REVIEW ARTICLE

Ras plasma membrane signalling platforms

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The plasma membrane is a complex, dynamic structure that provides platforms for the assembly of many signal transduction pathways. These platforms have the capacity to impose an additional level of regulation on cell signalling networks. In this review, we will consider specifically how Ras proteins interact with the plasma membrane. The focus will be on recent studies that provide novel spatial and dynamic insights into the micro-environments

that different Ras proteins utilize for signal transduction. We will correlate these recent studies suggesting Ras proteins might operate within a heterogeneous plasma membrane with earlier biochemical work on Ras signal transduction.

Key words: lipid raft, microdomain, plasma membrane, Ras, signal transduction, single-particle tracking.

INTRODUCTION

The biophysical and biological processes that contribute to plasma membrane heterogeneity and micro-compartmentalization include lipid–lipid, lipid–protein interactions and interactions of lipids and proteins with the submembrane actin cytoskeleton [1–4]. Ras GTPases are plasma-membrane-localized molecular switches that regulate multiple signal transduction pathways.

In the present review, we will consider how Ras proteins physiologically operate on this complex surface. Three isoforms of Ras, H-, N- and K-ras, are ubiquitously expressed in mammalian cells. These highly homologous GTPases interact with a common set of exchange factors and effector proteins to transduce signals from growth factor receptors and regulate cell proliferation, differentiation and apoptosis [5]. The G-domain of Ras (amino acids 1–166) is > 95 % conserved between isoforms and comprises the regions of the protein that bind guanine nucleotides, the switch 1 and switch 2 loops that undergo the major conformational changes on GTP–GDP exchange, and the binding surfaces for effectors, exchange factors and GAPs (GTPase-activated proteins). In contrast, the Ras C-terminal 24–25 amino acids of the HVR (hypervariable region) is poorly conserved between isoforms. The HVR comprises the sequences that direct post-translational processing, plasma membrane anchoring and trafficking of newly synthesized and processed Ras from the cytosolic surface of the ER (endoplasmic reticulum) to the inner surface of the plasma membrane [6,7]. The C-terminal CAAX motif of all three Ras proteins is post-translationally processed to generate an S-farnesyl cysteine carboxymethyl ester (Figure 1). The membrane anchor is completed by one or two adjacent S-palmitoyl cysteine residues in N- (Cys¹⁸¹) and H-ras (Cys¹⁸¹ and Cys¹⁸⁴), or a polybasic domain of six lysine residues (Lys¹⁷⁵–Lys¹⁸⁰) in K-ras [7–9].

These minimal membrane anchors (referred to as tN, tH and tK for targeting motif of N-, H- and K-ras) (Figure 1) are sufficient to

traffic and anchor heterologous proteins to the plasma membrane [10,11]. The linker region of the HVR connects the anchor sequence with the N-terminal G-domain [7] (Figure 1).

Interest in the biophysics and biochemistry of Ras–membrane interactions converges from multiple directions. First, different HVR anchors may target the Ras isoforms to different microdomains of the plasma membrane that differ in their lipid and protein content; if these different membrane micro-environments significantly influence Ras signal output, this hypothesis can account for the extensive biological differences between Ras isoforms [7]. Secondly, the Ras C-terminal membrane anchors are near mirror images of the N-terminal anchors of the Src-family kinases [12], thus insights from Ras may extend to the micro-localization and regulation of other lipid-modified signalling proteins. Thirdly, fluorescently labelled probes with Ras C-terminal anchors are increasingly being used as tools for SPT (single-particle tracking) and FRET (fluorescence resonance energy transfer) studies to explore the dynamics of plasma membrane micro- and nano-compartmentalization [13–15].

An intriguing and topical question is whether Ras proteins signal from platforms other than the plasma membrane. There is excellent evidence that endogenous and ectopically expressed Ras proteins are captured into endosomal compartments from where a signal output is generated [16–20]. It has also become clear that ectopically expressed Ras proteins and mutant Ras proteins with defective anchor sequences accumulate on Golgi and ER membranes, from where they are competent to generate a signal output [21–23]. However, the physiological significance of these ER and Golgi platforms is not fully resolved, in that it remains to be demonstrated formally whether they constitute important signalling platforms for endogenous Ras (see [7,24,25] for various discussions). The subject of endomembrane Ras signalling has been reviewed extensively (see, e.g., [7,24,25]) and it will not be discussed further here.

Abbreviations used: CaM, calmodulin; DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; EGF, epidermal growth factor; EM, electron microscopy; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; Gal-L11A, galectin-1-L11A; GAP, GTPase-activated protein; GFP, green fluorescent protein; HVR, hypervariable region; LUV, large unilamellar vesicle; MARCKS, myristoylated alanine-rich C-kinase substrate; MEK, mitogen-activated protein kinase/ERK kinase; pcCMT, prenylcysteine carboxyl methyltransferase; PDE δ , phosphodiesterase subunit δ ; PI3K, phosphoinositide 3-kinase; mRFP, monomeric RFP; RhoGDI, Rho guanine nucleotide dissociation inhibitor; SPT, single-particle tracking; tH, tN and tK, targeting motif of H-, N- and K-ras respectively; YFP, yellow fluorescent protein.

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Figure 1 Membrane anchors of Ras proteins

The fully processed C-terminal membrane anchors of H-, N- and K-ras are depicted in the upper panel. The C-terminal cysteine residue of all three Ras proteins is farnesylated and carboxymethylated (shown in red) as a result of the triplet of modifications directed by the C-terminal CAAX motif, i.e. farnesylation, removal of the AAX sequence and methylesterification. The anchor of N- and H-ras is completed by palmitoylation (shown in blue) of one or two cysteine residues, and of K-ras by a sequence of lysine residues (shown in blue). The anchor (shown in grey in the lower panel) is connected to the G-domain by the linker sequence of the HVR (shown in black). The linker comprises residues 166–180 in H- and N-ras, and residues 166–174 in K-ras. Structures of H-ras linker and N-ras anchor have recently been reported and are discussed in the text.

SPATIAL MAPPING OF RAS PROTEINS ON THE PLASMA MEMBRANE

Biochemical and plasma membrane fractionation experiments provided the first tentative evidence that H-ras and K-ras proteins might localize to different plasma membrane microdomains [26,27]. There is always a potential problem of experimental artifacts with membrane fractionation protocols, and definitive evidence was subsequently provided by EM analysis of intact plasma membrane sheets [28].

Direct visualization of the distribution of Ras proteins on intact two-dimensional sheets of apical plasma membrane sheets is possible using a combination of immunogold labelling and spatial statistics to interpret the immunogold point patterns (Figure 2). This approach is particularly well suited to probing microdomain structure because it interrogates scale lengths of 1–250 nm with nanometre precision [28–30]. EM (electron microscopy) analysis shows that constitutively activated H-rasG12V and K-rasG12V cluster in non-overlapping domains that are spatially distinct, because there is no significant co-localization of co-expressed mRFP (monomeric red fluorescent protein)–H-rasG12V and GFP (green fluorescent protein)–K-rasG12V [28] (C. Muncke, R. G.

Parton and J. F. Hancock, unpublished work). Recent estimates of the size of these domains suggest diameters that may be as small as 10 nm (S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work). The clustering of H-rasG12V and K-rasG12V is insensitive to acute cholesterol depletion, indicating that neither domain is a classical lipid raft [28]. The minimal membrane anchor of H-ras targets GFP (GFP–tH) and mRFP (mRFP–tH) to cholesterol-sensitive microdomains or nanoclusters with an average diameter of < 15 nm ([28], and S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work). Wild-type H-ras, but not H-rasG12V or K-rasG12V, co-localizes with a raft marker (GFP–tH) when both proteins are co-expressed, indicating that GDP-loaded H-ras, but not GTP-loaded H-ras, is localized to lipid rafts [28].

DYNAMICS OF RAS MOBILITY ON THE PLASMA MEMBRANE

The movement of individual proteins attached to the plasma membrane can be visualized in live cells using SPT. On the outer leaflet of the plasma membrane, single proteins can be labelled with a gold particle or a fluorophore. The trajectory of a gold

Figure 2 EM mapping of Ras proteins

(A) Example of a plasma membrane sheet prepared from BHK (baby hamster kidney) cells expressing GFP–H-rasG12V. The sheet is labelled with anti-GFP antibody coupled to 5 nm gold and imaged at 100 000 $\times$ magnification in an electron microscope. (B) Statistical analysis of multiple sheets is used to determine the extent of clustering and the radius of the clusters. In this type of analysis, if the K-function $[L(r) - r]$ curve leaves the confidence interval (99% CI: broken black lines), it indicates that the gold pattern is significantly clustered. The analysis shows that (i) H-rasG12V (red line) is clustered, (ii) knockdown of galectin-1 expression (blue line) causes a loss of H-rasG12V clustering, and (iii) ectopic expression of galectin-1 (green line) increases the radius of H-rasG12V clusters by 8 nm.

Figure 3 SPT of Ras proteins

Trajectories of Ras proteins monitored by SPT (reproduced with permission from Murakoshi, H., Iino, R., Kobayashi, T., Fujiwara, T., Ohshima, C., Yoshimura, A. and Kusumi, A. Single-molecule imaging analysis of Ras activation in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 7317–7322 © 2004 National Academy of Sciences, U.S.A.). (A) Inactive (GDP-loaded) YFP–H-ras and YFP–K-ras single-molecule trajectories were recorded at video rate (30 Hz) and followed for 1 s on the cell membrane. After EGF stimulation, trajectories of single-activated Ras molecules are followed by FRET between YFP–Ras and BODIPY[®]-TR–GTP (where BODIPY[®] is 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene and TR is Texas Red). Using this elegant technique, Kusumi and colleagues demonstrate that some activated Ras trajectories exhibit immobile periods. This is illustrated further in (B), where trajectories of YFP–Ras molecules are followed until they bind BODIPY[®]-TR–GTP, and the resulting FRET signal (red) is then tracked; three of the trajectories exhibiting FRET are immobile.

particle is tracked by light scattering, allowing for very fast imaging (one image every 25 μ s). The time resolution for fluorophore-labelled proteins is three orders of magnitude lower (one image every 33 ms) because of the time needed to excite the fluorophore and for the camera to collect sufficient photons for visualization [2]. Inner plasma membrane proteins cannot yet be labelled with gold particles, so all SPT studies on Ras proteins have been with GFP- or YFP (yellow fluorescent protein)-labelled Ras. SPT of the trajectories of individual Ras proteins reveals that approx. 7% of YFP–H-ras molecules in serum-starved cells have transient periods of immobility that last < 1 s [14]. These immobile periods are interspersed with mobile, apparently Brownian movement, periods in the plane of the membrane (Figure 3). The number of YFP–H-ras molecules that exhibit transient immobile periods increases up to ~25% after stimulation with EGF (epidermal

growth factor) [14]. Imaging using single-molecule FRET with BODIPY[®] (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)–GTP as acceptor and YFP–H-ras as donor shows that the majority of immobile YFP–H-ras proteins are GTP-loaded i.e. activated [14] (Figure 3). This immobile fraction is unaffected by cholesterol depletion, thus these immobile periods do not correspond to sequestration in cholesterol-rich rafts. Constitutively activated YFP–H-rasG12V also exhibits these periods of transient immobilization. At any point, approx. 25% of YFP–H-rasG12V proteins are immobile and approx. 75% are mobile, with the duration of the immobile periods again lasting < 1 s [14] (Figure 3). SPT of YFP–K-ras and YFP–K-rasG12V reveals very similar behaviours to those of YFP–H-ras and YFP–H-rasG12V: periods of immobility that are related to GTP loading, but are unaffected by cholesterol depletion [14]. One overall conclusion

from these data is that GTP loading of Ras proteins results in an increased probability of the protein becoming transiently immobile, but there is not a 1:1 correlation between the fraction of Ras molecules that are GTP-loaded and the fraction that are immobile: typically approx. 20 % of wild-type Ras is GTP-loaded in response to agonist stimulation, whereas > 85 % of RasG12V is constitutively GTP-loaded. This will be discussed further below.

INTERPRETING EM, SPT AND FRAP (FLUORESCENCE RECOVERY AFTER PHOTBLEACHING) DATA SETS WITHIN A COMMON CONCEPTUAL FRAMEWORK

EM immunogold mapping provides high-resolution (nanometre) spatial information on the location of Ras proteins on the plasma membrane, but with no temporal component, whereas SPT in living cells gives temporal information on the dynamics of single Ras proteins, but with lower spatial accuracy. FRAP measures the lateral diffusion and membrane affinity of a population of Ras proteins. We are therefore now at the fascinating stage of being able to combine these different data sets to formulate a model for how Ras proteins operate at the plasma membrane.

As an example, we will first discuss activated H-ras and K-ras. The most critical question is whether the immobile YFP-H-rasG12V and YFP-K-rasG12V molecules observed by SPT correspond to the clusters of H-rasG12V and K-rasG12V visualized by EM spatial mapping. We propose here that they do, and that this assumption is an excellent basis for uniting the spatial and dynamic data sets. First, consider that a recent analysis of the observed plasma membrane distributions of H-rasG12V and K-rasG12V expressed at different densities shows that the fraction of H-rasG12V and K-rasG12V molecules in clusters is 40 % and 46 % respectively, with the remainder of the proteins randomly distributed as monomers (S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work).

The SPT and EM data agree that a minority of H-rasG12V and K-rasG12V proteins are immobile (SPT) or in clusters (EM) at any given time (estimates are approx. 25 % from SPT compared with 40–46 % from EM). Conversely then, at any given time, the majority of H-rasG12V and K-rasG12V proteins (even though they are GTP-loaded) are not immobile or confined to clusters. This population of molecules appears as randomly distributed Ras proteins by EM and mobile Ras proteins by SPT. Furthermore after growth factor stimulation, approx. 25 % of YFP-H-ras spots double in fluorescent intensity [14]: the frequency of these brighter activation-induced clusters and their average molecular size again fits approximately with the EM analysis (the mean number of H-rasG12V proteins detected in a cluster is 2.5). The SPT results predict that the lateral diffusion of a population of activated H-rasG12V proteins should be slightly reduced compared with H-ras, a prediction confirmed by FRAP [32]. Also, the lateral diffusion of H-rasG12V and K-rasG12V measured by FRAP is unaffected by cholesterol depletion [32,33], consistent with conclusions from SPT, EM and earlier biochemical studies that neither H-rasG12V nor K-rasG12V is associated with lipid rafts [14,27,32]. Taken together, these results strongly suggest that the immobile YFP-H-rasG12V and YFP-K-rasG12V molecules observed by SPT correspond to the clusters of H-rasG12V and K-rasG12V visualized by EM spatial mapping (Figure 4). For simplicity, we will refer to these structures as the H-rasG12V and K-rasG12V immobile clusters.

What about lipid raft domains and Ras? First, we need to comment on terminology. Classically, lipid rafts have been viewed as stable pre-existing cholesterol-rich liquid-ordered domains into which proteins with appropriate anchors or transmembrane do-

main are able to partition [34–36]. An alternative view is that these domains are small, unstable assemblies of saturated lipids, sphingolipid and cholesterol, and that the formation or stability of these structures, while critically dependent on cholesterol, may also be determined in part by their constituent lipid-anchored proteins. The terms nanocluster (referring to the proteins in these domains) or unstable raft have been used to capture these ideas [2,37]. Developing these ideas further is beyond the scope of the present review, and the subject is somewhat controversial, but our own data offer support to the alternative model view of lipid rafts as cholesterol-dependent nanoclusters/unstable rafts (S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work). Against this background, EM shows that GDP-loaded wild-type H-ras co-localizes significantly with GFP-tH, which is a marker protein for lipid rafts/cholesterol-dependent nanoclusters ([28], and S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work).

Consistent with this spatial mapping data, FRAP studies report that the lateral diffusion of H-ras and GFP-tH is faster in cholesterol-depleted than in control cells, as would be expected if the proteins partitioned in slower-moving lipid rafts or cholesterol-dependent nanoclusters [32,38]. In contrast, the trajectories of GFP-tH and YFP-H-ras molecules tracked by SPT do not exhibit immobile periods that are sensitive to cholesterol depletion or show evidence of confinement to small (i.e. 15 nm) compartments [14,15]. How can these observations be reconciled with the EM and FRAP data? The simple answer is that the temporal resolution of fluorophore SPT is just not high enough to visualize highly dynamic transient interactions with cholesterol-dependent domains. For example, to appear immobile by SPT, a protein has to be resident for multiple (more than five) sequential images; if the frame rate is 30 Hz, then residency times would need to be greater than 165 ms for a protein to appear immobile. As the imaging time decreases and the temporal resolution of SPT increases, shorter residence times become possible to detect. One advantage of EM is that it provides an instantaneous snapshot of the plasma membrane, and so records whether proteins are clustered or not, irrespective of how fast the clusters are forming and dissolving. Thus collecting these ideas together, we suggest that, at any given time, approx. 30 % of GFP-tH and YFP-H-ras proteins exist in cholesterol-dependent clusters on the plasma membrane, but SPT illustrates that these must be highly dynamic structures with a lifetime that is < 165 ms (Figure 4). The combination of techniques therefore illuminates interesting differences between the types of domains or clusters with which Ras proteins interact.

THE H-rasG12V IMMOBILE CLUSTER

EM, FRAP and SPT all indicate that formation of H-rasG12V immobile clusters does not depend on cholesterol [14,28,32,33,38]. The lectin galectin-1 binds preferentially to GTP-loaded H-ras over GDP-H-ras, and has greater affinity for H-ras over N- and K-ras [39]. EM analysis shows that galectin-1 is required for the formation of H-rasG12V clusters: in cells depleted of galectin-1 H-rasG12V association with the plasma membrane is weakened, and the 60 % of H-rasG12V molecules that remain on the plasma membrane are distributed randomly [28].

Conversely, ectopic expression of galectin-1 increases the radius of the H-rasG12V clusters (Figure 2) (I. A. Prior, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work). Studies with SPT have recently identified Sur-8 as an additional scaffold that is required for YFP-H-ras to exhibit immobile periods ([14], and A. Kusumi, personal communication). Thus at least two scaffold proteins recruited from the cytosol play a critical

Figure 4 Model of Ras protein organization on the plasma membrane

The upper panel shows the theoretical outcome of 'simultaneously' analysing the plasma membrane of a cell expressing GFP-H-rasG12V by SPT and EM. In the SPT experiment, four Ras molecule trajectories are followed, two of these exhibit an immobile period. EM provides a snapshot of the positions of all of the GFP-H-rasG12V proteins present on the membrane when the imaging by SPT is terminated. In the overlay of the two experiments, note that the two immobile trajectories result from GFP-H-rasG12V proteins forming nanoclusters, whereas EM detects the two mobile GFP-H-rasG12V proteins as non-clustered random particles. The lower panel summarizes the characteristics of the two types of H-ras nanocluster discussed in the text. Activated H-ras forms a nanocluster that is stabilized by scaffolds, such as galectin-1 and Sur-8. The nanocluster becomes trapped by the actin cytoskeleton and immobilized. The cholesterol-dependent nanocluster, formed by inactive H-ras, is more transient than the activated H-ras nanocluster.

role in the formation and/or stabilization of the activated H-ras clusters. Effector-null H-rasG12V molecules (with mutations in the effector domain that block interactions with all known effectors) are immobilized with the same frequency as effector-competent H-rasG12V, thus the formation of immobile clusters is not driven by protein-protein interactions with effectors (A. Kusumi, personal communication). This is consistent with the conclusion of earlier biochemical work that effector interactions are not required to stabilize the association of H-rasG12V with non-raft plasma membrane [27]. The role of actin is more complicated. The formation of the H-rasG12V clusters visualized by EM is unaffected by the depolymerization of actin with latrunculin (S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work), whereas, according to SPT, latrunculin treatment decreases the number of immobile GTP-loaded H-ras proteins by 50 % [14]. It is therefore possible that actin does not play any direct role in forming H-rasG12V clusters, but the submembrane actin fence is involved, at least in part, in immobilizing the H-rasG12V clusters once they have formed.

THE K-rasG12V IMMOBILE CLUSTER

The structure of the K-ras cluster is less well characterized. EM, FRAP and SPT show that extraction of plasma membrane cholesterol does not prevent formation of the cluster. Immobilization is dependent on Sur-8, exactly as for H-ras (T. Kobayashi and A. Kusumi, personal communication). Electrostatic interactions with anionic lipids are required for K-ras plasma membrane binding [9,41,42], but whether K-ras reorganizes plasma membrane phospholipids in its immediate proximity as proposed for MARCKS (myristoylated alanine-rich C-kinase substrate)

[43–45], another polybasic tethered protein, is unknown at present. In the light of recent biochemical reports that galectin-3 regulates K-ras signal output [46], reminiscent of the effects of galectin-1 on H-ras signalling [39,47], it is tempting to speculate that galectin-3 may be an important scaffold for the K-ras cluster, although this awaits EM or SPT confirmation. In latrunculin-treated cells, K-ras clusters visualized by EM are partially destabilized, but are not completely destroyed (S. Plowman, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work), whereas SPT shows that they remain immobile [14]. Therefore either the actin fence is not involved in immobilizing the K-ras clusters or, possibly, the clusters could retain some affinity for micro-aggregates of actin that form after latrunculin treatment [14]. Further EM studies should be able to confirm whether the latter explanation is valid.

A NEW MODEL FOR RAS PLASMA MEMBRANE MICRO-ORGANIZATION

Taken together, these results best fit a molecular model where Ras proteins are involved in generating or driving the formation of the domains in which they operate, rather than interacting with pre-existing microdomains on the plasma membrane. Thus Ras proteins may operate in one sense like Rab proteins: marking plasma membrane sites for the molecular assembly of a signalling microdomain, akin to Rab or Arf (ADP-ribosylation factor) proteins marking a membrane site for the assembly of molecular machinery to bud or fuse vesicles. We envisage GTP-loaded Ras-recruiting scaffold proteins, such as galectin-1, galectin-3, Sur-8 and possibly other similar proteins, to assemble a microdomain, which interacts directly or indirectly with the submembrane

actin cytoskeleton and stops lateral diffusion (Figure 4). These microdomains are unstable, lasting on average < 1 s. Formation of the domain requires Ras to be GTP-loaded, but the inherent instability of the scaffolded complex limits its lifetime, since this appears to be independent of GTP hydrolysis, i.e. the immobile periods are the same for GTP-loaded Ras, which is sensitive to GAP, and RasG12V, which is not. Several important questions flow from this model. Are these clusters also Ras signalling centres? How is the affinity of H-ras for lipid rafts/cholesterol-dependent nanoclusters when GDP-loaded and non-raft clusters when GTP-loaded regulated? Ras–scaffold interactions are clearly important for stabilizing Ras clusters, but is the primary determinant of domain formation actually lipid–lipid interactions of the anchor with the membrane bilayer? A variety of diverse studies are beginning to shed light on these questions.

ARE THE RAS CLUSTERS SIGNALLING CENTRES?

The first indirect evidence in favour of the hypothesis that Ras clusters are signalling centres comes from biochemical analyses of galectin function. Ectopic expression of galectin-1 stabilizes the GTP-bound state of H-ras in response to EGF stimulation and alters effector output. Thus cells expressing GFP–H-ras and galectin-1 activate PI3K (phosphoinositide 3-kinase) more weakly and the Raf/MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase]/ERK cascade more strongly than cells expressing GFP–H-ras alone [47]. These effects on H-ras signal output may reflect the increased radius and perhaps therefore the stability of activated H-ras clusters induced by galectin-1 expression (Figure 2) (I. A. Prior, C. Muncke, R. G. Parton and J. F. Hancock, unpublished work). Knockdown of galectin-1 expression inhibits H-ras signalling [47], an effect that correlates with a loss of H-rasG12V clustering on the plasma membrane [28]. A somewhat similar, but less marked, effect of galectin-1 is also seen on the signal output in cells that express GFP–K-ras [47]. Galectin-1 may interact weakly with K-ras under conditions of high expression [39,47], and so might be expected to have some effect on K-ras clustering, although this has not yet been investigated. Galectin-3, however, clearly stabilizes K-ras GTP levels in response to EGF stimulation, and selectively potentiates PI3K activation by K-ras, but not by H-ras [46]. Galectin-3 is also recruited to the plasma membrane by activated K-ras, and interacts selectively *in vitro* with K-ras, but not with H-ras, in a prenyl- and GTP-dependent manner [46]. Again, these effects on K-ras signalling could reflect changes in the stability of K-ras clusters on the plasma membrane, although, at present, this remains speculative.

SPT studies have provided more direct insight into the dynamics of Ras and Raf interactions on the plasma membrane. In EGF-stimulated HeLa cells, FRET from YFP–Ras and GFP–Raf correlates temporally with biochemical activation of Raf, confirming that Raf is recruited via a direct interaction with activated Ras [48]. In cells that express GFP–Raf alone, EGF treatment stimulates recruitment of single GFP–Raf molecules to the plasma membrane; these molecules diffuse laterally after recruitment, but remain bound to the plasma membrane for only very short periods: on average, the binding period for a single GFP–Raf molecule is 0.41 s [48]. At sites of membrane ruffling, a significant fraction of GFP–Raf molecules (37%) have longer binding periods of 1.6 s; although, for the majority of GFP–Raf molecules (63%), it is the same as on the general plasma membrane (0.37 s) [48]. Ruffles also represent sites for persistent recruitment of a small pool of Raf (3–5%) for < 100 min after EGF stimulation [48]. This small degree of Raf activation

would be difficult to detect biochemically, but suggests that the assembly or persistence of Ras signalling platforms may exhibit some degree of heterogeneity over the plasma membrane. This phenomenon of Ras platform heterogeneity may also extend to the activation of other effectors: for example, PI3K shows prolonged Ras-dependent activation at the leading edge of migrating *Dictyostelium* [49].

Kusumi and colleagues have extended the analysis of Ras–Raf interactions at the single-molecule level by simultaneous imaging of mRFP–Raf (where mRFP is monomeric RFP) and YFP–H-rasG12V trajectories in living cells (T. Kobayashi and A. Kusumi, personal communication). Essentially they show that only during periods of H-rasG12V immobilization are mRFP–Raf molecules recruited to the plasma membrane. Importantly, they observe that mRFP–Raf molecules are recruited directly to Ras from the cytoplasm and not initially to other sites on the plasma membrane, whence they move laterally to engage an activated Ras protein (T. Kobayashi and A. Kusumi, personal communication). Occasionally Ras molecules leave the site of a Ras–Raf interaction 0.1–0.2 s before the Raf-1 protein returns to the cytosol, thus the duration of individual Raf protein engagement with the plasma membrane is on the same time scale as the duration of the Ras immobile periods < 0.5 s (T. Kobayashi and A. Kusumi, personal communication). The spatial resolution of the two-camera imaging is 20–30 nm, but single-molecule Ras–Raf FRET confirmed direct protein–protein interaction: $> 90\%$ of the FRET signals were immobile, and, again, the duration of the FRET signal was generally < 0.7 s (T. Kobayashi and A. Kusumi, personal communication). The same phenomenon is seen for the interaction of GTP-loaded Ras and the negative regulator GAP [14]. Following EGF stimulation, there is recruitment of GAP-334 molecules from the cytosol to the plasma membrane (GAP-334 is a C-terminal fragment of p120 GAP that contains the GAP catalytic domain). Once on the plasma membrane, a high fraction (82%) of these GAP-334 molecules are classified as immobile, i.e. they have been recruited to sites of immobile activated Ras [14].

As expression of mRFP–Raf increases, the fraction of mobile Raf proteins that exhibit lateral diffusion on the plasma membrane, not in complex with Ras, increases. However, several observations suggest that it is only the immobile Raf molecules that generate a signal: there is a temporal correlation between immobile Raf-1, but not mobile Raf-1, molecules on the plasma membrane in melanocytes after SCF (stem cell factor) stimulation. Similarly, in HeLa cells, the recruitment of immobilized Raf-1 coincides with Raf-1 kinase activation, but that of mobile molecules does not (T. Kobayashi and A. Kusumi, personal communication). Thus inactive Raf proteins may interact with the plasma membrane independently of Ras, although it remains formally possible that such interactions are largely a consequence of overexpression. Hibino et al. [48] did not report immobile Raf proteins, although this could be due to several factors, including time-averaging trajectories over long-time windows and an analysis based on the assumption that all Raf proteins are mobile, rather than the analysis by Kusumi and colleagues that trajectories can be resolved into immobile and mobile components ([14], and T. Kobayashi and A. Kusumi, personal communication). In summary, these SPT experiments provide strong evidence that the immobile Ras clusters are *bona fide* signalling platforms. The transient nature of Ras–Raf interactions at the molecular level in these platforms underscores the intrinsic instability of these signalling complexes. It has also led Kusumi and colleagues to the interesting speculation that this type of transient interaction of Ras and Raf may be the basis of digital signal generation ([14], and T. Kobayashi and A. Kusumi, personal communication).

These single-molecule studies require reinterpretation of earlier work that showed that endocytosis is required for efficient H-ras-dependent activation of the Raf/MEK/ERK signalling cascade [19]. Expression of dominant-interfering dynamin and Rab mutants that block clathrin-dependent and other endocytic pathways abrogates H-ras-, but not K-ras-, dependent Raf activation [19]. When taken together with observations that a fraction of H-ras and, to a lesser extent, K-ras decorate the cytosolic surface of endocytic vesicles, these data lead to a simple model where H-ras–Raf complexes must be trafficked into the endocytic pathway in order to complete the multistep process of Raf activation [19]. However, the very short duration of Ras–Raf interactions (0.4–1.4 s) on the plasma membrane ([14,48], and T. Kobayashi and A. Kusumi, personal communication) suggests that interaction of Raf proteins with endosomes may reflect direct recruitment of Raf from the cytosol to Ras-containing endosomes, rather than the endocytosis of Ras–Raf complexes generated on the plasma membrane. This model predicts that the micro-environment of H-ras on the endosome is more favourable to Raf activation than in the H-ras clusters on the plasma membrane, or that the duration of Raf engagement with endosomal signalling platforms is longer, i.e. more stable, than the 0.4–1.4 s periods of contact with the plasma membrane. Either way, the effect of the dynamin/Rab expression on Raf activation then translates into a direct influence of these regulators of endocytosis on the partitioning of H-ras between plasma membrane and endosomes, so regulating the relative number of plasma membrane and endosomal sites for Raf activation. The validity of this model will require, among other things, SPT to determine the interaction time of Raf molecules with endosomal vesicles: a technically demanding, but intriguing, challenge.

HOW DO H-RAS PROTEINS MODULATE THEIR LATERAL SEGREGATION?

Wild-type H-ras interacts reversibly with lipid rafts/cholesterol-dependent nanoclusters, whereas activated H-ras does not, and has a high probability of initiating the formation of a signalling cluster. How might this be achieved? A study of the spatial distribution and membrane affinity of a series of H-ras C-terminal mutants determined using a combination of EM spatial mapping and two-beam-size FRAP concluded that three forces seem to operate on H-ras at the plasma membrane [38]. The first is generated by the minimal membrane anchor that provides an attractive force for plasma membrane generally and for lipid rafts/cholesterol-dependent nanoclusters specifically. The second is a repulsive force generated, in some way, by the N-terminal G-domain which opposes the attractive force of the membrane anchor; the strength of this repulsive force is greater when H-ras is GTP-bound rather than GDP-bound. Finally, the third is an attractive force generated by the HVR linker (amino acids 166–180), adjacent to the minimal membrane anchor that provides affinity for non-raft clusters. The net effect of these various forces is that GTP-loading significantly decreases the affinity of H-ras for lipid rafts, and allows the attractive interactions of the HVR to dominate [38], allowing the formation of transient non-raft signalling clusters as described above. The biophysical and biochemical mechanisms that generate these forces remain to be elucidated. For example, how does the G-domain reduce membrane affinity? The definitive answer will need to await a crystal structure of fully processed Ras anchored to a membrane bilayer in the GTP- and GDP-bound conformations. Possibilities include a modulation of the extent to which the palmitate and/or prenyl groups insert into the lipid bilayer, or steric and/or electrostatic interactions of the G-domain with the inner leaflet of the plasma membrane.

BIOPHYSICS OF RAS MEMBRANE ANCHORING

The biophysics of Ras membrane binding has started to be investigated. The mechanism of membrane insertion of a C-terminal N-ras heptapeptide (GC¹⁸¹MGLPC¹⁸⁶) into a DMPC (dimyristoyl phosphatidylcholine) lipid bilayer has been solved using a combination of NMR and neutron-diffraction spectroscopy [50]. In the peptide, Cys¹⁸¹ was palmitoylated and Cys¹⁸⁶ was lipidated and methylsterified to replicate a processed Ras C-terminal cysteine residue. The C₁₆ lipid chains insert completely into the DMPC bilayer. The order parameter for the Ras lipid chains is lower than the surrounding lipids, reflecting the need to match chain length of the Ras lipids with the surrounding membrane. Since the peptide lacks charge, there is little Born repulsion, and the peptide chain penetrates the membrane surface. The hydrophobic peptide backbone localizes among the lipid headgroups at the lipid/water interface, with a significant amount of the peptide plus the apolar side chains of the hydrophobic methionine and leucine residues penetrating more deeply, perhaps to a depth of four to six carbons of the DMPC chains, and causing some disordering of these membrane lipids [50]. The peptide backbone has no identifiable secondary structure, thus multiple extended conformations are energetically permissible [50].

Molecular Dynamics simulations of the same lipidated peptide–DMPC interaction confirmed the experimental observations, concluding that insertion of the lipid chains is driven by hydrophobic interactions. However, once in place, the backbone peptide, which is inserted deep in the lipid bilayer contributes to the stability of the complex through hydrogen bonding between backbone amides and DMPC headgroups, and with the apolar side chains of Met¹⁸² and Leu¹⁸⁴ contributing further stability [51]. Interestingly, the simulations suggested that the monolayer is thinner where the peptide is inserted, suggesting that insertion of the peptide actually deforms the membrane [51]. A caveat for both of these analyses is that the farnesyl thioether was replaced by a hexadecyl thioether, although such a substitution probably has very little effect on Ras biology [52]. What inferences can be made from these studies in relation to H-ras? The sequence of the H-ras anchor peptide is nearly identical, except that Leu¹⁸⁴ is replaced with a cysteine residue that is palmitoylated. The H-ras anchor peptide is therefore even more hydrophobic than the N-ras anchor peptide, and, rather than the hydrophobic side chain of Leu¹⁸⁴ contributing to stability of membrane binding, there will be the additional palmitate. It is possible that this palmitate may drive the peptide even deeper into the bilayer and deform the membrane further.

In a complementary study, the partitioning of the N-ras prenylated palmitoylated heptapeptide between gel/liquid-ordered/fluid phases was investigated in simple two- or three-component membranes [53]. In binary mixtures of DMPC/DSPC (distearoyl phosphatidylcholine), the peptide preferentially partitions into DMPC-enriched fluid phases. Intriguingly, however, in ternary mixtures that included cholesterol, the peptide induces phase separation: the mixture of 1:1 DMPC/DSPC + 20 mol % of cholesterol at > 30°C is a homogenous liquid-ordered phase, but the addition of 2 mol % of N-ras prenylated palmitoylated heptapeptide induces the appearance of small fluid-phase domains on the vesicle surface, presumably due to the high affinity of the peptide for a fluid DMPC-rich environment [53]. Incidentally, the supplementary conclusion of this study, that the N-ras anchor preferentially localizes to fluid membrane environments [53], is consistent with spatial mapping data showing that wild-type GDP-bound N-ras clusters in cholesterol insensitive, non-raft domains (I. A. Prior, R. G. Parton and J. F. Hancock, unpublished work). Similar analyses of an Src peptide and other lipidated charged peptides show that hydrophobic repulsion from the positively

charged lysine residues in the peptide prevents complete insertion of the acyl chain into the lipid bilayer [54–58]. By analogy, the farnesyl chain of K-ras will not penetrate as deeply into the membrane as that of H- and N-ras, but electrostatic interactions between the lysine residue and negative charged phospholipid headgroups will contribute to membrane binding [59,60]. Taken together, these studies provide tantalizing evidence that the anchors of palmitoylated Ras proteins may help drive the formation of their own signalling clusters by a combination of lipid–lipid and protein–lipid interactions of the lipid-modified anchor peptide with the plasma membrane bilayer.

PRENYL-BINDING PROTEINS AND RAS–MEMBRANE INTERACTIONS

The interaction of Ras with the plasma membrane will be modified in the presence of prenyl-binding proteins. Two such proteins have been identified that have quite different effects on Ras plasma membrane association. PDE δ , originally identified as a subunit of phosphodiesterase, binds to a large number of small GTPases, including H-ras, N-ras, Rap1 and Rap2, but not K-ras [61,62]. The crystal structure of PDE δ shows that it has the same fold as RhoGDI (Rho guanine nucleotide dissociation inhibitor) [61]. The hydrophobic cavity in RhoGDI accommodates the prenyl group of Rho family GTPases, and a similar hydrophobic cavity is present in PDE δ [61]. It seems probable that the Ras farnesyl and not a palmitoyl group is accommodated in this pocket, because PDE δ does not bind non-prenylated Ras proteins, but does bind prenylated non-palmitoylated H-ras proteins [61]. Ectopic expression of PDE δ causes a partial redistribution of Ras, Rap and other GTPases from plasma membrane to cytosol, and incubation of recombinant PDE δ with membrane fractions *in vitro* solubilizes the same set of Ras proteins [62]. Thus PDE δ has the potential to modify the membrane interactions of its target GTPases, although what the biological role of this protein is remains unclear. However, it is interesting to note that the affinity of PDE δ for GDP-loaded H-ras is greater than that for GTP-loaded H-ras [61], consistent with a possible role for PDE δ in effecting changes in H-ras affinity for lipid raft and non-raft microdomains. Recent work suggests that galectin-1 is also a prenyl-binding protein [63]. The fold of galectin-1 is sufficiently similar to that of RhoGDI to allow an alignment of the structures and identification of a potential hydrophobic pocket in the galectin-1 protein [63]. Mutation of a single residue (Leu¹¹) in this putative pocket in galectin-1 generates an interesting dominant-interfering mutant. Expression of galectin-1-L11A (Gal-L11A) displaces H-rasG12V, but not wild-type H-ras, from the plasma membrane to the cytosol, with a concomitant reduction in H-rasG12V signal output [63]. This is a similar effect to a knockdown of endogenous galectin-1 expression, where some H-rasG12V is lost from the plasma membrane, and the remaining plasma-membrane-localized H-rasG12V is randomly distributed and not clustered [28,39,47]. Gal-L11A is not recruited to the plasma membrane by H-rasG12V [63], thus the interactions of the H-rasG12V farnesyl group with galectin-1 are required both to recruit galectin-1 on to the plasma membrane and to stabilize H-rasG12V in non-raft clusters. However, Gal-L11A (and, by inference, wild-type galectin-1) must make additional contacts elsewhere with H-ras in order to operate as an interfering mutant; candidate sites include the HVR and regions flanking the effector domain [47,63].

Sequestering the Ras farnesyl group changes the H-ras lipid anchor to two palmitates and, in the light of the biophysical studies described above, would be expected to lower membrane affinity,

and perhaps change the preference of the anchor for the type of plasma membrane lipids into which it partitions. It is also possible that the transient decreased membrane affinity observed on H-ras GTP loading is required to facilitate galectin-1 and PDE δ binding, or is a consequence of binding these and potentially other, as yet unidentified, prenyl-sequestering proteins. Presumably, the additional protein–protein interactions or protein–lipid interactions of the galectin-1–Ras and PDE δ –Ras complex determine the fate of the complex: clustering in signalling domains in the former case and unknown in the latter case. No other intracellular galectin-1-binding proteins have been reported as yet, but PDE δ has been identified as an effector of Arl2 and Arl3 proteins [61,64]; although whether these GTPases regulate H- and N-ras membrane interactions is presently unclear. A challenge now is to investigate precisely how these proteins modulate the biophysics of Ras membrane binding, an area that should be theoretically and experimentally accessible with the simplified model systems described above.

REGULATION OF RAS STRUCTURE BY THE PLASMA MEMBRANE

Crystal structures of the G-domain of Ras (amino acids 1–166) in the GTP- and GDP-bound states, and in complexes with effectors and exchange factors have been solved [65,66]. Nevertheless, information is still lacking about the structure of Ras proteins positioned in their biologically relevant environment of the plasma membrane. For example, does the binding of Ras proteins to membranes significantly alter the conformation of the G-domain? What is the structure of the HVR when stabilized on a membrane bilayer? How is the G-domain oriented with respect to the inner surface of the plasma membrane, and does this orientation change with GTP-loading? Recent work has now started to address this poorly understood area of Ras structural biology. Campbell and colleagues have studied the structure of non-palmitoylated full-length H-ras using NMR and CD [67]. They showed first that α -helix-5 extends to residue 172, but thereafter the C-terminus is conformationally flexible (consistent with the modelling of the C-terminal heptapeptide of N-ras). The presence of HVR residues beyond 166 does not change the overall fold of the G-domain, but introduces wide-ranging dynamic changes in the central β -sheet, which becomes more flexible, as well as to residues in and flanking the Switch 1 and Switch 2 regions that undergo the major conformational changes on GTP-binding [67]. In particular, the residues preceding Switch 1 become more ordered in the presence of the HVR. The additional attachment of a farnesyl group by *in vitro* farnesylation and the titration into the solution of LUVs (large unilamellar vesicles) composed of DOPC, engage the farnesyl group, and induce further, albeit as yet poorly characterized, changes in the overall structure of the protein [67].

Some of these changes may facilitate the interaction of H-ras with the Raf cysteine-rich domain [67–70]. These authors have not yet attempted similar studies with palmitoylated, fully CAAX-processed H-ras, nor have they used more complex mixtures of lipids for their LUVs, but the approach is clearly one with much promise to give important insights into how membrane interactions might regulate or tune the molecular structure of lipid-anchored proteins.

REVERSIBLE MEMBRANE INTERACTIONS

Throughout the present review, we have discussed regulation of the lateral segregation of Ras proteins on the plasma membrane: the time scales for changes in the distribution of Ras between

different plasma membrane microdomains is < 1 s, and faster still in the case of raft-like clusters. However, since Ras is not an integral membrane protein, there is a potential for Ras to be removed from the plasma membrane and be redistributed to the cytosol or intracellular membranes. The thioether linkage that attaches farnesyl to the protein backbone is stable, and consequently the half-life of farnesyl on Ras is the same as the half-life of the Ras protein itself, i.e. approx. 24 h [8,71]. In contrast, the thioester linkage that attaches palmitoyl to H- and N-ras is sensitive to hydrolysis by thioesterases. As a result of this, the palmitoyl groups turn over with a half-life of approx. 30 min on N-ras and approx. 1–2 h on H-ras [72,73]. Interestingly, the palmitoyl groups on constitutively activated H-rasG12V turn over faster than those on wild-type H-ras [73]. Farnesylation alone is insufficient to stably anchor Ras to the plasma membrane, so loss of palmitate will cause dissociation of H- and N-ras from the plasma membrane [8,9], unless de-palmitoylation occurs in the vicinity of a plasma-membrane-localized palmitoyltransferase [74] that immediately re-attaches palmitate. Inhibition of palmitoyltransferase using compounds such as 2-bromopalmitate causes redistribution of H-ras to the Golgi and ER [75]. This mislocalization reflects the ER-targeting function of the processed CAAX motif [10] and the localization of the mammalian Ras palmitoyltransferase that palmitoylates newly CAAX-processed H- and N-ras [76–79]. However, characterization of Ras thioesterases and further work on Ras palmitoyltransferases (reviewed in [80]) will be required to elucidate to what extent the concentration of H- and N-ras on the plasma membrane is actually regulated and perhaps used to control the relative Ras signal outputs from the plasma membrane and endomembranes. In this context, it has also recently been shown that a cycle of palmitoylation and de-palmitoylation is critically important for maintaining the fidelity of H- and N-ras targeting to Golgi and plasma membrane [81].

The carboxy group on the Ras C-terminal farnesyl cysteine is methylesterified by pcCMT (prenylcysteine carboxyl methyltransferase) in the final step of CAAX processing [82]. Prolonged inhibition of pcCMT with pharmacological inhibitors prevents Ras trafficking to the plasma membrane by blocking the methylation of newly synthesized Ras [83,84], and consequently inhibits Ras signalling [82,84]. There is no evidence to date, however, that Ras proteins are demethylated once CAAX processing has been completed to remove them from the plasma membrane.

A fascinating recent study shows that K-ras is acutely redistributed from the plasma membrane to internal membranes in hippocampal neurons stimulated with glutamate or NMDA (*N*-methyl-D-aspartate) (M. Fivaz and T. Meyer, personal communication). These agonists generate a high-amplitude calcium signal, a consequence of which is the recruitment of a cytoplasmic Ca^{2+} /CaM (calmodulin) complex to the plasma membrane, where it sequesters the polybasic farnesylated membrane anchor and releases K-ras from the membrane (M. Fivaz and T. Meyer, personal communication). CFP (cyan fluorescent protein) targeted to the plasma membrane by the C-terminal anchor of K-ras shows a similar translocation response, illustrating that this phenomenon involves only the lipid anchor (M. Fivaz and T. Meyer, personal communication). Ca^{2+} /CaM binds selectively to K-ras [86] and solubilizes K-ras from membranes *in vitro* [87]. Furthermore, inhibitors of Ca^{2+} /CaM selectively stimulate K-ras, but not H-ras, signal output [86,88]. Ca^{2+} /CaM also binds to MARCKS and Src [89–92], two proteins that are anchored by a combination of myristoylation and a polybasic domain. As with K-ras, Ca^{2+} /CaM can displace MARCKS from the plasma membrane [91,92]. The crystal structure of Ca^{2+} /CaM has a hydrophobic groove that can accommodate myristoyl groups [93] and, probably, in the light

of this recent study (M. Fivaz and T. Meyer, personal communication), also prenyl groups. One consequence of K-ras translocation will be to reduce K-ras signal output from the plasma membrane. Whether the Golgi-associated K-ras, or the K-ras associated with non-Golgi internal membranes, generates a signal output, and, if so, whether it is different from that generated by plasma-membrane-localized K-ras, akin to the case for H-ras and N-ras [21–23], remains as yet an open question.

CONCLUDING REMARKS

New techniques for imaging Ras proteins on intact plasma membranes have over the past 2 years given remarkable new insights into how these critical signalling GTPases are organized. Interpreting dynamic and spatial data with biophysical data from model membrane systems in a single conceptual framework has yielded new models for how Ras proteins might operate on the plasma membrane. The basic concept that the different lipid anchors on the Ras isoforms directs the GTPases into different signalling micro-environments or platforms remains valid, but, intriguingly, it is possible that Ras proteins could participate directly in the formation of these micro-environments rather than passively encountering them. As such, Ras proteins not only operate as critical signalling proteins, but are also proving to be incredibly useful tools to probe plasma membrane structure and function.

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