IQGAP2 is a scaffolding protein that plays crucial roles in various cellular processes including cell adhesion, migration, and signaling. It interacts with multiple Rho GTPases, including Cdc42 and Rac1, and IQGAPs contain several IQ motifs that mediate GTPase activity. IQGAPs play a critical role in mediating the interaction of Rho GTPases with downstream effectors and in regulating the cellular behavior of cancer cells.

Cdc42 and Rac1 are Rho GTPases that are involved in cellular processes such as cell motility, migration, and invasion. Cdc42 and Rac1 are activated by GTP and inactivated by the binding of GDP. This binding is regulated by the switch mechanism, where GDP binding prevents GTP binding, and vice versa. IQGAPs interact with Cdc42 and Rac1 through their IQ motifs, which mediate the interaction of the GTPases with downstream effectors. IQGAPs also interact with the RasGAP site of GRD2, facilitating the dimerization of IQGAP2.

Our study used atom molecular dynamics simulations, site-directed mutagenesis, and Western blotting to unravel the detailed mechanisms of Cdc42 and Rac1 interactions with IQGAP2. We observed that Cdc42 binding to the Ex-domain and the RasGAP site of the GTPase-activating protein (GAP)-related domain (GRD) of IQGAP and promote IQGAP dimerization. Only one Rac1 molecule might bind to the RasGAP site of GRD and may not facilitate the dimerization, and the exact mechanism of Cdc42 and Rac1 binding to IQGAP is unclear. Using all-atom molecular dynamics simulations, site-directed mutagenesis, and Western blotting, we unraveled the detailed mechanisms of Cdc42 and Rac1 interactions with IQGAP2. We observed that Cdc42 binding to the Ex-domain of GRD of IQGAP2 (GRD2) releases the Ex-domain at the C-terminal region of GRD2, facilitating IQGAP2 dimerization. Cdc42 binding to the Ex-domain promotes allosteric changes in the RasGAP site, providing a binding site for the second Cdc42 in the RasGAP site. Of note, the Cdc42 "insert loop" was important for the interaction of the first Cdc42 with the Ex-domain. By contrast, differences in Rac1 insert-loop sequence and structure precluded its interaction with the Ex-domain. Rac1 could bind only to the RasGAP site of apo-GRD2 and could not facilitate IQGAP2 dimerization. Our detailed mechanistic insights help decipher how Cdc42 can stimulate actin polymerization in metastasis.

The Rho GTPase family is a subfamily of the Ras GTPase superfamily. Rho GTPases regulate multiple cellular processes, such as growth, survival, cell invasion, cell motility, and vesicle trafficking, by interacting with effector molecules. Rac1 is one of the most studied Rho GTPases and mediates actin polymerization and membrane ruffling, which are important in cell migration and invasion. IQGAPs are scaffolding proteins that play a critical role in mediating the interaction of Rho GTPases with downstream effectors and in regulating the cellular behavior of cancer cells. IQGAPs interact with multiple Rho GTPases, including Cdc42 and Rac1, and promote cell motility and invasion.
accompanied by conformational changes in two regions, the switch I and II motifs (Fig. 1A). These regions serve as platforms for selective interactions with structurally and functionally diverse effectors, such as p21-activated kinase 1 (PAK1); p67phox, a member of the NADPH oxidase family; and IQ motif-containing GTPase-activating proteins (IQGAPs) (21, 22).

IQGAP is a ubiquitously expressed eukaryotic scaffold protein that is found in many species. The first identified and most-studied is human IQGAP1, a 189-kDa and 1657-amino acid–long protein (23). IQGAP1 plays a role in cell motility, cell–cell attachment, protein trafficking, transcription, regulation of the cytoskeleton, and microbial pathogenesis (24, 25). Cdc42 activity and intrinsic GTP hydrolysis are inhibited upon binding to IQGAPs, which stabilize its active form (26, 27). Cdc42 liberation fuels its signaling. Consistent with this, overexpression of IQGAP1 in mammalian cells promotes metastasis by stimulating actin polymerization mediated by Cdc42 (12, 15, 26, 28). In addition to Cdc42 and Rac1, IQGAPs interact with over 100 molecules, including calmodulin (CaM),4 mitogen-activated protein kinase pathway proteins such as extracellular signal-regulated kinase 2 (ERK2), growth factors, chemokine receptors, actin, β-catenin, Yes-associated protein 1 (YAP1), small molecules such as Ca\(^{2+}\), and more (23, 29–31). These interactions occur through five major domains of IQGAPs: the calponin homology domain for actin and Ca\(^{2+}\)-CaM binding, the

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4The abbreviations used are: CaM, calmodulin; IQGAP, IQ motif–containing GTPase-activating protein; WASP, Wiskott–Aldrich syndrome protein; MD, molecular dynamics; C-term, C-terminal region of GRD2; RMSF, root mean square fluctuation; RMSD, root mean square deviation; WISP, weighted implementation of the suboptimal paths; H-bond, hydrogen bond; MM, molecular mechanics energies; GBSA, generalized Born surface area continuum solvation; CRIB, Cdc42/Rac binding; PVDF, polyvinylidene difluoride; PDB, Protein Data Bank.
WW domain for ERK2 and phosphoinositide 3-kinase binding, IQ motifs for CaM and myosin light chain binding, GRD for Cdc42 and Rac1 binding, and the RasGAP C-terminal domain for E-cadherin and β-catenin binding (32–35). Some of the binding partners compete or interfere with each other for binding to IQGAPs. For example, CaM binding causes allosteric Cdc42 release from GRD and decreases actin binding to the calponin homology domain (34). Free, active Cdc42 can then promote actin polymerization through Wiskott–Aldrich syndrome protein (WASP) and Arp2/3. IQGAP dimerization can also help to simultaneously bind to competing targets (36).

Despite the high degree of sequence identity, Cdc42 and Rac1 bind to IQGAP1 and IQGAP2 in different ways and perform distinct cellular functions. Two molecules of Cdc42 can bind to IQGAP1 and IQGAP2. The crystal structure of the GRD of IQGAP2 in complex with Cdc42 has been solved recently. The structure reveals two different Cdc42-binding sites in GRD2, the Ex-domain and the RasGAP sites (Fig. 1B) (16). The Ex-domain is composed of the N- and C-terminal regions of GRD2. Because IQGAP2 dimerization through GRD2s is promoted by Cdc42 binding, the complex has dimerized GRD2s and four Cdc42s bound to these GRD2s (16, 37). On the other hand, it was speculated that only one Rac1 molecule can bind to GRD, and there has been no evidence that it facilitates IQGAP dimerization (16); however, the exact mechanism of Rac1-GRD2 interaction and the differing stoichiometry have not been identified. If the assumption for Rac1-GRD interaction is correct, the reason behind the difference in binding of Cdc42 and Rac1 to GRD2 needs to be understood. To elucidate the mechanisms of these interactions, we carried out molecular dynamics (MD) simulations of Cdc42-GRD2 and Rac1-GRD2 complexes and performed site-directed mutagenesis and Western blot experiments to validate their predictions. We observed that the insert loop and switch I of Cdc42 are mainly responsible for Cdc42 binding to the Ex-domain. This binding releases the Ex-domain at the C-terminal region of GRD2 (hereafter referred to as C-term) to facilitate dimerization. However, the

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**Figure 2.** A, RMSF plots of apo-GRD2 (left) and GRD2 from Ex-mode Cdc42-GRD2 complex (right). Green lines cover the RasGAP site residues, and the remaining residues (cyan lines) belong to the Ex-domain. In the right panel, the red ellipse represents the residues between 1124 and 1128 that conformationally change upon Ex-mode Cdc42 binding. In the right panel, C-term residues show higher fluctuation than 10 Å (up to 20 Å); however, to clearly observe and compare fluctuation profiles of GRD2s from different complexes, the y axis limit is determined as 10 Å. B, time series of snapshots of Ex-mode Cdc42-GRD2. Pink molecules are Cdc42s, and green molecules are GRD2, whereas cyan-highlighted residues are the C-terms of the Ex-domain of GRD2s.
orientation of the insert loop of Rac1 does not allow a Rac1–Ex-domain interaction; thus, the C-term cannot be released. Essentially, Cdc42 binding to the Ex-domain induces allosteric changes in the RasGAP site to enable the second Cdc42 to bind to this site, whereas apo-GRD2 is already available for Rac1 binding.

Here we unravel the underlying mechanism of the different stoichiometry that is involved in the binding of Cdc42 and Rac1 to GRD and of IQGAP2 dimerization. We then suggest how the mechanism can help to elucidate the key question of the roles of Cdc42 and Rac1 in actin polymerization in metastasis.

Results

Cdc42 insert-loop binding to the Ex-domain induces allosteric changes in the RasGAP site that facilitate GRD dimerization

The crystal structure of the GRD2 dimer bound to four Cdc42s (PDB entry 5CJP) provided a base structure for this study. In the structure, dimerization occurs between the Ex-domains of two GRD2s. Especially, the C-terminal portions
(residues 1204–1246) are mainly involved in the dimerization, and they intertwine with each other (Fig. 1B).

Atomistic MD simulations were performed on the Ex-domain bound to the Cdc42 (hereafter referred to as Ex-mode Cdc42)-GRD2 complex for 500 ns. During the simulation, the bound C-term vigorously fluctuates compared with the C-term of apo-GRD2 (Fig. 2A, apo-GRD2 in the left panel and Ex-mode Cdc42–bound GRD2 in the right panel). Root mean square fluctuation (RMSF) calculations show that the residues in C-term (residues 1204–1246, right panel in Fig. 2A) fluctuate much more than the other residues of GRD2 upon Ex-mode Cdc42 binding. Because of this fluctuation, this region undergoes conformational changes; it is released and freely moves (Fig. 2B). Throughout the simulation, the C-term points in different directions. To confirm that the conformations with released C-term are highly populated throughout the Ex-mode Cdc42–GRD2 simulation, we performed clustering analysis with respect to the root mean square deviation (RMSD). We clustered the conformations based on pairwise best-fit RMSD. After the clustering analysis, we observed that C-term is released in 90% of the conformations (data not shown). This indicates that C-term–released conformations are highly populated throughout Ex-mode Cdc42–GRD2 simulation. These findings suggest that Cdc42-induced release of the C-term provides a platform for interacting with the C-term of the second GRD2 and facilitates dimerization.

In the crystal structure study, it was suggested that Ex-mode binding must precede Cdc42 binding to the RasGAP site (hereafter referred to as RasGAP mode Cdc42); however, how the binding of the first Cdc42 facilitates the binding of the second was unknown (16). Throughout our Ex-mode Cdc42-GRD2 simulation, as well as the fluctuation of C-term, we observed changes in the RasGAP site that enable the RasGAP mode Cdc42 binding. To elucidate how the binding of the Ex-mode Cdc42 allosterically affects the conformation of the RasGAP site, we conducted a dynamical network analysis using weighted implementation of the suboptimal paths (WISP) algorithm (38). The algorithm can identify the shortest primary communicating pathway through nodes that are protein residues of interest and edges connecting the nodes. In this analysis, we calculated 100 optimal pathways between selected residue pairs on Ex-mode Cdc42 and GRD2. The residue pair selection is made by considering residues that are in those regions that are mostly involved in the interactions and have high positive cross-correlation. We calculated the cross-correlation of the atomic fluctuations throughout the simulation. Positively correlated residues with a high correlation coefficient move in the same direction. We chose numerous residue pairs, but we could obtain pathways only between five of them. All possible pathways obtained by WISP start with Ex-mode Cdc42 residue Asn-132 and terminate at GRD2 residues 1124–1128 (Fig. 3), which have a high fluctuation profile (Fig. 2A, residues
in red ellipse). The frequently involved residues in each of the 500 pathways (100 pathways for each five residue pairs) are Gln-983, Ile-1122, Arg-982, Asp-1123, and Ile-1121 of GRD2 (data not shown). Allosteric changes in residues 1124–1128 of GRD2, initiated by Asn-132 of Ex-mode Cdc42, prepare the RasGAP site for Cdc42 binding.

**Rac1 cannot interact with the Ex-domain because of differences in sequence and orientation of its insert loop**

Analysis of the interaction interface of Ex-mode Cdc42 with GRD2 identifies the regions and residues playing key roles. Stable salt bridges and hydrogen bonds (H-bonds) between Ex-mode Cdc42 and GRD2 generally involve insert-loop and switch I residues of Cdc42 (Fig. 4A). Residue Asn-132 of the insert loop, which is identified as the starting point of the allosteric changes in the RasGAP site, forms a stable hydrophilic interaction throughout the simulation that confirms its importance. Previous experimental studies also showed that Asn-132 residue plays an important role in the stability of Cdc42-GRD binding (6, 16). It has been shown that mutations in this residue significantly affect binding affinity of Cdc42 to GRD1 (6). Also, it has been reported that Asn-132 is involved in stable interac-
The insert loop is a Rho GTPase-specific \( \alpha \)-helix structure, rich in both positively and negatively charged amino acids. This loop plays an important role in the interactions of Rho GTPases (6, 7). Taken together, this analysis suggests that the binding mechanism of Ex-mode Cdc42 to GRD2 exploits the highly charged insert loop and switch I residues and that this interaction, especially that involving residue Asn-132, induces allosteric changes in the RasGAP site to facilitate the RasGAP mode Cdc42 binding. The Ex-mode Cdc42-GRD2 interaction then allosterically releases the C-term and increases its fluctuation to induce GRD2 dimerization. We modeled the Ex-mode Rac1-GRD2 interaction using PRISM web server, which is a template-based protein-protein interaction prediction tool (39). 500-ns-long atomistic MD simulations were performed on the Ex-mode Rac1-GRD2 complex. We first observed that the orientation of the insert loop of Rac1 differs from that of Cdc42 (Fig. 4B, left). Because of this difference, fewer stable interactions formed between Rac1 and GRD2 compared with the Ex-mode Cdc42-GRD2 (Fig. 4B, right). Although some insert-loop residues are involved in salt-bridge and H-bond formation, stable Cdc42 interactions outnumber the stable Rac1 interactions. When we compared the structure and the sequence of Cdc42 and Rac1 in detail, we observed that there are some differences between the insert loops of Cdc42 and Rac1, both in sequence and structure. The sequence of the insert loop of Cdc42 is 120RDDPSTIEKLAKN132, and that of Rac1 is 120RDDKDTIEKLKEK132 (Fig. 1A). There is a 38% difference between the sequences of the insert loops of Cdc42 and Rac1 (5 of the 13 amino acids differ). The polar Asn-132 residue of Cdc42 (Fig. 4A, right) was observed as an important residue in our analyses, inducing allosteric changes and involved in hydrophobic interaction; however, this residue is replaced with positively charged Lys-132 in Rac1. Previous experimental studies showed that mutating Asn-132 of Cdc42 to Lys decreases the affinity of Cdc42 for IQGAP1 (6). Also, positively charged Lys-131 in Cdc42, which forms a salt bridge with Asp-1067 of GRD2 (Fig. 4A, right), is replaced by negatively charged Glu-131 in Rac1. These two changes result in disruption of the stable interactions between Ex-mode Cdc42 and GRD2. The five different amino acids affect Rac1 interactions and change the structure of its insert loop. The allosteric effects induced by residue 132 of Cdc42 cannot be observed in the RasGAP site of

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**Figure 6.** A, RMSF plots of apo-GRD2 (left) and GRD2 from Ex-mode Rac1-GRD2 complex (right). Green lines cover the RasGAP site residues, and the remaining residues (cyan lines) belong to the Ex-domain. B, time series of snapshots of Ex-mode Rac1-GRD2. Gray molecules are Rac1, and green molecules are GRD2, whereas cyan-highlighted residues are the C-terms of the Ex-domain of GRD2s.
GRD2 after the Ex-mode Rac1 interaction. The changes in amino acid sequence in the Rac1 insert loop prevent it from inducing allosteric changes in the RasGAP site.

To compare the interaction strengths of these complexes, we calculated the binding free energy of Ex-mode Cdc42-GRD2 and Ex-mode Rac1-GRD2 using molecular mechanics energies combined with the generalized Born surface area continuum solvation (MM-GBSA) method (40). Our calculated binding free energies for the Ex-mode Cdc42-GRD2 and the Ex-mode Rac1-GRD2 interactions are $-23.9 \pm 9.2$ and $-6.6 \pm 8.2$ kcal/mol, respectively (Fig. 5). Compared with the Ex-mode Cdc42-GRD2 interaction, the Ex-mode Rac1-GRD2 interaction is significantly weaker, suggesting that because of the differences in the insert loop, Rac1 has a small number of residues involved in the intermolecular interaction with the Ex-domain of GRD2.

Consistent with these results, we could not observe release of the C-term throughout the simulation. Also, the fluctuation profile of GRD2 is similar to that of apo-GRD2 (Fig. 6A, left). Unlike the GRD2 from Ex-mode Cdc42-GRD2 complex (Fig. 2A, right), the C-term does not fluctuate vigorously (Fig. 6A, right part of the right panel). The C-term is not released but is folded inward, which closes the dimerization interface (Fig. 6B). Clustering analysis shows that the folded C-term conformation is highly populated throughout this simulation, and folded C-term populates 96.2% of all conformations (data not shown). As a result of this folding, exposure of the dimerization interface decreases, and the C-terms of the GRD2s cannot interact with each other. This finding also suggests that Rac1 cannot bind to the Ex-domain and cannot facilitate dimerization.

**Cdc42 and Rac1 binding to the RasGAP site involve switch I and switch II residues and cannot induce C-term release**

To observe the nature of RasGAP mode binding, we simulated RasGAP mode Cdc42-GRD2 complex. We observed that the binding mechanisms of Cdc42 to the RasGAP site of GRD2 are significantly different from those of Ex-mode Cdc42. In the case of the Ex-mode Cdc42-GRD2 interaction, the insert loop plays an important role in stabilizing the interaction (Fig. 4A). However, the insert loop of Cdc42 is less involved in the RasGAP mode Cdc42-GRD2 interaction (Fig. 7A). Instead, the salt bridges and H-bonds established between RasGAP mode Cdc42 and GRD2 mostly engage switch I and switch II residues. The binding mechanism of Rac1 to the RasGAP site of GRD2 shares similarities with the RasGAP mode Cdc42-GRD2 interaction, indicating that the RasGAP mode Rac1-GRD2 interaction also employs switch I and switch II residues (Fig. 7B). These findings also indicate that Rac1 can bind to the RasGAP site unlike the Ex-domain, because of stable interactions between RasGAP mode Rac1 and GRD2. Although binding free energy calculations imply that RasGAP mode Rac1 binding is weaker than RasGAP mode Cdc42 binding ($-8.1 \pm 10.2$ kcal/mol).
Another similarity between RasGAP mode Cdc42 and Rac1 binding is the motion of the C-term. In both cases, we did not observe release of C-term, unlike in the case of Ex-mode Cdc42 binding, and a folded C-term appears throughout both simulations (Fig. 8). This indicates that the RasGAP mode binding of neither Cdc42 nor Rac1 is sufficient to induce C-term release and facilitate GRD2 dimerization. We conclude that Ex-mode Cdc42 binding is likely to be required for GRD dimerization, because this is the only case we observed that releases the C-term.

The Y1106A mutation causes complete loss of interactions of both Cdc42 and Rac1 with GRD2

We investigated energetically important residues in GRD2 to mutate and observe their effect on the Cdc42 and Rac1 binding. We observed that there are 10 hot spots, which are the residues contributing to the binding free energy most, in GRD2 using the HotRegion web server (41). We chose Tyr-1106 to mutate, because it has the highest pair potential among the other hot spot residues. Then we performed MD simulations after mutating in silico residue Tyr-1106 to alanine under the same conditions as the previous simulations of RasGAP mode Cdc42-GRD2 and RasGAP mode Rac1-GRD2 complexes. Because Tyr-1106 is a RasGAP site residue, we simulated RasGAP mode complexes. In silico mutations were performed using the “mutate” function of Discovery Studio version 4.1. We observed that the interaction of mutated GRD2 with Cdc42 and Rac1 is considerably weaker than the interaction of wildtype GRD2 (Fig. 9A). The binding free energy for RasGAP mode Cdc42-GRD2 (wildtype) interaction is $-43.1 \pm 14.1$ kcal/mol; following mutation to alanine, it is $-10.9 \pm 11.5$ kcal/mol. A similar decrease in binding strength is observed in the mutated RasGAP mode Rac1-GRD2 interaction; for the wildtype RasGAP mode Rac1-GRD2 interaction, it is $-8.1 \pm 10.2$ kcal/mol, whereas for the mutant, it is $5.3 \pm 11.8$ kcal/mol. Also, in the mutated complexes, there are fewer stable salt bridges and H-bonds than in the wildtype interactions (Fig. 9, B and C). Tyr-1106 forms hydrophilic interactions in wildtype Cdc42-GRD2 and Rac1-GRD2 complexes. Additionally, upon mutation, stable interactions of some of the nearby residues observed in the wildtype simulations, such as Arg-1107 and Asp-956 (forming stable salt bridges with RasGAP Cdc42 residues (Fig. 7A)) and Asp-952 and Lys-966 (forming stable salt bridges with RasGAP Rac1 residues (Fig. 7B), are eliminated.

To validate the model, we generated a GST-tagged construct comprising the C-terminal half of IQGAP1 in which Tyr-1193 (which corresponds to Tyr-1106 in GRD2) was changed to Ala (termed Y1193A). We compared the abilities of the wildtype and the mutated IQGAP1 proteins to bind to purified Cdc42 and Rac1 in vitro. As observed previously by scintillation proximity assays (6), GST-pulldown analysis showed that the
C-terminal half of wildtype IQGAP1 binds Cdc42 and Rac1 (Fig. 10). By contrast, mutating a single amino acid (Tyr-1193) in IQGAP1 causes a complete loss of the Rac1-GRD1 interaction and markedly attenuates binding of Cdc42 to GRD1 (Fig. 10). Together, these data argue for the importance of the Tyr-1106 residue in IQGAP2 and provide a possible explanation for the complete loss of Cdc42 and Rac1 interactions with GRD2.

Discussion
Here, we provide possible mechanisms for the interactions of Cdc42 and Rac1 with GRD2 in atomistic detail (16). We observe that Cdc42 stably binds to the Ex-domain of GRD2 with high affinity, promoting high fluctuations of the C-term of the Ex-domain. The fluctuations release the C-term. The C-term plays a role in dimerization of IQGAP via its GRD. In GRD2s, they intertwine and stabilize the dimer structure; their release upon Cdc42 binding to the Ex-domain induces dimerization. Despite the availability of a crystal structure containing the GRD2 dimer and four molecules of Cdc42s that bind the Ex-domain and RasGAP site of each GRD2, the mechanism involving conformational changes facilitating the binding of the second Cdc42 was unclear. Through atomic-level conformations and allosteric pathway analysis, we showed that upon binding of Ex-mode Cdc42, allosteric changes are induced in the RasGAP site. These allosteric changes create a suitable binding interface for the binding of Cdc42 to the RasGAP site.

Rac1 is observed to only bind to the RasGAP site of GRD; however, it has been unclear whether Rac1 can bind to the Ex-domain and can promote dimerization (6, 42). When replacing Ex-mode Cdc42 with Rac1, with ~70% sequence identity, the C-term cannot be released; instead, it folds. The interaction interface for dimerization cannot be created when the C-term is folded; therefore, Rac1 cannot induce dimerization. Also, we do not observe allosteric changes in RasGAP site upon Ex-mode Rac1-GRD2 interaction. This is consistent with the observation that Rac1 can bind to the RasGAP site of apo-GRD2 and does not require allosteric changes to bind this site. Our energy calculations also showed that the binding affinity of Rac1 to the Ex-domain is considerably lower than the affinity of Cdc42 to the Ex-domain. Consistent with this, there are fewer stable interactions between Ex-mode Rac1 and GRD2 than between Ex-mode Cdc42 and GRD2. Detailed analysis indicates that the insert loop, which is Rho GTPase–specific and important for effector binding, plays a role in Cdc42–Ex-domain interaction. There are some sequence differences between the insert loops of Cdc42 and Rac1, which may decrease the interactions between Rac1 and the Ex-domain. All of these findings imply that Rac1 cannot interact with the Ex-domain and that neither facilitated dimerization nor allosteric changes are induced by

Figure 9. A, binding free energies of RasGAP mode Cdc42-GRD2Y1106A and RasGAP mode Rac1-GRD2Y1106A in kcal/mol calculated by the MM-GBSA method. B and C, stable salt bridges formed between RasGAP mode Cdc42 and GRD2Y1106A (B) and RasGAP mode Rac1 and GRD2Y1106A (C). Green molecules are GRD2, and pink/gray molecules are Cdc2/Rac1. In the residue label, switch I residues (*) and switch II residues (**) are indicated. Dashed lines, salt bridges. The mutated Ala-1106 residue is highlighted by boldface type.
Rac1. These results support a 1:1 binding mode of Rac1 and GRD. They also explain the difference between the binding modes of Cdc42 and Rac1 by revealing the importance of the insert loop in the Ex-mode Cdc42-GRD2 interaction and show how (and possibly why) the insert loop of Rac1 differs from that of Cdc42.

Cdc42 and Rac1 are both known to bind to the RasGAP site of IQGAPs. However, the RasGAP site of apo-GRD2 provides a more suitable interaction interface for Rac1, whereas allosteric changes in RasGAP are required for RasGAP mode Cdc42 binding. Although their interaction interfaces on the RasGAP site differ, the Cdc42 and Rac1 interactions with the RasGAP site share some similarities. In both cases, a large number of stable interactions are formed, and these interactions mostly include switch I and switch II residues of Cdc42 and Rac1. Because these regions of Cdc42 and Rac1 are almost identical, these GTPases interact with the RasGAP site in a similar manner. Inducing C-term folding but not release is the other similarity in the RasGAP mode Cdc42 binding and RasGAP mode Rac1 binding. The C-term release that is observed in the case of the Ex-mode Cdc42-GRD2 interaction cannot be observed in any of the RasGAP mode binding. This suggests that Ex-mode Cdc42 binding induces dimerization by releasing the C-term; however, alone, RasGAP mode Cdc42 and RasGAP mode Rac1 binding are not sufficient to facilitate dimerization. With these findings, we also enhance understanding of IQGAP dimerization and provide an explanation as to why Cdc42, but not Rac1, can induce the dimerization.

Thus, the mechanism of the interactions of Cdc42 and Rac1 with GRD2 can now be understood. Our atomistic MD simulation data, combined with mutagenesis experiments, yield realistic models of Cdc42-GRD2 and Rac1-GRD2 interactions. The data provide insights into the mechanism of how Cdc42 can induce GRD2 dimerization and can allosterically change RasGAP site to create interaction interface for Cdc42 binding (Fig. 11A), whereas Rac1 can bind only to the RasGAP site and cannot facilitate dimerization (Fig. 11B). Cdc42 interaction with IQGAP is crucial because of their collective roles in cell motility (3, 4). IQGAP binds and stabilizes Cdc42 in its GTP-bound active form, blocking GTP hydrolysis (26).

Little is known about the detailed mechanisms of Cdc42 and Rac1 in actin polymerization (43, 44), although progress is being made (45). Their functions differ; Cdc42 enhances filopodia (containing parallel bundles of filamentous (F)-actin) formation, whereas Rac1 promotes lamellipodia (17, 46, 47). There is evidence that IQGAP1 is overexpressed in cancer, that it affects actin polymerization (48), and that it promotes migration of tumor cells (19). GTP-bound Cdc42 and IQGAP1 regulate filopodia formation. Cdc42 activates N-WASP by interacting with its Cdc42/Rac binding (CRIB) motif and releasing N-WASP’s autoinhibited conformation; IQGAP1 C-terminal half activates N-WASP (49). Studies showed that Cdc42 and IQGAP1 bind to N-WASP and activate it synergistically (43). Activated N-WASP then recruits actin and Arp2/3, which nucleate filaments (3, 50). Cdc42-IQGAP binding stabilizes its active form, which facilitates Cdc42-dependent N-WASP activation. Actin polymerization by Rac1 differs, with lamellipodia (mesh) formation induced by Rac1 interaction with WAVE, a WASP-family verprolin homologous protein, and is not facilitated by IQGAP1 (51). Still, exactly how the different binding mechanisms of Cdc42 and Rac1 to IQGAP affect their actin-related functions is unclear. One possibility explored here is through stabilizing and releasing active Cdc42. It is possible that the 2:1 stoichiometry of Cdc42 binding to IQGAP1 is more stable than the 1:1 stoichiometry for Rac1. Which factor induces Cdc42 release from IQGAP and the mechanism regulating IQGAP1 binding to Cdc42 and Rac1 are also still open questions. Calmodulin may be one possibility.

To conclude, a major reason for the community focus on the Rho GTPases, particularly Cdc42 and Rac1, has been their effects on the actin cytoskeleton and thus tumor cell metastasis. However, exactly how they influence actin polymerization has been enigmatic. The emergence of the insert loop distinguishing them from the Ras family was established as critical, but the mechanism, mediated by IQGAP scaffolding protein, has been obscure. Here we provide a deeper understanding at the conformational level. Elucidating the interaction mechanism of Cdc42 with IQGAP is likely to facilitate targeting these molecules for therapeutic purposes.

Dynamic multiprotein assemblies shape the spatial structure of cell signaling (52, 53). Coordination, efficiency, and
robustness are essential. IQGAP provides an exquisite example of how a scaffolding protein can carry out these tasks. Its dynamics and allosteric response not only link key pathways; they can also control pathway cross-talk and signaling by fine-tuning responses, in this case to distinct members of the same family, with key consequences for actin polymerization.

Experimental procedures

Initial configurations of Cdc42-GRD2 and Rac1-GRD2 complexes

The structure including four Cdc42s bound to GRD2 dimer (PDB entry 5CJP) was directly used to perform Ex-mode Cdc42-GRD2 (chain A and chain E) and RasGAP mode Cdc42-GRD2 (chain C and chain E) simulations. For Ex-mode Rac1-GRD2 complex, the crystal structure of wildtype Rac1 (PDB entry 3TH5) was used. The structures were modeled using PRISM web server (39); the crystal structure of Cdc42-GRD2 (PDB entry 5CJP) was used as template, and we obtained initial configurations for Ex-mode Rac1-GRD2 and RasGAP mode Rac1-GRD2 complexes. GNP, a GTP analog bound to Rac1, was changed to GTP to be consistent with GTP-bound Cdc42-GRD2 complexes.

Atomistic MD simulations

A total of seven initial configurations were subject to the MD simulations in an aqueous environment: one apo-GRD2, two Cdc42-GRD2 complexes (Ex-mode Cdc42-GRD2 and RasGAP mode Cdc42-GRD2), two Rac1-GRD2 (Ex-mode Rac1-GRD2 and RasGAP mode Rac1-GRD2) complexes, one RasGAP mode Cdc42-mutated GRD2 (Y1106A) complex, and one RasGAP mode Rac1-mutated GRD2 (Y1106A) complex. The TIP3P water model was used to create the isometric unit cell box containing the protein complex.

Minimization was performed for 10,000 steps. In the production runs, the Langevin temperature control maintained the constant temperature at 310 K, and the pressure was kept at 1 atm. A total of 3.5 μs of simulations, each with 500 ns, were performed using the NAMD parallel-computing code (54) on a Biowulf cluster at the National Institutes of Health (Bethesda, MD) and on a yunus cluster at Koc University (Istanbul, Turkey). RMSD and free energy (ΔG) plots as a function of time show that 500-ns-long simulations are long enough to observe the behavior of our systems (Fig. S1 and Fig. S2). To exempt initial transients, the first 100-ns trajectories were removed, and thus averages were taken afterward. The simulated trajectories were analyzed using the CHARMM all-

Figure 11. Interaction of GRD2 with Cdc42 (A) and Rac1 (B and C). A, schematic diagram illustrating the release of C-term and allosteric change in RasGAP site upon Ex-mode Cdc42 binding. Allosteric change in the RasGAP site induces binding of the second Cdc42 to RasGAP site, and GRD2 with two Cdc42s can dimerize. B, Rac1 cannot bind to the Ex-domain and cannot induce allosteric change in the RasGAP site and release of C-term. C, Rac1 can bind to the RasGAP site of apo-GRD2 but cannot induce the release of C-term. Therefore, Rac1 cannot facilitate GRD2 dimerization.
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The beads were removed, and 3 glutathione-Sepharose beads. After incubating for 1 h at 4 °C, the proteins migrated to the expected positions on SDS-PAGE and evaluated by SDS-PAGE and Coomassie staining. All of the proteins were expressed in Escherichia coli and isolated using glutathione-Sepharose (GE Healthcare) chromatography. Further details of the calculation protocol have been reported in our previous studies (58). The electrostatic and non-polar contributions to the solvation free energy are obtained from the GB approximation within the CHARMM program (55).

**Protein expression and purification**

The generation of the GST-tagged C-terminal half of IQGAP1 (IQGAP1-C, amino acids 864–1657) has been described previously (34). IQGAP1-C with Tyr-1193 mutated to Ala (Y1193A) was generated using site-directed mutagenesis. His-Cdc42-Q61L and His-Rac1-Q61L (constitutively active forms of Cdc42 and Rac1, respectively) were generated from pGEX-Cdc42-Q61L and pGEX-Rac1-Q61L (residues 1–184 for Cdc42 and 1–192 for Rac1; generously donated by Darenca Owen, University of Cambridge, Cambridge, UK) by exciting them and inserting the plasmids into pRSET. DNA sequencing confirmed the sequences of the constructs. The GST fusion proteins were expressed in *Escherichia coli* and isolated using glutathione-Sepharose (GE Healthcare) chromatography essentially as described previously (34). His-Cdc42-Q61L and His-Rac1-Q61L were purified with nickel-nitrilotriacetic acid–agarose (Qiagen), following the company’s protocol. The GST fusion proteins superfamily: evolutionary tree and role of conserved amino acids. J. Cell Biol. 283, 1692–1704 CrossRef Medline

Further details of the calculation protocol have been reported in our previous studies (58). The electrostatic and non-polar contributions to the solvation free energy are obtained from the GB approximation within the CHARMM program (55).

**MM-GBSA calculations**

The average binding free energy is calculated as follows,

$$\Delta G_b = \langle \Delta G_{mol} \rangle + \langle \Delta G_{gas} \rangle - T \Delta S$$

where $\langle \Delta G \rangle$ is the average binding free energy, $\Delta G_{mol}$ is the solvation energy contribution, $\Delta G_{gas}$ is the gas phase contribution, and $T \Delta S$ is the entropic contribution. Angle brackets represent the average along the simulations. The change in binding free energy due to the complex formation is calculated as follows.

$$\Delta G_b = G_b^{\text{complex}} - (G_b^{\text{Cdc42-Rac1}} + G_b^{\text{GRD2}})$$

Further details of the calculation protocol have been reported in our previous studies (58). The electrostatic and non-polar contributions to the solvation free energy are obtained from the GB approximation within the CHARMM program (55).

**References**

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