Direct Attack on RAS: Intramolecular Communication and Mutation-Specific Effects
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Abstract

The crystal structure of RAS was first solved 25 years ago. In spite of tremendous and sustained efforts, there are still no drugs in the clinic that directly target this major driver of human cancers. Recent success in the discovery of compounds that bind RAS and inhibit signaling has fueled renewed enthusiasm, and in-depth understanding of the structure and function of RAS has opened new avenues for direct targeting. To succeed, we must focus on the molecular details of the RAS structure and understand at a high-resolution level how the oncogenic mutants impair function. Structural networks of intramolecular communication between the RAS active site and membrane-interacting regions on the G-domain are disrupted in oncogenic mutants. Although conserved across the isoforms, these networks are near hot spots of protein–ligand interactions with amino acid composition that varies among RAS proteins. These differences could have an effect on stabilization of conformational states of interest in attenuating signaling through RAS. The development of strategies to target these novel sites will add a fresh direction in the quest to conquer RAS-driven cancers. Clin Cancer Res; 21(8); 1810–8. ©2015 AACR.

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Introduction

RAS GTPase is a master signaling protein at the hub of numerous signal transduction pathways, responding to upstream signals from a variety of sources and interacting with a wide range of effector proteins to regulate cell proliferation, survival, migration, and apoptosis (1). Signal transduction pathways involving RAS are illustrated in McCormick’s overview of this CCR Focus series (2). The three major isoforms of RAS, KRAS, NRAS, and HRAS together are mutated in about 20% of human cancers, primarily in the active site at residues G12, G13, and Q61 near the γ-phosphate of the guanosine triphosphate (GTP) substrate (Fig. 1). RAS GTPases are, therefore, among the most urgent targets for the treatment of cancer. Yet, to date, there are no drugs in the clinic that directly target RAS, and thus, these proteins have come to be thought of as undruggable. The current state of directly drugging the G-domain of RAS is the subject of this review. Targeting post-translational modifications at the C-terminus is discussed by Cox and colleagues (3) in this CCR Focus series, while alternative strategies focused on altered metabolic pathways driven by RAS mutants and synthetic lethality approaches are reviewed by Kimmelman (4) and Downward (5), respectively.

RAS functions as an on/off switch regulated by guanine nucleotide exchange factors (GEF), such as SOS, to facilitate loading of GTP turning the signal on, and by GTPase-activating proteins (GAP), such as p120GAP and neurofibromin (NF1), that increase the very slow RAS intrinsic GTP hydrolysis rate to turn the signal off (6). This cycle is represented in Fig. 1 in the article by Cox and colleagues (3). Although this canonical cycle is the regulatory holy grail for RAS and related GTPases, a high-resolution view reveals variations that embellish and complement this cycle based on detailed structural features. RAS is composed of the core G-domain containing the catalytic machinery and a lipidated C-terminal hypervariable region with low homology between isoforms, which tethers the G-domain to the membrane and drives localization to specific microdomains (7). The G-domain of RAS is composed of a 6-stranded β-sheet flanked by 6 α-helices and 10 connecting loops with two switch regions, switch I and switch II, that have different conformations in the GDP (guanosine diphosphate) and GTP-bound states (Fig. 1 and Fig. 2; ref. 8). Residues in the P-loop interact closely with the nucleotide. Within the range of conformations accessed by the switch regions in RAS–GTP, switch II has two major states: One in which it is open, away from the nucleotide (state 1) and the other where it is closed over the nucleotide (state 2; Fig. 2A; ref. 9). Switch I must be in state 2 to interact with effector proteins (10). Switch II contains the catalytic residue Q61 and is normally highly disordered (11), resulting in a very slow hydrolysis rate in the absence of GAPs (12). We have shown an association between allosteric modulation and intrinsic hydrolysis in the presence of RAF that would be expected to take place independent of GAPs (13–16). In our current model, the GTP hydrolysis rate is expected to be increased in the RAS/RAF complex by binding of Ca²⁺ at a remote allosteric site, resulting in a conformational shift from a catalytically impaired T state (disordered active site) to the competent R state in which the catalytic residue Q61 is ordered in the active site (Fig. 2B). Note that accompanying this disorder to order transition is a shift in helix3/loop 7 toward helix 4. Given the canonical GTPase cycle, major points of interference expected to result in signal attenuation through RAS are the inhibition of exchange of GDP to GTP catalyzed by GEFs and the inhibition of interaction between...
RAS–GTP and its effector proteins (Fig. 3). Stabilization of distinct conformational states opens new opportunities for targeting RAS directly. Switch I stabilized in state 1 would have greatly diminished interaction with effectors and switch II stabilized in the ordered R state would promote intrinsic hydrolysis (Fig. 3). This review first focuses on the accomplishments achieved so far in terms of small-molecule inhibitors that directly interact with RAS. We then turn to an analysis of the structural details associated with RAS–GTP conformational states and communication between remote areas of the G-domain, as well as the effect of oncogenic mutations. This structural understanding suggests unexplored strategies to move forward in new directions.

**The State of Small Molecules That Directly Bind RAS**

The problem of drugging RAS for treatment of human cancers is a major challenge for the academic and industry research communities, and the limited success attained so far has recently converged in a call for renewed efforts to conquer this seemingly insurmountable problem (17, 18). Numerous reviews on this topic have been published in the last 2 years (19–27). These articles outline the diverse strategies used to target RAS and related signaling pathways, including direct targeting. The picomolar affinity of RAS for GDP and GTP, coupled with sub-millimolar concentrations of these nucleotides in the cell, make the prospect of developing drugs that effectively compete with the nucleotide generally unrealistic. Furthermore, the lack of other well-defined active site pockets have left us with limited options for directly targeting RAS. The exchange of GDP for GTP, catalyzed by GEFs, turns on signaling through RAS, promoting interaction with effector proteins (1). Hydrolysis of GTP to GDP is the key reaction through which RAS signaling is turned off and is the center of action for oncogenic mutations at G12, G13, and Q61 that impair both intrinsic and GAP-catalyzed hydrolysis (28). The challenge of directly targeting the RAS G-domain, thus, amounts to three approaches, all of which have been attempted with little or moderate success: increasing hydrolysis rates of mutant RAS (29, 30), inhibiting the exchange of GDP for GTP (31–39), or interfering with effector binding and activation (40–49). Recent efforts based on high-throughput screening of fragments to identify compounds that affect downstream signaling, accompanied by crystal structures that allowed structure-based optimization in some cases, have for the first time provided a high-resolution view of how small molecules are accommodated in binding pockets near the active site to interfere with RAS signaling (33–35, 39).

Within this cohort of structures are covalent inhibitors of KRASG12C (33, 35), a mutant commonly found in lung adenocarcinoma (28), bringing to the forefront the prospect of selectively targeting this specific mutant with minimal effect on wild-type RAS. This kind of specificity will be difficult to achieve with other oncogenic mutants due to their less reactive side chains. One of the two covalent series of inhibitors consists of nucleotide analogues that bind in place of GDP (33), and the other series of inhibitors bind in a pocket between switch II and helix 3 (35). Other compounds for which there are crystal structures are not selective for particular mutants and bind at a site on the outer side of switch II (34, 39). All of these compounds with high-resolution crystal structures bind to the GDP-bound form of RAS on either side of switch II, inhibiting nucleotide exchange catalyzed by GEFs (Fig. 4A). Given that oncogenic RAS mutants retain RAS in its GTP-bound form or promote rapid exchange in the absence of GEFs (50), it has been proposed that nucleotide exchange inhibitors may be best used in cancers bearing wild-type RAS (22). However, an increasing understanding of the role of the wild-type protein in promoting oncogenic RAS-driven cancers (51, 52) suggests that...
GEF inhibitors may have a role to play in these types of cancers as well.

Targeting RAS–GTP has produced a few low-affinity inhibitors of RAS–effector interactions accompanied by NMR information on the general binding area for the compounds near switch I (Fig. 4B, refs. 44–46). One of the compounds has a second binding site near loop 7 and the C-terminus, visible in a crystal structure of RAS (44). These compounds stabilize switch I in an open conformation that disfavors interaction with effector proteins. This mode of binding illustrates a strategy associated with stabilization of conformational states that takes advantage of controlling accessibility to functionally important conformers. In RAS–GTP, when switch I is open in state 1 Y32 is far from the active site where it obstructs the binding of RAF–RBD (RAS-binding domain) and other effectors, while in the closed state 2 Y32 becomes part of the active site, allowing interaction with effector proteins (9, 10). A recent crystal structure of HRAS in the GTP-bound form in which switch I is in state 1 reveals a binding pocket between switch I and the nucleotide that may be used for structure-based ligand development (53). This is only one angle on the more general strategy of stabilizing conformational states that may offer windows of opportunity for direct targeting. RAS is full of this type of opportunity throughout its small but complex structure, where intramolecular communication networks work between distant sites on the RAS G-domain have been discovered in the past few years (13, 54–56), but remain largely unexplored. To open new opportunities for drugging RAS, we must understand these structural features in depth, their relative importance in KRAS, NRAS, and HRAS, as well as the ways in which specific mutations at G12, G13, and Q61 affect them in terms of hydrolysis rates and communication with the membrane.

**Intramolecular Communication in the Structure of Wild-type Isoforms of RAS Bound to GTP**

The Ras G-domain is subdivided in two lobes (57). The first (residues 1–86), identical in sequence between the Ras isoforms, contains the nucleotide-binding site, including the P-loop (residues 10–17), as well as the switch I (residues 30–40) and switch II (residues 60–76) regions that recognize effector and regulatory proteins, and thus is referred to as the effector lobe (58). The second contains several membrane-interacting regions that link elements of allosteric communication between the active site and the membrane (13, 55, 59), and thus is referred to as the allosteric lobe (58). This lobe is 90% identical between the isoforms, with most differences residing in sites of protein–membrane interactions, thus conferring specificity to the way in which the G-domain of KRAS, NRAS, and HRAS interacts and communicates with the membrane (60).

A challenging aspect of directly targeting RAS in its GTP-bound form is the fact that switch I and switch II are disordered in solution (11, 58, 61), as is also the case in RAS–GDP (62), making it difficult for small molecules to bind. When bound to RAF–RBD, switch I is in state 2 and Y32 interacts with the γ-phosphate of GTP via a bridging water molecule (Fig. 5). This brings Y32 into reach of a water-mediated H-bonding network that leads to N85 at the N-terminal end of helix 3 (56) and onto nucleotide-sensing residues R161 and R164 in helix 5 and D47 and E49 in the effector lobe loop L3 (55). This water-mediated H-bonding network is highly conserved in HRAS–GTP, but not present in HHRAS–GDP, with switch I providing a structural venue for communication between the γ-phosphate of GTP and the membrane through the allosteric lobe (56).

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**Figure 2.** Conformational states associated with RAS–GTP. The RAS structures are shown in ribbon diagram with switch I and switch II labeled. The elements of secondary structure discussed in the text are also labeled. All structures are bound to the GTP analogue GppNHp, shown explicitly. A, switch I in state 1 is represented by the HRAS structure with PDB code 4EFL (pink), and state 2 is represented by the HRAS structure with PDB code 3K8Y (green). B, switch II in the T state is represented by the HRAS structure with PDB code 2RGE (blue), and the R state is represented by the HRAS structure with PDB code 3K8Y (green). Note that the model in the T state (blue) does not contain the N-terminal end of switch II (including G66) due to disorder in that part of the structure. Also note the Ca2⁺ and acetate bound in the allosteric site in the R state structure (green), with accompanying shift in helix 3/loop 7 (α3/ L7) toward helix 4 (α4).
The GTP-bound orientation of RAS with respect to the membrane is mediated through R128 and R135 in helix 4 in response to the nucleotide sensor residues (55, 59), and thus this intramolecular network that connects switch I to the allosteric lobe may have an impact on the orientation of the G-domain.

Switch II also has been observed in distinct conformations associated with T and R states of allosteric modulation of the active site, given a state 2 conformation of switch I (16). Although normally disordered in crystals in the T state, switch II becomes highly ordered by binding of small molecules at a remote allosteric site found between helix 3, loop 7, and helix 4 (Fig. 2B; ref. 13). In crystals in which switch I has the conformation observed in the RAS/RAF–RBD complex (15), binding of calcium at the allosteric site results in a shift of helix 3 toward helix 4 and away from switch II, making room for the switch II helix to become ordered (13). This results in positioning of R68 at the center of an H-bonding network that facilitates placement of catalytic residue Q61 in the active site. Thus, in its effector-bound R state, switch II is connected to the membrane-interacting allosteric lobe through an H-bonding network to the allosteric site.

Residues in the intramolecular communication networks from switch I and switch II in the effector lobe to the membrane interacting allosteric lobe described above are conserved in the three isoforms (Fig. 5), implicating the interlobal connectivity of the G-domain in the fundamental mechanisms through which RAS functions.

**Figure 3.**

The GTPase cycle, including the nuances of conformational states. RAS is shown in cyan, with switch I in light blue and switch II in yellow. The GEF SOS is in a dark brick color and GAP is in pink-gray. Black crosses indicate opportunities for blocking interactions that result in signal propagation through RAS, and the thick black arrow associated with stabilization of the R state indicates enhanced hydrolysis of GTP. GEF and covalent inhibitors target RAS–GDP, while RAF inhibitors, inhibitors that stabilize state 1 (state 1 inhibitors), or potential inhibitors that stabilize the R state to stimulate hydrolysis, target RAS–GTP. Note the distinction between inhibitors that prevent binding of RAF to RAS in state 2 and the state 1 inhibitors that stabilize a RAS conformation that intrinsically does not bind RAF.

**Binding Pockets on the Allosteric Lobe of RAS Differ between the Isoforms**

Screening of large compound libraries resulted in the identification of molecules that inhibit RAS signaling, as summarized above. In a very different approach, a smaller group of generic compounds or probe molecules have been used with both experimental and computational methods to identify the location of binding site hot spots on RAS–GTP in search of pockets that can be explored in drug discovery (58, 63). In addition to identifying known sites on the effector lobe of RAS–GTP, this work identified hot spots of protein–ligand interactions on the allosteric lobe that are not far from the elements involved in the interlobal communication networks discussed above. These hot spots are key targets of opportunity, as they are not identical between the isoforms (60) and to date most have been unexplored.

The locations of identified hot spots on the RAS–GTP effector lobe include the binding pocket near switch I known as the binding site for RAF–RBD (site 1) and the binding site on the outer side of switch II (site 2), while the binding site between switch II and helix 3 is found in the interlobal region (site 3; Fig. 5A). Sites 2 and 3 are also found to bind small molecules in RAS–GDP (Fig. 4A). These three sites, as well as the site near loop 7 mentioned above (Fig. 4B), are identified as high consensus sites because they are found repeatedly in several studies (25). Site 3 between switch II and helix 3 is of note because, in spite of it being...
a strong hot spot of protein–ligand interaction, every small molecule known to bind at this site in RAS–GTP is observed to stabilize an anticatalytic conformation associated with the T state of the allostERIC switch (58, 64). Thus, targeting the site between switch II and helix 3 might inadvertently result in prolonged signaling through RAS–RAF in some situations. Because RAF does not bind switch II (15), a compound at this site would be unlikely to have a significant effect on RAF binding while abrogating hydrolysis of GTP, working against the goal of inhibiting RAS signaling. Although no natural binding partners have been identified to interact with RAS at site 3, importin-β occupies it in RAN GTPase to halt hydrolysis during transport of cargo into the nucleus (65).

All of the sites identified on the allostERIC lobe other than the already mentioned site near loop 7 (site 5) have been classified as low consensus sites because they have been found in a single study (58) and no compounds from large library screens have been found to bind at those sites. Interestingly, however, the amino acid composition of these sites varies among the isoforms (60) and they are found conspicuously close to the elements of the interlobal communication networks in RAS–GTP. Figure 5 shows the sites of interest in the allostERIC lobe and their relationship to the H-bonding networks that connect the two lobes. One of the sites is between helices 3 and 4, adjacent to the allostERIC site (site 6; Fig. 5A), and compounds that bind there interact with the side chain hydroxy group of Y137 (58), whose main chain carbonyl group coordinates the Ca²⁺ in the R state of the allostERIC switch (13). Two other sites are between helices 4 and 5 and contain helix 4 residues R128 (site 7) and R135 (site 8), respectively (Fig. 5B). Residues R128 and R135 help maintain an orientation of the RAS G-domain with respect to the membrane conducive to effector binding and were shown to abrogate signaling when mutated in cells (59). Another low consensus hot spot identified by the same study with an experimental approach (58) is at the interface between the effector and allostERIC lobes and is known to bind RAF–CRD (cystein-rich domain), the second RAS-binding domain found in RAF (site 4; Fig. 5B; ref. 66). Binding at this site can potentially have an effect on the switch I to helix 5 communication network associated with nucleotide sensing residues in helix 5 near the membrane or affect the orientation of the G-domain at the membrane surface.

**Effects of Oncogenic Mutations on Intramolecular Communication Networks in RAS–GTP**

It is well known that oncogenic RAS mutants are insensitive to GAPs (67–70). This is a difficult problem to overcome, as the mutations themselves severely perturb the active site and impair the ability of GAPs to enhance GTP hydrolysis even upon binding RAS (58, 71). If it is indeed the case that the duration of the RAS–RAF interaction is regulated by intrinsic hydrolysis activated by membrane interaction and calcium binding at the allostERIC site (13, 14, 16, 56), there an exciting window of opportunity exists for targeting signaling through RAS–RAF enhanced by some oncogenic mutants. This is of interest because the RAS–RAF–MEK–ERK pathway is a potent mitogenic pathway and an aggressive driver of several types of cancers (72). Intrinsinc hydrolysis is dependent on an active site with an intact communication network to the allostERIC site that requires the R state positioning of helix 3/loop 7 and correct placement of the R68 link to Q61 through water-mediated H-bonds (Fig. 5A). It has been shown that both G12V and Q61L can attain the R and T states of the allostERIC switch under circumstances where switch I is in state 2 (14), as it is when bound to RAF, and it is likely that other oncogenic mutants can as well.

In structures available for the G12 and Q61 mutants, those in which switch I is in state 2 and the allostERIC switch is in the R state (14, 34, 35, 67, 73, 74), with the exception of G12D, the bridging water molecule seen in the wild-type protein is not present and Y32
makes a direct H-bond with the γ-phosphate of GTP (Fig. 6). The arrangement in wild-type RAS is shown in Fig. 6, with the bridging water molecule within H-bonding distance of Y32, the γ-phosphate, and of Q61. RASG12V and RASQ61L, superimposed on the wild-type, are representative examples of the direct H-bond between Y32 and the γ-phosphate in most oncopgenic mutants (Fig. 6A). This results in a severed connection between switch I and the helix 5 nucleotide sensor residues associated with membrane orientation (54). In the G12D mutant, for which there are two crystal structures, the bridging water molecule is either replaced with one of the side-chain oxygen atoms of the D12 residue or is present and D12 replaces Q61 in its interaction with the bridging water molecule (Fig. 6B). Contrary to what is observed in all other oncopgenic mutants, the water-mediated network from the γ-phosphate to the nucleotide sensing residues in helix 5 is intact in both structures we have discussed, switch I is stabilized in state 2 by interactions with other proteins (15, 30, 76, 77). In the crystal complex (15) or in the RAS/NORE1A complex (78) and switch II crystal contacts in the conformation found in the RAS/RAF complex (14, 15, 73). When bound to RAF, abolishing even the slow hydrolysis rate tendency to stabilize the T state in the anticatalytic conformation of the G12D and Q61 mutants directly impair the chemistry of GTP hydrolysis by either placing a negative charge right over the nucleotide or removing an important catalytic residue. Furthermore, Q61L is also unique in that it has a strong tendency to stabilize the T state in the anticatalytic conformation when bound to RAF, abolishing even the slow hydrolysis rate observed in the uncomplexed protein (14, 15, 73).

In the absence of binding partners, both switch I and switch II are disordered in solution (11, 61), and they become stabilized by interactions with other proteins (15, 30, 76, 77). In the crystal structures we have discussed, switch I is stabilized in state 2 by crystal contacts in the conformation found in the RAS/RAF–RBD complex (15) or in the RAS/NORE1A complex (78) and switch II is less constrained by crystal contacts. This may not always be the case. Therefore, in general one must be vigilant in taking into account the effects of crystal contacts. Remarkably for RAS, most of the switch I conformations stabilized by crystal contacts are also observed in a biologically relevant complex (64). Given a careful structural analysis, the kind of information discussed herein will be invaluable in obtaining insights leading to specific targeting of the nearby allosteric lobe sites to promote the shift in helix 3. Full wild-type rates will not be achieved because of the perturbed active site. However, it has been argued that a 30- to 50-fold enhancement of the hydrolysis rate may result in significant anticancer activity (75), and it is worth testing whether this is possible for the G12V and G12C mutants. The G12R and Q61H mutants displace residue 61 from the active site, abrogating connectivity to the allosteric lobe, and are less likely to respond to ligand binding aimed at promoting the R state. The G12D and Q61L mutants directly impair the chemistry of GTP hydrolysis by either placing a negative charge right over the nucleotide or removing an important catalytic residue. Furthermore, Q61L is also unique in that it has a strong tendency to stabilize the T state in the anticatalytic conformation when bound to RAF, abolishing even the slow hydrolysis rate observed in the uncomplexed protein (14, 15, 73).
RAS mutants. Although focusing on the allosteric site and nearby pockets on the allosteric lobe (sites 5 and 6) may target RAS in a mutant-specific fashion, targeting the sites near R128 and R135 between helices 4 and 5 (sites 7 and 8) is likely to work more generally by disrupting the functional orientation of the G-domain relative to the membrane, which is specific for each of the isoforms (54, 55, 79, 80), possibly superseding the effects of particular mutations.

Conclusions
Targeting RAS against cancers has been a challenge for the past 25 years. Yet recent progress in the discovery of small molecules that bind directly to the G-domain with inhibitory effects on its function is exciting and encouraging. For RAS–GTP, focusing on the RAS–effector interaction at the active site by stabilizing state 1 is particularly promising, as this is the most direct way to inhibit RAS signaling, although nonspecific with respect to particular isoforms. In-depth understanding of the structure and function of RAS and its interaction with the membrane opens new avenues for targeting isoforms or specific mutations that are in need of further development. Targeting the allosteric lobe will require assays that detect binding at those sites in high-throughput screens, and the hypotheses derived from structural analysis of specific mutants regarding the activation of the allosteric switch need to be confirmed. Another unexplored area of opportunity is interaction between the C-terminal hypervariable region and switch II in K-RAS4B with calmodulin, which results in signal attenuation (81–84). Although promotion of protein–protein interaction is not trivial, it is not unprecedented (85) and should be explored in the future. Although treatment of RAS-driven cancers will continue to require a cocktail of inhibitors of multiple pathways (86), the rapidly increasing knowledge of RAS structure and function fuels the vision that among available inhibitors in the future will be a drug that directly binds to RAS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Marcus

Grant Support
This work was supported by a grant from the National Science Foundation (MCB-1244203; to C. Mattos).
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