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A Minimal Rac Activation Domain in the Unconventional Guanine Nucleotide Exchange Factor Dock180[†]

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ABSTRACT: Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by catalyzing the exchange of bound GDP for GTP, thereby resulting in downstream effector recognition. Two metazoan families of GEFs have been described: Dbl-GEF family members that share conserved Dbl homology (DH) and Pleckstrin homology (PH) domains and the more recently described Dock180 family members that share little sequence homology with the Dbl family and are characterized by conserved Dock homology regions 1 and 2 (DHR-1 and -2, respectively). While extensive characterization of the Dbl family has been performed, less is known about how Dock180 family members act as GEFs, with only a single X-ray structure having recently been reported for the Dock9−Cdc42 complex. To learn more about the mechanisms used by the founding member of the family, Dock180, to act as a Rac-specific GEF, we set out to identify and characterize its limit functional GEF domain. A C-terminal portion of the DHR-2 domain, composed of approximately 300 residues (designated as Dock180$^{DHR\text{-}2c}$), is shown to be necessary and sufficient for robust Rac-specific GEF activity both in vitro and in vivo. We further show that Dock180$^{DHR\text{-}2c}$ binds to Rac in a manner distinct from that of Rac-GEFs of the Dbl family. Specifically, Ala^{27} and Trp^{56} of Rac appear to provide a bipartite binding site for the specific recognition of Dock180$^{DHR\text{-}2c}$, whereas for Dbl family Rac-GEFs, Trp^{56} of Rac is the sole primary determinant of GEF specificity. On the basis of our findings, we are able to define the core of Dock180 responsible for its Rac-GEF activity as well as highlight key recognition sites that distinguish different Dock180 family members and determine their corresponding GTPase specificities.

Members of the Rho family of GTPases regulate a wide range of cellular activities, including cell-cycle progression, gene transcription, cell migration, cell polarity, and vesicular trafficking, through their abilities to bind to multiple downstream effectors (*1*−*4*). Rho GTPases switch between two states, the GDP-bound inactive state and the GTP-bound active state. Tight regulation of Rho GTPases and their nucleotide-bound state is important for mediating their different cellular functions (*5*). Three main classes of regulatory proteins for Rho GTPases have been identified and characterized. Guanine nucleotide exchange factors (GEFs)[1] include ∼70 mammalian/human proteins that promote the exchange of GDP for GTP on Rho GTPases. GTPase-activating proteins (GAPs) catalyze the hydrolysis of the bound GTP to GDP, and Rho GDP dissociation inhibitors (GDIs) slow nucleotide exchange while serving to sequester Rho GTPases in the cytoplasm (*6*−*8*).

Two families of GEFs have been discovered for Rho GTPases, which we here term the Dbl (*9*) and Dock180 superfamilies (*10*−*12*). The Dbl family members all possess two tandem conserved domains, the Dbl homology (DH) and pleckstrin homology (PH) domains (*13*−*18*). The DH domains of Dbl-GEFs are directly responsible for catalyzing GDP−GTP exchange activity, while the PH domains are important for the localization of these proteins to the plasma membrane. More recently, Dock180-related proteins have been shown to catalyze nucleotide exchange on Rac and/or Cdc42 despite the absence of primary sequence homology to DH domains (*19*, *20*). The functional domain for this unconventional class of GEFs was originally suggested to consist of ∼500 residues located within the C-terminal half of Dock-180-related proteins and has been termed the Dock180 homology region 2 or DHR-2 domain.

Dock180 family members have been implicated in multiple biological pathways that include cell phagocytosis (*21*, *22*), cell migration (*21*, *23*−*25*), tumor suppression (*26*), and axonal outgrowth (*27*, *28*). Dock180 is the founding member of the family and functions as a Rac-specific GEF. Full-length Dock180 shows much lower GEF activity than the isolated DHR-2 domain, apparently because of autoinhibition (*29*, *30*). Relief of the autoinhibited state in cells has been suggested to result upon binding to the accessory protein Elmo (*19*, *31*−*34*).

Recently, X-ray crystal structures were reported for the DHR-2 domain of one member of the Dock180 family, Dock9 (Zizimin 1), bound to different nucleotide-bound states of Cdc42 (*35*). This work has provided a first glimpse of how a Dock180 family member functions in catalyzing GDP−GTP exchange. However, as Dock9 is a Cdc42-specific GEF, we still do not have a detailed picture of how a Rac-specific GEF for the Dock180 family functions and confers specificity for Rac. Moreover, Dock180

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[1]Abbreviations: DHR-2, Dock180 homology region 2; Dock180$^{DHR\text{-}2c}$, C-terminus of DHR-2 in Dock180 (residues 1335−1657); DH, Dbl homology; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor; Mant-GDP, 2′,3′-*O*-(*N*′-methylanthraniloyl)guanosine 5′-*O*-diphosphate.

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appears to exhibit higher catalytic GEF activity, compared to Dock9, suggesting that the founding member of this GEF family may possess some important distinguishing features with regard to its mechanism of action.

In this study, we set out to define a limit functional domain for Dock180 as an important first step for mechanistic studies and ultimately for high-resolution structural characterizations. During the course of our efforts to obtain an active limit functional domain for Dock180, we modeled the domain structure of the C-terminus of DHR-2 and isolated a stable region from Dock180 that can be expressed in high yield and exhibits full Rac-GEF activity. Here we show that this fragment, designated as Dock180^{DHR-2c} (or sometimes simply DHR-2c), is fully active despite lacking the upstream helical domain that purportedly mediates dimerization of DHR-2 domains in Dock9 and has been suggested to be necessary for the full activation of its GEF activity (*35*). We provide evidence that this defined region of DHR-2 harbors specific recognition sites that allow for the discrimination between Rac and Cdc42 by Dock subfamilies A (Rac-specific) and D (Cdc42-specific) (*20*). Moreover, by specifically comparing the activity of the tandem DH-PH domains of the Rac-specific GEF Tiam-1 with that of the Rac-specific Dock180^{DHR-2c}, we define a key contact made by Ala^{27} in Rac that is necessary together with Trp^{56} for Dock180^{DHR-2c} recognition and is absent in the functional coupling of DH-PH domains to Rac.

EXPERIMENTAL PROCEDURES

Plasmid Constructs. The Dock180 plasmid was a gift from M. Matsuda (Kyoto University, Kyoto, Japan). To obtain the clone of the full-length DHR-2 domain (amino acids 1178−1657), a polymerase chain reaction (PCR) was performed using the Dock180 plasmid as template DNA and primers 5′-GCGGATCCATGGAAAGGCTTTTGGAT-3′ and 5′-CGGAATTCTCACGATGAGAGGGAAGAGA-3′. The PCR product was cloned into the pET28a plasmid (Novagen) using BamHI and EcoRI restriction sites. The constructs encoding the DHR-2n (residues 1178−1334) and DHR-2c (residues 1135−1657) subdomains were generated by PCR using DHR-2 as the template and cloned into both pET28a and pKH-3 plasmids. The different Rac and Cdc42 point mutants, and the Dock180$^{DHR-2c/M1524L}$ mutant, were generated using the QuikChange site-directed mutagenesis kit (Stratagene).

Protein Expression and Purification. Single colonies of *Escherichia coli* BL21(DE3) containing target plasmids were inoculated in 10 mL of LB medium with 50 μg/mL kanamycin or 100 μg/mL carbenicillin (RPI) and cultured overnight at 37 °C. These cultures were subsequently used to inoculate 1 L of LB medium with antibiotic in a shaking incubator at 37 °C. The large-scale cultures were incubated to an OD_{600} of 0.6 and induced with IPTG (RPI) (final concentration of 200 μM) at room temperature overnight. Bacteria were harvested by centrifugation at 5000 rpm for 10 min, and the pellets were resuspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 500 mM NaCl] with 10 μg/mL leupeptin and 10 μg/mL aprotinin. For DHR-2, DHR-2c, and DHR-2n, the suspensions were sonicated on ice and the resulting lysates were cleared by centrifugation at 20000*g* for 30 min at 4 °C. The supernatants were collected and incubated with nickel-chelating beads (Amersham) for 30 min on ice. The beads were washed with 100 mL of lysis buffer containing 40 mM imidazole until no significant protein was detected in the wash buffer. The proteins were eluted with lysis buffer containing 200 mM imidazole, concentrated to ~200 μM, and stored at −80 °C for further use. Rac and Cdc42 were expressed as GST fusion proteins (*36*) using procedures similar to those described above, and the supernatants were collected and applied to a glutathione-Sepharose column. After binding, the beads were washed extensively with lysis buffer, and the proteins were then eluted using the same buffer with 10 mM glutathione adjusted to neutral pH. The eluted proteins were applied to PD-10 desalting columns (GE Healthcare) to remove glutathione and concentrated to ~300 μM. Mant-GDP-Rac and Mant-GDP-Cdc42 were prepared by mixing Rac or Cdc42 with a 10-fold excess of Mant-GDP in lysis buffer with 10 mM EDTA for 10 min followed by the addition of excess $MgCl_2$ to quench the excess EDTA. The mixture was applied to a PD-10 desalting column equilibrated in lysis buffer to remove unbound Mant-GDP.

In Vitro GEF Assays. All fluorescence measurements were taken using a Varian Eclipse fluorescence spectrophotometer. Samples were stirred continuously and thermostated at 25 °C in HMA buffer [20 mM Hepes (pH 8.0), 5 mM $MgCl_2$, and 1 mM NaN_3]. In vitro GEF assays used Mant-GDP as a probe to monitor the extent of nucleotide exchange on GTPases. Mant-GDP was added to HMA buffer to a final concentration of 1 μM. Different concentrations of Rho GTPases (Rac and Cdc42) and their mutants were added to the solution together with various concentrations of GEF proteins. The Mant-GDP fluorescence changes were monitored at 25 °C using an excitation wavelength of 340 nm and an emission wavelength of 440 nm. All measurements were repeated at least three times. When the turnover rates of the GEF proteins were measured, Rac was preloaded with Mant-GDP and the decrease in fluorescence was detected as different concentrations of Rac were mixed with GEF proteins and excess GDP.

GST-Rac Pull-Down Assays. To check the binding of DHR-2c to Rac, GST-Rac (0.3 nmol) was prebound to 15 μL of glutathione-Sepharose beads while the same amount of DHR-2c was added to the beads in the presence of 5 mM EDTA. The negative control tube contained beads and DHR-2c (no GST-Rac). The mixtures were rotated at 4 °C for 30 min and then centrifuged at 16000*g* for 1 min. The supernatant was discarded. The beads were washed (three times) with buffer and loaded onto an sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel. To check the nucleotide binding preference of different DHR-2c constructs, glutathione-Sepharose beads preloaded with GST-Rac were mixed with DHR-2c and excess GDP or GTPγS in EDTA (final concentration of 10 mM)-containing buffer. Excess $MgCl_2$ (20 mM) was added to the solution after 15 min and the mixture incubated for an additional 15 min. The beads were washed as described above, and the binding was detected by Colloidal Blue staining following SDS−PAGE.

Indirect Immunofluorescence. The cells were transfected with the plasmids of interest and then plated on acid coverslips overnight. The cells were fixed on the coverslip in 4% formaldehyde in PBS for 20 min at room temperature and rinsed (three times) with PBS. Triton X-100 (0.1%) was added to permeabilize the cells, after which the cells were rinsed with PBS (three times). Incubations were performed with primary and secondary antibodies in PBS supplemented with 2% BSA. The cells were again washed with room-temperature PBS (three times) after each incubation.

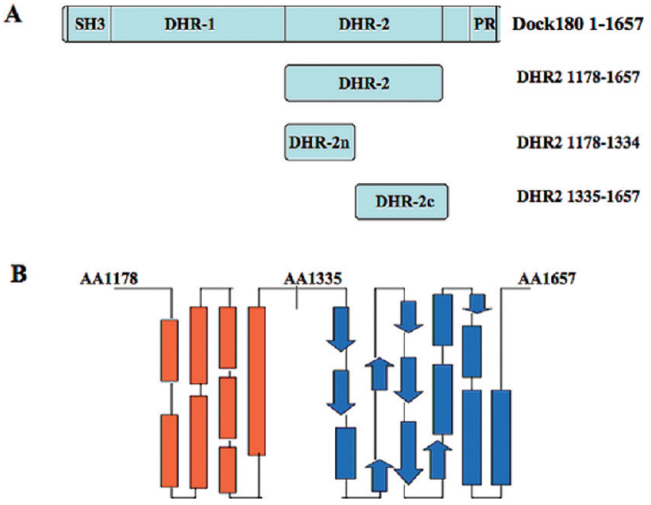

FIGURE 1: Dock180 constructs examined in this study. (A) Schematic representation of the Dock180 and DHR-2 constructs that were used to study the in vivo and in vitro activation of Rac. (B) Secondary structure prediction of the DHR-2 domain of Dock180 using J-pred.

PBD Assays. Cells transfected with plasmids of interest were harvested with MBL buffer (magnesium-containing lysis buffer) that contained 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Whole cell lysates were cleared by centrifugation, and the supernatants were used to assess the total amount of Rac. The remaining extract was combined with 2 volumes of lysis buffer and the recombinant Rac/Cdc42-binding domain of Pak [i.e., designated as GST-PBD (Upstate) (*37*)] coupled to glutathione beads and incubated for 30 min at 4 °C. The beads were washed (three times) with lysis buffer and eluted in SDS−PAGE sample buffer. Aliquots of both total cell extracts and the eluents from the PBD beads were immunoblotted with the anti-Rac polyclonal antibody (Santa Cruz) and visualized with ECL reagents from Amersham following the manufacturer's instructions.

RESULTS

Identifying a Stable and Functionally Active GEF Domain for Dock180. The functional DHR-2 domain was originally suggested to comprise a conserved region present in all Dock180 family members. It is composed of approximately 500 amino acids (residues 1111−1657 for Dock180), with sequence alignment of all the DHR-2 domains revealing a low degree of primary sequence identity among different members (i.e., 18% between Dock180 and Dock9) (*20*). Although we and others have found that the bacterially expressed, complete DHR-2 domain from Dock180 is active in vitro, it is relatively unstable and easily denatured, whereas a shorter construct lacking the 70 N-terminal amino acids of DHR-2 yielded a more stable domain [amino acids 1178−1657 (Figure 1A)] that could be expressed in bacteria in good yield and possessed nucleotide exchange activity. However, further purification of this DHR-2 construct for biochemical and structure−function studies revealed that it exhibited a tendency to aggregate, especially in solutions containing < 300 mM NaCl. Thus, it has been difficult to routinely use this domain in nucleotide exchange experiments for comparison with other known GEFs.

Further secondary structure analysis and homology modeling of DHR-2, which included our taking advantage of the recently

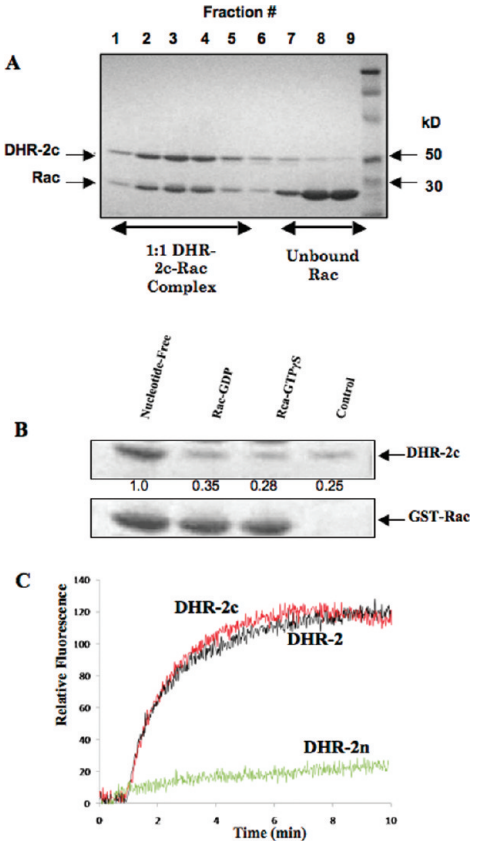

FIGURE 2: Dock180^{DHR-2c} activates Rac in vitro and in vivo. (A) Complex between the purified Dock180^{DHR-2c} domain (designated DHR-2c in the figure) and Rac. Colloidal Blue-stained SDS−PAGE was performed on fractions obtained from G75 gel filtration chromatography. The samples shown were obtained from successive 4 mL fractions of the gel filtration elution profile. (B) Preference of DHR-2c in binding to nucleotide-free Rac. Colloidal Blue-stained SDS−PAGE of GST-Rac affinity precipitation of GDP- or GTPγS-bound Rac. Each of the GST-Rac bead samples was incubated with 1 μg of purified DHR-2c, washed, and analyzed via SDS−PAGE and Colloidal Blue staining. (C) The C-terminal domain of the DHR-2 domain of Dock180 (DHR-2c) (600 nM) (red) shows a similar ability to stimulate GDP-Mant-GDP nucleotide exchange on Rac (600 nM), as an equivalent amount of full-length DHR-2c (black). Mant-GDP (1 μM) was added to initiate nucleotide exchange.

published X-ray crystal structures for the DHR-2 domain of Dock-9 bound to Cdc42 (*35*), suggested that the organization of the entire DHR-2 domain can be resolved into two distinct regions, which we designate here as DHR-2n (i.e., the helix-rich N-terminus, amino acid residues 1178−1334) and DHR-2c [amino acid residues 1335−1657 (Figure 1A,B)]. The expressed DHR-2c domain of Dock180 (Dock180^{DHR-2c}) was purified to homogeneity and observed to bind to its cognate GTPase target, Rac, in a manner typical for a GEF, such that the nucleotide-free form of GST-Rac binds the recombinant Dock180^{DHR-2c} much more tightly than either the GDP- or GTPγS-bound forms of Rac (Figure 2A,B). In addition, this ~37 kDa domain exhibited robust GEF activity toward purified Rac in fluorescence-based assays monitoring the exchange of GDP for the nucleotide analogue Mant-GDP and exhibited a molar specific activity equal to that of the full-length DHR-2 domain (Figure 2C). Thus, Dock180^{DHR-2c} fully accounts for the GEF activity of the DHR-2 domain in Dock180. In our in vitro assays, we found no evidence that the DHR-2n domain of Dock180 contributes to the overall GEF activity in contrast to the Cdc42-specific GEF, Dock9, which was suggested to require the helix-rich DHR-2n

domain for full activity (*35*). Further truncations at either end of Dock180$^{DHR\text{-}2c}$ yielded proteins that were difficult to isolate because of stability problems and thus could not be used to measure GEF activity. During the process of purifying each of the two subdomains of DHR-2, we also discovered that DHR-2n is not stable in a low-salt solution and appears to be responsible for the aggregation of the DHR-2 domain, as the removal of this N-terminal segment from DHR-2 eliminated the problem, with Dock180$^{DHR\text{-}2c}$ being stable and fully active under these same conditions.

Characterizing the Highly Specific GEF Activity of Dock180$^{DHR\text{-}2c}$ toward Rac. To obtain catalytic activity and turnover rates for the GEF activity of Dock180$^{DHR\text{-}2c}$, we assayed nucleotide exchange under conditions of excess Rac. Fluorescence experiments were performed using recombinant Rac that had been preloaded with Mant-GDP and purified by gel filtration to remove any free nucleotide. Different concentrations of the purified Mant-GDP-Rac complex were mixed with excess GDP, and then 20 nM Dock180$^{DHR\text{-}2c}$ was added to initiate the exchange of the fluorescent nucleotide for unlabeled GDP. The concentration of Dock180$^{DHR\text{-}2c}$ in these assays was much lower than that of Rac so the Michaelis−Menten approximation could be used in subsequent analyses. The release of Mant-GDP from Rac (i.e., because of its exchange for excess unlabeled GDP) caused the fluorescence to decrease, thus providing a real-time measurement of nucleotide exchange, i.e., the catalyzed dissociation of the bound nucleotide. We obtained a series of fluorescence traces for which we performed an initial velocity analysis, yielding a lower limit for k_{cat} of ~10 min^{-1} for the Dock180$^{DHR\text{-}2c}$-catalyzed nucleotide exchange activity (Figure 3).

We next tested the ability of Dock180$^{DHR\text{-}2c}$ to promote the activation of Rac in cells, i.e., following its transient transfection into Cos-7 cells. Figure 4A shows the results obtained when hemagglutinin (HA)-tagged Dock180$^{DHR\text{-}2c}$ was expressed in Cos-7 cells and the lysates were tested for their relative amounts of activated, endogenous GTP-bound Rac, following pull downs using GST fused to the limit Rac-binding domain of its effector Pak3 (GST-PBD) (*37*). Figure 4A (top panel) shows that Rac-GTP was pulled down with GST-PBD from lysates that contained expressed HA-Dock180$^{DHR\text{-}2c}$, whereas no detectable Rac was precipitated from control lysates. Dock180$^{DHR\text{-}2c}$ appeared to be as effective as the full-length DHR-2 domain in promoting the activation of endogenous Rac in cells, whereas as expected, the GEF-defective Dock180$^{DHR\text{-}2c/V1534A}$ showed no cellular activity. Further evidence of the in vivo coupling of Rac and Dock180$^{DHR\text{-}2c}$ is provided by the colocalization of Myc-Rac and HA-Dock180$^{DHR\text{-}2c}$ observed in cotransfected cells as shown in Figure 4B (top panels). Similarly, the DHR-2 domain of Dock180 was localized to membrane ruffles (Figure 4B, middle and bottom panels). The incorporation of the DHR-2 and Dock180$^{DHR\text{-}2c}$ domains into membrane ruffles (lamellipodia) was somewhat unexpected, given that it had been suggested that the conserved DHR-1 domain, which contains a putative site for interaction with PtdIns(3,4,5)P_3, might be necessary for the membrane binding of full-length Dock180 family members (*38*).

Trp^{56} of Rac Is Required for Dock180$^{DHR\text{-}2c}$ Binding and Catalysis. To explore the factors underlying the selectivity of the GEF activity of Dock180$^{DHR\text{-}2c}$ toward Rac versus Cdc42, we began by evaluating the importance of position 56 that is a tryptophan residue in Rac and a phenylalanine residue in Cdc42. From the first report of the X-ray crystal structure of the complex between the DH and PH domains of Tiam-1 and Rac (*39*), a wealth of crystallographic data has provided additional detail regarding how Rho GTPases bind to Dbl family GEFs and where in their primary structure the specificity for GEFs resides. In the case of Rac, these critical contact sites are primarily located in switch I and switch II, as well as in the two or three strands that lie between the switch regions. Tyr^{32}, Asp^{38}, and Asn^{39} of switch I, together with Gln^{61} and Gln^{64} of switch II, are all critical either for GEF binding or for activation, as substitutions for any of these residues severely impair the ability of Dbl family members to catalyze nucleotide exchange (*40*).

Strand β_3 of Rac, which is just upstream of Trp^{56} (Phe^{56} in Cdc42) in switch II, is particularly important, as it is this residue that is primarily responsible for Rac specificity in DH domain-mediated nucleotide exchange. Thus, the W56F mutant of Rac is not activated by Rac-GEFs of the Dbl family, while conversely, the F56W mutant of Cdc42 enables it to be recognized by these same Rac-specific GEFs (*40*, *41*). However, the primary structures of members of Dock180 family members bear little similarity to Dbl family GEFs, thus raising the question of whether the DHR-2 domain of Dock180 binds to a similar region on Rac as the DH domain of Dbl-GEFs. In addition, whether Trp^{56} of Rac is equally critical for Dock180 recognition is controversial, as it has been reported that the W56F mutant of Rac can still interact with the DHR-2 domain of Dock180 (*19*) as well as being partially activated by the DHR-2 domain of Dock2 (*42*). In contrast, when Trp^{56} of Rac is mutated to phenylalanine, which is the corresponding residue in Cdc42, we find that Rac W56F is activated to < 10% of the level of wild-type Rac by Dock180$^{DHR\text{-}2c}$ (Figure 5A, left panel; the right panel shows the purified recombinant wild-type Rac and the Rac mutants used in these and other experiments described below). We confirmed that the Rac W56F mutant is able to fully load Mant-GDP when treated with EDTA; however, unlike wild-type Rac, it appears to be incapable of forming a stable complex with Dock180$^{DHR\text{-}2c}$, based on GST-Rac pull-down experiments (Figure 5B). As we will describe further below, our data clearly demonstrate the important role that Trp^{56} in Rac plays in the specificity of DHR-2 binding and activation, similar to what has been observed for the DH domain of the Dbl family GEF Tiam-1.

Using the Dock9−Cdc42 X-ray crystal structure as a guide (*35*), we set out to identify critical residues within Dock180$^{DHR\text{-}2c}$ that make contact with Trp^{56} of Rac. The candidate residues from the DHR-2 domain of Dock9 that interact with Cdc42 are Leu^{1940} and Gln^{1943}. Specifically, the methyl group of Leu^{1940} in Dock9 interacts with the phenyl ring of Phe^{56}. This leucine residue is conserved in members of Dock subfamily D (Dock9−11, which are specific GEFs for Cdc42), while in the Rac-specific subfamily A (Dock1, -2, and -5, Dock1 being Dock180), the corresponding residue is Met^{1524} (Figure 5C). To test the degree to which the leucine−phenylalanine interaction in the Dock9−Cdc42 complex and the methionine−tryptophan contact in the Dock180−Rac complex encode the specificity for these interactions, we mutated the corresponding Met^{1524} residue to leucine in Dock180$^{DHR\text{-}2c}$. As shown in Figure 5D, changing the methionine to leucine decreased the GEF activity of Dock180$^{DHR\text{-}2c}$ by ~80%. Conversely, the methionine-to-leucine substitution at position 1524 in Dock180$^{DHR\text{-}2c}$ resulted in a 5-fold increase in GEF activity toward the Rac W56F mutant (Figure 5E). Taken together, these results confirmed a point of specific discrimination between Rac and Cdc42 toward Dock180 (vs Dock9): the indole ring of Trp^{56} of Rac interacts with the methyl thioether side chain of Met^{1524} of Dock180, while the extra methyl group of the leucine residue at the corresponding position in Dock9 is

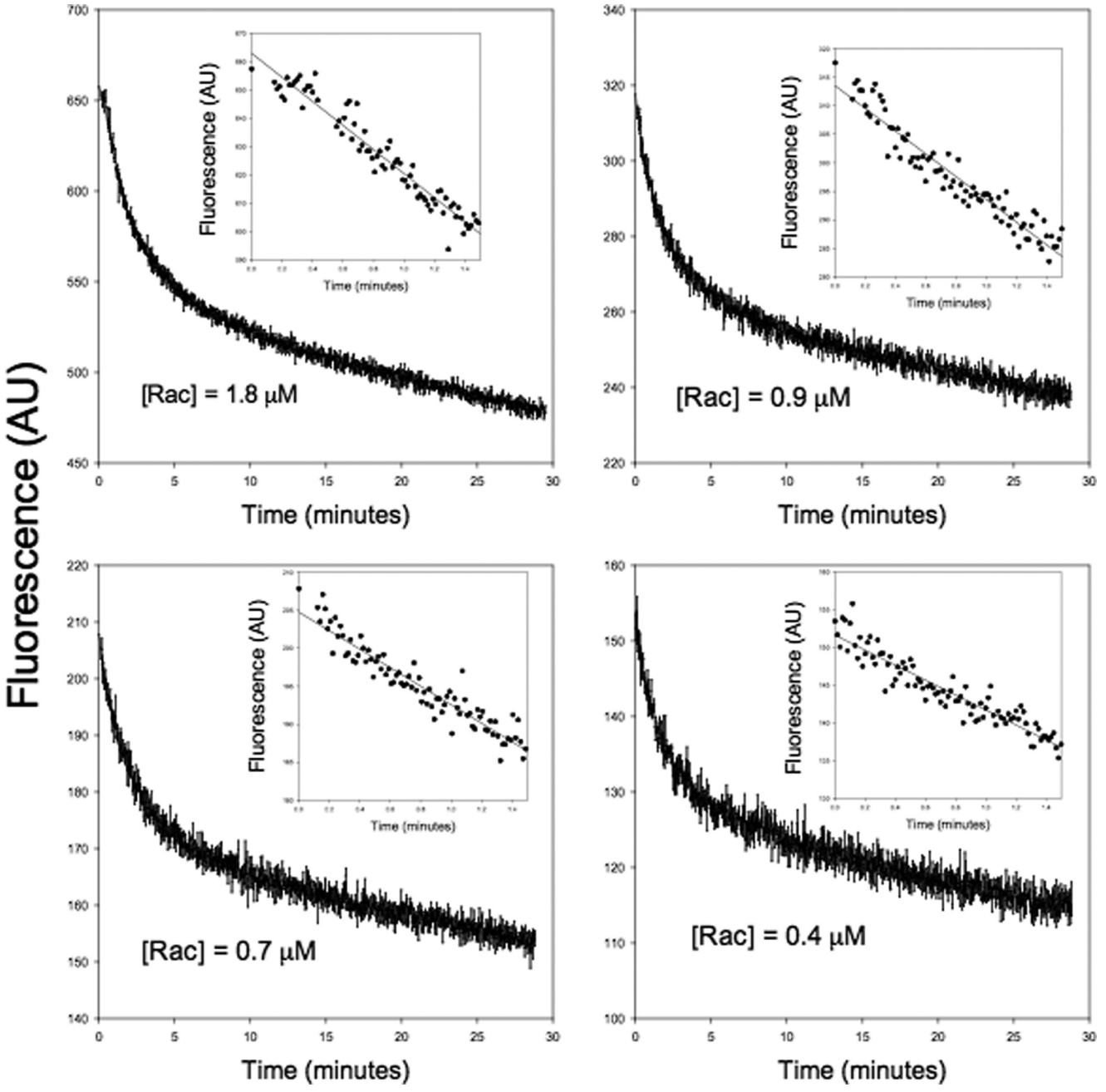

FIGURE 3: Kinetics of Mant-GDP dissociation catalyzed by Dock180$^{DHR\text{-}2c}$. The decreasing fluorescence emission at 440 nm (excitation at 340 nm) shows the rate of Dock180$^{DHR\text{-}2c}$ (DHR-2c)-catalyzed Mant-GDP dissociation when the indicated concentrations of Rac were assayed. The concentration of DHR-2c was 20 nM in all experiments shown. To initiate the dissociation of Mant-GDP, 20 μM unlabeled GDP was added. Insets show linear fits to the initial fluorescence changes.

a key hydrophobic site of interaction for the phenyl ring of Phe^{56} in Cdc42.

Comparison of Rac-Specific GEF Activity by Tiam-1 and Dock180$^{DHR\text{-}2c}$. Although there are similarities between the set of residues in Rac that are important for binding DHR-2 and DH domains, we wanted to further define these residues and investigate their relative contribution to the overall GEF activity. The data in panels A and B of Figure 6 again show that Trp^{56} of Rac is necessary for Dock180$^{DHR\text{-}2c}$ binding and activation, similar to what has been observed for the DH-domain of Tiam-1. However, the complementary substitution (i.e., Phe to Trp) at position 56 in Cdc42 is not sufficient to allow for GEF activity by Dock180$^{DHR\text{-}2c}$, although this Cdc42 mutant can be strongly activated by the DH-PH domains of Tiam-1 (Figure 6C,D). Moreover, although the M1524L mutant of Dock180$^{DHR\text{-}2c}$ can activate the W56F mutant of Rac, the introduction of this Cdc42-specific residue into Dock180$^{DHR\text{-}2c}$ conferred no detectable GEF activity when it was tested with wild-type Cdc42 (data not shown). Thus, Trp^{56} is necessary but not sufficient for the specific binding and GEF activity of Dock180 toward Rac, indicating that additional contacts are necessary for full GEF activity.

Structural comparisons and primary sequence alignments between Cdc42 and Rac highlight other candidate residues, which may play critical roles in mediating Dock180$^{DHR\text{-}2c}$-catalyzed nucleotide exchange on Rac. These residues, which are shown in the Dock9−Cdc42 structure to be sites of contact in Cdc42 that are not conserved in Rac and to interact with

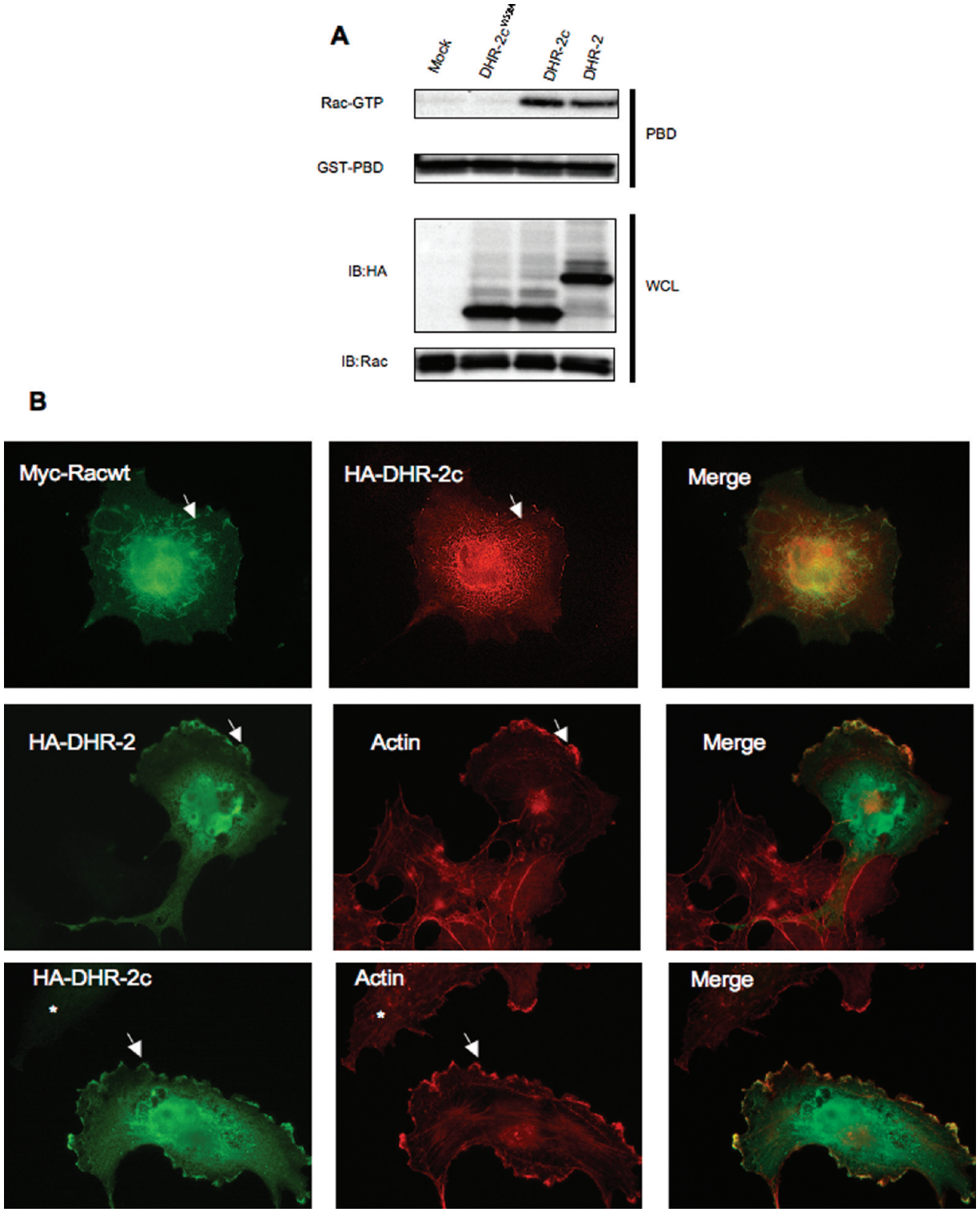

FIGURE 4: Comparisons of the cellular localization and GEF activities of Dock180^{DHR-2} and Dock180^{DHR-2c}. (A) PBD assay comparing the ability of Rac to be activated in cells by Dock180^{DHR-2} (DHR-2), Dock180^{DHR-2c} (DHR-2c), and the GEF-defective Dock180$^{DHR-2c/V1534A}$ (DHR-2c V1534A). GTP-bound endogenous Rac was affinity precipitated with PBD beads from Cos-7 cell lysates. The top panel shows the relative amounts of Rac-GTP pulled down under the described conditions, as indicated by Western blotting with an anti-Rac polyclonal antibody. The second panel from the top shows the GST-PBD inputs. The third panel from the top shows the relative expression of the HA-DHR-2 and HA-DHR-2c constructs as detected by Western blotting with the anti-HA antibody, and the bottom panel shows the relative expression of endogenous Rac in the different transfected cells. (B) Immunocytochemistry of ectopically expressed Rac and DHR-2 constructs and their localization to membrane ruffles and lamellipodia in Cos-7 cells. The top panels show colocalization of ectopically expressed Myc-Rac and HA-DHR2c to membrane ruffles (arrows). We have examined 100 cells expressing DHR-2c, with the vast majority (> 75%) showing DHR-2c at the leading edge. The next two sets of panels show the similar localization of DHR-2 (middle) and DHR-2c (bottom) with actin predominantly at the indicated leading edge lamellipodia. Nonspecific binding of the anti-HA antibody to nontransfected cells is negligible as illustrated by the nonstained cell indicated in the bottom panels with an asterisk.

nonconserved residues in the respective DHR-2 domains, are Ala[27], Gly[30], Ser[41], and Asn[52] in Rac. To test whether these residues played a significant role in the selectivity of the GEF, Ser[41] and Asn[52] in Rac were mutated to their corresponding

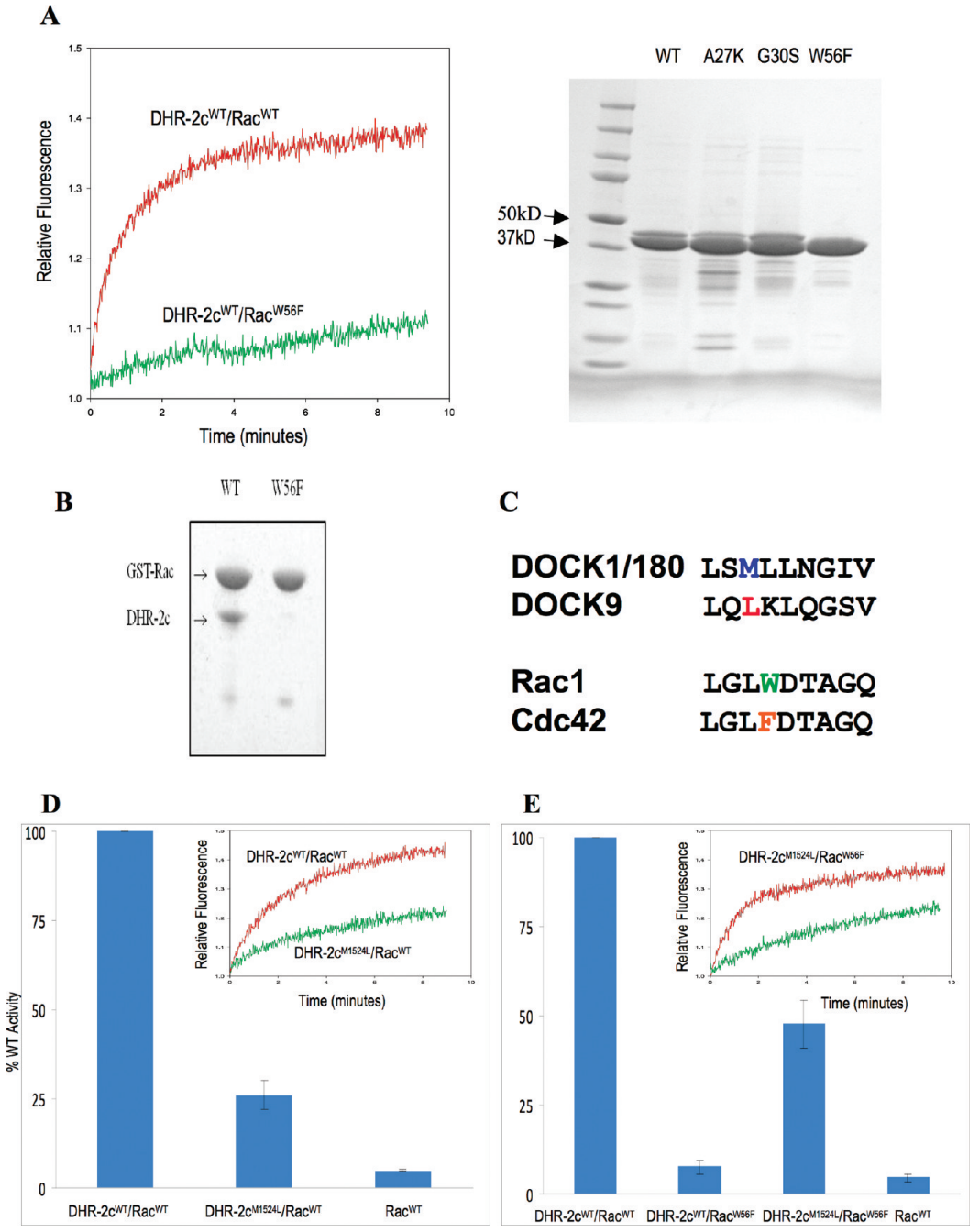

FIGURE 5: Met^{1524} of Dock180^{DHR-2c} (DHR-2c) specifically recognizes Trp^{56} of Rac. (A) The Rac W56F mutant is ineffective in coupling to DHR-2c. In the left panel, DHR-2c (60 nM) was mixed with either 600 nM wild-type Rac or Rac W56F and 1 μM Mant-GDP in HMA buffer. The relative rates of nucleotide exchange on the Rac proteins catalyzed by wild-type DHR-2c are shown by changes in Mant fluorescence. The right panel shows SDS−PAGE and Colloidal Blue staining of wild-type Rac and the Rac mutants used in the experiments shown in this figure. (B) The Rac W56F mutant is incapable of forming a stable complex with DHR-2c. The GST-Rac bead samples were incubated with 1 μg of purified DHR-2c, washed, and analyzed by SDS−PAGE and Colloidal Blue staining. (C) Sequence comparisons of key residues within the DHR-2 domains of Dock subfamilies A and D. (D) Mutation of methionine 1524 of DHR-2c reduces its GEF activity. DHR-2c and the DHR-2c M1524L mutant (60 nM each) were mixed with 600 nM Rac and 1 μM Mant-GDP in HMA buffer, and the relative rates of nucleotide exchange were assayed by changes in Mant fluorescence. The inset shows fluorescence traces reflecting the exchange reactions quantified in the figure. (E) DHR-2c and DHR-2c M1524L (240 nM each) were mixed with 600 nM Rac and 1 μM Mant-GDP in HMA buffer and assayed for nucleotide exchange following changes in Mant fluorescence. The inset shows fluorescence traces reflecting the exchange reactions quantified in the figure.

residues in Cdc42, Ala^{41} and Thr^{52}, respectively. We found that neither residue had a detectable effect on the ability of Dock180^{DHR-2c} to catalyze nucleotide exchange (see Table 1 of the Supporting Information). Residues 27 and 30 of Rac or Cdc42 have been suggested to mediate selective recognition of Rac by the DHR-2 domain of Dock2 (*42*). To test the importance of these residues for the recognition of Rac by Dock180^{DHR-2c}, we introduced the A27K and G30S substitutions (i.e., Rac-to-Cdc42 changes at these positions) into Rac. Figure 6A shows the results of fluorescence nucleotide exchange assays demonstrating that both of these Rac mutants were impaired in their ability to functionally couple to Dock180^{DHR-2c}. The Rac A27K mutant was ~20% effective, and thus only marginally better than the Rac W56F mutant, whereas the G30S mutant was ~30% as effective as wild-type Rac in coupling to Dock180^{DHR-2c}. In contrast to their essential role in Dock180^{DHR-2c}-mediated nucleotide exchange,

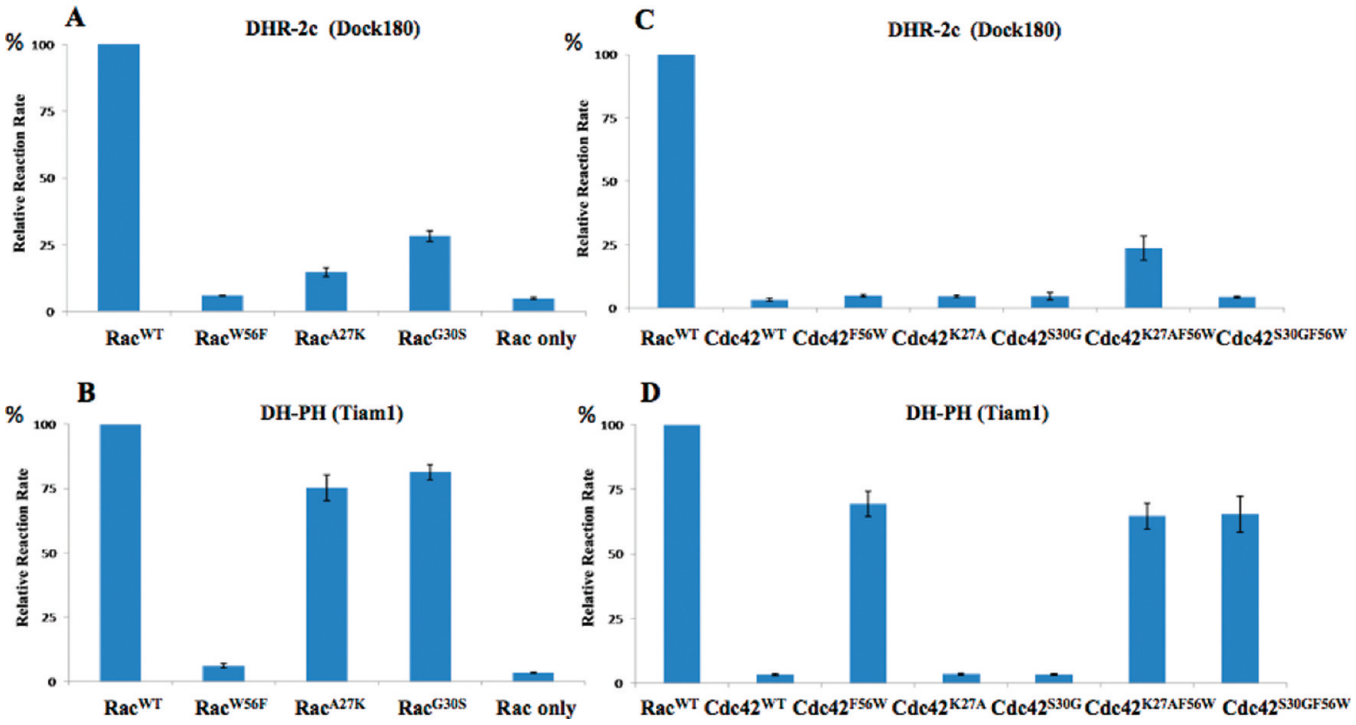

FIGURE 6: Comparisons of the abilities of the Rac-specific Dock180^{DHR-2c} and Tiam-1^{DH-PH} to activate Rac and Cdc42 mutants. Dock180^{DHR-2c} (180 nM) and Tiam-1^{DH-PH} (15 μM) have similar catalytic properties toward the same concentration of Rac^{WT} and were used in all subsequent experiments. The concentration of Rac, Cdc42, or their mutants was kept at 600 nM. Bars show the relative nucleotide exchange activity of the Rac and Cdc42 mutants compared to the wild-type proteins and the standard error of three trials. Note that the A27K and G30S mutants of Rac exhibit weakened abilities to be activated by Dock180^{DHR-2c}, while they still can be fully activated by Tiam-1^{DH-PH} (A and B). Residues 27 and 56 of Rac or Cdc42 are both critical for specific Dock180 GEF recognition (C), while the single Cdc42 F56W mutant can be nearly fully activated by the DH-PH domain of Tiam-1 (D). Note that the Cdc42 K27A/F56W double mutant can be partially activated by Dock180^{DHR-2c}.

when we tested the Rac A27K and Rac G30S mutants with the DH-PH domains of Tiam-1, we found that both Rac mutants were activated to a degree comparable to that of wild-type Rac (Figure 6B). Taken together, these data confirm a key contact site in Rac for Dock180^{DHR-2c} that does not play a significant role in binding Dbl family GEFs.

To determine whether these two residues (i.e., at positions 27 and 30), along with position 56, are sufficient for specific recognition by Dock180^{DHR-2c}, we mutated these sites in Cdc42 either individually or in combination to provide Rac contact points in a Cdc42 background. Figure 6C shows that the double mutant S30G/F56W of Cdc42 was incapable of being activated by Dock180^{DHR-2c}. However, the K27A/F56W mutant of Cdc42 exhibited a partial ability to recognize Dock180^{DHR-2c} (Figure 6C). Unlike the Cdc42 F56W mutant, which was nearly 80% effective compared to wild-type Rac in coupling to Tiam-1 (Figure 6D), the K27A/F56W mutant of Cdc42 was only ~25% as effective as wild-type Rac in its interaction with Dock180^{DHR-2c}. Thus far, we have not found any other residue that contributes to the specific recognition of Dock180^{DHR-2c}.

In addition to these residues that are divergent between Cdc42 and Rac, we also mutated several residues common to Cdc42 and Rac that have been shown to be necessary for binding the DH-PH domain of Tiam-1. In the β2-β3 and switch II regions, the G54A and Q61L mutants of Rac are unable to be activated by Dock180^{DHR-2c} (Table 1 of the Supporting Information). Together with the results obtained for the Rac W56F mutant of Rac, we conclude that the β3 and switch II regions of Rho GTPases make up the critical area for the binding of both the Dock180 and Dbl families of GEFs. However, in the switch I region, the binding site for Rac in Dock180^{DHR-2c} appears to be different from its binding site on the DH domain of Tiam-1. In addition to Ala^{27} and Gly^{30} of Rac, the Rac Y32A mutant also responds differently to the DHR-2c and DH domains, as the nucleotide exchange activity of this mutant is not responsive to DH-PH domains but it is still able to be activated by Dock180^{DHR-2c}. The Rac E39A mutant, which contains another substitution at the end of switch I, is unable to be activated by either Dock180^{DHR-2c} or the DH-PH domain of Tiam-1 (Table 1 of the Supporting Information). Taken together, these data suggest that the switch I region in GTPases plays an important role in the specific binding of both Dock180 and Dbl family GEFs, but significantly, the residues that make up the contact site are different for these two families of GEFs.

DISCUSSION

The Rho family GEFs are critical cellular regulatory proteins, given the important roles that their target GTPases play in a wide variety of cellular and biological functions ranging from actin cytoskeletal changes to cell migration and invasion to cell-cycle progression, differentiation, and developmental events. Two classes of upstream activators (GEFs) for the Rho GTPases have been identified. One family of GEFs, for which the founding member is the oncogenic Dbl protein, has been extensively characterized, and a good deal of structure−function information is now available regarding how different members of the Dbl family functionally engage their target Rho GTPases. Less is known, at least from a biochemical and structural perspective, about the second family of Rho-GEFs for which Dock180 is the prototype.

The recent X-ray crystal structure for the DHR-2 domain from one member of the Dock180 family, Dock9, bound to its GTPase

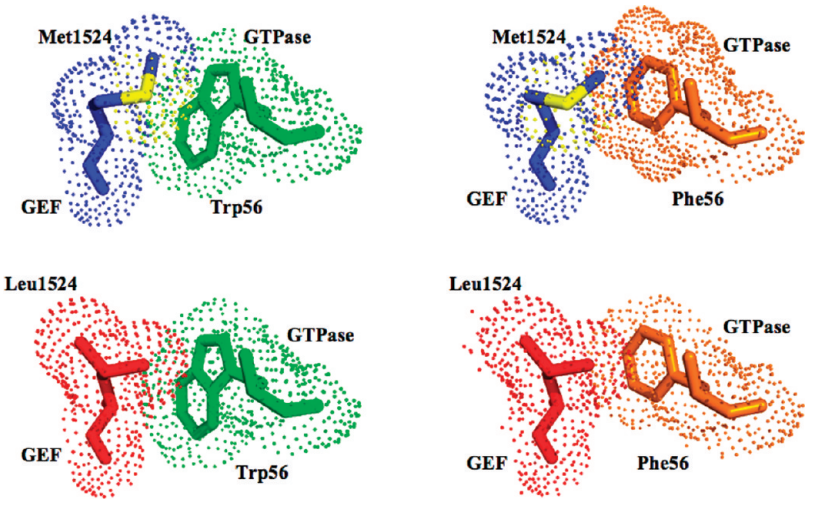

FIGURE 7: Structure of the modeled complexes showing the interaction between residue 1524 of Dock180$^{DHR\text{-}2c}$ and residue 56 of Rac1. The side chains are shown as both sticks and dots: Met^{1524} (blue), Leu^{1524} (red), Trp^{56} (green), and Phe^{56} (orange). The sulfur of Met^{1524} is colored yellow. The homology model for the Dock180$^{DHR\text{-}2c}$ domain (residues 1336−1615) and wild-type Rac1 (residues 1−177) was obtained using the structures of the Dock9 DHR-2 domain and Cdc42 from the structure of their complex (Protein Data Bank entry 1WM9) as a template using the Swiss-model server (*43*−*45*). The homology-modeled structures were superimposed on the structure of the complex of Dock9 DHR-2 and Cdc42 using PyMOL (DeLano Scientific LLC). The structure of this complex was energy-minimized first by steepest-descent and then by conjugate-gradient methods using Gromacs (version 4.0) (*46*) to remove any steric clashes. Mutations were made using the mutagenesis module on PyMOL, keeping the side chain dihedral angles the same as in the wild-type complexes. The structures for the mutant complexes were again energy minimized to yield the final structures. The interactions between residues were calculated either using the PIC server (*47*) or the CSU software (*48*).

target, Cdc42, was recently determined and has begun to shed some light on how this Cdc42-specific GEF couples to different nucleotide-bound states of Cdc42. However, some important questions have remained unanswered regarding whether the Rac-specific GEF Dock180 uses a mechanism to activate Rac similar to that used by Dock9 to activate Cdc42, as well as those used by Rac-specific GEFs from the Dbl family, such as Tiam-1.

As a means for developing biochemical strategies to address these mechanistic issues, we set out to delineate a limit functional domain for Dock180 and examine whether its ability to catalyze nucleotide exchange is mechanistically comparable to that of the DH-PH domains of Dbl GEFs. Our earlier modeling based on secondary structure predictions led us, in a trial and error manner, to the discovery of a limit functional subdomain of DHR-2 (Dock180$^{DHR\text{-}2c}$). This fully active subdomain is unexpectedly small in light of the recently published X-ray structures for the Dock9−Cdc42 complexes, as it consists of only the 300 C-terminal residues of DHR-2. The limit domain, Dock180$^{DHR\text{-}2c}$, can bind and activate Rac in vitro with a rate of catalysis that is essentially identical to that for the full-length DHR-2 domain of Dock180. It also is able to activate Rac in cells, while maintaining specificity as it is completely incapable of coupling to Cdc42. This differs from the findings regarding the regulation of Dock9 activity, as the N-terminal helix-rich domain is necessary for the full activation of Dock9 by providing the binding site for homodimerization with a second Dock9 DHR-2 domain. Interestingly, even under optimal dimerization conditions, the apparent k_{cat} reported for Dock9 (~0.6 min^{-1}) is significantly lower than what we have measured for either the DHR-2 domain of Dock180 or Dock180$^{DHR\text{-}2c}$ (i.e., ~10 min^{-1}). The apparent high rate of turnover of Dock180−Rac complexes could have important implications for the full-length Dock180 protein in vivo and a significant influence on the rate of phagocytosis and cell engulfment and thus will be an interesting subject for further study.

Mutagenesis of Dock180$^{DHR\text{-}2c}$ has enabled us to identify residues in Rac that are essential for Dock180 recognition. In addition, we found that the selective nature of Rac recognition of Dock180 is mediated by Trp56 of Rac, as is the case for Rac specificity for the Dbl family GEF Tiam-1, therefore indicating a similarity in their use of position 56 as a means of GTPase discrimination. While the importance of position 56 in Rac and Cdc42 is common to both the Dbl and Dock180 families of GEFs, we also demonstrate here the critical role of a second contact site on Rac at Ala^{27} and, to a lesser extent, Gly^{30}. Replacement with either residue in Rac with the corresponding residue in Cdc42 dramatically abrogates the nucleotide exchange activity of Dock180$^{DHR\text{-}2c}$ for Rac (Figure 6A). Using Cdc42 as a GTPase scaffold, we found that replacement of Cdc42 residues 27 and 56 (lysine and phenylalanine, respectively), but not residues 30 and 56, led to the restoration of a modest degree of coupling to Dock180$^{DHR\text{-}2c}$, as this Cdc42 double mutant showed ~25% of the nucleotide exchange activity of wild-type Rac (Figure 6C). Using the crystallographic data provided by the structure for the DHR-2 domain of Dock9 bound to Cdc42, we examined the importance of the residue from Dock180$^{DHR\text{-}2c}$ that makes contact with residue 56 of Rac and found that Met^{1524} of Dock180 is critical for Rac recognition, as it is for Cdc42 recognition by Dock9, where the methionine is replaced with leucine at residue 1941. On the basis of the structural data, it would appear that Arg^{1367} from Dock180$^{DHR\text{-}2c}$ makes contact with Ala^{27} of Rac and further contributes to the specificity of GTPase recognition.

As an initial attempt toward understanding how Dock180 discriminates between Rac1 and Cdc42, we used homology modeling for the complex between Dock180$^{DHR\text{-}2c}$ and Rac1, and their mutants, using the X-ray structure of the Dock9−Cdc42 complex as a template. Within the modeled complex of wild-type Dock180$^{DHR\text{-}2c}$ and wild-type Rac, there appears to be a strong interaction between the aromatic ring of Trp^{56} in Rac (Figure 7, top left, green ring) and the sulfur of Met^{1524} in Dock180$^{DHR\text{-}2c}$ (Figure 7, top left, yellow), with the distance between the ring centroid and the sulfur being ~4 Å. This interaction may be weakened in the complex between wild-type Dock180$^{DHR\text{-}2c}$ and the Rac W56F mutant (Figure 7, top right),

as the model suggests that the center of the phenylalanine ring (orange) and the sulfur from the methionine (yellow) would not be as proximally positioned as the corresponding moieties for a tryptophan-methionine pair, thus possibly accounting for the ineffectiveness of wild-type Dock180^{DHR-2c} as a GEF for the Rac W56F mutant. Also, within the complex between wild-type Dock180^{DHR-2c} and wild-type Rac, there are considerable hydrophobic contacts between the sulfur atom of Met^{1524} and the indole ring of Trp^{56}. When Met^{1524} in Dock180^{DHR-2c} is changed to leucine, the strong interaction that occurred between the sulfur from the methionine and the tryptophan ring is lost while a few hydrophobic contacts are maintained between the leucine at position 1524 and Trp^{56} (Figure 7, bottom left), which might account for this mutant having slightly less activity than wild-type Dock180^{DHR-2c}. Surprisingly, the model for the complex between the Dock180^{DHR-2c} M1524L mutant and the Rac W56F mutant (Figure 7, bottom right) is very similar to that of the complex between Dock180^{DHR-2c} M1524L and wild-type Rac, making it difficult to rationalize why the exchange activity for the former complex is higher. In this regard, we are in the process of obtaining structural information for the complex between Dock180^{DHR-2c} and wild-type Rac using X-ray crystallography, which should shed light on this question as well as provide further insights into the mechanism of GEF activity by Dock180.

In conclusion, we have been able to define a minimal region harboring full nucleotide exchange activity within the DHR-2 domain of Dock180 and provide the molecular basis by which the founding member of the Dock180 family of GEFs specifically couples with the Rac GTPase. The establishment of a minimal functional domain for the Dock180 protein now sets the stage for structural and biochemical studies that aim to further probe the mechanism by which this GEF exhibits its catalytic activity as well as to learn more about how other parts of the Dock180 protein are used to regulate this activity in cells to initiate Rac-mediated signal transduction pathways.

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SUPPORTING INFORMATION AVAILABLE

Rac amino acids important in specifying nucleotide exchange activity for GEF domains Dock180^{DHR-2c} and Tiam1^{DH-PH}. This material is available free of charge via the Internet at http://pubs.acs.org.

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