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The disordered hypervariable region and the folded catalytic domain of oncogenic K-Ras4B partner in phospholipid binding

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Abstract

The C-terminal hypervariable region (HVR) of the splice variant *KRAS4B* is disordered. Classically, the role of the post-translationally-modified HVR is to navigate Ras in the cell and to anchor it in localized plasma membrane regions. Here we propose additional regulatory roles, including auto-inhibition by shielding the effector binding site in the GDP-bound state and release upon GTP binding and in the presence of certain oncogenic mutations. The released HVR can interact with calmodulin. We show that oncogenic mutations (G12V/G12D) modulate the HVR-phospholipid binding specificity, resulting in preferential interactions with phosphatidic acid. The shifts in the conformational preferences and binding specificity in the disordered state exemplify the critical role of the unstructured tail of K-Ras4B in cancer.

Keywords

K-Ras4B; K-Ras4A; *KRAS4B*; plasma membrane; phospholipids; Ras drugs; oncogenic mutations; calmodulin; lung cancer

Introduction

The paradigm that dominated the field of structural biology for many years declared that to exert function, protein molecules must have a rigid structural fold consisting of helices, sheets, turns, and loops. However, the free energy landscape describes proteins as ensembles of states, which are in a dynamic equilibrium. It emphasizes that macromolecules are

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dynamic objects that always sample a gamut of interconverting structures with varying energies and that function can be achieved by distinct states within the ensemble with a subsequent population shift [1]. In the disordered state, the differences in energy among the conformations are small and the barriers are low. The flexible polypeptide chains do not form stable structural elements but rather adopt multiple conformational states [2] with the interconversion rates subject to environmental regulation, fine-tuned to respond to the needs of the cell [3]. The redistribution of the conformational propensities of the unstructured elements through stabilization of the relevant states is a fundamental regulatory mechanism.

Here, we explore how the function of oncogenic K-Ras4B is affected by its natively unstructured C-terminal tail, named the hypervariable region (HVR). Based on the literature and new experimental evidence we suggest that the HVR conformations and their functional consequences are greatly influenced by the protein context, in addition to the membrane environment. The HVR can be sequestered by the globular catalytic domain of GDP-bound K-Ras4B or released from the sequestered state when the catalytic domain binds GTP [4]. It can specifically interact with the calcium modulator protein calmodulin [5–7], selectively associate with plasma membrane lipids [8,9], critically contribute to dimer formation at the membrane [10–14], become phosphorylated by protein kinase C (PKC) [15]; and be segregated at spatially distinct plasma membrane signaling platforms [16].

Below, we propose that the structural flexibility of the HVR is critical to the diversity of K-Ras4B functions and show that the distribution of the ensemble of its disordered states readily shifts in the presence of oncogenic mutations. These mutations in the catalytic domain alter the preferred HVR conformations and thereby change the HVR-phospholipid binding specificity of oncogenic versus wild type K-Ras4B. HVR interactions which are preferred in the wild type (e.g. with phosphatidylserine (PS), phosphatidylinositol 4,5-bisphosphate (PIP₂), and phosphatidylinositol (3,4,5)-triphosphate (PIP₃)), appear significantly attenuated in oncogenic G12V and G12D mutants, which instead preferentially interact with phosphatidic acid (PA). The disordered state of HVR may be shifted in the presence of mutations to populate different states, affecting oncogenic mechanisms.

KRas4B is the Ras isoform most frequently expressed in cancer

K-Ras4B is one of four classical proto-oncogenic Ras GTPases (H-Ras, N-Ras, and splice variants K-Ras4A, and K-Ras4B) that are frequently mutated in cancer [17]. Ras GTPases are highly homologous in their catalytic domains but differ significantly in their HVRs. The amino acid sequences of the HVRs are less than 15% homologous and bear different post-translational modifications (PTMs). The HVR of K-Ras4B is lysine-rich, carboxymethylated and farnesylated [8]. The HVRs of other Ras isoforms are palmitoylated, in addition to the C-terminal methylation and farnesylation [17,18]. Ras proteins cycle between the active GTP-bound and inactive GDP-bound states to regulate cell proliferation, survival, and migration [19,20]. Nucleotide loading is achieved with the help of guanine nucleotide exchange factors (GEFs) that promote the active state of Ras. GTP hydrolysis is aided by GTPase activating proteins (GAPs) that turn off Ras function. Oncogenic mutations occur predominantly in the N-terminal catalytic domain where they inhibit GTP hydrolysis and attenuate binding of GAPs [21]. GTP-bound oncogenic Ras is constitutively active. Through

the C-terminal HVR, it recruits to the plasma membrane and activates effector proteins. These initiate signaling cascades to regulate cellular processes.

While all four Ras GTPases are susceptible to mutagenesis in cancer, and all are expressed in the various tissues, K-Ras4B is mutated more frequently than other Ras isoforms [22]. Why mutant K-Ras is more prevalent in cancer in comparison with other isoforms is unclear. K-Ras4B appears to function differently from the other Ras proteins in normal physiological processes as well as in pathogenesis [23]. Although direct experiments are needed to test its proposed role in proliferation and growth, we believe that this differential action in oncogenesis could at least partly be related to its unique interaction with calmodulin, which was suggested to play a key role in K-Ras driven cancers [7], as well as additional K-Ras specific regulators, such as RASSF2 tumor suppressor [24] and complementary signaling pathways. The difference in sequences between K-Ras4B and other isoforms centers on the HVR domain, shown to be key contributor to calmodulin binding [6].

Direct and catalytic domain-mediated HVR effects in K-Ras4B

Knockout of K-Ras but not H-Ras is embryonic lethal [25]. Differential activation of effector proteins by K-Ras *versus* H-Ras is widely known [23,26,27]. Inherent biochemical differences may also exist. For example, Sur8 in *Drosophila* and in particular RASSF2 in lung tumor cell line specifically interact only with K-Ras *in vitro* [24,28]. These interactions are mapped to the effector binding domain of K-Ras, which is identical to that of H-Ras. While the H-Ras V12P34G mutant transforms NIH 3T3 fibroblasts with the same potency as the V12 mutant, the same mutation in K-Ras significantly reduces its oncogenic activity [29]. Surprisingly, the P34 mutation is in the region of absolute homology between all Ras proteins. One possible explanation for this differential sensitivity of Ras proteins to mutations in the effector domain is the influence of the HVR. Incidentally, the HVR of H-Ras transiently interacts with the catalytic domain and induces perturbations in the structure and dynamics propagating throughout the whole protein [30]. Calmodulin and guanine nucleotide exchange factor Smg-GDS specifically interact with K-Ras4B predominantly through the HVR [31,32]. GTP binding to the conserved portion particularly of the oncogenic mutant protein significantly enhances these interactions. The HVR of K-Ras4B may induce changes in the whole K-Ras4B protein, creating a structurally and dynamically unique system with features that might be used for drug design [4].

The dynamic role of the HVR in membrane microdomain-selective localization

Microdomain selective localization of Ras proteins may account for some of the functional variation [33,34]. Isoform specific HVRs dispatch palmitoylated H-Ras and N-Ras through the exocytic pathway to the plasma membrane, while polybasic K-Ras4B is trafficked around the Golgi, possibly through a microtubular route [35]. In the plasma membrane, the positively charged HVR directs K-Ras4B to disordered microdomains within the plasma membrane, whereas H-Ras populates either the disordered plasma membrane or the cholesterol-rich lipid rafts in a GDP/GTP-dependent manner. Together with the catalytic

domain, the HVR can define specific nucleotide-dependent H-Ras and N-Ras orientations on the plasma membrane, thereby providing an important regulatory mechanism [36–38]. Interestingly, solution NMR studies found that when K-Ras4B is tethered to thiol-reactive lipids which were incorporated into PS containing nanodiscs, the effector-binding region of activated K-Ras4B is occluded by the membrane [39], suggesting that the occlusion promotes an auto-inhibited state of K-Ras4B. Auto-inhibition is released by binding of the Raf isoform ARaf and Ral-GDS Ras binding domains (RBDs) that reorient K-Ras4B on the membrane and release K-Ras4B from the occluded state. Importantly, oncogenic G12D, as well as two mutations associated with Noonan syndrome, relieve the occluded orientation by directly altering the electrostatics of two membrane interaction surfaces. The D153V and G12D mutations increase the rate of association of ARaf-RBD with lipid bilayer-tethered K-Ras4B [4]. Regardless of the nucleotide type (GDP or GTP) which is loaded onto the catalytic domain, the altered HVR conformation can relieve the auto-inhibition at the membrane (Figure 1a). In the active state, the effector binding site is exposed, while in the inactive state it is buried by the membrane-interacting HVR. The exposure of the effector binding site at the membrane can promote calmodulin binding (Figure 1b). Oncogenic mutations in K-Ras4B may shift the HVR conformation toward an open active state, which can be populated at specific domains of plasma membrane (Figure 1c), bolster dimer formation (Figure 1d), and help recruit effectors through the exposed binding sites and in the case of Raf also dimerization [12].

The HVR and the catalytic domain function in concert to regulate K-Ras4B

Together, accumulating evidence suggests an emerging paradigm for auto-regulation of K-Ras4B. In this paradigm, the HVR and the catalytic domain function in concert to enable specific protein-protein interactions and to fine-tune K-Ras4B orientation on the plasma membrane. The HVR is influenced not only by oncogenic mutations, but also by changes in the conformation of the catalytic domain that are dictated by GTP or GDP binding (Figure 2). We reasoned that if the HVR is modulated by the context of the catalytic domain, it is possible that different conformations of the catalytic domain can also affect phospholipid binding selectivity. The established view of oncogenic K-Ras4B is that it does everything the wild type protein can do, only it does it better. This view suggests that, with the exception of targeting of oncogenic G12C K-Ras4B with thiol-reactive agents [40], designing drugs that can distinguish between the oncogenic mutant and the wild type proteins is a daunting task. The resulting drugs will likely inhibit both oncogenic and wild type K-Ras4B leading to significant side effects. If the oncogenic mutants of K-Ras4B display binding selectivity towards phospholipids, to which the wild type protein binds weakly, it may be possible to design inhibitors that preferentially dislodge oncogenic mutant K-Ras4B from the membrane.

Can oncogenic mutations alter selective lipid binding?

To test the hypothesis that oncogenic mutations in K-Ras4B might bind phospholipids selectively we used phosphoinositide (PIP) strips (purchased from Life Technologies). These hydrophobic membranes are spotted with different phospholipids to determine lipid-protein interactions through a protein-lipid overlay experiment. We incubated the same

concentrations of recombinant wild type, G12D, and G12V K-Ras4B-GDP with the PIP strips. The G12D mutation is the most common in cancer, while the G12V mutation is associated with the most aggressive cancer [41]. In all cases, the extent of GDP loading was found to be greater than 93% by liquid chromatography/mass spectrometry (LC/MS). After incubation, the PIP strips were extensively washed with Tris buffered saline (TBS) and K-Ras4B binding to phospholipids was determined by Western blotting. The assay showed that wild type K-Ras4B-GDP interacts weakly and nonspecifically with anionic phospholipids, including phosphatidylinositol (PI), phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), PIP₂, PIP₃, PA, and PS (Figure 3a). The weak and nonspecific binding to anionic phospholipids is consistent with the sequestered state of HVR by the catalytic domain of K-Ras4B-GDP [4,6]. Strikingly, the GDP-bound G12V and G12D K-Ras4B proteins interact strongly and selectively with PI3P, PI4P, PI5P, and PA (Figure 3b,c). This result suggests that the HVR is altered by the context of the catalytic domain of G12V and G12D K-Ras4B-GDP. Alternatively, the catalytic domains themselves might be responsible for phospholipid binding specificity.

To address this possibility we studied phospholipid binding of catalytic domains (1-166) of wild type, G12V, and G12D K-Ras4B-GDP. The results showed that wild type K-Ras4B₁₋₁₆₆-GDP binds some of the same phospholipids as the full-length protein, including PI3P, PI4P, PI5P, and PS (Figure 3d). This observation suggests that HVR in the context of wild type GDP-bound K-Ras4B contributes to binding PI, PI(3,4)P₂, PIP₂, PIP₃, and PA phospholipids. G12V K-Ras4B₁₋₁₆₆-GDP was able to bind strongly and selectively to PI5P and PA (Figure 3e). No phospholipid binding was observed for G12D K-Ras4B₁₋₁₆₆-GDP (Figure 3f), implying that in this oncogenic mutant HVR is solely responsible for interactions with phospholipids. Although no significant structural differences between oncogenic mutants of K-Ras4B were observed by X-ray crystallography [42], the structures may be different in solution. A possible reason for the structural difference is the previously proposed hydrogen bonds between D12 and Y32 and Q61 that do not exist in the G12V mutant [42]. Analysis of X-ray structures of the GDP-bound mutants suggests that the change in side-chain orientation of Q61 might propagate through the Switch I region and cause occlusion of R68 in G12D K-Ras4B-GDP [43], while this arginine residue is solvent exposed in G12V K-Ras4B-GDP [42]. It is thus possible that the exposure of R68 in G12V K-Ras4B-GDP enables binding to PI5P and PA.

Previous studies identified anionic PS [44], PIP₂ and PIP₃ [45] as phospholipids directly interacting with the wild type and oncogenic K-Ras4B in the plasma membrane. Although the negatively charged PA potentiated K-Ras4B binding to liposomes containing anionic lipids, its direct involvement in K-Ras4B binding to the membrane has not been demonstrated [44]. Indeed, the same PIP strip assay as used by us found that HVR attached to green fluorescent protein binds PI3P, PI4P, PI5P, PIP₂, and PIP₃ but not PA and PS. These observations suggest that HVR is greatly influenced by the context of the protein, to which it is attached. In cells, the interactions of HVR with phospholipids may be further influenced by its post-translational modifications and by protein-protein binding [8,46–48].

PS and PIs can regulate K-Ras4B association with the plasma membrane

The roles of PS and PIs in the regulation of K-Ras4B association with the plasma membrane are known. For example, a change in the membrane surface potential following depletion of PIP₂, PS, and PI4P in the process of phagosome formation sequesters K-Ras4B into the cytosol [44]. Staurosporin, a blocker of PS endosomal recycling, redistributes wild type and oncogenic K-Ras4B to endosomes [49,50]. Voltage-induced membrane depolarization reorganizes PS and PIP₂ and causes enhanced nanoclustering of wild type and oncogenic K-Ras4B [51,52]. While PS is an important player in K-Ras4B binding to the plasma membrane, depletion of both PIP₂ and PIP₃ is required for dissociation of K-Ras4B clusters [45]. The proposed function of PA is to bring together the activators and effectors of Ras to maximize the efficiency of signal propagation [53]. For example, plasma membrane association of Ras GEF Son of sevenless (Sos) through direct PA binding of the Sos plekstrin homology domain enhances GTP loading and signaling of Ras [54]. Raf-1 kinase, an important effector of Ras can also be recruited to the plasma membrane through direct interactions with PA [55,56]. Taken together, although K-Ras4B binding to PA has not been investigated its potential significance in regulation of K-Ras4B signaling is not surprising.

Conclusions

Our results suggest that oncogenic mutations in K-Ras4B significantly affect phospholipid binding specificity. We find that the previously reported interactions with the phospholipids PS, PIP₂, and PIP₃ are mostly characteristic of wild type K-Ras4B; however, binding to these phospholipids is significantly attenuated in oncogenic G12V and G12D mutants, which instead preferentially interact with PA. Selective association of the two mutants with PA is due to positive and negative contributions from their catalytic domains and due to the modulatory effects of these domains on HVR. We suggest that the mutations shift the disordered state of the K-Ras4B HVR, which now selectively bind PA. Which specific conformations now preferentially populate the ensemble is not clear, nor the variability among the mutations; however detailed conformational studies could help elucidate such details. Based on the current data, we hypothesize that oncogenic mutations in K-Ras4B affect HVR sequestration and release by the catalytic domain.

This underscores the importance of detailed structural studies of p21 Ras proteins. Elucidating the unique conformational features of K-Ras4B in its inactive, GDP-bound form may help to develop pharmaceutical agents stabilizing the inactive state of K-Ras. Conversely, understanding the structural specifics of K-Ras in its GTP-bound form will help to design K-Ras inhibitors. Stabilizing the auto-inhibitory interactions between K-Ras4B and the membrane might be an attractive target for anticancer drug discovery.

The unstructured HVR in K-Ras4B plays several important roles that are dictated by the conformational changes in the catalytic domain. It is sequestered by the GDP-loaded catalytic domain and is released upon GTP binding. The released HVR interacts with calmodulin and enhances binding to Raf-1. The finding that oncogenic mutants of K-Ras4B modulate the membrane binding HVR to acquire the ability to selectively interact with PA in the plasma membrane further exemplifies the critical role of this unstructured tail in K-

Ras4B function in cancer. Finally, our tale provides unique illustration of a natively unstructured state modulated by oncogenic mutations of a key membrane-anchored protein in cancer.

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Highlights

- The C-terminal hypervariable region (HVR) of KRAS4B is structurally disordered
- Classically, the HVR role is to anchor Ras in the membrane
- We suggest that the K-Ras4B HVR can also act to autoinhibit K-Ras4B
- The K-Ras4B HVR conformation may depend on the nucleotide bound
- The K-Ras4B HVR conformation may also depend on oncogenic mutations
- Oncogenic mutations can shift the HVR-lipid binding selectivity

Figure 1.

Cartoons representing HVR-mediated K-Ras4B membrane interactions. (A) The inactive and active states of K-Ras4B at the membrane containing phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol 4,5-bisphosphate (PIP₂), and phosphatidylinositol (3,4,5)-triphosphate (PIP₃). Active K-Ras4B liberates the HVR, exposing the effector binding site, while in the inactive state the catalytic domain is autoinhibited by the HVR, burying the effector binding site. (B) Calmodulin can easily access the liberated HVR from the catalytic domain of active K-Ras4B, competing with the membrane for the HVR interaction which results in K-Ras4B extraction from the membrane. (C) Oncogenic mutants of K-Ras4B selectively interact with membrane containing phosphatidic acid (PA)-rich domain, while wild type K-Ras4B preferentially binds PS, PIP₂, and PIP₃ in the plasma membrane. In the presence of mutations, the PA-rich domain may promote folding of the HVR, shifting from the disordered state. The illustrations are for the active state. (D) Active, oncogenic K-Ras4B mutants form a dimer through the allosteric lobe interface, exposing the effector binding sites for Raf interactions. Here the membrane domain is PA-rich. In the case of a membrane raft, the two farnesyls can interact which would promote dimer formation [8]. In the cartoons, the balloon represents the K-Ras4B catalytic domain, the blue thread tied to the balloon represents the HVR, and the yellow sawtooth represents the farnesyl.

Figure 2.

The HVR configurations in the signaling cycle of K-Ras4B. Ras acts as a binary signal switch cycling between active (GTP-bound) and inactive (GDP-bound) states. In the inactive state, the HVR of K-Ras4B-GDP is sequestered by the catalytic domain, while in the active state the HVR of K-Ras4B-GTP is released from the catalytic domain. Ras's effectors bind the GTP-bound Ras in the active state. The son of sevenless (SOS) nucleotide exchange factor (GEF) activates Ras, while GTPase-activating protein (GAP) inactivates Ras via the GTP→GDP hydrolysis. Ras with oncogenic mutations, such as G12C, G12V, and G12D, aborts the hydrolysis reaction, retaining in a constitutively active GTP-bound state. The oncogenic K-Ras4B mutations completely release their HVRs from the catalytic domains promoting membrane anchoring and dimerization for the initiation of downward signaling.

Figure 3.

Investigation of phospholipid binding selectivity for full length (a) wild type, (b) G12V, and (c) G12D K-Ras4B-GDP. The same for the catalytic domain (1-166) of (d) wild type, (e) G12V, and (f) G12D K-Ras4B-GDP. 1 mL of 30 μ M purified recombinant K-Ras4B-His6 protein was used for all experiments under identical conditions. The PIP Strips (Life Technologies) were incubated with the proteins overnight at 4 °C followed by addition of the anti-His6 primary and anti-mouse IgG secondary antibodies and thorough washing with TBS. Assignments of the dots in the assay are shown in the top left panel with numbers labeling the dot position. The phospholipid names corresponding to the dot numbers are shown on the lower left.