A New View of Ras Isoforms in Cancers

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Abstract

Does small GTPase K-Ras4A have a single state or two states, one resembling K-Ras4B and the other N-Ras? A recent study of K-Ras4A made the remarkable observation that even in the absence of the palmitoyl, K-Ras4A can be active at the plasma membrane. Importantly, this suggests that K-Ras4A may exist in two distinct signaling states. In state 1, K-Ras4A is only farnesylated, like K-Ras4B; in state 2, farnesylated and palmitoylated, like N-Ras. The K-Ras4A hypervariable region sequence is positively charged, in between K-Ras4B and N-Ras. Taken together, this raises the possibility that the farnesylated but nonpalmitoylated state 1, like K-Ras4B, binds calmodulin and is associated with colorectal and other adenocarcinomas like lung cancer and pancreatic ductal adenocarcinoma. On the other hand, state 2 may be associated with melanoma and other cancers where N-Ras is a major contributor, such as acute myeloid leukemia. Importantly, H-Ras has two, singly and doubly, palmitoylated states that may also serve distinct functional roles. The multiple signaling states of palmitoylated Ras isoforms question the completeness of small GTPase Ras isoform statistics in different cancer types and call for reevaluation of concepts and protocols. They may also call for reconsideration of oncogenic Ras therapeutics. Cancer Res; 76(1); 18–23. ©2015 AACR.

Introduction

Numerous studies have focused on the genes and gene products of small GTPase Ras isoforms, HRAS, NRAS, and KRAS, the most frequently mutated isoform in human cancers (1, 2). The KRAS gene has two splice variants, K-Ras4A and K-Ras4B (Fig. 1A). Mutations in K-Ras4B, which are also present in K-Ras4A, are common in cancer. The expression level of K-Ras4A in tumors was believed to be substantially lower, retaining the attention of the community almost exclusively on the K-Ras4B variant. Recent observations by Tsai and colleagues (3) may however lead to reevaluation of our views. The quantitative RT-PCR assay for K-Ras4A and K-Ras4B message that the authors developed allowed them to measure the absolute amounts of the two transcripts. They observed that K-Ras4A was widely expressed in all their human cancer cell lines. In particular, in human colorectal tumors, the amounts equaled those of K-Ras4B (Fig. 2). Their analysis revealed that the C-terminus of K-Ras4A contains the CAAX motif, a palmitoylation site, and a bipartite polybasic region (PBR, Fig. 1B), and that unlike K-Ras4B, K-Ras4A does not bind to the cytosolic chaperone δ-subunit of cGMP phosphodiesterase type 6 (PDE6δ). They concluded that efforts to develop anti-K-Ras drugs that interfere with membrane trafficking should consider the distinct mechanisms of both K-Ras splice variants.

These remarkable observations couple with earlier pioneering studies on H-Ras (4), leading to important conclusions on oncogenic Ras research. These question the completeness of recorded statistics of isoforms in distinct cancer types (5, 6) and call for reassessment of procedures and protocols for tallying oncogenic mutations. In light of these observations, we contend that classifying Ras isoforms solely by their sequences may be erroneous; their signaling states in the tissue should also be catalogued. We note that even though our hypothesis is in agreement with all currently available observations, to date it has not been directly tested.

K-Ras4A Is in Between K-Ras4B and N-Ras

Sequence comparisons of H-Ras, N-Ras, K-Ras4A, and K-Ras4B indicate that the catalytic domains (residues 1-166) are almost identical. K-Ras4A1–166 differs from its K-Ras4B1–166 counterpart in only four amino acids (Fig. 1A), located at the edges of helix 5, which is believed to adjoin the membrane. The hypervariable regions (HVR) of all isoforms at the C-terminal domain differ markedly (Fig. 1B; ref. 7). Importantly, this results in distinct interactions of the HVRs with the membrane (Fig. 1C). Even though direct experimental evidence is lacking, current data suggest that this may differentially restrict Ras orientations (8, 9) thereby encoding selective effector recruitment and signaling. K-Ras4A is most similar to K-Ras4B. Their HVRs are highly positively charged, with that of K-Ras4B containing an additional positively charged patch. The lysine residues in K-Ras4B form an almost continuous stretch; in K-Ras4A, they are fewer and divided into regions, the common polybasic region 1 and 2 (PBR1, PBR2; ref. 3). The lipid posttranslational modification patterns at the C-terminal differ as well (Fig. 1B). H-Ras has two palmitoyl groups and a farnesyl, N-Ras one palmitoyl and farnesyl, K-Ras4A similarly one palmitoyl and farnesyl, and K-Ras4B only a farnesyl group. Thus, like K-Ras4B, K-Ras4A has a positively charged HVR, albeit to a lesser extent, and a farnesyl group. PBR2, closer to the C-terminal and just

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following the palmitoylated C180 and prior to the farnesylated C185, is more important to K-Ras4A membrane association than PBR1. Tsai and colleagues (3) observed that substitution of the basic residues in common polybasic region 1 and 2 (PBR1 + 2) with glutamine generated a K-Ras4A mutant that localized on the plasma membrane similarly to N-Ras. Of
particular note, elimination of the palmitoylation through a C180S mutation mislocalized K-Ras4A in internal membranes. However, the mutant trafficked to the plasma membrane, unlike palmitoylation-deficient C181S N-Ras. Along similar lines, inhibiting palmitoylation of K-Ras4A with 2-bromopalmitate failed to block its expression on the plasma membrane, which was not the case with N-Ras.

Taken together, K-Ras4A may have two functional states (Fig. 1C): state 1 has polybasic regions and farnesyl; state 2 also has palmitoyl. As the stoichiometry of the palmitoylation...
K-Ras4A state 1, but not state 2, may bind calmodulin

Calmodulin plays an important role in oncogenic K-Ras biology (16–19), and the race is on to obtain a crystal structure, so far without success. Calmodulin temporarily downregulates activation of Raf and amplifies PI3K activation (19, 20). Calmodulin may selectively bind to oncogenic K-Ras4B (21), sequestering its farnesyl from the membrane. Current data point to the farnesyl docking into a hydrophobic pocket in calmodulin with the negatively charged calmodulin surface creating a favorable environment for the positively charged HVR (22). While additional data and controls in studies of oncogenic K-Ras4B-calmodulin interaction need to be assembled and implemented, the observation by the Philips laboratory that K-Ras4A can associate with the membrane in the absence of the palmitoyl raises the question of whether calmodulin also interacts with K-Ras4A state 1. If this is indeed the case, as would be expected in its K-Ras4B-like state, it raises the possibility of therapeutic targeting of calmodulin/K-Ras4A/PI3K in adenosarcoma signaling similar to calmodulin/K-Ras4B/PI3K (19). With a positively charged HVR and a farnesyl, and no sterically obstructing palmitoyl, K-Ras4A possesses the hallmark of calmodulin binding, albeit with a lower affinity. Thus, K-Ras4A may bind calmodulin in K-Ras-, but not N-Ras-driven cancers.

K-Ras4A may generate signaling redundancy in therapeutics

The observations made in the Tsai and colleagues article (3) further raise the question of why K-Ras4A? As K-Ras4B−like (state 1), the positively charged HVR avidly interacts with the negatively charged membrane, but not as avidly as K-Ras4B, as N-Ras−like (state 2), the lower HVR charge can have an advantage in neutral membranes, though with lesser avidity than N-Ras. The K-Ras4A HVR sequence places it between K-Ras4B and N-Ras, suggesting that its posttranslational modification pathway in the cell (farnesyl-only or farnesyl−palmitoyl) decides its functional fate. This leads us to speculate that K-Ras4A is the oldest Ras species that could fulfill both functions. As to the question of why has it been retained by evolution, one reasonable possibility is to create redundant pathways (23). This functional redundancy is the problem we currently face when targeting K-Ras and N-Ras cancers (24).
Finally, this raises the question of why unlike K-Ras4B, K-Ras4A does not bind to the cytosolic chaperone δ-subunit of cGMP phosphodiesterase type 6 (PDE6δ), whereas the more dissimilar N-Ras HVR sequence does (3). We believe that this requires further examination.

H-Ras, with two palmitoyls and a farnesyl, also exists in two states

A decade ago, a pioneering study (4) observed that H-Ras, with two palmitoyls and a farnesyl, may also exist in two states—N-Ras–like singly palmitoylated and doubly palmitoylated H-Ras. This compelling discovery can explain the heterogeneity and the expression of oncogenic H-Ras isoforms in largely N-Ras–driven cancers, such as AML (13, 14). The sequences of N-Ras and H-Ras do not appear to possess distinctive features. The palmitoylation half-life of Ras proteins varies, and has been shown to be shorter for oncogenic H-Ras12V than for WT H-Ras (7, 25, 26), with palmitoylation/depalmitoylation linked to the GTP/GDP cycle (25). Regulation of the cleavage of each of the two palmitoyl–protein thioester linkages of H-Ras (4) can take place through FKBP12-catalyzed prolyl isomerization, indicating that depalmitoylation is enzymatic (27). Thus, our thesis is that in the singly palmitoylated state, oncogenic H-Ras can substitute for N-Ras, which is singly palmitoylated. The two distinct states of oncogenic H-Ras may explain its occurrence in AML; in this cancer, the N-Ras–like singly palmitoylated state, but not the doubly palmitoylated state, may be expressed.

Implications for Different Types of Cancers

Functional redundancy versus specificity has been an ongoing question in cellular signaling (23, 28, 29). Perhaps the most significant conclusion is the emerging higher functional complexity of Ras isoforms and splice variants than previously thought. Even though the frequency of Ras gene mutations varies across cancer types, the preferential occurrence of oncogenic K-Ras versus N-Ras in colorectal carcinoma cannot be explained solely on this basis. N-Ras (28) and H-Ras (30) are expressed in mouse colorectal cancer cells; despite this, K-Ras mutations are six times more frequent than N-Ras, and H-Ras mutations are not present. However, expression levels may not indicate the effective local concentration at the membrane, which also depend on the HVR posttranslational states, local membrane composition, presence of certain scaffolding proteins such as galectin 1, etc. In light of the new findings, the current statistics of mutant occurrences in distinct cancers may need to be revised and broken down into tissue- and cell-specific Ras isoform states. The interactions, mechanisms, and signaling pathways may differ.

To date, in cancer cell line analysis, K-Ras4A has been taken as a homogeneous entity. On the basis of the observations reported in the literature, we point out that this common perception may be mistaken. K-Ras4A occurrence in oncogenic K-Ras4B–driven adenocarcinomas, like colorectal, pancreatic, and lung cancers may mirror a K-Ras4B–like state (it is only farnesylated), whereas K-Ras4A occurrence in N-Ras-driven cancers, such as melanoma and acute myeloid leukemia may reflect the N-Ras–like state (farnesylated i-palmitoylated). This is important because of the clinical and biologic implications. It emphasizes that the recorded statistics of isoforms in distinct cancer types does not account for their functional states in specific tumors that may differ according to the tumor type. K-Ras4A in melanoma or AML may not be identical to K-Ras4A in pancreatic ductal adenocarcinoma, lung, or colorectal cancer. This calls for a reanalysis of procedures and mutational data, and re-evaluating treatments. This also holds for the less frequent farnesylated and doubly palmitoylated H-Ras, which may also exist in two states, N-Ras–like singly palmitoylated and doubly palmitoylated H-Ras, although oncogenic H-Ras is not as frequent compared with mutational activation of N-Ras and K-Ras (1). Here our thesis is that isoform identification in cancer rests not only on its sequence, but on its possible tissue-specific functional state. How is the differential regulation between states 1 and 2 accomplished? Among the several possible mechanisms, we favor one whereby the two states preferentially act in distinct cancers, that is, are tissue-dependent. However, there is no direct evidence either way, and this requires experimental studies.

Future Prospects

Nature often reexploits useful mechanisms. Thus, the mechanism proposed here for accomplishing distinct isoform functions, which are dictated by differing membrane trafficking motifs, can be general. The recent elegant work of Nishimura and Linder (31), who described two states of processing for one of the Cdc42 HVR splice variants, that are also relevant to distinctions among other highly related cancer-associated small GTPases such as the RaLA and RaLB isoforms (32), may provide examples.

Direct experimental evidence at the cellular or biochemical level for different functions of any Ras isoform, including K-Ras4A versus K-Ras4B, is still lacking. The evidence for differential functions comes from isoform utilization in Ras-driven cancer (where K-Ras mutations predominate) and from transgenic models where isoforms do not always substitute for each other, as for example observed in the differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation, and tumor progression in the colon (28). Notwithstanding, the emerging picture conforms to the general principle adopted by evolution, where single genes are adapted for different functions by extending isoform heterogeneity.

Recent reports in the literature support the call for reevaluation of hypotheses and approaches used to calculate the statistics of the occurrence of Ras isoforms in different cancer types. Enhanced tumor isoform and mutational analysis protocols that are able to quantitatively distinguish among isoform species may better forecast cancer progression and guide platforms for therapeutics. Tsai and colleagues (3) utilized a newly generated antibody that detected K-Ras4A but not K-Ras4B. This emphasizes the importance of tool and protocol availability to identify the different isoforms and functional states of K-Ras. Thus, challenges are posed to the community to develop methods to accurately and completely determine and decipher molecular data, and resolve the treatment implications (33). The critical importance of completeness emphasizes the enormity of the challenge. Improved methods will more accurately identify drug targets, advance innovative concepts, unveil mechanisms, and decipher redundant pathways. Pursuing these has the potential of inspiring new research directions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Two Functional States of K-Ras4A

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