

Oncogenic mutations on Rac1 affect global intrinsic dynamics underlying GTP and PAK1 binding

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ABSTRACT Rac1 is a small member of the Rho GTPase family. One of the most important downstream effectors of Rac1 is a serine/threonine kinase, p21-activated kinase 1 (PAK1). Mutational activation of PAK1 by Rac1 has oncogenic signaling effects. Here, although we focus on Rac1-PAK1 interaction by atomic-force-microscopy-based single-molecule force spectroscopy experiments, we explore the effect of active mutations on the intrinsic dynamics and binding interactions of Rac1 by Gaussian network model analysis and molecular dynamics simulations. We observe that Rac1 oncogenic mutations are at the hinges of three global modes of motion, suggesting the mechanical changes as potential markers of oncogenicity. Indeed, the dissociation of wild-type Rac1-PAK1 complex shows two distinct unbinding dynamic states that are reduced to one with constitutively active Q61L and oncogenic Y72C mutant Rac1, as revealed by single-molecule force spectroscopy experiments. Q61L and Y72C mutations change the mechanics of the Rac1-PAK1 complex by increasing the elasticity of the protein and slowing down the transition to the unbound state. On the other hand, Rac1's intrinsic dynamics reveal more flexible GTP and PAK1-binding residues on switches I and II with Q61L, Y72C, oncogenic P29S and Q61R, and negative T17N mutations. The cooperativity in the fluctuations of GTP-binding sites around the p-loop and switch I decreases in all mutants, mostly in Q61L, whereas some PAK1-binding residues display enhanced coupling with GTP-binding sites in Q61L and Y72C and within each other in P29S. The predicted binding free energies of the modeled Rac1-PAK1 complexes show that the change in the dynamic behavior likely means a more favorable PAK1 interaction. Overall, these findings suggest that the active mutations affect intrinsic functional dynamic events and alter the mechanics underlying the binding of Rac1 to GTP and upstream and downstream partners including PAK1.

SIGNIFICANCE Rac1 acts as a binary molecular switch between the GTP-bound active and GDP-bound inactive states and has important roles in oncogenic signaling. Through an integrated computational and experimental approach at the single-molecule level, we explore the mutation-dependent dynamic regulation of the small GTPase Rac1 in atomistic detail. Here, we elucidate that active mutations P29S and Q61R, as well as Q61L and Y72C, affect the intrinsic dynamic events underlying the binding of Rac1 to GTP and upstream and downstream partners through an allosterically regulated interplay between important functional regions on Rac1. The findings here posit an intrinsic dynamic mechanism that could be utilized in drug design efforts.

INTRODUCTION

Rac1 (Ras-related C3 botulinum toxin substrate 1) is an important GTPase in all eukaryotic organisms, regulating

cytoskeletal organization and cell motility in response to extracellular signals (1,2). Associated with the plasma membrane, Rac1 acts as a binary molecular switch between the GDP-bound inactive (off) and GTP-bound active (on) states (3). The activation and inactivation cycle is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and GDP-dissociation inhibitors (GDIs) (Fig. 1). GEFs activate Rac1 to transmit an incoming signal through a series of fast reactions: catalysis of GDP release, transient stabilization of the nucleotide-free state during GDP/GTP exchange, and catalysis of GTP binding. GAPs inactivate Rac1 by accelerating the weak intrinsic hydrolysis of GTP to GDP through stabilization of the charged

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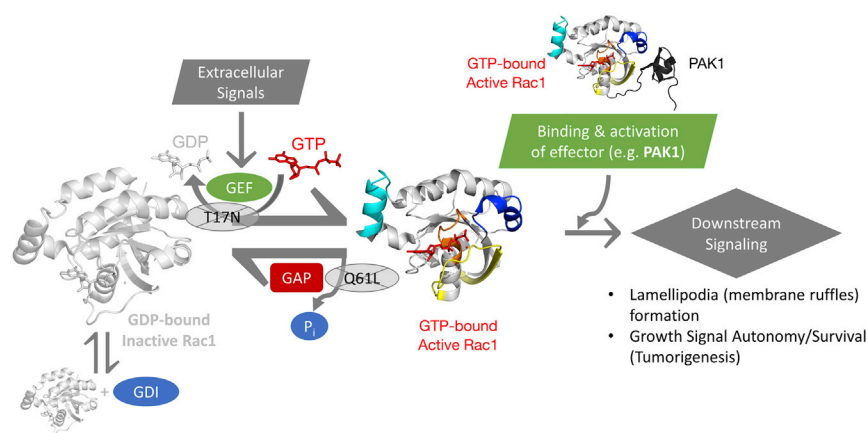


FIGURE 1 Rac1 activation and deactivation cycle. To see this figure in color, go online.

intermediate and reduction of the activation barrier for hydrolysis (3–6). On the other hand, the negative regulators of the Rho family, namely RhoGDIs, inhibit both nucleotide dissociation of GDP and hydrolysis of GTP and can help Rac1 release from the membrane (Fig. 1; (7)). GDIs interact with both GDP- and GTP-bound GTPases, with a lower affinity toward the latter (8). One of the most important downstream effectors of Rac1 is PAK1 (p21-activated kinase 1, belonging to the Pak family) (9). PAKs are serine/threonine kinases that interact with multiple partners to regulate essential cellular processes such as cytoskeletal regulation, motility, and apoptosis (10).

Rac1 is involved in various steps of cancer, including its “player” role in cancer cell migration and invasion, as well as its “driver” role in regulating tumor metastasis and progression (11–19). In this study, we focus on the oncogenic mutational positions P29, Q61, and Y72, as well as one negative control position, T17 (Fig. 2). Rac1 is the third most frequently mutated proto-oncogene in melanoma (after Braf and NRas, identified in 9% of ultraviolet-light-related melanomas) with the hotspot somatic missense mutation P29S (19–22). P29S is a fast-recycling (i.e., spontaneously active) mutant with gain-of-function property because it increases the ability to switch from the inactive to the active state through enhanced GDP-GTP exchange. Without losing its GTPase function, this increases the binding events to downstream effectors (20,21). Q61R is a prostate-cancer-associated gain-of-function mutation in Rac1 (23–25) and potently induces Rac1 activation, as in P29S (26). Q61L, however, is a constitutively active loss-of-function mutation that is oncogenic in KRas, but not in Rac1. Q61L prevents GTPase activity by blocking GAP-stimulated hydrolysis of GTP to GDP (4). Fast-recycling mutants might be more efficient oncogenic drivers than constitutively active mutants because of their ability to mimic normal signaling (19). Y72C is a rare oncogenic mutation related to liver carcinoma listed in the COSMIC database (23). Y72C is considered as a case study here because of a lack of information about the functional mechanism leading to its oncogenicity. T17N, used as a negative test case, is a dominant-

negative mutation on the p-loop that prevents GTP binding and reduces GDP binding (2). Thus, T17N is in either a nucleotide-free or inactive state, causing competitive binding to GEFs with higher affinity than the wild-type to block activation (27). Both dominant-negative and constitutively active mutants have important roles in the relation of the Rho GTPase family (including Rac1) with oncogenesis (27).

There are four main functional sites in Rac1 protein: the insert region, the p-loop, and the switch loops (switches I and II) (Fig. 2 A). The insert region is specific to the Rho subfamily and regulates signaling activity by interacting with GEFs (28), GDIs (29), and downstream effectors (30). Although being distant from the binding site, the insert region was shown to be important in GDI binding, which leads to the inhibition of GDP dissociation by stabilizing the bound nucleotide (29,31). The p-loop coordinates the γ -phosphate of the guanine nucleotides (GDP and GTP) and Mg^{2+} ion during the nucleotide binding of Rac1 (28). The p-loop was also shown to have a role in GAP binding along with the switch loops (32). Switch I, also known as the “effector region,” is mainly responsible for the interaction of Rac1 with downstream effectors (e.g., PAK1 and MLK3). Switch I also includes some important nucleotide-binding residues. Switch II is important for the specific recognition and activation of Rac1 by GEFs and/or inactivation by GAPs (33). Overall, the residues on both switch loops participate in upstream and downstream signaling through PAK1, GAP, GEF, and GDI binding, thus having a crucial role in Rac1 activity (28,33).

The conformation of the switch loops changes dramatically upon GTP binding with hydrogen bonds forming between the γ -phosphate and residues T35 (switch I) and G60 (switch II) (7). During GTP hydrolysis, it is important to position T35 such that the affinity for the nucleotide binding is enhanced via the coordination of the main chain carbonyl of T35 with both the Mg^{2+} ion and the γ -phosphate (7). Mg^{2+} is considered as a “gatekeeper” of nucleotide exchange because it is involved in the regulation of both nucleotide binding and hydrolysis kinetics. In addition,

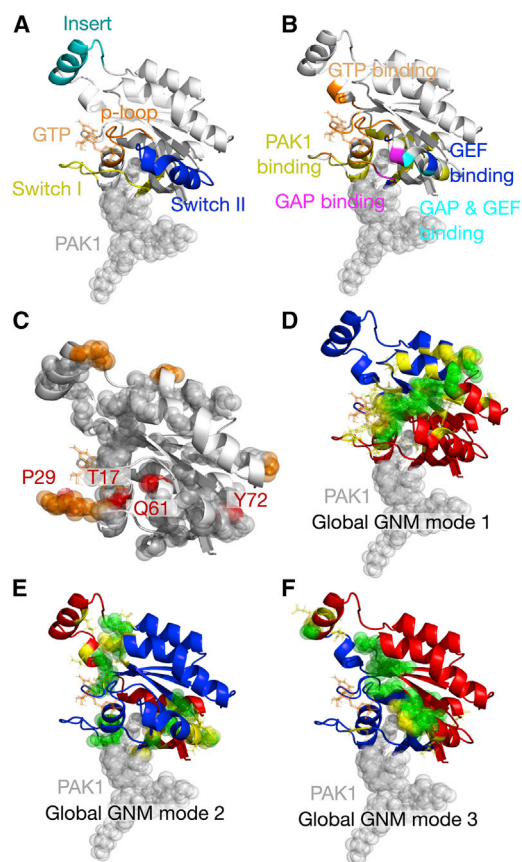


FIGURE 2 Dynamic and functional domains and oncogenic mutations on Rac1. (A) Functional domains: p-loop (orange: residues 10–17, GTP binding), switch I (magenta: residues 26–45, downstream effector), switch II (yellow: residues 59–74, activation loop), and insert (cyan: residues 124–135) regions are shown as spheres on the cartoon representations of Rac1 structure. (B) GTP-binding residues (orange: residues 10–17, 28, 32, 34, 35, 60, 116, 118, 119, and 158–160) and PAK1-binding residues (magenta: residues 20, 21, 23–25, 27, 31, 33, 34, 36–47, 56, 58, 63, 64, 66, 67, 70, 165, 166, 169, 170, 173, and 174) are shown. Rac1-PAK1 binding interface was extracted from Cdc42-PAK1 and Rac3-PAK1 complex structures (PDB: 1E0A and 2QME) using HotRegion server (<http://prism.cccb.ku.edu.tr/hotregion/>). Common with PAK1 binding are some GAP binding (yellow: residues 36, 37, and 63) and GEF binding (blue: residues 38, 39, 61, and 66), and both GEF- and GAP-binding regions (cyan: 64 and 67) are shown as spheres. (C) All oncogenic mutations corresponding (white spheres) and not corresponding (orange spheres) to the first three slowest modes and the selected case study mutations T17, P29, Q61, and Y72 (red spheres) are shown. (D–F) Dynamic domains of Rac1 of the first three slowest modes of motion are shown; red and blue display two dynamic domains moving in opposite directions, and green regions are hinge sites. The oncogenic mutations are shown in yellow close to or on the hinge sites (spheres are on the hinges, and lines are nearby residues). To see this figure in color, go online.

Mg^{2+} stabilizes both nucleotide-bound states of Rac1. Its coordination with T17, T35, and D57 can keep Rac1 in the GDP-bound inactive state, whereas both GDP and Mg^{2+} are destabilized during GEF binding and activation of Rac1 (34,35). On the other hand, Mg^{2+} binding enhances the intrinsic GTP hydrolysis rates up to 10-fold, and its presence significantly increases the rates of the RhoGAP-catalyzed hydrolysis reactions (35). So, new interactions

introduced with GTP binding change the structure and dynamics of the switch loops, which are unstructured or highly flexible otherwise (7). Moreover, the switch loops display significantly reduced flexibility upon effector binding (36).

Exploring the structural and dynamic alterations underlying functional variations with disease-related genetic mutations is of significant interest toward drug development in cancer treatments (37). Active mutations likely change the dynamics of the off and on states as well as the transition pathway in between, shifting the populations in favor of certain states. The computation of conformational heterogeneity of mutant GTPases (e.g., Ras and Rac1) is important yet challenging because the entire conformational transition pathway can only be observed using extended classical molecular dynamics (MD) simulation timescales (microseconds or milliseconds) or accelerated MD simulations (38–40). In the scope of this work, we focus on the GTP-bound (on) state of Rac1 and the effects of active mutations on its intrinsic dynamics and binding interactions, particularly PAK1-binding. Two of these Rac1 mutants already known to be fast recycling (spontaneously active, P29S) and constitutively active (Q61L) have increased population in the GTP-bound state (41,42).

Binding is an intrinsic dynamic phenomenon. The cooperative motion favored by the intrinsic dynamics has been shown to have close similarity to structural changes observed during binding actions, i.e., global motions underlie functional structural changes in different states (liganded, complexed, or free) observed in experiments (43–46). These conformational motions are usually energetically favorable fluctuations driven by low-frequency global modes. The ligands and oligomeric interactions modulate the equilibrium conformation by affecting the energetic profile along these few global modes, which trigger allosteric interactions. The association of active mutations with the three global dynamic modes that we show in Rac1 provides us with a mechanistic framework. Although we focus on the Rac1-PAK1 interaction by atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) experiments, we seek the response of internal dynamics of Rac1 to the active mutations with respect to the intrinsic binding behavior by MD simulations and the structural modeling of wild-type and mutant Rac1s in complex with PAK1.

MATERIALS AND METHODS

Experimental methods

We performed AFM-based SMFS experiments to probe the unbinding dynamics of Rac1 against PAK1. Specifically, we measured the single-molecular interactions of wild-type as well as T17N, Q61L, and Y72C mutants of Rac1 with PAK1 (we only used p21-binding domain of PAK1 in our experiments). In this method, the free energy landscape (FEL) of the molecular pair can be perturbed by directly applying force on individual molecules so that the influence of force on transition rates from one state to another can be measured. Preparation and immobilization of molecules, the experimental setup, and data

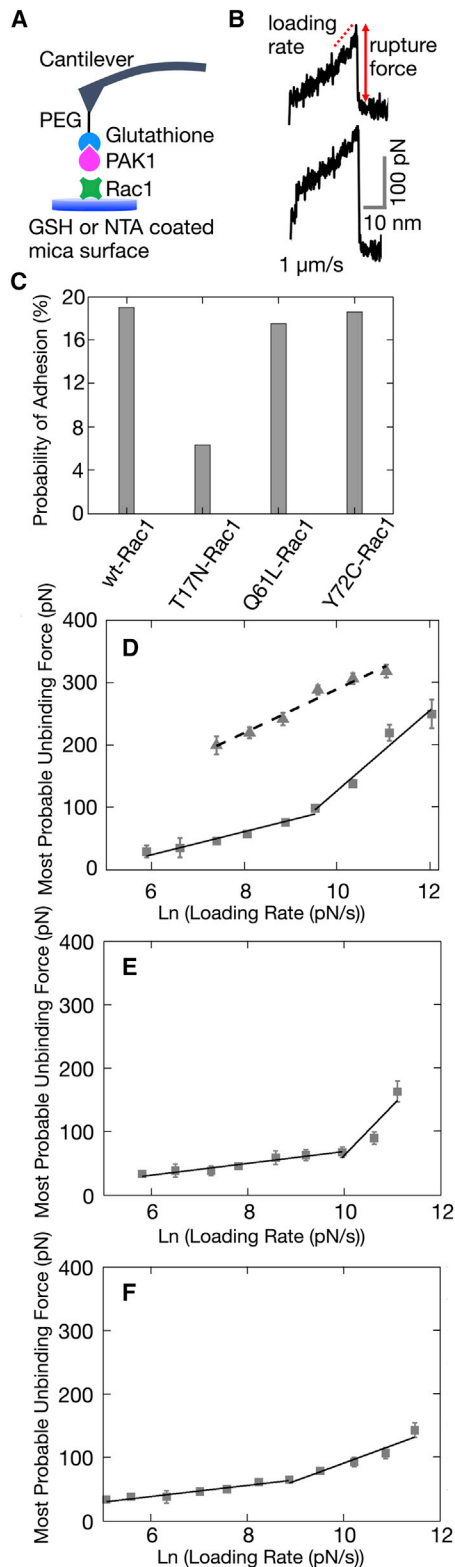


FIGURE 3 The design of AFM pulling experiments. (A) A schematic representation of Rac1-PAK1 experiment setup is given. (B) Example experimental force-distance traces of low (top) and high (bottom) unbinding forces for wt Rac1-PAK1 complex at 1 $\mu\text{m/s}$ pulling velocity are shown. Calculation of loading rate and rupture force is indicated on the exemplary curve. The polymeric extension profile of the curves is due to the stretching

interpretation are explained in detail in [Supporting materials and methods](#). The experimental setup and the multistep functionalization of AFM tips and surfaces are depicted in [Fig. 3 A](#) (the details are explained in [Supporting materials and methods](#)). We assessed the specificity of the measured forces via three different control experiments (see [Fig. S1](#)). We determined the most probable rupture force of the Rac1-PAK1 complexes at a given loading rate by calculating the median of the force histograms (for the details of data interpretation, see the corresponding sections and [Eq. S1](#)).

Computational methods

We mapped the known oncogenic mutations on Rac1 using the data in COSMIC and cBioPortal ([23–25](#)) to the wild-type Rac1 structure (Protein Data Bank, PDB: 3TH5) to observe the extent of the association of the disease-related mutations with the predicted global hinge sites considering three slowest modes using the Gaussian network model (GNM) ([Fig. 2, D–F; \(47,48\)](#)). Then, we selected the case-study mutations and performed MD simulations for the wild-type and mutant Rac1 structures. The starting structures for the simulations are the GTP-bound wild-type Rac1 (PDB: 3TH5), P29S mutant Rac1 (PDB: 3SBD), Q61L mutant Rac1 (PDB: 4GZL), Q61R, Y72C mutant Rac1 (in silico), and also the nucleotide-free T17N mutant Rac1 (PDB: 3B13). We performed a 2.4- μs -long simulation including four (100-ns-long) parallel runs for the wild-type and five mutant Rac1 structures. We analyzed an MD-sampled ensemble of conformations in detail to identify and characterize the shift and variations between the wild-type and mutant Rac1 structures (see [Table S1](#) and [Supporting materials and methods](#) for details). We also modeled the complexes of the representative Rac1 conformations in each simulated system (wild-type (wt), P29S, Q61L, Q61R, Y72C, and T17N Rac1) with the available PAK1 structure (PDB: 1E0A) to complement the MD analysis and results ([Table S2](#)).

RESULTS AND DISCUSSION

Mechanistic role of active mutations at global hinge sites

Disease-related genetic mutations are likely to be associated with the global hinge sites that coordinate the collective functional motion of Rac1 ([46,49,50](#)). We observe $\sim 90\%$ (61 out of 68) of the Rac1 oncogenic mutations at or near the principle hinge axes of the three slowest, i.e., global, modes of motion revealed by GNM, providing a dynamic rationale for not being sporadic in the structure ([Figs. 2, C–F](#) and [S2](#)), as observed previously ([50](#)).

of the polyethylene glycol linker. (C) The probability of adhesion of wild-type, T17N, Q61L, and Y72C Rac1-PAK1 observed during experiments is given. (D) Most probable rupture forces of wild-type Rac1-PAK1 complex depending on the loading rates are shown, with triangle data points for the state 1 and square data points for state 2 with the SEMs and the fitted linear relation is shown with straight and dashed lines, respectively. Two distinct linear regimes describe the best relation for state 2, corresponding to the two distinct activation energy barriers of the dissociation reaction of the complex. (E) Rupture force spectra of Q61L Rac1-PAK1 complex are shown in square data points with the SEMs and the fitted linear relation is shown with straight line. Two distinct linear regimes are fitted to the relation corresponding to the two distinct activation energy barriers of the complex dissociation reaction. (F) Rupture force spectra of Y72C Rac1-PAK1 complex are shown in square data points with the SEMs and the fitted linear relation is shown with straight line. Two distinct linear regimes are fitted to the relation corresponding to the two distinct activation energy barriers of the complex dissociation reaction. To see this figure in color, go online.

We selected three of the oncogenic mutational positions (P29, Q61, and Y72) and one negative control position (T17) that are at or near these global hinge sites, at functional and binding sites of Rac1, and have known diverse effects on Rac1 function. These mutations are, namely, the dominant-negative T17N, constitutively active Q61L, and oncogenic P29S, Q61R, and Y72C. Their positions on Rac1 and their proximity to the binding sites are as follows (Fig. 2, A–C): T17 on the p-loop is a nucleotide-binding residue also interacting with an Mg^{2+} ion. P29 on switch I is close to both the GTP- and the PAK1-binding regions. Q61 on switch II is a GEF-binding residue and close to GTP-, GAP-, PAK1-, and GAP-binding residues. Lastly, Y72 on switch II is close to PAK1-binding residue L70.

The effects of perturbations introduced by T17N, P29S, Q61L/R, and Y72C mutations on the intrinsic dynamics of Rac1 by concentrating on the binding regions of Rac1 with GTP, upstream proteins (GEF, GDI, and GAP), and downstream partner PAK1 are analyzed through extensive MD simulations. Particularly, the effect of Q61L and Y72C mutations specifically on Rac1-PAK1 binding mechanics has been subjected to a more elaborate analysis with SMFS experiments using AFM and molecular modeling of the complex structure.

GTP-loaded wild-type Rac1-PAK1 is a multistate complex

The measured force curves with AFM-based SMFS experiments (experimental setup depicted in Fig. 3 A) initially show a characteristic increasing force as a result of flexible stretching of the polyethylene glycol linker and then a sudden decrease in force due to the unbinding of Rac1-PAK1 complex (Fig. 3 B). We observe a similar probability of adhesion in the GTP-loaded wt, Q61L, and Y72C Rac1-PAK1s under similar experimental conditions. It is, however, significantly lower for the nucleotide-free dominant-negative T17N Rac1-PAK1, which indicates a loss of binding ability of Rac1 to PAK1 with T17N, which we used as a negative control case (Fig. 3 C). A bimodal distribution in the unbinding forces of wt Rac1-PAK1 appears within the range of measured loading rates, whereas it becomes unimodal with Q61L and Y72C. The bimodal distribution of the unbinding forces can be explained by the existence of two different states in the mechanics of wt Rac1-PAK1 (namely state 1 and state 2 for the lower and higher unbinding forces, respectively). These two states may correspond to different conformational and/or dynamical states of either Rac1 itself or the Rac1-PAK1 complex structure. The measured rupture forces between 200 and 300 pN of Rac1-PAK1, which will be referred as state 1, are observed only in wt Rac1. The forces observed in this state are even higher than the unfolding forces of Ig domains from cardiac titin (~ 200 pN) at similar loading rates (Fig. 3 D, triangles; (51)). The measured rupture forces of wt Rac1-PAK1 in the range of

20–200 pN also appear for the Q61L and Y72C Rac1-PAK1s at similar loading rates (Fig. 3, D–F; Fig. S4). This state will be referred as state 2. Higher rupture forces indicate that state 1 of wt Rac1 is mechanically a more stable but probabilistically less-sampled state (Fig. S3A).

Change in the mechanics of Rac1-PAK1 complex upon selected active mutations

The dynamic force spectra obtained from the most probable rupture forces at each loading rate interval for wt, Q61L, and Y72C Rac1-PAK1s are shown in Fig. 3, D–F (see also Fig. S4), respectively. The linear increase in rupture forces with logarithmically increasing loading rates is in agreement with the phenomenological Bell-Evans model (52,53). Although a single-barrier model is sufficient to describe the unbinding FEL of state 1 of wt Rac1-PAK1 dissociation, the unbinding FELs of state 2 of wt, Q61L, and Y72C Rac1-PAK1s possess multiple barriers in the range of measured loading rates. The outer and inner barriers are dominant at low and high loading rates, respectively.

Table 1 displays the natural dissociation rate (k_{off}) and the effective distance to the transition state (x_β) for each case according to the Bell-Evans model. With multiple energy barriers, only the rate-limiting slowest step (the outer barriers with significantly lower k_{off} -values than the inner barriers) is considered for further discussion. The lower the dissociation rate, the higher the activation energy barrier in the unbinding FEL and the longer the lifetime of the complex. As such, the unbinding rate of the mutants shifts toward state 1, whereas unbinding forces resemble state 2 of wt Rac1 in the dynamic force spectrum.

The distance from the ground state (x_β) to the transition state along the unbinding pathway also changes with mutations (Table 1). The higher the distance to the transition state is, the wider the energy barrier. Because the FEL is a rough surface, a wider barrier is directly related to the abundance of intermediate states. Alternatively, higher distance to the transition states may also imply a higher elasticity of the protein itself because the protein may extend as the ligand pulls away (54). Relatively shorter distances for both states of wt Rac1-PAK1 interaction suggest a highly structurally

TABLE 1 Energy landscape parameters

Ligand-receptor		Loading rate range (pN/s)	x_β (nm)	k_{off} (s^{-1})	$\Delta\Delta E$ ($k_B T$) ^a
Wild-type Rac1-PAK1	state 2	3.5×10^2 – 1.4×10^4	0.22	5.5	
		1.4×10^4 – 1.7×10^5	0.06	46.2	
	state 1	1.6×10^3 – 6.2×10^4	0.12	0.2	
Q61L Rac1-PAK1		3.2×10^2 – 2.1×10^4	0.44	1.2	1.5
		2.1×10^4 – 6.5×10^4	0.05	121.0	–1.0
Y72C Rac1-PAK1		1.6×10^2 – 7.1×10^4	0.47	0.5	2.4
		7.1×10^4 – 9.6×10^4	0.15	28.5	0.5

^a $\Delta\Delta E$ is relative to wild-type Rac1-PAK1 binding energy.

coordinated unbinding process of the complex. The higher distance to the transition state observed in the Q61L and Y72C mutants indicates an increased elasticity either locally in the binding interface, which might be related to the variable spatial accessibility of the Rac1 binding sites, or globally in the whole Rac1 structure.

We thus anticipate that there should be a change in the internal dynamic cooperativity in Rac1-PAK1 complex with the mutations, which we explore with the intrinsic dynamics of Rac1 by MD simulations in the following sections.

Intrinsic dynamics of Rac1 revealed by MD simulations

Binding is an intrinsic dynamic phenomenon. The intrinsic dynamics of allosteric proteins are defined by their topology of inter-residue contacts and favor cooperative motions that are plausibly structural changes approximated to their allosteric and binding actions. Moreover, the structure of the Rac1-PAK1 complex has not been experimentally determined yet, so we try to enlighten the mutation-driven changes in binding interfaces of Rac1 through analyzing intrinsic dynamic events. We seek here the structural and dynamic response of Rac1 to the active mutations. To this end, extensive MD simulations have been performed on the GTP-bound wt, Q61L, Y72C, P29S, and Q61R and GTP-free T17N Rac1s. Except for the dominant-negative T17 mutant, Rac1s do not undergo significant conformational changes but display subtle dynamical changes (Fig. S5), as will be described below.

Mutations modify the allosteric communication among binding interfaces of Rac1

Rac1 acts through its interactions with GTP/Mg²⁺, upstream proteins (GEF, GDI, and GAP), and downstream partner PAK1. The active mutations affect the global dynamics of Rac1 underlying the cooperativity of allosteric binding interactions with the change in the local interactions and hydrogen bonding and residue conformational preferences, particularly at critical binding residues with Mg²⁺/GTP and upstream and downstream partners, as presented below.

Rac1's interactions with Mg²⁺ ion and GTP

The active mutations, except Y72C, allosterically change the position of Mg²⁺ and the stability of GTP with redistribution of hydrogen bonds around GTP (Fig. 4; Figs. S6, A and B, S7, and S8).

The nucleotide controls the positioning of switch loops through binding to the pocket between the p-loop (G10-T17), switch I (F28, Y32, P34, T35), switch II (Q60), helix 6 (K116, D118, and L119), and residues S158-L160 (Fig. 2 B; (3)). T17, T35, and D57 of Rac1, in coordination with Mg²⁺, are critical for the activation cycle of Rac1. Although the distances of Mg²⁺ from these three residues display a unimodal distribution for wt Rac1, the multimodal distances observed in mutant Rac1s display the change in the relative proximity of Mg²⁺ with GTP and Rac1 and also the change in H-bonding. We observe that H-bond occupancy of T17 (main) and T35 (side) with GTP decreases with compensating new H-bonds between K16 (side) and S29 (main)

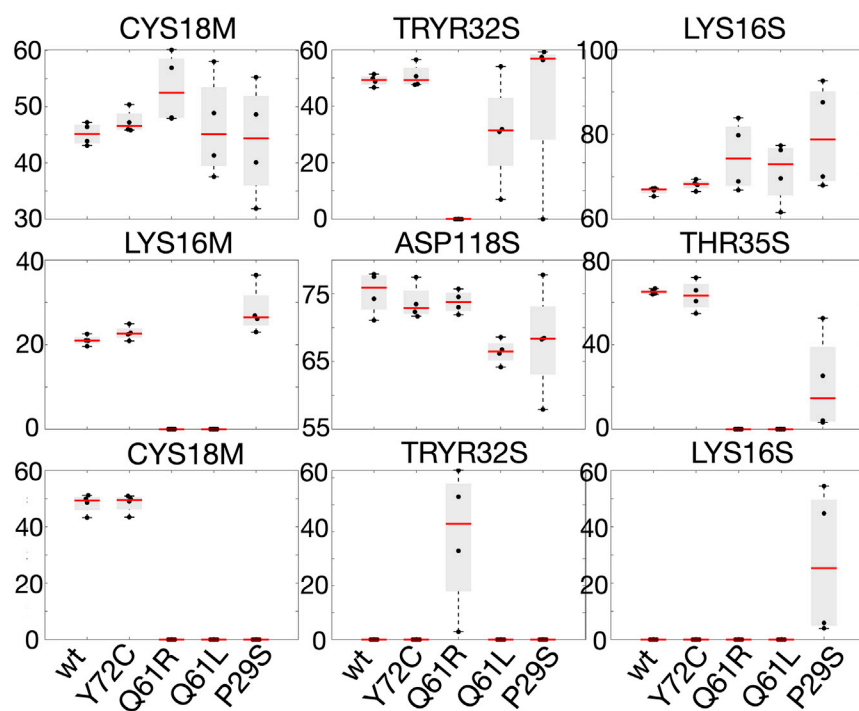


FIGURE 4 Box plot of H-bonding occupancies between Rac1 and GTP at different systems. The red line is the median; the boxes show the 25th and 75th percentiles, and whiskers represent $2.7 \times$ the standard deviation. The H-bonds with an occupancy higher than 20% are considered. There is a significant decrease in the occupancies of H-bonds between GTP-T17 and GTP-T35 with P29S, Q61L, and Q61R. The H-bond between GTP-Y32 also decreases upon Q61L and diminishes with Q61R, whereas GTP-K16 (main) also diminishes upon Q61L/R mutations. The occupancy of H-bond between GTP-K16 increases, and a new H-bond is formed between GTP-R61 with Q61R. Similarly, the occupancy of H-bond between GTP-K16 (main and side) increases, and a new H-bond is formed between GTP-S29 with P29S. To see this figure in color, go online.

and GTP in P29S Rac1 (Fig. 4). This new S29-GTP H-bond observed in P29S Rac1 was previously shown to alter the structure into a Ras-like switch I conformation with increased effector activation (20). Likewise, Q61L and Q61R Rac1s, especially for Q61L, also show a significant decrease in H-bond occupancies of T17 (main) and T35 (side) with GTP (Fig. 4). However, Q61R gains a new compensating H-bond between R61 and GTP, reflecting a distinct behavior between two different mutations of the same position. We speculate that these phenomenal changes in H-bonding with interatomic distances around GTP-binding and Mg^{2+} -coordinating residues alter the coordination of the nucleotide in a nonhydrolyzable manner in the constitutively active Q61L, whereas Q61R Rac1 can withstand this effect and still stabilize the nucleotide in a hydrolyzable state with the help of the newly gained H-bond. On the other hand, although D57 approaches both GTP and Mg^{2+} significantly, it does not form a H-bond with GTP; instead, it forms a new H-bond with GTP-binding and Mg^{2+} -coordinating residue T35 in the proximity of PAK1-binding interface only in Q61L Rac1. Y72C Rac1 behaves similar to wt Rac1 in terms of residue distances from Mg^{2+} /GTP and H-bonds with GTP.

Together with distances and H-bonding, the side-chain dihedral angles of Mg^{2+} -coordinating and GTP-binding T17 and T35 also show distinctive characteristics (Fig. S10). The median values of χ_1 angle residues T17 and T35 shift with all mutations except Y72C and most significantly with T17N Rac1 (Fig. S10), implying higher variation or flexibility of these residues with altered orientations and explaining the loss of H-bonds with GTP around this region with all of the active mutations except Y72C. Moreover, T35 has two states in Q61L Rac1 and three states in P29S Rac1 (Fig. S10). In parallel, we observe a higher flexibility of Y32 and T35 in P29S, Q61L, and Q61R Rac1s, whereas T35 is slightly more restricted in Y72C Rac1.

Change in mechanics at the upstream and downstream binding partners

Rac1's upstream and downstream binding partners, as well as GTP, share some of the same Rac1 residues in their interactions. V36 and F37 on switch I and D63, Y64, and L67 on switch II, being also PAK1-binding sites, are seen as the predominant contacts with GAP on the Rho family GTPases (Fig. 2 B; (7)). Moreover, the mutation of D38 and N39 on switch I and Q61, Y64, R66, and L67 on switch II lead to loss of GEF binding, indicating their involvement in GEF binding (55). Also, W56 is crucial for the recognition by GEFs, and Y32, D65, L70, and S71 are important in the catalysis of efficient exchange of nucleotides (GDP to GTP) during activation (55). On the other hand, T35, V36, Y64, R66, L67, L70, P73, H103, and H104, as based on Cdc42, were suggested as GDI-interacting residues (31).

Flexibility at a given region of a protein structure is reflected by its range of fluctuations in the backbone and/or side dihe-

dral angles. The difference of backbone dihedral angle mean-square fluctuations of each mutant with respect to wt is given in Fig. S11. The main difference in the backbone flexibility is observed mainly at switch regions, which comprise the binding interfaces of both GTP and upstream and downstream partners, including PAK1-binding interfaces.

PAK1, GEF, GDI, and GAP can competitively bind to Rac1 using shared interface residues such as V36-N39 on switch I and Q61, N63, Y64, R66, and L67 on switch II. Among common binding residues, the most profound difference is observed in the side-chain conformations of V36 and F37 (Fig. S12). The χ_1 (first side-chain dihedral angle) angles of PAK1- and GAP-binding V36 cluster into two orientations in wt and P29S Rac1, whereas only one of these orientations is sampled in Y72C and Q61L Rac1, one that is rarely sampled in Q61R and T17N Rac1. (Fig. S12; Table S3). The NMR structure of Cdc42 in complex with PAK1 (PDB: 1E0A) significantly samples the same orientation as Y72C and Q61L. F37 has a single state, which is the only sampled state of Cdc42 in complex with PAK1, with a very low variability in wt and Y72C Rac1s (Fig. S12; Table S3). The backbone flexibility reflected by the fluctuations of backbone angles of these two residues supports the importance of these sites in competitive upstream and downstream binding (Fig. S13). The flexibility of V36 and F37 is significantly higher in the P29S Rac1, whereas they are slightly more rigid in Q61L and Y72C compared with wt. Knowing that Q61L is unfavorable for GAP binding and the mutant has a defect in the GTP hydrolysis function, we can deduce that V36 and F37 rigidify, and the side chains sample a position more suitable for PAK1 binding with Q61L and Y72C and for GAP binding with Q61R mutations.

Among the PAK1-only binding interface, the most profound increase in backbone flexibility is observed in residues T25, N26, Y32, P34, M45, V46, A59, G60, D63, and Y64 in all mutants compared with wt (see Fig. S14). T25 and N26 have higher flexibility in Y72C and T17N Rac1, and a relatively subtle increase is observed in P29S compared with wt, whereas no change is observed in the case of Q61L/R. For T17N Rac1, increased flexibility is expected because these residues are in the vicinity of the nucleotide binding pocket, but for the Y72C mutation, this change is an allosteric effect of oncogenic mutation. On the other hand, I33, P34, M45, and V46 have higher flexibility in Q61L, P29S, and T17N compared with wt, Y72C, and Q61R Rac1. Increased flexibility in these regions indicates a mechanically softer binding interface. This result agrees with the longer distance to the transition state obtained from SMFS experiments for Q61L and Y72C mutants compared with wt.

GTP-independent intrinsic dynamics of Rac1

The dominant-negative T17N mutant, which has no bound nucleotide, behaves similarly to the active mutants (even more intensified) in terms of cooperativity with respect to wt Rac1 (Fig. 5). Moreover, for almost all residues, the

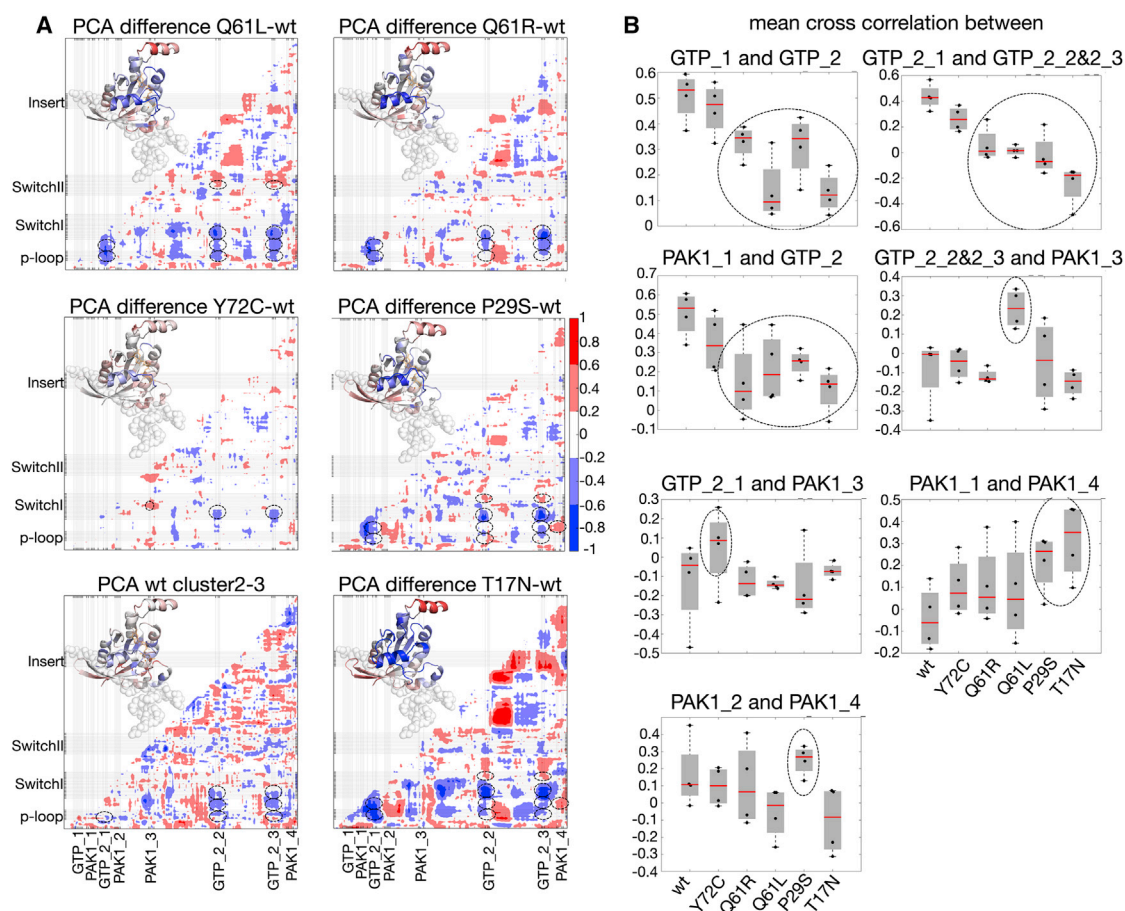


FIGURE 5 Correlation differences of mutants from wild-type Rac1 and the differences between the two wild-type clusters. (A) On correlation difference maps, Rac1 structures are colored by average correlations for GTP-binding residues 28, 32, 34, 35, 60, 116, 118, 119, and 158–160. The residues corresponding to the regions on the *x* axis are listed below. GTP_1 (p-loop): residues 10–17. GTP_2 is composed of three subregions as GTP_2_1: residues 28, 32, 34, and 35; GTP_2_2: residues 116, 118, and 119; and GTP_2_3: residues 158, 159, and 160. PAK1_1: residues 20, 21, 23, 24, and 25; PAK1_2: residues 41–45; PAK1_3: 66, 67, and 70; and PAK1_4: 169, 170, 173, and 174. Transparent spheres on the bottom of the structures represent PAK1 structure taken from PDB: 1E0A. The encircled regions show significant differences in the mutants with respect to wt Rac1, when all the parallel runs are considered. (B) Box plot of Rac1's mean cross correlations coming from parallel runs for different regions at different systems is given. The red line is the median; the boxes show the 25th and 75th percentiles, and whiskers represent $2.7\times$ the standard deviation. To see this figure in color, go online.

nucleotide-free T17N mutant Rac1 spans a wider backbone conformational space, having the highest degree of flexibility with respect to wt and active mutant Rac1s, and interestingly encapsulates the conformational variation of the mutant Rac1s (Fig. S11). The tendency of the nucleotide-free form to approach the GTP-bound states in terms of free energy surfaces was also demonstrated for Rho GTPases previously (56). Thus, it can be concluded that activating mutations can affect the dynamics of Rac1 in a GTP-independent intrinsic manner.

Changes in fluctuations and cooperativity

Significant changes in the occupancy of H-bonds and the interatomic distances in the vicinity of the nucleotide binding pocket affect the positional fluctuations of GTP (Figs. S7–S9; see details in Supporting materials and methods) with mutations. T17N Rac1 shows the highest residue fluctuations overall, but especially around GTP-binding resi-

dues because of the lack of a bound nucleotide (Fig. S15). Higher residue fluctuations of GTP are also observed in the binding cavity of P29S, Q61L, and Q61R Rac1s (Fig. S6 B). Although switch I in P29S and Q61L displays the highest fluctuations, switch II in Y72C displays the most significant restriction in its fluctuations (Fig. S15). Specifically, the Y32-T35 region on switch I that is crucial in nucleotide stabilization is fairly immobile in wt and Y72C Rac1s. This region gets more mobile with P29S and Q61R and gains the highest mobility in Q61L (around a root mean-square fluctuation value of 1.5 Å) (Fig. S15).

GTP-binding residues within wt Rac1 are intercorrelated such that the nucleotide-binding p-loop (G10-T17) and $\alpha 1$ region (G15-T25) are strongly correlated with three other regions containing the GTP-binding residues: the beginning of switch I (GTP-binding residues F28, Y32, P34, and T35); the K116, D118, and L119 region; and the S158-L160 region (Fig. 5 B; see wt in GTP_1 versus GTP_2 and

PAK1_1 versus GTP_2 regions). The $\alpha 1$ region partially contains PAK1-binding residues (L20, I21, and Y23-T25) between the p-loop and switch I, so GTP- and PAK1-binding residues are also cooperative in wt Rac1. The mutants show a commonality in the behavior of correlation changes with respect to wt Rac1, but some changes are more prominent in certain regions for different mutants. When the correlation differences from wt Rac1 are considered, it is immediately observed that the p-loop and $\alpha 1$ PAK1-binding residues (named as GTP_1 and PAK1_1, respectively, in Fig. 5) lose cooperativity with the other GTP-binding regions (GTP_2 in Fig. 5) in all mutations, most significantly with Q61L and T17N (Fig. 5, A and B, GTP_1 versus GTP_2 and GTP_2_2 and 2_3 versus PAK_1_1 regions).

On the other hand, some of the GTP-binding residues are not correlated with some PAK1-binding residues, and some PAK1-binding regions are not correlated with each other in wt Rac1 (Fig. 5 B; see wt in box plots for GTP_2_2 and GTP_2_3 versus PAK1_3, GTP_2_1 versus PAK1_3, PAK1_1 versus PAK1_4, and PAK1_1 versus PAK1_4 regions) but show increased correlations with certain mutations (see P29S, Q61L, and Y72C in respective plots in Fig. 5 B and maps in Fig. 5 A). Q61L also has increased correlations of GTP-binding residues with PAK1-binding residues at the end of switch II (R66, L67, L70), including the critical PAK1-, GEF-, GDI-, and GAP-binding residue L67 (Fig. 5, A and B, GTP_2_2 and 2_3 versus PAK_1_3 region). One of the most remarkable and unique changes with the Y72C mutation is the increase in cooperativity around switch II PAK1-, GEF-, GDI-, and GAP-binding residue L67 and switch I GTP-binding residue T35 (Fig. 5, A and B, GTP_2_1 versus PAK1_3 region).

Binding energies of modeled Rac1-Pak1 complexes from representative MD-sampled conformations

MD-sampled conformations are clustered to disclose whether there are distinctly behaving conformational ensembles by wt and mutant Rac1s. wt Rac1 forms two main relatively evenly populated ensembles. The mutants, however, show either a single cluster (Y72C and Q61R) or multiple clusters, with a major one comprising $\sim 70\%$ of

the population (P29S and Q61L) (Fig. S17; Table 2). Moreover, the dominant-negative T17N Rac1, without a bound nucleotide, has six clusters with one dominating conformational state. Within the time window of MD simulations, we observe a shift in the conformational space with the mutations such that the correlated residue motions of the mutants with respect to wt Rac1 reveal significant changes, especially around the GTP-binding residues, that are similar to the correlation differences of two clusters of wt Rac1 (Fig. 5). Rac1-GTP H-bonding occupancies of different clusters are also subject to changes with mutations that vary among different clusters of mutations (Fig. S18).

The representatives of wt and mutant Rac1 clusters are then used to model Rac1-PAK1 complexes and predict plausible effects of the mutations on the binding behavior based on their predicted binding energy and free energy of dissociation (see Table 2). The predicted binding energies of the modeled complex structures are estimated by the global binding energy calculated at the end of the flexible refinement step of the PRISM algorithm (59,60) by employing FiberDock (flexible induced-fit backbone refinement in molecular docking) (57). FiberDock scores and ranks the putative complexes by including a variety of energy terms, such as desolvation (atomic contact) energy, vdW interactions, partial electrostatics, hydrogen and disulfide bonds, π -stacking, and aliphatic interactions. Moreover, the free energy of assembly dissociation ΔG^{diss} (in kcal/mol) values of the modeled complex structures are predicted by the PDBePISA (Proteins, Interfaces, Structures and Assemblies) server (58). The average predicted binding energies of the two most populated wt Rac1 clusters are -104 (cluster 2, with 53%) and -93 (cluster 3, with 38%) with the corresponding average free energy of dissociation values 6.5 and 5.7 kcal/mol (Fig. S17 and Tables 2 and S2; the details of clustering Rac1s and modeling Rac1-PAK1 complex structures are discussed in Supporting materials and methods).

The consistent binding behavior with lower binding energy (stronger binding) and higher energy of dissociation (more difficult dissociation) as well as the mutant Rac1s assuming mainly the cooperative behavior of one of the clusters of wild-type Rac1, along with SMFS observations,

TABLE 2 Ensembles of Rac1 conformations and binding properties of the modeled Rac1-PAK1 complexes

Structure_cluster#	Member % in the cluster	Parallel MD runs represented in the cluster	Average global binding energy of the top 10 cluster members (FiberDock, unitless (57))	Average ΔG^{diss} of the top 10 cluster members (PDBePISA, kcal/mol (58))
wild-type_cluster 2 (state 1)	53.3	1-2-3-4	-104	6.5
wild-type_cluster 3 (state 2)	38.2	1-2-3-4	-93	5.7
Y72C_cluster 1	100	1-2-3-4	-91	5.9
Q61R_cluster 1	100	1-2-3-4	-96	5.9
Q61L_cluster 1	67.6	1-3-4	-106	5.5
Q61L_cluster 2	17	2	-86	6.8
P29S_cluster 1 (state 1)	70.7	1-2-3	-97	5.6
P29S_cluster 2 (state 2)	25	4	-82	3.8
T17N_cluster1	60	1-2-3	-51	3.1

Modeled Rac1 represents cluster averages.

permit us to map the correspondence between the two most populated clusters (cluster 2 and cluster 3) of the MD simulations and two proposed states: state 1 and state 2, respectively, from AFM experiments in wt Rac1. This is also shown in a recent work (56), in which GTP-bound Rho GTPases were observed to have intermediate states and constitutively active G14V mutant RhoA (G12V in Rac1) was observed to shift the protein toward a state in which switch I has increased flexibility, which may result in the promotion of arbitrary effector binding through induced fit and hence activation of downstream effectors in a less selective or specific manner (56).

CONCLUSIONS

The GNM analyses with MD simulations show the deformation of global modes of motion with the functional mutations, namely dominant-negative T17N, constitutively active Q61L, oncogenic P29S and Q61R, and Y72C. This deformation modifies Rac1's intrinsic dynamics and affects binding interactions with GTP, PAK1, GEF, and GAP.

In the specific case of PAK1, Q61L and Y72C mutations in Rac1 display a shift in the dynamic ensemble as such to favor PAK1 binding in interaction with altered GTP-binding behavior, as supported by AFM-based SMFS experiments. The unbinding behavior of the Rac1-PAK1 complex reveals the existence of at least two dynamic states, state 1 and state 2, of wt Rac1-PAK1, which is reduced to a single state with Q61L and Y72C mutations. A decrease in the dissociation rate similar to state 1 yet similar unbinding forces with respect to state 2 of wt Rac1 imply a shift of the dynamic state toward a more stable Rac1-PAK1 complex with the active mutations. The active Q61L and oncogenic Y72C mutations also alter the mechanics of the molecule(s). The distance to the transition state in the unbinding energy landscape increases. So, the elasticity of the protein increases, resulting in a slower transition to the unbound state. The change in mechanics is more drastic in Y72C than Q61L. This demonstrates that active mutations, especially oncogenic ones, alter the mechanics of Rac1 and affect its interactions with other molecules. The change in the mechanical properties is supported by the results obtained from extensive atomistic MD simulations. Analyses of the backbone and side-chain angle rotation, together with H-bonding network and correlated motions within and among GTP- and PAK1-binding interfaces, reveal more flexible GTP- and PAK1-binding residues on switch I and switch II with Q61L and Y72C, as well as oncogenic P29S and Q61R and negative T17N. The predicted binding free energies of the modeled Rac1-PAK1 complexes indicate a more favorable PAK1 interaction. Our concerted results suggest that the active mutations affect intrinsic functional dynamic events and alter the mechanics underlying the binding of Rac1 to GTP and upstream and downstream partners, including PAK1.

In conclusion, although the positions of the mutations appear seemingly sporadic, they have the commonality of aligning with the global hinges and have the dynamic capacity to affect the natural functional cycle of Rac1. Thus, in response to any perturbations to Rac1's intrinsic dynamics, both upstream and downstream interactions are affected because of an allosterically regulated interplay between the functionally important regions on Rac1, leading to the activation of cell signaling networks.

SUPPORTING MATERIAL

Supporting Material can be found online at <https://doi.org/10.1016/j.bpj.2021.01.016>.

AUTHOR CONTRIBUTIONS

S.E.A. and F.S. performed computational and experimental research and contributed equally to this work. S.E.A., F.S., H.T., and T.H. designed research, analyzed data, and wrote the manuscript.

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