Biochimica et Biophysica Acta 1853 (2015) 841–849

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Ras nanoclusters: Versatile lipid-based signaling platforms ☆

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ARTICLE INFO

Article history:
Received 17 June 2014
Received in revised form 5 September 2014
Accepted 8 September 2014
Available online 16 September 2014

Keywords:
Ras proteins
Nanoclusters
Spatial cross talk
lateral segregation
cholesterol
phosphatidylserine

ABSTRACT

Ras proteins assemble into transient nanoclusters on the plasma membrane. Nanoclusters are the sites of Ras effector recruitment and activation and are therefore essential for signal transmission. The dynamics of nanocluster formation and disassembly result in interesting emergent properties including high-fidelity signal transmission. More recently the lipid structure of Ras nanoclusters has been reported and shown to contribute to isoform-specific Ras signaling. In addition specific lipids play critical roles in mediating the formation, stability and dynamics of Ras nanoclusters. In consequence the spatiotemporal organization of these lipids has emerged as important and novel regulators of Ras function. This article is part of a Special Issue entitled: Nanoscale membrane organisation and signalling.

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1. Introduction

Ras proteins are small GTPases that oscillate between an active GTP-bound and inactive GDP-bound state to function as plasma membrane localized molecular switches in growth factor regulated signaling pathways [1,2]. The level of Ras.GTP is normally tightly regulated by restricted, controlled access to exchange factors that catalyze GTP binding and GTPase activating proteins (GAPs) that enhance GTP hydrolysis to return activated Ras back to the inactive ground state. This control is subverted in ~15% of all human tumors which express Ras proteins with oncogenic mutations that prevent GAP action and lock Ras in the GTP-bound state [3,4]. Three major Ras isoforms, H-, K- and N-Ras are ubiquitously expressed in mammalian cells. K-Ras has two alternative splice variants: K-Ras4A and K-Ras4B. Since K-Ras4B is the ubiquitously expressed splice variant, we will focus mainly on K-Ras4B in this review, and all references to K-Ras hereafter imply K-Ras4B unless otherwise stated. All these Ras proteins share a common set of exchange factors and effectors, but the efficacy with which each isoform activates a specific effector varies significantly [1,3]. Isoform signaling specificity is encoded not by the highly conserved Ras G-domains (amino acids 1–165) that directly interact with effector proteins, but by the highly divergent C-terminal hypervariable regions (HVR, amino acids 166-188/89) [5,6]. The HVR contains a linker region and a C-terminal membrane-anchoring domain, which undergoes posttranslational modification to attach different lipid anchors to each Ras isoform (Fig. 1). Complex interactions between plasma membrane constituents and the different Ras lipid anchors, which are further modified by the activation state of the G-domain, determine the spatial distribution of Ras proteins on the plasma membrane. In this review we will consider how plasma membrane lipids and Ras interact to generate multiple types of signaling nanocluster on the plasma membrane, each with distinct lipid compositions. These newly defined Ras-lipid interactions can account for the different efficacies with which each isoform recruits and activates effectors to explain isoform-specific signaling. These same Ras-lipid interactions also lead to intriguing emergent control networks for Ras signaling platforms.

2. Ras proteins have a complex spatiotemporal distribution on the plasma membrane

Quantitative imaging techniques, such as electron microscopy (EM)-spatial mapping (Fig. 2) [7–10], fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer (FLIM-FRET) [10–12], single particle tracking [13] and fluorescence recovery after photobleaching (FRAP) [10,14], reveal a highly dynamic spatiotemporal organization of Ras proteins on the plasma membrane. Approximately 40% of Ras proteins exist in immobile nanodomains, termed nanoclusters, with the remaining proteins freely diffusing as mobile monomers (Fig. 3 and Table 1) [8,9]. Ras nanoclusters are ~9 nm in radius and contain ~6-7 Ras proteins per nanocluster. Ras nanoclusters turnover rapidly with lifetimes in the order of 0.1-1 s [9,13], thus nanoclusters are constantly forming and disassembling.

Recent work suggests that Ras dimer formation is a critical prerequisite for the assembly of the larger nanoclusters [15–19]. Mathematical

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http://dx.doi.org/10.1016/j.bbamcr.2014.09.008
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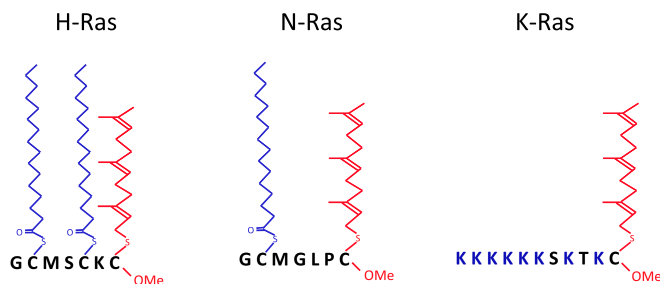

Fig. 1. Membrane anchors of three Ras proteins. After posttranslational modification all Ras isoforms are farnesylated and methyl esterified on a C-terminal cysteine. H-Ras is then dual-palmitoylated on Cys 181 and Cys 184 while N-Ras is mono-palmitoylated on Cys 181. K-Ras is not further lipid-modified but has a polybasic domain consisting of 6 contiguous lysines, plus two other lysines.

modeling shows that cooperativity between Ras monomers and dimers is a required parameter to replicate K-Ras nanocluster formation *in silico* [15]. MD simulations also reveal that H-Ras lipid anchors spontaneously form excess dimers as well as nanoclusters on a lipid bilayer, suggesting that dimers possess intrinsic stability [16]. This is further supported by computational modeling and experiments using purified N-Ras [17] or H-Ras [18] on model bilayers, which again show that Ras proteins form spontaneous dimers with a conformational orientation that is optimal for effector interactions. Furthermore, CRAF, a downstream effector of Ras, dimerizes upon binding to K-Ras.GTP on the plasma membrane [19], indirectly supporting the existence of K-Ras dimers. Preliminary results also suggest that the maintenance of a monomer pool may require nanocluster assembly to uncouple dimers followed by nanocluster disassembly to regenerate monomers (Fig. 4).

Within this framework H-, K- and N-Ras isoforms each form distinct, spatially non-overlapping nanoclusters, thus the Ras isoforms laterally segregate with high fidelity on the plasma membrane [8,11,12,16,20–22]. In addition Ras proteins exhibit guanine nucleotide-dependent segregation such that each isoform assembles into spatially distinct, non-overlapping GTP and GDP nanoclusters (Table 1) [8,11,12,16,20–22]. Ras nanocluster formation is biologically important because nanoclusters are the sole sites for effector recruitment and activation, and is therefore essential for signal propagation [1,9,23]. Extensive signaling experiments as well as computational modeling show that the fraction of Ras proteins in nanoclusters is fixed over a multi-log range of expression levels [9,23]. One result of this non-equilibrium behavior is that increasing the number of K-Ras.GTP molecules on the plasma membrane leads to a linear increase in the number of K-Ras.GTP nanoclusters. This coupled with switch-like activation of the RAF-MEK-ERK cascade in nanoclusters allows the Ras nanocluster system to operate as an analog-digital-analog (ADA) converter for high-fidelity signal transmission (Fig. 5) [23–26].

3. Structural basis of Ras plasma membrane localization and spatial segregation

Ras membrane anchors: the minimal membrane anchors of each Ras isoform are required and sufficient to target and anchor their cognate isoform to the plasma membrane. These anchors comprise a common fanesyl-cysteine-methyl ester attached posttranslationally at the extreme C-terminus of the Ras proteins and one of three different "second" signals to complete the anchor [27,28]. These alternate second signals comprise palmitoylation on Cys 181 and Cys 184, for H-Ras, and palmitoylation on Cys 181 alone for N-Ras [28] (Fig. 1). In contrast, K-Ras is not further lipidated but possesses a polybasic domain (PBD) consisting of six contiguous lysines (aa175-180) [27,29] (Fig. 1). Each of these membrane anchors visualized by GFP tag undergoes trafficking to the plasma membrane and organizes into non-overlapping nanoclusters [8]. The minimal membrane anchors of H-

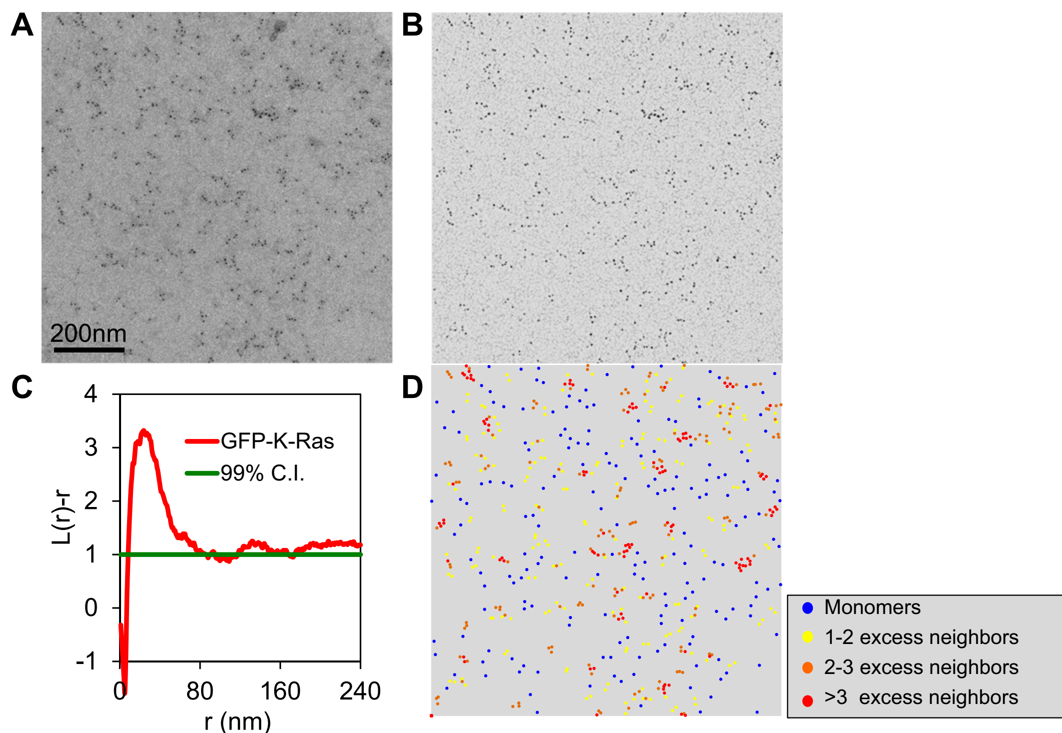

Fig. 2. Electron microscpy (EM) combined with spatial mapping. An intact plasma membrane sheet of BHK cells expressing GFP-K-Ras was labeled with 4.5 nm gold nanoparticles coupled to anti-GFP antibody. EM image was acquired using a transmission EM (**A**) and further processed using ImageJ (**B**). The spatial distribution of the gold particles was analyzed using Ripley's K-function ($L(r)$-r), showing that the pattern is highly clustered (**C**). A local L(r) was then calculated for each point in the image in B and used to constuct a heat map, which codes each particle according to the excess number of neighbors (over that expected for a random pattern of the same density) detected within a radius of 15 nm (**D**). This allows a visualization of clusters.

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