relationship to calculate the distance between the Mant and sNBD probes:

$$E = (R_0/R)^6/[1 + (R_0/R)^6] \quad (4)$$

where R_0 is the Förster distance for the Mant/sNBD donor/acceptor pair. Using an R_0 value of 28.2 Å for this donor/acceptor pair (see the Experimental Procedures section), we determined that a distance of 28 ± 3 Å separated the Mant moiety at the nucleotide binding site of Cdc42 from the sNBD moiety attached to lysine 481 of GBD-ACK.

For the case of DEAC-Cdc42, the binding of unlabeled GBD-ACK resulted in an ~8% quenching of the DEAC fluorescence. However, the binding of sNBD-GBD-ACK caused a 31% quenching of the DEAC fluorescence (Figure 7, upper panel). This decrease in fluorescence was prevented by an excess of either unlabeled Cdc42-GTP or unlabeled GBD-ACK (data not shown). From eq 3, we calculated an efficiency of energy transfer of about 25% (Figure 7, lower panel). Using an R_0 value of 34 Å for the DEAC/sNBD donor/acceptor pair (described in the Experimental Procedures section), we then measured a distance of 41 ± 4 Å between the DEAC adduct attached to lysine 150 of Cdc42 and the sNBD attached to lysine 481 of GBD-ACK. Thus, the reporter group attached to GBD-ACK, within a Cdc42/GBD-ACK complex, is significantly closer to the nucleotide-binding site compared to the conformationally sensitive lysine 150 of Cdc42. This is in fact consistent with the tertiary structure predicted for the Cdc42/GBD-ACK complex, as solved by NMR spectroscopy (*28*; see below).

We next examined whether the binding of Cdc42-GTP to sNBD-GBD-ACK could influence the fluorescence of the sNBD reporter group, as this might be indicative of Cdc42-induced conformational changes within the effector protein. As shown in Figure 8 (upper panel), the addition of Cdc42-GTP to sNBD-GBD-ACK resulted in a striking 5-fold increase in sNBD fluorescence. The fluorescence enhancement was accompanied by a significant blue shift in the emission maximum from 545 to 530 nm (Figure 8, lower panel). Given that we have found that the attachment of the sNBD reporter group to GBD-ACK does not influence its binding to activated Cdc42, it seems unlikely that these changes in fluorescence represent direct effects of Cdc42 on the sNBD moiety. Rather, it appears that they are the outcome of a Cdc42-induced conformational change within the GBD-ACK that enhances the hydrophobicity of the environment immediately surrounding the sNBD reporter group.

DISCUSSION

In this study, we have demonstrated that the binding of effectors to Cdc42 can cause changes in the fluorescence of reporter groups at the nucleotide-binding site, as well as at lysine 150 of Cdc42. In the case of GBD-ACK, we observed that the binding of this target/effector caused a 20% increase in the Mant fluorescence at the nucleotide-binding site of Cdc42, and an ~12% decrease in the sNBD fluorescence at lysine 150. Considering the fact that GBD-ACK comprises only 64 residues, it is quite remarkable that its binding to Cdc42 results in distinct fluorescence changes at two sites separated by as much as 30 Å. This suggests that the binding of GBD-ACK to a particular site on Cdc42 could result in the transduction of conformational changes across the entire GTP-binding protein.

The binding of GBD-PAK and GBD-IQ to Cdc42 also resulted in fluorescence changes at both the nucleotide-binding site of Cdc42 and lysine 150. PAK binds Cdc42 through a domain that contains the CRIB motif, as does ACK. Like GBD-ACK, GBD-PAK induced a decrease in the sNBD fluorescence of sNBD-Cdc42-GTP, although the

magnitude of the fluorescence change caused by PAK was greater (20% versus 12% for ACK). The binding of GBD-PAK to Cdc42-MantdGTP, however, induced an almost identical increase in Mant fluorescence as that observed for GBD-ACK. IQGAP binds to Cdc42 through a domain that does not bear any sequence similarity to the CRIB motif; instead, the GBD-IQ sequence shows similarity to the Ras-GTPase-activating protein. The binding of GBD-IQ to sNBD-Cdc42-GTP caused a slight increase (5%) in sNBD fluorescence and a more significant increase (35%) in the fluorescence of the Mant moiety within the Cdc42-MantdGTP complex. These results indicate that the mode of binding of IQGAP to Cdc42 is distinct from that for either ACK or PAK. This is further borne out by our recent finding that the binding affinity of IQGAP was decreased for a Cdc42 mutant in which the Rho insert domain (a region unique to the Rho subfamily of GTP-binding proteins, consisting of residues 122−134 in Cdc42) was deleted, while the binding of PAK to this Cdc42 mutant was unaffected (*17*, *29*). Overall, these results suggest that the fluorescence assays we have developed provide a sensitive readout for comparing the binding of Cdc42 to its different target/effector proteins.

The presence of a single reactive lysine residue on GBD-ACK provides for a number of possibilities for monitoring the interactions between this effector and Cdc42. An sNBD moiety was attached to the unique lysine residue of GBD-ACK (lysine 481), and fluorescence resonance energy transfer measurements were made in order to estimate its distance to the nucleotide binding site of Cdc42, occupied by MantdGTP. On the basis of the efficiency of energy transfer, we determined that a distance of approximately 28 Å separated the two sites. We also found that a distance of 41 Å separated the DEAC reporter group attached to lysine 481 of GBD-ACK from the sNBD moiety attached to lysine 150 on Cdc42. We then examined the conformational changes that occurred in the vicinity of lysine 481 of GBD-ACK as a result of its binding to Cdc42. The addition of Cdc42-GTP to sNBD-GBD-ACK induced a 5-fold increase in sNBD fluorescence, as well as a blue shift in the emission maximum from 545 to 530 nm. Taken together, these results indicate that the environment of the reactive lysine residue on GBD-ACK becomes more hydrophobic upon the formation of a Cdc42/GBD-ACK complex.

The NMR solution structure of Cdc42 complexed to ACK-1 (residues 504−545) has been recently solved (*28*). The structure depicts ACK making extensive contacts with Cdc42, especially within its switch I and switch II regions. The close proximity of ACK to the nucleotide binding site is probably responsible for the increase in the Mant fluorescence seen upon ACK binding. Interestingly, ACK does not appear to be in close proximity to lysine 150 of Cdc42, suggesting that the sensitivity of the sNBD fluorescence of sNBD-Cdc42 to ACK binding reflects conformational changes transmitted across the Cdc42 molecule. The results of our fluorescence energy transfer distance measurements regarding the proximity of specific sites on Cdc42, relative to lysine 481 on GBD-ACK, are also consistent with the NMR structure. In particular, arginine 537 of ACK-1, which is the equivalent residue to lysine 481 of ACK-2, is in closer proximity to the nucleotide-binding site of Cdc42, than it is to lysine 150 (*28*). In addition, the increase in the hydrophobicity of the environment surrounding lysine 481 of GBD-ACK, which occurs upon Cdc42-GTP binding, could be due to the formation of a hairpin centered around phenylalanine 478 (phenylalanine 534 in ACK I), which would result in the vicinity of lysine 481 adopting a more globular conformation.

Thus, in summary, the results presented here suggest that the binding of Cdc42 to its target/effector molecules results in conformational changes within both the GTP-binding protein and the effectors. The ability of GBD-PAK to cause relatively widespread conformational changes within Cdc42 has also been suggested from NMR studies (*30*). The underlying reason for effector-induced conformational changes within the GTP-binding protein remains to be determined, although an intriguing possibility is that the binding of a specific effector may need to influence the ability of the GTP-binding protein to recruit or activate other effector/target molecules. It has been recently shown through NMR that the binding of Cdc42 to a limit domain of PAK causes significant conformational changes within the target/effector molecule (*31*). This is as expected, because it has been well established that the binding of activated Cdc42 or Rac to PAK eliminates an autoinhibitory intramolecular interaction within the target/effector and thereby causes a significant stimulation of serine/threonine kinase activity (*11*, *13*). However, the fact that Cdc42 appears to induce conformational changes within the GBD-ACK is interesting, because Cdc42 does not affect ACK tyrosine kinase activity in vitro (*15*), although it is necessary for the activation of ACK-2 in cells. It may be that Cdc42-induced conformational changes within ACK-2 release it from a negative regulator and/or enable the binding of substrates. Future structural studies of full-length ACK-2 (i.e., complete with its tyrosine kinase domain), alone and complexed to Cdc42, should shed light on how the GTP-binding protein-induced conformational change influences ACK activity.

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REFERENCES

1. Macara, I. G., Lounsbury, K. M., Richards, S. A., McKiernan, C., and Bar-Sagi, D. (1996) *FASEB J. 10*, 625−30.
2. Symons, M. (1996) *Trends Biochem. Sci. 21*, 178−81.
3. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) *Mol. Cell. Biol. 15*, 1942−52.
4. Nobes, C. D., and Hall, A. (1995) *Cell 81*, 53−62.
5. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) *J. Biol. Chem. 270*, 27995−8.
6. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell 81*, 1159−70.
7. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell 81*, 1137−46.
8. Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995) *Cell 81*, 1147−57.
9. Olson, M. F., Ashworth, A., and Hall, A. (1995) *Science 269*, 1270−2.
10. Abo, A., Qu, J., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998) *EMBO J. 17*, 6527−40.
11. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) *J. Biol. Chem. 270*, 22731−7.

12. Martin, G. A., Bollag, G., McCormick, F., and Abo, A. (1995) *EMBO J. 14*, 1970−8.
13. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) *Nature 367*, 40−6.
14. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim, L. (1993) *Nature 363*, 364−7.
15. Yang, W., and Cerione, R. A. (1997) *J. Biol. Chem. 272*, 24819−24.
16. Hart, M. J., Callow, M. G., Souza, B., and Polakis, P. (1996) *EMBO J. 15*, 2997−3005.
17. McCallum, S. J., Wu, W. J., and Cerione, R. A. (1996) *J. Biol. Chem. 271*, 21732−7.
18. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) *J. Biol. Chem. 269*, 18727−30.
19. Burbelo, P. D., Drechsel, D., and Hall, A. (1995) *J. Biol. Chem. 270*, 29071−4.
20. Leonard, D. A., Evans, T., Hart, M., Cerione, R. A., and Manor, D. (1994) *Biochemistry 33*, 12323−8.
21. Nomanbhoy, T. K., Leonard, D. A., Manor, D., and Cerione, R. A. (1996) *Biochemistry 35*, 4602−8.
22. Nomanbhoy, T., and Cerione, R. A. (1998) *Methods Mol. Biol. 84*, 237−47.
23. Feltham, J. L., Dotsch, V., Raza, S., Manor, D., Cerione, R. A., Sutcliffe, M. J., Wagner, G., and Oswald, R. E. (1997) *Biochemistry 36*, 8755−66.
24. Nassar, N., Hoffman, G. R., Manor, D., Clardy, J. C., and Cerione, R. A. (1998) *Nat. Struct. Biol. 5*, 1047−52.
25. Leonard, D. A., Satoskar, R. S., Wu, W. J., Bagrodia, S., Cerione, R. A., and Manor, D. (1997) *Biochemistry 36*, 1173−80.
26. Hiratsuka, T. (1983) *Biochim. Biophys. Acta 742*, 496−508.
27. Haas, E., Katchalski-Katzir, E., and Steinberg, I. Z. (1978) *Biochemistry 17*, 5064−70.
28. Mott, H. R., Owen, D., Nietlispach, D., Lowe, P. N., Manser, E., Lim, L., and Laue, E. (1999) *Nature 399*, 384−388.
29. Wu, W. J., Leonard, D. A., Cerione, R. A., and Manor, D. (1997) *J. Biol. Chem. 272*, 26153−8.
30. Guo, W., Sutcliffe, M. J., Cerione, R. A., and Oswald, R. E. (1998) *Biochemistry 37*, 14030−14037.
31. Stevens, W. K., Vranken, W., Goudreau, N., Xiang, H., Xu, P., and Feng, N. (1999) *Biochemistry 38*, 5968−5975.

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