Cell. Mol. Life Sci. 64 (2007) 2575 – 2589
1420-682X/07/192575-15
DOI 10.1007/s00018-007-7133-8

Cellular and Molecular Life Sciences

Review

Ras proteins: paradigms for compartmentalised and isoform-specific signalling

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Received 16 March 2007; received after revision 29 May 2007; accepted 15 June 2007
Online First 13 July 2007

Abstract. Ras GTPases mediate a wide variety of cellular processes by converting a multitude of extracellular stimuli into specific biological responses including proliferation, differentiation and survival. In mammalian cells, three *ras* genes encode four Ras isoforms (H-Ras, K-Ras4A, K-Ras4B and N-Ras) that are highly homologous but functionally distinct. Differences between the isoforms, including their post-translational modifications and intracellular sorting, mean that Ras has emerged as an important model system of compartmentalised signalling and membrane biology. Ras isoforms in different subcellular locations are proposed to recruit distinct upstream and downstream accessory proteins and activate multiple signalling pathways. Here, we summarise data relating to isoform-specific signalling, its role in disease and the mechanisms promoting compartmentalised signalling. Further understanding of this field will reveal the role of Ras signalling in development, cellular homeostasis and cancer and may suggest new therapeutic approaches.

Keywords. Ras, GTPase, oncogenesis, isoform, compartmentalisation.

Introduction

In humans three Ras proto-oncogenes encode four isoforms (H-Ras, K-Ras4A, K-Ras4B and N-Ras) that function as molecular switches sitting near the top of a complex web of signalling cascades [1]. Ras proteins predominantly sit on the inner leaflet of the plasma membrane and are switched on when a wide range of cell surface receptors become activated. Active Ras operates as an adaptor protein, recruiting effectors to membranes, where they can interact with proteins and lipids to generate intracellular signals. Ras activation promotes cell proliferation, differentiation and survival amongst many other cell regulatory functions. These proteins have been subjects of intensive scrutiny since their transforming potential was first identified in rats more than 40 years ago [2]; in humans, mutations causing inappropriate Ras activation are present in 30% of cancer cases [3].

Ras proteins are GTPases that are inactive when GDP-bound and become activated when a guanine nucleotide exchange factor (GEF) stimulates GDP dissociation, allowing rapid replacement by the more abundant GTP. The archetypal Ras GEF is Sos; however, nine Ras GEFs have been identified so far, most of which are also able to activate other GTPases. Allosteric changes caused by GTP binding increase the affinity of effector interactions before this is reversed by GTP hydrolysis stimulated by GTPase-activating proteins (GAPs). GAPs stimulate the intrinsic GTPase activity of Ras 10 000-fold. Onco-

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genic Ras mutations interfere with the ability of GAPs to interact with Ras, producing constitutively active Ras proteins. In the same way that multiple GEFs have been identified, eight GAPs have been characterised, providing a range of possibilities for initiating down-regulation of Ras signalling.

Raf and phosphatidylinositol 3-kinase (PI3K) were the first two Ras effectors identified and have been the main focus of research investigating Ras function. Raf promotes cell proliferation and differentiation through the MAP kinase (MAPK) pathway, whilst PI3K generates anti-apoptotic signalling. Since then, at least 20 effectors have been identified; many of these other proteins are GEFs for other GTPases, providing links to pathways regulating cytoskeletal organisation, membrane trafficking, cell migration, cell cycling and transcription.

The majority of Ras activators and effectors consist of groups of closely related protein families facilitating signalling convergence and divergence. Given the potential complexity of these interactions, it is clear that there must be finely tuneable mechanisms to ensure that the correct pathways are engaged depending on the strength and type of initial input. Recent work has focussed on the role that individual Ras isoforms might play in specifying distinct signalling outputs. H-, K- and N-Ras are ubiquitously expressed and possess almost complete sequence homology or identity along most of their length. Nevertheless, several lines of evidence point to isoform-specific signalling; this will be discussed together with the relevant regulatory mechanisms that are now being identified. Important amongst these are the roles of post-translational modifications and compartmentalisation of signalling. Ras has become one of the key model systems for research in this area, and Ras research informs many other areas of cell biology and cell signalling. This review will highlight recent insights into Ras biology and describe how the highly homologous Ras isoforms have the flexibility to tune distinct outputs.

1. Isoform-specific Ras signalling

Genetic studies reveal non-overlapping functions. Ras isoforms were initially assumed to be functionally redundant due to their high degree of sequence homology; however, many studies point to specific roles for each isoform. Early evidence for isoform-specific roles came from analysis of the mutation rates of Ras isoforms associated with different types of cancer. Activating mutations of individual Ras isoforms are strongly linked to certain cancers; for example, almost 90% of pancreatic and 50% of colon cancers have mutated K-Ras, whilst acute leukaemias often possess activated N-Ras [4].

More compelling evidence has come from work with knockout mice, which revealed that H-, N- and double N-, H- homozygous Ras knockouts apparently develop normally with no detrimental impact on long-term survival [5–7]. Similarly, the K(A)-Ras splice variant is dispensable for mouse development [8]; in contrast, K(B)-Ras knockout mice died during embryogenesis between days 12 and 14 [5, 9]. These results indicate that only K(B)-Ras is required during embryogenesis and that there must be redundancy in signalling between the other isoforms during this period. An intriguing recent study has suggested that the issue of Ras isoform redundancy is more complex than first thought: Potenza and colleagues generated HrasK1 mice in which H-Ras was substituted for K-Ras such that they expressed no K(A)- or K(B)-Ras in any tissue; these mice survived to adulthood with no developmental problems during embryogenesis [10]. Whilst K-Ras is not intrinsically essential for development, adult mice did develop cardiomyopathy, highlighting a unique role for K-Ras in cardiovascular homeostasis. This study implies that it is the specific pattern of K-Ras expression rather than unique signalling pathways associated with K-Ras that are critical during development. This might be expected because the first 85 amino acids of all the Ras isoforms, covering the site of interaction with all known Ras effectors, are identical (Fig. 1). This work, however, begs questions such as: can this result be explained in terms of pure isoform abundance at critical stages of development or are there additional factors? Can N-Ras also substitute for K-Ras?

An important feature of Ras isoforms is their differential localisation, which is discussed in depth later; briefly, however, H-Ras and K(B)-Ras represent the two most cell surface-localised isoforms, whereas in many cell types, N-Ras is a prominent endomembranous component (Fig. 1). HrasK1 mice expressed significantly more H-Ras than control mice, whereas our own unpublished data suggest that H-Ras is normally only a minor isoform in cells derived from many tissues. Therefore, in HrasK1 mice, substitution of one strongly plasma membrane-localised isoform for another maintains essential signalling, suggesting a model in which it is not the amount of a Ras isoform that is expressed but the amount of Ras occupying a particular compartment that is important. The fact that H-Ras was not able to completely substitute for K-Ras function in adult mice points to a critical aspect more subtle than gross localisation; this may involve differential microdomain association, which will be discussed in subsequent sections.

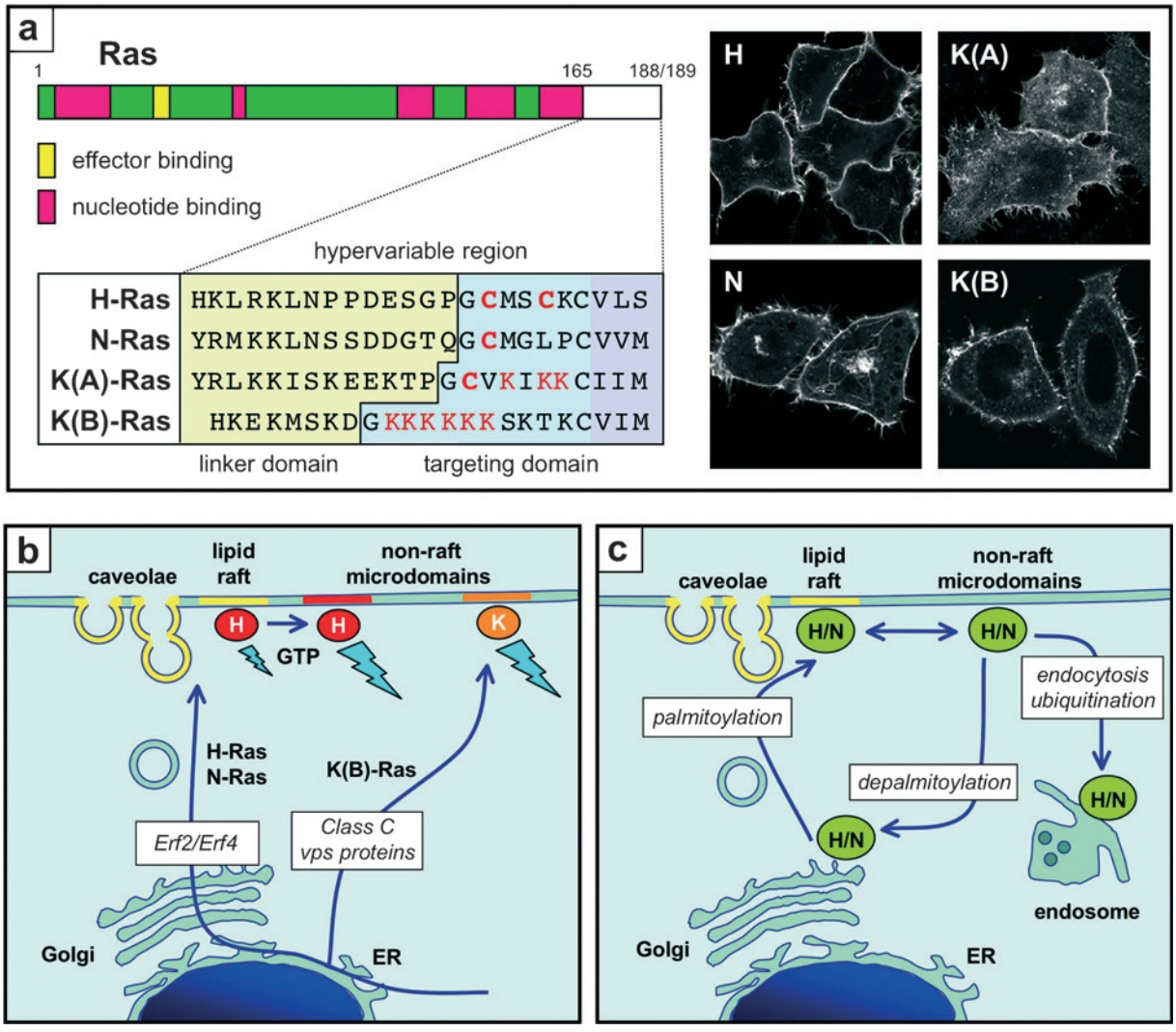

Figure 1. The Ras hypervariable region (HVR) enables membrane targeting and differential localisation. (*a*) Almost complete sequence conservation between Ras molecules exists in the N-terminal region that contains all of the sites for effector interactions and nucleotide binding; the Ras HVR represents the only area of sequence divergence. Processing of the C-terminal CAAX motif results in farnesylation of all Ras isoforms. A second signal consisting of palmitoylated cysteine(s) or polybasic stretches of lysine residues (highlighted in red) stabilise membrane binding and regulate different steady-state cellular localisation. (*b*) Palmitoylated Ras isoforms traffic via the conventional secretory pathway, whereas K(B)-Ras moves *via* a poorly understood pathway to the cell surface. H-Ras exists in dynamic equilibrium between raft and non-raft microdomains; exit from lipid rafts is needed for efficient H-Ras signalling. K(B)-Ras operates from distinct non-raft microdomains. (*c*) A cycle of palmitoylation and depalmitoylation regulates H- and N-Ras localisation; alternative mechanisms promote trafficking to the endocytic system.

Ras isoform germline mutations. Knockout studies have revealed critical roles for Ras isoforms in normal mouse development; recent data has highlighted similarly important isoform-specific functions in human embryonic development. Research into the causes of neurofibromatosis type 1 (NF1) has already established that aberrant Ras signalling induces developmental defects. NF1 is a familial cancer syndrome caused by loss of or inactivating mutations of the tumour suppressor Ras GAP – neurofibromin [11]. The disease is characterised by skin pigmentation problems and a predisposition to developing many benign and malignant peripheral neuronal tumours.

NF1 is a member of a group of neuro-cardio-facial-cutaneous (NCFC) syndromes involving mutations of members of the Ras signalling pathway resulting in increased Ras-Raf signalling [11]. These disorders share similar phenotypic features including short stature, short neck, cardiovascular defects, facial anomalies, learning difficulties and predisposition to developing cancers. Genetic screening of patients with Noonan syndrome has identified gain-of-function mutations in PTPN11 (50%), Sos1 (20%) and

K-Ras (5%) [12–15]. H-Ras is mutated in >85% of Costello syndrome cases, and K-Ras (7%), B-Raf (>35%) and MEK (13%) are mutated in cardio-facial-cutaneous (CFC) syndrome [16–18]. Interestingly, the germline Ras mutations were often distinct from the classical oncogenic point mutations, resulting in less potent activation [11]. This was particularly the case for K-Ras, supporting observations with mouse models conditionally expressing oncogenic K-Ras, which was lethal when induced during embryogenesis [19]. In contrast, H-Ras mutations associated with Costello syndrome are the same as those that promote oncogenesis, indicating far more tolerance of oncogenic H-Ras during embryogenesis and development. The spectrum of Ras mutations, isoform-specific associations with these syndromes and incomplete overlap of phenotypic outputs point to a complex relationship between the pattern of Ras isoform signalling and maintenance of the normal developmental program.

Oncogenic isoform signalling. Low-level ectopic expression of constitutively active Ras isoforms and analysis of downstream signalling revealed that H-Ras is a more potent activator of PI3K, whereas K(B)-Ras is a more potent Raf and Rac activator [20, 21]. More recent *in vitro* studies employing isogenic colorectal cancer cells lines engineered to overexpress oncogenic versions of N-, H- or K-Ras revealed that K-Ras is by far the most potent inducer of a transformed phenotype [22]. In normal human fibroblasts, the consequence of hyperactive Ras signalling is activation of the ARF/p53 tumour suppressor pathway and induction of replicative senescence [23, 24]. Deletion of components of the tumour suppressor pathway such as ARF is needed to generate the hyperproliferative state and induce carcinogenesis [25]. However, in contrast to these *in vitro* studies in which supra-physiological levels of Ras were expressed, mouse embryonic fibroblasts (MEFs) derived from mice engineered to express endogenous levels of oncogenic K-Ras are immortalised and do not become senescent [19, 26].

Clearly, cellular context and expression levels are important factors in determining experimental outcomes. For this reason, the mouse models that have been developed in which oncogenic Ras isoform mutants are conditionally expressed at endogenous levels will be invaluable tools for dissecting the role of Ras in oncogenesis [19, 27–30]. One model using conditional H-Ras^{G12V} expression revealed that H-Ras signalling is necessary for induction and maintenance of melanomas in a mouse model also deficient for the tumour suppressor INK4a [31]. However, most studies have focussed on the role of K-Ras in tumourigenesis, because analysis of Ras mutations has indicated that this isoform is frequently mutated across a broad spectrum of cancers. All studies agreed that induction of oncogenic K(B)-Ras (G12V or G12D mutations) promotes widespread development of lung adenomas or adenocarcinomas [27–30]. Additionally, withdrawal of oncogenic K-Ras expression resulted in loss of tumours, indicating a reliance on K-Ras signalling for maintenance of the tumours, as seen earlier with H-Ras-induced melanomas [27]. Finally, expression of oncogenic Ras during embryogenesis was embryonic lethal [19, 26]; when combined with the knockout mouse data, this indicates that a precise pattern of regulated Ras isoform signalling is required for normal development.

Whilst there is some consensus on the consequences of oncogenic K-Ras signalling, recent studies from two groups highlight how subtly different approaches can generate significantly different outcomes. Specifically, depending on the type of expression system employed, oncogenic K-Ras induces only lung cancer [19] or additionally stimulates a wide variety of epithelial hyperplasias, pancreatic neoplasia and defective erythrocytic differentiation [26, 32, 33]. Interestingly, MEFs derived from these mice showed attenuated PI3K and MAPK activation in response to serum, although pharmacological inhibition of these pathways revealed that they are still required for the observed phenotypic changes [26]. This unexpected lack of effector activation was also observed in other studies of pancreatic and leukaemia cells harbouring oncogenic K-Ras [34–37]. Therefore, whilst most studies examine Ras signalling following acute growth factor stimulation or ectopic overexpression of Ras isoforms, endogenous oncogenic Ras appears to achieve its effects through an essentially undetectable trickle of effector activation.

Microarray profiling Ras isoform-specific regulation of gene expression represents a natural extension of this work. Recent studies have examined genes regulated by Ras activation following overexpression of oncogenically mutated K(B)-Ras in rat ovarian epithelial cells [38] or activated versions of each isoform overexpressed in rat embryonic fibroblasts [39]. Another recent study compared gene expression profiles of mouse and human models of lung cancer bearing endogenous levels of mutated K(B)-Ras to identify a K-Ras gene expression signature in transformed cells [40]. Whilst overexpression studies typically identified approximately 200 genes differentially up- or down-regulated by oncogenic Ras expression, comparative analysis of large human and mouse cancer data sets indicated that fewer than 100 gene targets may represent the endogenous oncogenic K(B)-Ras gene expression signature [38–40].

Although most work has focussed on K-Ras, a comparison of isoform-specific regulation of gene expression revealed only 26 genes whose expression is differentially regulated by Ras isoforms. This finding implies that there is an almost complete functional overlap and is at odds with the phenotypic data described earlier [39]. Given the lessons that have been learned from analysis of oncogenic Ras in promoting tumourigenesis, it would seem to be vital that any comparison of differential Ras isoform signalling is conducted in cells expressing endogenous amounts of each isoform. To date, no genome-wide direct comparison of endogenous H-, K- and N-Ras regulation of gene expression has been conducted, although a partial screen using MEFs from homozygous H- and N-Ras knockout mice has provided a promising starting point [41]. By comparing transcriptional profiles of the knockout cell lines with a wild-type control, Castellano and colleagues identified only 12 genes differentially regulated in H-Ras knockouts. In contrast, 96 genes with altered expression were observed in N-Ras knockouts. Whilst these genes are involved in a broad spectrum of cellular functions, an overriding theme appeared to be that many N-Ras targets are involved in cellular immunity and apoptosis.

In summary, data from mouse knockouts and Ras-related genetic disorders as well as analysis of oncogenic signalling have revealed specific roles for each isoform in development, cellular homeostasis and promotion of disease. A significant and parallel area of Ras research has been engaged in trying to understand the mechanistic basis for these differences in Ras isoform signalling.

2. Compartmentalisation of Ras

The Ras HVR promotes membrane binding. H-, K-, and N-Ras are almost identical and ubiquitously expressed yet they are capable of preferentially engaging distinct signalling pathways. The only area of significant sequence divergence between isoforms lies in the C-terminal hypervariable region (HVR) [42]. This short (23–24 aa) stretch terminates with a CAAX motif that undergoes a series of post-translational modifications that promote membrane binding (Fig. 1). Firstly, the cysteine is prenylated by farnesyl protein transferase, facilitating binding to the endoplasmic reticulum (ER). Next the AAX motif is cleaved off by Ras-converting enzyme (Rce1), and finally the farnesylated cysteine is methylated by isoprenyl cysteine carboxymethyl transferase (Icmt) [43].

The farnesyl group provides weak membrane binding affinity that is stabilised by a second signal motif essential for correct trafficking and microlocalisation of each isoform [44]. For K(B)-Ras the second signal is a polybasic domain, formally a hexa-lysine sequence between residues 175–180, although the processed 20-residue K(B)-Ras HVR possesses 11 lysines in total for an HVR net positive charge of +9. For the other three isoforms, this consists of palmitoylation of adjacent cysteine residues – Cys180 [K(A)-Ras], Cys181 (N-Ras), Cys181 and Cys184 (H-Ras). K(A)-Ras also possesses a basic patch adjacent to the palmitoylated cysteine that is also likely to promote membrane binding. Mutagenesis of 4 or more lysines in the K(B)-Ras hexalysine stretch or the palmitoylated cysteines in H-Ras inhibits the ability of Ras to stably interact with membranes [44]. Similarly, mutation of the farnesylated cysteine prevents subsequent post-translational modifications and renders Ras cytosolic, inhibiting the potential for Ras activation [45]. For this reason, farnesyl transferase inhibitors were promoted as potential anti-Ras chemotherapeutic drugs; however, their effectiveness was undermined by the ability of both K(B)-Ras and N-Ras to be alternatively prenylated by geranylgeranyl transferase [46]. The utility of these drugs as anti-Ras therapies was further diminished by the fact that the only isoform that they are particularly effective against, H-Ras, is rarely mutated in human cancers [4].

Ras isoforms display differential trafficking and localisation. Classically, Ras function is associated with the cell surface; however, a major new area of Ras research involves understanding why intracellular pools of Ras exist and the mechanisms modulating Ras isoform localisation. The minimal sequence containing the farnesylated cysteine and the second signal is called the targeting domain, and these have been extensively used to analyse Ras membrane trafficking. GFP-tagged H-Ras and K(B)-Ras targeting domains display overlapping distributions with their respective full-length proteins, indicating that the second signals are both necessary and sufficient for correct localisation. These motifs are required for plasma membrane localisation of Ras and specify trafficking of isoforms through distinct pathways to the plasma membrane. Both H-Ras and N-Ras predominantly traffic through the conventional secretory pathway, whereas K(B)-Ras traffics via an uncharacterised Golgi-independent pathway to the cell surface (Fig. 1b) [47, 48]. In yeast and adipocytes, palmitoylated Ras isoforms can also utilise a Golgi-independent pathway to reach the plasma membrane [49, 50]. Intriguingly, this pathway in yeast requires class C vps proteins that are classically associated with regulating endosome fusion [51]. The steady-state distribution of the iso-

forms varies; whilst all Ras isoforms exhibit plasma membrane localisation, the extent of endomembranous labelling varies amongst isoforms and between cell types. N-Ras often exhibits a prominent Golgi pool, which is seen for the other isoforms to varying degrees, with N≥H, K(A)>>K(B) (Fig. 1).

Several mechanisms that regulate precise Ras compartmentalisation have been identified. *In vitro* experiments using the processed K(B)-Ras HVR and liposomes demonstrated that incorporation of negatively charged lipids (phosphatidylserine or phosphatidylinositols) significantly increases the avidity of K(B)-Ras binding [52, 53]. In addition, cellular microinjection with an anionic lipid probe consisting of a D-amino acid version of the K(B)-Ras HVR revealed that the cell surface has the highest concentrations of negatively charged lipids [53]. The polybasic domain of K(B)-Ras therefore ensures almost complete plasma membrane localisation of K(B)-Ras via electrostatic interactions that have an exquisite surface potential-sensing capability. Indeed, K(B)-Ras HVR constructs have been used to map localised changes in surface potential caused by hydrolysis or displacement of anionic lipids during phagocytosis [54]. In these studies, phagocytosis induced a decrease in surface potential, resulting in a localised loss of signalling molecules such as K(B)-Ras, Rac1 and c-Src, whose anchoring is stabilised via electrostatic interactions.

A beneficial consequence of these interactions is that K(B)-Ras may be able to concentrate lipids useful for signal transduction such as phosphatidylinositol-4,5-bisphosphate (PIP_2), a substrate required for phosphatidylinositol-3 kinase (PI3K) – Akt signalling. Studies of MARCKS protein, which also interacts with anionic lipids via a basic/hydrophobic cluster, revealed the potential microdomain-forming capacity of this motif. Theoretical calculations estimate that the MARCKS cluster will sequester bivalent PIP_2 even when univalent anionic lipids such as phosphatidylserine are present at a 100-fold excess [55].

Importantly, K(B)-Ras interactions with the cell surface are highly dynamic; elegant *in vivo* experiments by Silvius and colleagues revealed that K(B)-Ras can accumulate on an intracellular platform for which it is engineered to have an inducible high affinity within minutes [56]. Whilst the cell surface is the default location for K(B)-Ras, other proteins were recently shown to stimulate translocation to intracellular membranes by modulating HVR interactions with the membrane. Calmodulin has an established role in destabilising protein electrostatic interactions with membranes; this is likely due to interactions with the basic cluster of amino acids inhibiting interactions with anionic lipids [55]. Calmodulin binds to the K(B)-Ras HVR and, in rat hippocampal neurons, promotes redistribution to the Golgi apparatus [57–59]. An alternative mechanism is provided by protein kinase C, which phosphorylates K(B)-Ras on serine 181. The introduction of the strong negative charge partially neutralises the charge of the adjacent polybasic residues, causing dissociation from the cell surface and accumulation on the ER, Golgi and outer mitochondrial membrane (Fig. 2b) [60]. The benefits of dynamic localisation will be discussed later; first we will see that H-Ras and N-Ras are similarly capable of being relocated in response to specific signals.

The localisation of H-Ras and N-Ras is primarily determined by the presence and position of the palmitoyl groups. Mutagenesis of the H-Ras palmitoylated cysteines 181 and 184 revealed that H-Ras monopalmitoylated on cysteine 184 is trapped within the Golgi complex; in contrast, mono-palmitoylation of cysteine 181 enables cell surface localisation [61]. Importantly, palmitoylation, unlike farnesylation, is labile, with half-lifes estimated to be a few minutes to a few hours depending on the methods used for analysis [62–64]; these are much shorter than the 21-h half-life of H-Ras, meaning that palmitoylated Ras isoforms must go through many cycles of acylation and deacylation. This cycle seems to be important for trafficking to and from the Golgi complex (Fig. 1c). Recent elegant fluorescence experiments revealed that depalmitoylated H-Ras and N-Ras rapidly traffic from the cell surface to the Golgi via a non-vesicular pathway [64, 65]. The palmitoyltransferase complex responsible for palmitoylating Ras was recently identified as DHHC9/GCP16 (Erf2/Erf4 in yeast), and these have also been localised to the Golgi [66–68]. Taken together, these data suggest a model where palmitoylation in the Golgi stabilises Ras-membrane interactions, facilitating trafficking to the plasma membrane before depalmitoylation causes Ras to become cytosolic and traffic back to the ER/Golgi. ER/Golgi localisation is independent of protein-protein interactions and appears to rely solely on farnesylation, possibly through lipid-protein interactions.

An alternative intracellular destination for H- and N-Ras is the endosome. Following growth factor stimulation, H-Ras but not K(B)-Ras can be seen to accumulate on early/recycling endosomes [69]. Bar-Sagi and colleagues recently demonstrated that H-Ras di-ubiquitination also promotes redistribution to endosomes (Fig. 1c) [70]. In these studies K(B)-Ras was not ubiquitinated; more intriguingly, H-Ras ubiquitination was independent of activation state and represented only 1–2% of total H-Ras. It will be interesting to determine the lability of this modifica-

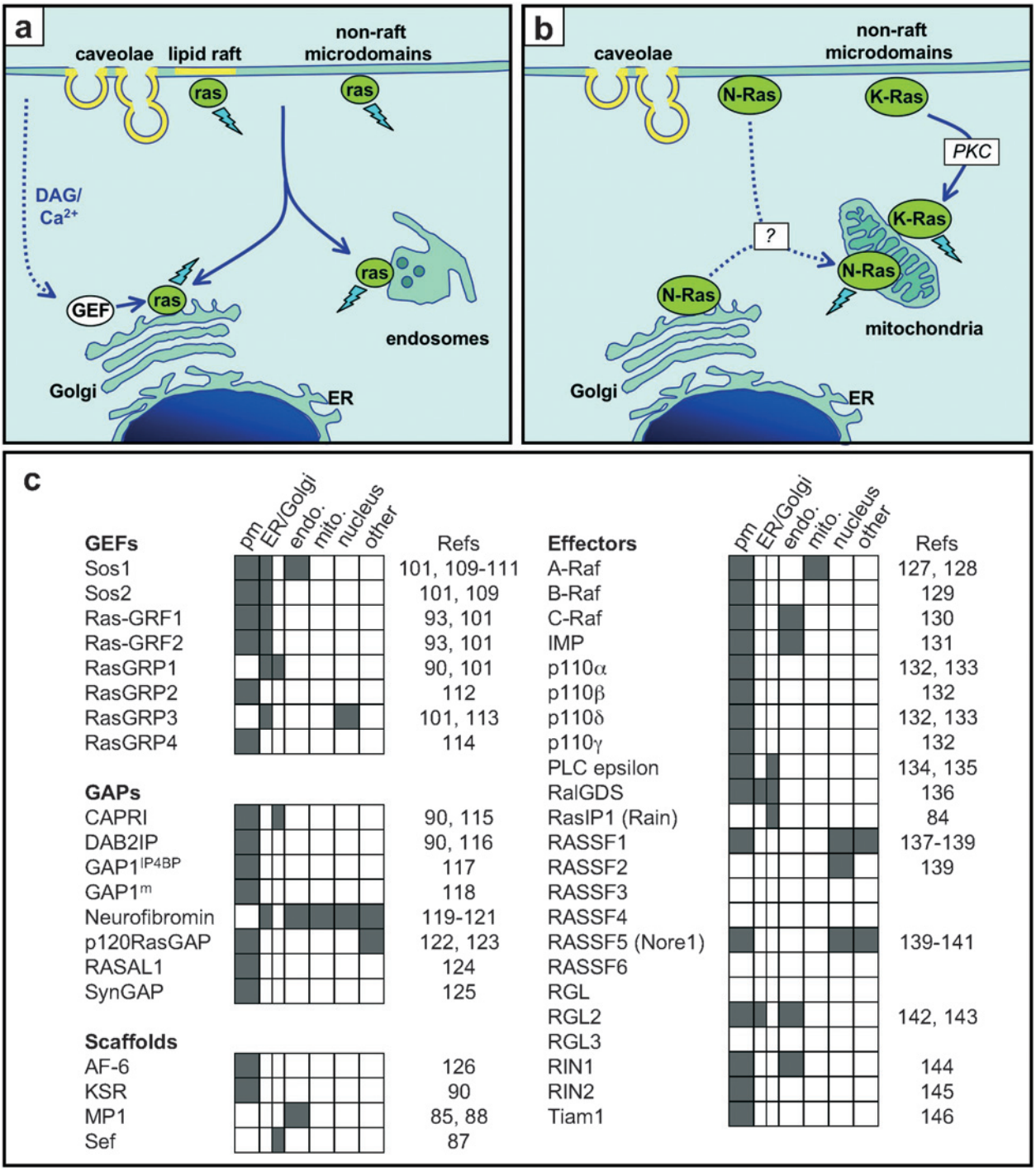

Figure 2. Ras operates from multiple platforms within the cell. (*a*) Ras has been localised to and is proposed to signal from surface and endomembrane compartments. (*b*) Mitochondria have recently been identified as sites for N- and K(B)-Ras signalling, engaging pathways involved in retrograde signalling to the nucleus and apoptosis, respectively. (*c*) Ras regulators and effectors are also differentially localised. Filled squares indicate positive identification of localisation to a membranous compartment based on immunofluorescence, subcellular fractionation or indirect biochemical analysis. Many of these proteins exhibit dynamic exchange with the cytosol. Unfilled squares indicate a lack of evidence for occupancy of this organelle.

tion on H-Ras, how this is regulated and to what extent this is seen in other cell types.

Finally, differential Ras isoform lateral microlocalisation within the cell surface has been characterised using a combination of subcellular fractionation and microscopy techniques. The cell surface is proposed to consist of a mosaic of functional microdomains that concentrate proteins and lipids, facilitating processes

such as cell signalling [71]. A well-known, although controversial, type of microdomain is the lipid raft; these are believed to concentrate proteins, including many signalling proteins that are able to intercalate into the highly ordered lipid structure of the microdomain [72, 73]. Ras isoforms with their mixture of membrane-anchoring motifs are predicted to have differing affinities for rafts; farnesyl groups are unsaturated and therefore not suited to packing in with the saturated lipids present in a raft. This means that K(B)-Ras should have no particular affinity for rafts, whereas the saturated palmitoyl groups of the other isoforms are predicted to drive strong raft association. Experiments have revealed that whilst H-Ras is initially targeted to lipid rafts, GTP binding promotes translocation into non-raft domains [74, 75]; this movement requires the presence of the first seven amino acids of the linker domain, which somehow transmit the N-terminal conformational changes associated with GTP binding into alterations in HVR interactions with lipid rafts [75, 76]. This is likely to involve modulation of palmitoyl interactions with the membrane, since H-Ras mutagenesis experiments revealed that mono-palmitoylation on cysteines 181 or 184 has opposing effects on raft affinity [61].

Electron microscopy revealed that once activated, H-Ras moves out of lipid rafts and clusters into other cell surface signalling domains. These microdomains do not rely on cholesterol for their integrity (unlike lipid rafts) but instead require galectin-1 to stabilise them [75]. Galectin-1 is recruited from the cytosol by activated H-Ras through a putative interaction between the Ras farnesyl group and a hydrophobic pocket [77]. Importantly, whilst H-Ras is in a dynamic equilibrium between lipid raft and non-raft domains, K(B)-Ras occupies another type of non-raft microdomain (Fig. 1b) [75, 78]: K(B)-Ras signalling microdomains are not cholesterol-dependent but, like lipid rafts, require actin for their structural integrity [79]. Further evidence for Ras microdomain occupancy came from single-particle tracking of Ras, which revealed that H-Ras and K(B)-Ras become transiently immobile upon activation, thus supporting the idea that Ras associates with signalling platforms [80]. In summary, H- and K(B)-Ras appear to operate from distinct microdomains within the cell surface, and Ras activation state may be critical for directing association with particular microdomains.

These observations have been important not only for understanding Ras biology, but also for studies of cell surface organisation. Ras proteins represent an important model system, because localisation has been characterised at such a high resolution and using a variety of complementary techniques. Modelling indicates that whilst Ras signalling domains are tiny ($<$15 nm) and occupancy in these domains is short-lived ($<$75 μs), they can still act as protein concentrators facilitating protein-protein interactions [81]. New techniques enabling direct observation of protein dynamics within these domains are needed to validate these models; however, they do provide a framework for designing new experimental protocols and equipment with sufficient resolution.

3. Compartmentalised Ras signalling

Plasma membrane. We have seen that individual Ras isoforms preferentially associate with different organelles and microdomains depending upon the type of membrane anchor, post-translational modification and activation state. A prevailing model within the Ras field is that differential localisation enables Ras isoform-specific signalling by bringing isoforms into contact with distinct pools of effectors and activators. Early evidence for compartmentalised Ras isoform signalling came from simple membrane perturbation studies using drugs or dominant-negative proteins to reduce cell surface cholesterol content. These treatments disrupt the integrity of lipid raft microdomains and resulted in inhibition of H-Ras but not K(B)-Ras activation of the Raf-MAP kinase signalling cascade [82]. Electron microscopy subsequently confirmed the differential Ras isoform distribution; however, to date, effectors or facilitators of Ras signalling have not been similarly mapped. For example, a prediction based on observations that H-Ras is a more potent activator of PI3K than K(B)-Ras, while K(B)-Ras is a more potent Raf activator, is that these proteins or their lipid substrates will preferentially co-localise with either activated H- or K(B)-Ras. Other studies did, however, reveal how microdomain occupancy has a profound effect on the efficiency of signalling; for example, H-Ras mutants that were unable to escape from lipid rafts showed impaired Raf and PI3K activation [74]. This implies that not all microdomains have the same capacity to sustain signalling and that correct and dynamic microlocalisation significantly enhances signal propagation.

Ras signalling: ER/Golgi, endomembrane. Whilst there is no dispute that Ras operates from the cell surface, the extent of intracellular Ras activation and how it might be regulated is still under debate. Effectors and facilitators of Ras signalling have been localised to the Golgi and endosomes, indicating that these must be active sites for Ras signalling. A summary of known and often dynamic interactions of Ras effectors with cellular membrane compartments is included in Fig. 2: these include the effectors

Rain1 on the Golgi and the activated Raf-MEK-ERK cascade on endosomes [83, 84]. Further examples include scaffolds for the Raf-MAPK cascade; whilst KSR is recruited to the cell surface, Sef is localised on the Golgi and p14-MP1 on endosomes [85–88].

Perhaps the best method to analyse endomembranous Ras signalling is to directly observe Ras activation as it happens within the cell. Several advanced fluorescence-based approaches have been developed to detect Ras activation *in vivo*. These fluorescence resonance energy transfer (FRET)-based probes use the GTP-dependent interaction of the Raf-RBD (Ras-binding domain) with Ras [89]. Some studies have revealed endomembrane Ras activation in addition to the cell surface pool [64, 90–93], whilst others only detect plasma membrane-localised Ras activation [94–96]. One criticism of the evidence for endomembranous Ras activation is that it is normally seen only with overexpression of Ras. However, new studies have characterised the ability of T cell ligands to selectively engage compartment-specific Ras-MAPK signalling and influence different phenotypic outputs. Mark Philips and colleagues examined T cell receptor (TCR) signalling through the Ras pathway and found that the locations of Ras activation could be shifted depending on the type of ligands used to activate cell surface receptors [97]: whilst the TCR normally activated Golgi-localised Ras, co-stimulation *via* the integrin LFA-1 also activated Ras on the plasma membrane, revealing that despite the presence of overexpressed Ras, T cells have distinct mechanisms for activating Ras in different compartments. Furthermore, studies of thymocyte selection in which Ras was not overexpressed revealed that endomembrane Ras signalling is necessary for positive selection, whereas negative selection results in cell surface recruitment of the Ras-MAPK signalling cascade [98].

A complementary strategy being used to probe the function of intracellular pools of Ras is the targeting of a constitutively active mutant of Ras to specific organelles and cell surface microdomains [91, 99]. The results have been mixed: for example, Matallanas and colleagues showed that Golgi-restricted Ras poorly activates MAPK and Akt in comparison with cell surface- and ER-localised Ras and is dispensable for promoting cell proliferation [99]; an earlier study using Golgi-targeted Ras showed clear induction of proliferation and cellular transformation [90]. Despite this dispute over the precise role of intracellular pools of Ras, a recent study using targeted Ras constructs in fission yeast revealed that compartmentalised Ras signalling is evolutionarily conserved. In this case, surface-localised Ras supported pathways regulating mating, whereas endomembrane Ras supported pathways controlling morphology [100].

Importantly, endogenous Ras activation has been visualised, albeit with contradictory results [91, 94]. Whilst further work is clearly required to resolve these differences, Philips and colleagues used their ability to detect endogenous ER/Golgi Ras activation to investigate how this might be regulated [91]. They found that EGF produces rapid (onset within 1 min, 10 min duration) cell surface Ras activation in COS-1 cells, followed by a prolonged (onset within 10 min, 60 min duration) activation of Golgi Ras. These are believed to be the palmitoylated Ras isoforms, since K(B)-Ras is not observed on the Golgi; in Jurkat T cells, low-grade TCR activation specifically activated N-Ras [92]. Subsequent studies revealed GEFs and GAPs to be regulated by calcium, thereby modulating Golgi Ras function. Growth factor receptor-mediated activation of Src and subsequently phospholipase Cγ generates Ca^{2+} and diacylglycerol (DAG), which in turn activate the RasGRP and RasGRF families of GEFs [90, 93, 97, 101]. Ras GRP1 and 3 promote Golgi Ras activation, whilst Ras GRF1 and 2 activate Ras on the ER (Fig. 2a; [93, 101]).

Further insight into the potential mechanisms of Golgi Ras activation came in recent overexpression studies in which H-Ras palmitoylation was experimentally controlled. Inhibition of the palmitoylation/depalmitoylation cycle prevented accumulation and activation of H-Ras on the Golgi [64]. In this model, Ras activation stimulates depalmitoylation and trafficking to the Golgi, where a new and distinct pool of effectors may be subsequently activated. However, to date GTP-bound cytosolic Ras has not been experimentally detected. Additionally, single-particle analysis studies have revealed that the time course of activation of an individual Ras molecule is typically less than a second [80]. This means that guanine nucleotide turnover and effector interactions must occur within a compartment and are not sustained whilst Ras is trafficked to other regions of the cell. Finally, the localisation of RasGRP1 to the Golgi and the absence of Golgi Ras activation when this GEF is depleted argue for localised Ras activation rather than translocation of an active pool from another membrane compartment [90, 101].

In summary, whilst there is still some debate about the extent and targets of ER/Golgi Ras signalling, the evidence from several groups that this pool of Ras is biologically relevant is compelling. Importantly, these studies have provided clear evidence for selective activation of cell surface versus endomembrane Ras generating distinct phenotypic outputs.

Ras signalling: endosomes, mitochondria and nucleus. Other locations sustain Ras activity (Fig. 2b). Ras is observed on endosomes in response to ubiquitination

or cell stimulation [70, 83]; this might be expected to help down-regulate Ras signalling, but endosomal Ras also appears to be active [96, 102, 103]. Growth factor receptor endocytosis is necessary for efficient activation of the Raf-MAPK cascade and promotion of differentiation versus proliferation [104]. Isoform-specific signalling was seen when inhibition of dynamin-dependent endocytosis selectively inhibited oncogenic H-Ras but not K-Ras activation of Raf [69]. Mitochondria have also been identified as sites of Ras activation. The electrostatic switch promoting K(B)-Ras translocation to mitochondria was required for induction of Bcl-X_L-dependent apoptosis [60]. Importantly, N-Ras also localises to mitochondria, and cells lacking N-Ras or K(B)-Ras display abnormal mitochondrial morphology [105]. Analogous to this role in modifying organelle structure, overexpression of activated H-Ras but not other Ras isoforms causes gross vacuolarisation and expansion of the ER, resulting in cell cycle arrest [106]. Finally, an H-Ras splice variant, $p19^{ras}$, is localised to the cytosol and nucleus because it lacks the C-terminal HVR [107]. Nuclear $p19^{ras}$ inhibits the transcriptional activity of the p53 homologue p73 through modulation of MDM2-p73 interactions [108].

These data reveal that there are few locations from which combinations of Ras isoforms do not signal; however, there is still only limited evidence for isoform-specific functions linked to particular organelles. The majority of endomembrane signalling appears to impinge upon tumour suppressor or cell death pathways, whilst classical cell surface signalling promotes proliferation and differentiation. The challenge remains to understand when and how these Ras pools are engaged and to identify their full suite of targets.

Summary and future perspectives

Ras has a well-characterised central role in oncogenesis; however, it has also recently emerged as an important general model system for compartmentalised signalling and reversible post-translational regulation of signalling. These modifications variously regulate GDP/GTP loading, Ras isoform membrane trafficking and effector interactions, providing key avenues for the design of new therapeutic interventions. Understanding how different inputs to common signalling pathways result in divergent outputs represents one of the great challenges in cell biology. Research in the Ras field, specifically compartmentalisation of signalling and post-translational Ras regulation, is beginning to resolve some of the mystery.

Important model systems allowing analysis of endogenous Ras isoform signalling are shedding new light on the *in vivo* role of Ras in cellular homeostasis and cancer initiation, progression and maintenance. MEFs from knockout mice and new techniques such as acute knockdown using RNA interference (RNAi) promise a new phase in the analysis of effector interactions. Whilst most studies have focussed on an individual isoform, often K(B)-Ras, it is likely that attention will turn towards a more comparative analysis of Ras isoform engagement with downstream pathways or regulation of phenotypic outputs. To this end, it will also be useful to determine the abundance of individual isoforms across tissues and cell lines to allow proper interpretation of their relative contributions to these outputs.

Finally, there are varied and compelling data pointing to isoform-specific Ras signalling; the mechanism allowing this is largely proposed to be due to differential localisation allowing engagement with different pools of activators and effectors. At present, whilst differential and dynamic Ras localisation has been well characterised, there are relatively few examples of Ras isoforms being able to signal differently in one compartment versus another. Similarly, there is still plenty of work to do to map the relative abundance of effectors in specific microdomains and endomembrane compartments. Whilst there are still many avenues to pursue and unanswered questions, it is clear that Ras signalling has to be considered in terms of the contributions of individual isoforms rather than as a collective whole and that mechanistic insights are likely to be relevant to many other signalling pathways.

Acknowledgements. We gratefully acknowledge Cancer Research UK, the North West Cancer Research Fund and the Royal Society for funding work in our laboratory and thank members of the laboratory for helpful discussion.

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