

RAS PROTEINS: DIFFERENT SIGNALS FROM DIFFERENT LOCATIONS

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Ras signalling has classically been thought to occur exclusively at the inner surface of a relatively uniform plasma membrane. Recent studies have shown that Ras proteins interact dynamically with specific microdomains of the plasma membrane as well as with other internal cell membranes. These different membrane microenvironments modulate Ras signal output and highlight the complex interplay between Ras location and function.

HYPERVARIABLE DOMAIN

The carboxy-terminal 25 amino acids of H-ras, N-ras and K-Ras proteins, in which sequence homology is less than 15% between any two isoforms, compared with 90–100% over the amino-terminal sequences.

CAAX MOTIF

(where C is cysteine, A is aliphatic and X is any amino acid). A carboxy-terminal tetrapeptide that is common to all Ras proteins. It directs a triplet of post-translational modifications.

FRET

(fluorescence resonance energy transfer). The fluorescence energy that is transferred from one fluor excites a neighbouring fluor that then re-emits the energy at a third wavelength. Transfer occurs only if the two fluors are close, so FRET can be used to monitor real-time protein–protein interactions in living cells.

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Ras proteins are small GTPases that regulate cell growth, proliferation and differentiation. The basic mechanics of Ras signalling have been researched extensively over the past two decades. As a result, we now have a detailed biochemical understanding of how the Ras GTPase is loaded with GTP and how, in this activated state, Ras binds and activates an increasing catalogue of effector proteins¹. Many of these mechanisms are understood at the molecular level. Transgenic^{2–5} and cell-biological studies^{6–10}, complemented by clinical observations¹¹, indicate strongly that the different Ras isoforms — H-ras, N-ras and K-ras (in this review, K-ras is used to refer to the ubiquitously expressed K-ras4B, and not to the alternatively spliced isoform K-ras4A) — generate distinct signal outputs, despite interacting with a common set of activators and effectors (BOX 1). These biological differences are probably accounted for by the carboxy-terminal 25 amino acids of the HYPERVARIABLE DOMAIN (HVR), which is the only region that differs significantly in sequence between the otherwise highly homologous Ras isoforms (FIG. 1). The HVR contains the protein sequences that are necessary for Ras to associate with the inner plasma membrane. Almost 20 years ago, mutation of the CAAX MOTIF in the HVR was shown to abolish plasma-membrane localization and signalling of Ras¹². The model that localization of Ras to the plasma membrane was stable and essential for normal biological function became firmly established. Recent studies using live-cell imaging, fluorescence resonance energy transfer (FRET), fluorescence recovery after PHOTOBLEACHING (FRAP) and electron microscopy, however, have shown

that the interaction of Ras with the plasma membrane is highly dynamic and that Ras is present on endosomes and other intracellular membranes, such as the endoplasmic reticulum (ER) and Golgi. Furthermore, Ras can generate a signal output when it is attached to non-plasma membrane.

Models of plasma-membrane microstructure have also been evolving rapidly over the past 5–6 years. The plasma membrane is no longer considered to be a uniform lipid bilayer, but rather a complex mosaic of distinct MICRODOMAINS. These developments have raised intriguing questions about how Ras and other signalling proteins interact with this non-homogeneous membrane surface. Recent advances in visualizing and quantifying the interactions of Ras with distinct plasma-membrane microdomains and other subcellular compartments have yielded new insights into how membrane microenvironments further regulate signalling mechanisms that have previously been viewed simply in terms of protein–protein or protein–lipid interactions. It is therefore timely to review these exciting recent studies in the context of earlier work and to assess to what extent the old model of Ras signalling only at the plasma membrane might need to be updated.

Ras trafficking to the plasma membrane

Many steps to membrane localization. Ras proteins are synthesized as cytosolic precursors that undergo post-translational processing to be able to associate with cell membranes. The initial triplet of modifications is directed by the carboxy-terminal CAAX motif and is

Box 1 | Ras signalling pathways

Ras is activated by guanine nucleotide exchange factors (GEFs) that release GDP and allow GTP binding. Many RasGEFs have been identified¹. These are sequestered in the cytosol until activation by growth factors triggers recruitment to the plasma membrane or Golgi, where the GEF colocalizes with Ras. Some RasGEFs activate different Ras isoforms with varying efficiency^{1,107,108}. Active GTP-bound Ras interacts with several effector proteins: among the best characterized are the Raf kinases, phosphatidylinositol 3-kinase (PI3K), RafGEFs and NORE/MST1 (for a detailed review see REF. 1). Ras activates Raf-1 by recruiting it in a complex with 14-3-3 to the plasma membrane. A multistep activation process is initiated¹⁰⁹ that includes dephosphorylation of serine 259 (REFS 110,111) and phosphorylation of numerous residues including serine 338 and tyrosine 341 (REF. 112). Lipid modulators including phosphatidyl serine and phosphatidic acid are also involved, possibly in displacing 14-3-3 and providing a Ras-independent anchor for Raf-1^{94,97,113,114}. B-Raf has a higher intrinsic kinase activity than Raf-1 but is still activated when recruited to the plasma membrane by Ras^{112,115}. Activated Raf activates mitogen-activated protein kinase (MAPK) and extracellular-signal regulated kinase (ERK) kinase (MEK)1 and MEK2 by serine phosphorylation. Activated MEK, in turn, phosphorylates and activates ERK/MAPK, which has several substrates. Ras also binds directly to the p110 subunit of PI3K and upregulates lipid kinase activity¹¹⁶. As with Raf, several plasma-membrane-localized lipids and kinases can contribute to PI3K activation. These basic mechanisms apply to all three Ras proteins, but in intact cells, H-ras and K-ras activate Raf-1 and PI3K with varying efficiencies: K-ras is the more potent activator of Raf-1, and H-ras the more potent activator of PI3K^{6,7}. These signalling differences are not reproduced *in vitro*¹¹⁷, which indicates that the complexities of plasma-membrane microstructure, and exactly where on the membrane the signalling complex is assembled, are important factors in regulating signal output. Biochemical differences between Ras isoforms translate into biological differences; this is best shown by the different phenotypes of Ras transgenic mice. A *K-ras*-knockout is embryonic lethal, whereas *H-ras*, *N-ras* and *H-ras-N-ras* double knockouts survive normally^{2,4,5}. Biological assays of Ras function, including cell growth, transformation and survival, also show differences between isoforms^{7,9}. GTPase activating proteins (GAPs) terminate Ras signalling by stimulating intrinsic GTPase activity: oncogenic Ras point mutants (such as G12V and Q61L, where G is glycine, V is valine, Q is glutamine and L is leucine) are resistant to GAPs and are constitutively activated¹.

common to all Ras proteins^{12–15} (FIG. 2). First, protein farnesyl transferase, a cytosolic enzyme, attaches a farnesyl group to the cysteine residue of the CAAX motif¹⁶. Second, the farnesylated CAAX sequence targets Ras to the cytosolic surface of the ER where an endopeptidase, Rce1 (Ras and a-factor converting enzyme), removes the AAX tripeptide^{17–19}. Third, the α -carboxyl group on the now carboxy-terminal farnesyl-cysteine is methylated by isoprenylcysteine carboxyl methyltransferase (Icmt)^{20,21}. K-ras is more efficiently methylated than H-ras or N-ras, but it is unclear why this is the case²². Finally, after methylation, Ras proteins take one of two routes to the cell surface, which is dictated by a second targeting signal that is located immediately amino-terminal to the farnesylated cysteine^{23,24}. H-ras and N-ras undergo PALMITOYLATION on cysteine residues in their HVRs and enter the exocytic pathway, trafficking through the Golgi to the plasma membrane^{22,25}. K-ras, which has a polylysine sequence instead of cysteine residues, bypasses the Golgi and reaches the plasma membrane by an as-yet-unknown mechanism^{22,25}. The correct processing of the CAAX motif is essential for efficient forward transport of Ras, because deletion of Rce1 or Icmt impairs the ability of

Ras to engage either trafficking pathway, which results in mislocalization to the cytosol^{17,18,26–28}.

Moving H-ras and N-ras along. The key enzyme for H-ras and N-ras trafficking beyond the ER is Ras palmitoyltransferase (RPT), which was recently cloned from *Saccharomyces cerevisiae*^{29,30}. RPT is an ER-localized heterodimer³¹: one subunit, Erf2, is a 41-kDa integral membrane protein that has several homologues in mammals and yeast. Although a mammalian homologue of the second subunit, Erf4/Shr5, which is 24 kDa, has yet to be identified^{29–32}, the available data indicate that there is an ER-localized RPT in mammalian cells. H-ras and N-ras proteins in which the palmitoylation sites have been mutated from cysteine to serine accumulate predominantly in the ER and to some extent in the Golgi^{22,25}. Inhibition of RPT activity using the inhibitor 2-bromo-palmitate causes a similar ER accumulation of newly synthesized H-ras and N-ras, whereas treatment with BREFELDIN A (BFA) has no detectable effect on the palmitoylation of newly synthesized H-ras or N-ras^{25,33}. Exactly how palmitoylation operates as a trafficking signal is unclear. In mammalian cells it is possible that the addition of this second lipid moiety simply increases membrane affinity, which allows the palmitoylated proteins to be captured in the bulk-flow exocytic pathway to the *trans* Golgi, in which more precise sorting might occur. Certainly, the affinity of non-palmitoylated Ras proteins for the ER is very low. ER association is clearly seen when live cells are imaged²², but hypotonic lysis and cell fractionation is sufficient to dislodge non-palmitoylated proteins from the ER into the soluble cytosolic fraction^{14,23}, which indicates that these non-palmitoylated Ras proteins are probably in a dynamic equilibrium with a soluble pool.

Moving K-ras along. In contrast to H-ras and N-ras, which are distributed throughout the exocytic pathway, K-ras cannot be visualized in the Golgi at levels that are detectable by immunofluorescence or electron microscopy^{22,25}. In addition, a temperature of 15°C, treatment with BFA or co-expression of DOMINANT-NEGATIVE Sar1 (a small GTPase) or ADP-ribosylating factor 1 (Arf1) — all of which significantly inhibit the trafficking of newly synthesized H-ras and N-ras to the plasma membrane — have no such effect on K-ras^{22,25} (S. Roy & J.F.H., unpublished data). Several mechanisms have been proposed for K-ras transport: first, the polybasic domain drives the simple diffusion of K-ras down an electrostatic gradient to the negatively charged plasma membrane³⁴; second, an as-yet-undefined chaperone protein, which is perhaps analogous to RhoGDI (RhoGDP dissociation inhibitor) that solubilizes RHO-FAMILY PROTEINS out of cell membranes, is involved; and third, it is a microtubule-dependent process. Evidence for the latter mechanism comes from studies showing that K-ras — but not H-ras, Rap1A or Rho-family GTPases — binds to TAXOL-stabilized microtubules *in vitro*. This interaction requires the polybasic domain, prenylation and methylation of K-ras^{35,36}. Exactly how a direct interaction between K-ras

PHOTBLEACHING

The irreversible destruction, by any one of several different mechanisms, of a fluorophore that is under illumination.

FRAP

(fluorescence recovery after photobleaching). The time taken for a bleached area of membrane to re-fluoresce. FRAP can be used to calculate the lateral mobility of a green fluorescent protein (GFP)-tagged membrane-associated protein on the condition that recovery does not occur by exchange with a soluble pool of protein.

MICRODOMAINS

Sites within the plasma membrane that have a distinct lipid and/or protein composition.

PALMITOYLATION

The post-translational modification of a protein with palmitic acid. On Ras proteins, palmitic acid is attached as a thioester to the thiol (-SH) group of cysteine residues (this is known as S-acylation).

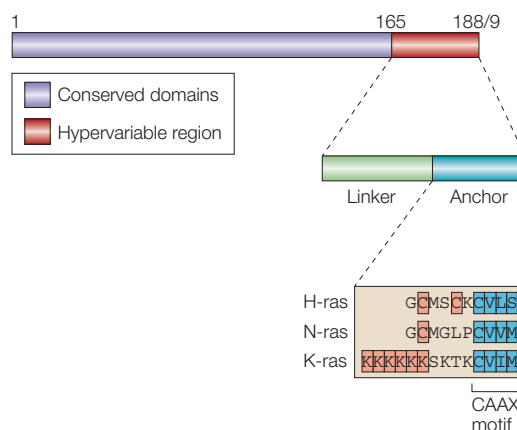
BREFELDIN A

A reagent that is used to disassemble the Golgi, probably by inhibiting the GTP-loading of ADP-ribosylation factor. In sensitive cells the Golgi is dispersed and trafficking of proteins through the exocytic pathway is inhibited.

DOMINANT-NEGATIVE

A defective protein that retains interaction abilities and so distorts or competes with normal proteins.

Figure 1 | Domain structure of the Ras proteins. The amino-terminal catalytic domains (amino acids 1–165) of H-ras, N-ras and K-ras are highly conserved (90–100% identical). The carboxy-terminal sequences diverge significantly and are referred to collectively as the hypervariable domain (HVR). The HVR comprises the well-characterized anchor sequences that also operate as Ras trafficking signals. The minimal Ras anchor comprises the carboxy-terminal CAAX motif in addition to a second signal. The second signal (shown for each in orange) comprises a single palmitoylation site (C181) in N-ras, two palmitoylation sites in H-ras (C181 and C184) and a polybasic domain of six contiguous lysine residues in K-ras (K175–K180). In this figure (and throughout this review) K-ras refers to the ubiquitously expressed K-ras4B, and not to the alternatively spliced isoform K-ras4A (not shown). The protein sequence between the anchor and the conserved domain constitutes the linker domain.



and microtubules could deliver the protein to the cell surface or keep it there is unclear. Nevertheless, an intact microtubule network must somehow be involved, because taxol treatment selectively mislocalizes newly synthesized K-ras in part to multivesicular structures that resemble late endosomes^{25,35}.

Intriguingly, in *S. cerevisiae*, the trafficking of palmitoylated Ras2 resembles that of both K-ras and H-ras: Ras2 traffics to the plasma membrane through the classical secretory pathway and a non-classical pathway, but it is the non-classical pathway that requires palmitoylation³¹.

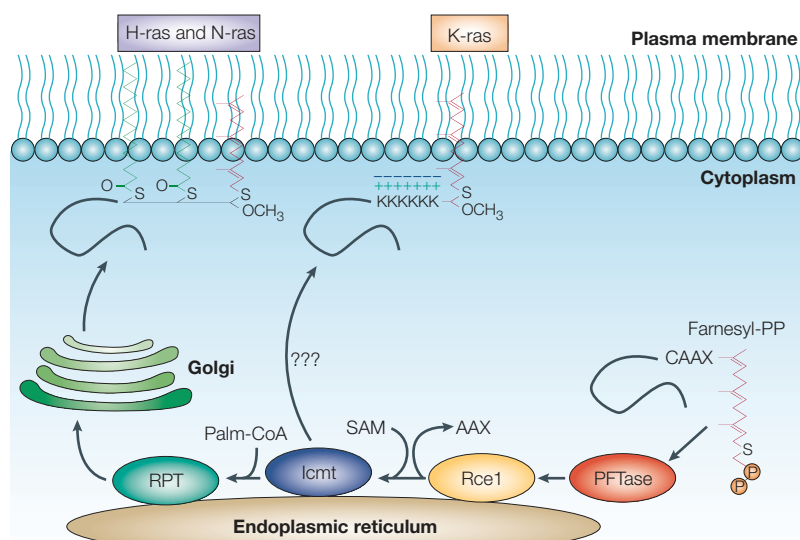


Figure 2 | Membrane trafficking of mammalian Ras proteins. CAAX processing is sequential. It is initiated in the cytosol by protein farnesyl transferase (PFase), which adds a farnesyl group (farnesyl-PP) to the cysteine residue of the CAAX motif (where A is aliphatic and X is any amino acid). Processing is completed on the cytosolic leaflet of the endoplasmic reticulum (ER) by the enzymes Rce1 (Ras and a-factor converting enzyme) and Lcmt (isoprenylcysteine carboxyl methyltransferase), which remove the AAX tripeptide and methylate with S-adenosylmethionine (SAM), respectively. K-ras then exits the ER and traffics to the plasma membrane through a poorly characterized pathway that bypasses the Golgi. H-ras and N-ras, after palmitoylation with palmitoyl CoA (Palm-CoA) by an ER-localized palmitoyltransferase (RPT), traffic through the classical secretory pathway through the Golgi to the plasma membrane. The trafficking of alternatively spliced K-ras4A has not been reported, although as it is palmitoylated on C180 and has no polybasic domain it would be expected to follow H-ras and N-ras. Farnesylation is a stable modification of Ras. Palmitoylation and methylation are not stable modifications, but no specific Ras thioesterase or methylase — the enzymes that would remove palmitate or the methyl group — have so far been identified. At present, only one Ras palmitoyltransferase has been characterized. Preliminary data indicate that this enzyme palmitoylates newly synthesized, CAAX processed H-ras and N-ras. However if Ras de-palmitoylation occurs in the Golgi and/or plasma membrane then other, as-yet-unidentified, palmitoyltransferases might also be located in these subcellular compartments. If not, then de-palmitoylated H-ras and N-ras would have to continuously cycle through the ER for re-palmitoylation.

Understanding protein palmitoylation. Palmitoylation is important for H-ras and N-ras trafficking and their plasma-membrane microlocalization, but a series of intriguing and unanswered questions about this lipid modification remains. For example, there are two cysteine palmitoylation sites in H-ras, but only a single site in N-ras. Mutation of either H-ras site decreases palmitate incorporation¹⁴, but to what extent one or the other or both residues are normally palmitoylated is not known. It is also possible that the palmitoylation status of H-ras is different in different membrane compartments³⁷. Fluorescent images from several groups indicate that more N-ras than H-ras is Golgi-associated at an equivalent level of expression. This qualitative result has not been formally quantified, but it could reflect the more efficient post-Golgi transport of doubly palmitoylated H-ras; especially if observations on the palmitoylated GAD65 (glutamate decarboxylase 2, pancreatic islets and brain, 65-kDa isoform) protein³⁸ can be extrapolated to Ras. Palmitoylation is an unstable modification — it turns over with a half-life of 20 min in N-ras³⁹, but with more complex kinetics in H-ras⁴⁰. At present, it is not known where in the cell palmitate turnover occurs, what the identity of the thio-esterase that is responsible is, and how Ras would re-access an ER-associated RPT for re-palmitoylation if there are not other, as-yet-unidentified, RPTs in the plasma membrane⁴¹. The turnover of palmitic acid on H-ras is stimulated by S-nitrocysteine. Treatment of cells with this agent decreases GTP-binding to oncogenic mutant H-rasG12V, which indicates that there is a possible link between Ras activation and the regulation of palmitoylation⁴⁰. There is a tantalizing analogy here with the α -subunits of heterotrimeric G proteins, which undergo increased de-palmitoylation when GTP-loaded⁴², and which, when de-palmitoylated, are more sensitive to the action of regulators of G-protein

Box 2 | Techniques that are used to study Ras plasma-membrane microlocalization

Each method that is used to investigate the microlocalization of Ras and other plasma-membrane proteins has advantages and limitations.

Density gradients

The biophysical properties of lipid rafts render them insoluble in 1% Triton X-100 at 4°C (REFS 44,118). Lipid rafts and associated proteins can then be 'floated away' from detergent-soluble proteins on density gradients. However, because Ras proteins are highly soluble in 1% cold Triton X-100, other detergent-free methods have been developed^{49,51,119}. These include combinations of cell shearing, sonication and the application of alkaline carbonate to disrupt the plasma membrane into vesicles that are small enough to be resolved into light (that is, raft-enriched) from dense (disordered) membranes on density gradients. Appropriate markers for raft and non-raft membrane are required to verify adequate resolution, but even then, interpretation is limited to one of co-fractionation with a membrane marker, rather than definitive assignment to a specific microdomain.

Fluorescence

This method has been used to colocalize Ras with caveolin and raft markers¹²⁰, but the resolution is low (>200 nm) when compared with the diameter of caveolae and rafts (<70 nm) and can therefore cause problems with interpretation. Outer-leaflet rafts can be crosslinked to generate structures that are readily resolved by light microscopy and this leads to patching of inner-leaflet raft proteins¹²¹. The use of quantitative methods, for example, cross correlation analysis, to assess colocalization formally is important¹²².

Electron microscopy

Immunogold labelling is the only technique that can definitively assign proteins to caveolae. One limitation of electron microscopy (EM) has been that caveolae are the only microdomain with a well-defined ultrastructure. However, new EM methods using plasma-membrane sheets^{123,124} and statistical analysis of immunogold patterns can assign proteins to microdomains that lack specific ultrastructure⁵⁷. Lipid rafts and other non-raft domains can be distinguished. EM resolution is high (1 nm).

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) is a technique that measures the lateral diffusion of green fluorescent protein (GFP)-tagged Ras proteins in live cells. Diffusion rates for different Ras isoforms differ significantly from those expected of freely diffusible membrane proteins: so Ras proteins interact with several membrane domains that restrict lateral mobility^{56,125}. The use of live cells excludes the artefacts that sometimes arise from cell fixation or fractionation, and illustrates that the interaction of Ras with specific microdomains is highly dynamic.

RHO FAMILY PROTEINS

Ras-related GTPases that are involved in controlling the polymerization of actin.

TAXOL

An antitumour agent that enhances the polymerization of tubulin and the subsequent stabilization of microtubules, thereby inhibiting mitosis and blocking the cell cycle.

LIPID RAFTS

The biophysical properties of certain long-chain saturated fatty acids packed together with cholesterol drive the spontaneous formation of small, relatively stable, structures that are known as lipid rafts. Lipid rafts phase separate from the more loosely packed phospholipids of the membrane bilayer.

LIQUID-ORDERED

A term that is used to describe the tightly packed molecular structure of a lipid raft: an intermediate physical state between fluid and gel.

signalling (RGS) proteins that stimulate GTP hydrolysis⁴³. Taking these observations together, it is clear that the regulation of Ras palmitoylation would be an interesting way of modulating H-ras and N-ras localization and therefore signal output. Addressing whether there is such regulation in mammalian cells requires further progress in unravelling the enzymology of protein palmitoylation.

Microlocalization at the plasma membrane

The plasma membrane comprises a complex mosaic of microdomains⁴⁴. The best characterized of these are LIPID RAFTS and caveolae, although it has been proposed that there are other lipid-based microdomains⁴⁵. The significance of this mosaic structure lies in the lateral segregation it can impose on signalling proteins — these can be concentrated or excluded from specific microdomains to increase the specificity and efficiency of signalling⁴⁴. As a general rule, palmitoylated peripheral membrane proteins can associate with lipid rafts because the saturated palmitate packs well into the LIQUID-ORDERED raft structure, whereas unsaturated, branched chain prenyl groups do not⁴⁶. The different carboxy-terminal lipid anchors on N-ras, H-ras and K-ras — a farnesyl group with one or two palmitate groups or a polybasic domain, respectively — would therefore be expected to sort, or target, to different plasma-membrane microdomains.

Ras isoforms in lipid rafts? Early work showed that H-ras, and the downstream components — the serine/threonine protein kinase Raf, MAPK and ERK kinase (MEK) and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) — of one Ras effector pathway were present in lipid raft/caveolin-enriched fractions on sucrose gradients^{47–49}. However, not all of the plasma membrane was always analysed, so quantifying the extent of raft association was difficult. Biochemical and electron microscopic (EM) analysis of isolated caveolae also showed that all of the elements of the Raf–MEK–ERK/MAPK cascade were present⁵⁰. Recently, a more detailed description of Ras plasma-membrane microlocalization has emerged from a combination of complementary methods (BOX 2). Quantitative EM analysis of intact plasma-membrane sheets shows that neither H-ras nor K-Ras is particularly enriched in caveolae — rather, these Ras isoforms are localized predominantly to morphologically featureless plasma membrane⁵¹. Of the Ras that is in caveolae, there is more H-ras than K-ras⁵¹. GTP- and GDP-loaded K-ras is predominantly (~85%) associated with non-raft plasma membrane; H-ras is partially (~50%) localized to lipid rafts when GDP-loaded, but it is essentially absent from rafts when it is GTP-loaded^{51,52}. Other protein sequences that are adjacent to the membrane anchor, the HVR linker domain (FIG. 1), are required for activated H-ras to redistribute from lipid

rafts to DISORDERED PLASMA MEMBRANE^{51,53}. The model that has been proposed to account for these results (FIG. 3) envisages that K-ras is localized outside lipid rafts, but that H-ras is in a dynamic equilibrium between lipid rafts and other non-raft sites of the plasma membrane, with the equilibrium regulated by GTP loading^{51,54}. The initial delivery of H-ras to lipid rafts could require sorting in the exocytic pathway, probably at the level of the Golgi, as shown recently for TC10, a Rho protein that has a similar membrane anchor to that of H-ras⁵⁵.

Ras isoforms outside lipid rafts. If activated H-ras and K-ras are both localized outside lipid rafts as shown in FIG. 3, are they in the same, or different, microdomains, or are they randomly associated with disordered membrane? This question has recently been answered. FRAP analysis of green fluorescent protein (GFP)-tagged Ras in the plasma membrane of live cells shows that the lateral mobility of activated K-ras and H-ras is restricted at low levels of expression, but increases at high levels of expression⁵⁶. The simplest interpretation of these data is that activated H-ras and K-ras interact with saturable sites in the plasma membrane. These sites are probably distinct, because the saturation curves for activated H-ras and K-ras are different. The sites are not lipid rafts, because identical results are obtained in cells with depleted cell-surface cholesterol⁵⁶. By contrast, the lateral mobility of GDP-loaded H-ras increases in cholesterol-depleted cells⁵⁶, which is consistent with the model that only inactive H-ras interacts with lipid rafts (FIG. 3). Data obtained using a totally different experimental approach⁵⁷ confirmed that activated H-ras and K-ras cluster in spatially distinct microdomains that do not contain lipid-raft markers. Furthermore, these clustering patterns are not disrupted by cholesterol depletion, so neither the activated K-ras nor the activated H-ras microdomain is a lipid raft⁵⁷. In summary, these studies provide compelling evidence that activated H-ras and K-ras operate in different, non-raft, microdomains of the plasma membrane.

Mechanisms of microlocalization

The results discussed above pose interesting questions as to the nature of the non-raft microdomains that are occupied by K-ras and H-ras. For example, are the sites stable entities, like the prototype lipid raft, or are they transient structures, and what is the mechanism of their formation?

Generating an acidic microdomain? Without further work, only speculation is possible, but work examining the interaction of polybasic peptides with model membranes is relevant to this discussion. The binding of myristoylated alanine-rich C-kinase substrate (MARCKS) to membranes requires cooperation between a long polybasic domain in the protein and an amino-terminal myristate⁵⁸. The membrane-bound polybasic domain attracts acidic phospholipids such as phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) and phosphatidyl serine through electrostatic interactions, which effectively generates an acidic microdomain^{59,60}.

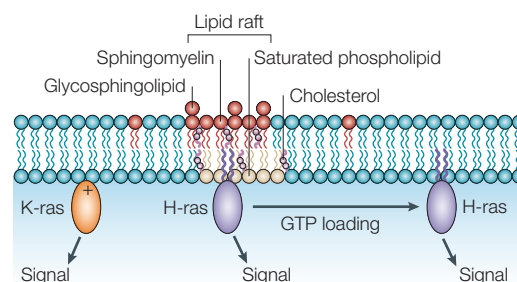


Figure 3 | Plasma-membrane microlocalization of H-ras and K-ras. K-ras is associated predominantly with non-raft plasma membrane, irrespective of its activation state^{51,52,56}. H-ras is distributed approximately equally between raft and non-raft plasma membrane, but GTP loading increases the fraction of H-ras in non-raft membrane. Oncogenic H-rasG12V, which is loaded with GTP constitutively, is localized almost exclusively to non-raft membrane. GTP loading therefore regulates the lateral segregation of H-ras in the plasma membrane. It is important to stress that H-ras and K-ras are highly mobile in the plasma membrane⁵⁶, so the localizations shown need to be thought of more in terms of residence times — that is, GDP-H-ras has affinity for raft and non-raft membrane, exchanges constantly between, and has equal residence time in, both sites; GTP-H-ras has a higher affinity for, and therefore a greater residence time in, non-raft membrane. These concepts are developed further in FIG. 4. H-ras signalling requires access to lipid rafts. This can be rationalized in the context of a dynamic model: GTP-H-ras initiates Raf-1 activation within lipid rafts, but completes the process outside. The main limitation of this simple model is that it is one-dimensional and offers no insight into whether the non-raft sites occupied by K-ras and activated H-ras are distinct.

Binding of calcium/calmodulin to the polybasic domain or phosphorylation by protein kinase C (PKC) reverses the electrostatic interaction with PtdIns(4,5)P₂ (REFS 58,60). The possible analogy with K-ras is intriguing, more so because recent work has shown that the activation and signalling by K-ras is also inhibited by calcium/calmodulin binding^{61,62}. Calcium/calmodulin selectively binds to K-ras and no other Ras isoform, and whereas the binding involves many regions on Ras, the isoform selectivity is provided by the HVR⁶¹. So calcium/calmodulin binding could regulate the interactions of K-ras with acidic microdomains that are generated by other polybasic proteins or even perhaps by K-ras itself (FIG. 4). Interestingly, the length of the prenyl group that is attached to K-ras is crucial for the correct microlocalization of K-ras. If K-ras is made into a substrate for geranylgeranyl transferase 1 by replacing the wild-type CAAX motif with a CCIL sequence (where I is isoleucine and L is leucine), the mutant protein no longer clusters at the plasma membrane⁵⁷. The generation of acidic microdomains by polybasic proteins might therefore depend on the presence of a lipid group, such as myristate or farnesyl, that has a relatively low affinity for membranes, rather than one that has a high affinity, such as geranylgeranyl.

HVR-galectin interactions? In addition to the carboxy-terminal membrane anchor, the activation state and the linker domain of the HVR also contribute to H-ras

DISORDERED PLASMA MEMBRANE
Plasma membrane that is not organized in liquid-ordered lipid rafts.

microlocalization. Deletion of the linker domain allows normal trafficking of H-ras to the plasma membrane, but confines activated H-ras to lipid rafts^{51,53}. These data indicate that the HVR might interact with other plasma-membrane proteins to regulate the lateral

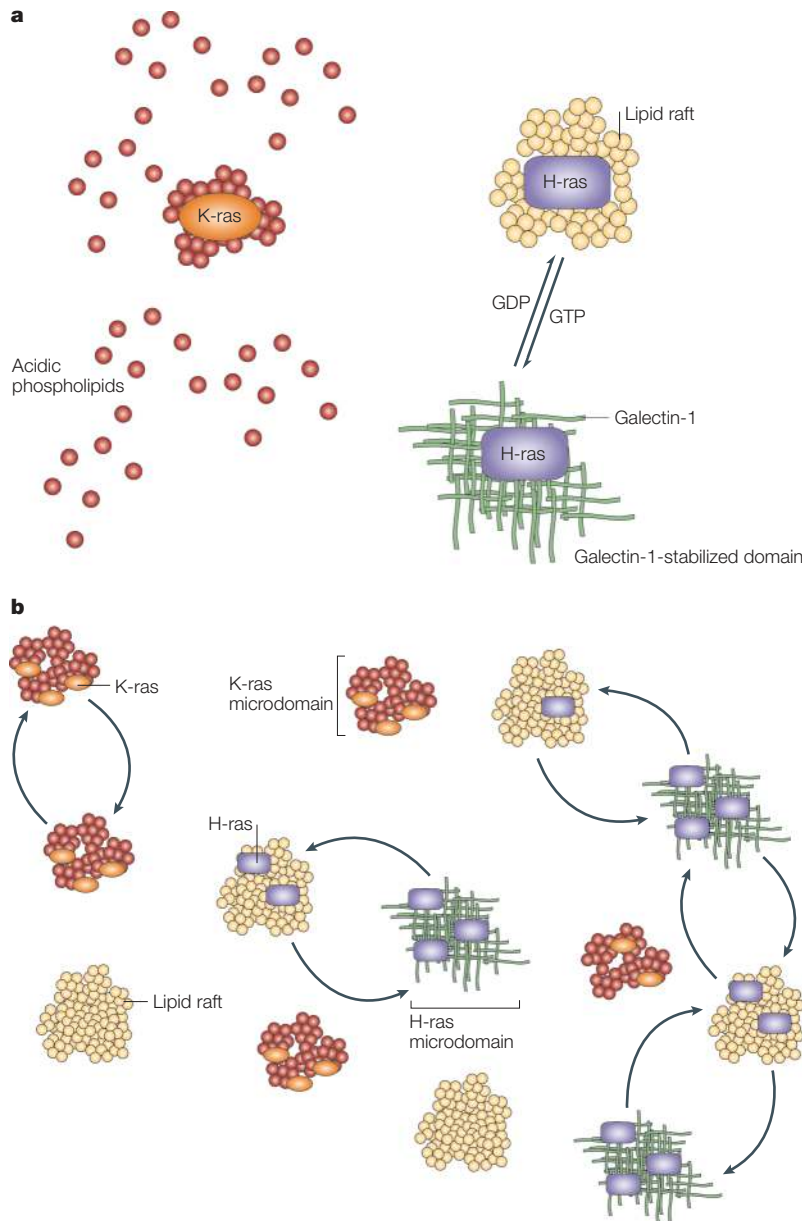


Figure 4 | K-ras and H-ras occupy distinct non-raft microdomains. a | Activated H-ras and K-ras occupy different non-raft plasma-membrane microdomains. The nature of these microdomains remains speculative. The radius of the K-ras microdomains is ~16 nm, compared with a radius of 22 nm for inner-plasma-membrane lipid rafts⁵⁷. One interesting possibility is that the polybasic domain of K-ras attracts acidic phospholipids and initiates the formation of a microdomain with a distinct lipid composition (left). Galectin-1 stabilizes the activated H-ras microdomain (right). As galectin-1 is predominantly cytosolic, we speculate that H-ras, on exiting lipid rafts, recruits galectin-1 and that this complex drives the formation of a transient signalling centre. GTP-hydrolysis might trigger disassembly. **b** | Lipid rafts and H-ras and K-ras microdomains have no fixed spatial relationship to each other. H-ras and K-ras proteins are laterally mobile, so they must constantly exchange between their respective binding sites in the plasma membrane (FIG. 3). Implicit in this hypothesis is that there must also be a random or transiting fraction of H-ras and K-ras within the plasma membrane *en route* between sites⁵⁷. These models are the starting point for an explanation of signalling differences between the Ras isoforms.

segregation of H-ras. A possible candidate here is **galectin-1**. Although it was originally identified as a secreted lectin, a substantial intracellular pool of galectin-1 has been identified⁶³. Galectin-1 can be crosslinked in a complex with activated H-ras and, to a lesser extent, activated K-ras, but not with N-ras or inactive H-ras or inactive K-ras⁶³. Downregulation of galectin-1 expression abolishes H-rasG12V clustering at the plasma membrane⁵⁷, and, concomitant with the consequent random distribution of H-rasG12V over the plasma membrane, there is partial mislocalization of H-rasG12V to the cytosol^{57,63}. Similar effects are not seen with activated K-rasG12V. So galectin-1 could be an essential component of the plasma-membrane microdomains that are occupied specifically by activated H-ras. It is tempting to speculate that these microdomains are not permanent structures, but that when activated H-ras exits rafts, it recruits galectin-1 to form a transient microdomain that is then further stabilized by other membrane or cytosolic components (FIG. 4). The analogy here is with **Rab5**, a member of the RAB PROTEIN family, which progressively recruits a series of binding partners and scaffolds that function cooperatively to generate a functional membrane domain, albeit in this case on the endosome⁶⁴. One prediction of such a model is that galectin-1 overexpression should stabilize H-ras GTP loading — an observation that has recently been made⁶⁵.

H-ras–phosphodiesterase- δ interactions. Another protein that interacts with the carboxyl terminus of H-ras is phosphodiesterase- δ (PDE δ). Originally identified as a component of cyclic GMP phosphodiesterase, PDE δ interacts strongly in YEAST TWO-HYBRID ASSAYS with H-Ras and **Rho6**, weakly with Rap1A and the GTP-binding protein **Rheb** (for Ras homologue enriched in brain 2)^{66,67} and not at all with K-ras (M. Hanzal-Bayer, personal communication). Farnesylation and the HVR linker domain are required for PDE δ binding, and the interaction is stronger for GDP-bound than GTP-bound H-ras⁶⁷. PDE δ is a structural homologue of RhoGDI^{66,67}, and preliminary cell-biology experiments indicate that ectopic expression of PDE δ might result in the redistribution of H-Ras from the plasma membrane to cytosol⁶⁶. At present, it is unclear whether PDE δ regulates H-Ras trafficking to the plasma membrane or H-Ras–plasma-membrane interactions, but sequestration of the H-ras prenyl group into a hydrophobic pocket⁶⁷ is probably involved in either process. Of note, however, is that PDE δ is itself an effector of the Arf-like small GTPases **Arl2** and **Arl3**. This prompts speculation that PDE δ –Ras interactions might, in turn, be regulated by Arl2 or Arl3 (REF 67). Arl binding might therefore endow further specificity on PDE δ –GTPase interactions.

Ras signalling from different microdomains

Differential lateral segregation of Ras proteins within the plasma membrane offers a plausible mechanism for how these highly homologous proteins might generate distinct signal outputs. If Ras microdomains

differ in their concentration of protein or lipid cofactors, or in their substrates for Ras effectors, then signal output from each microdomain will clearly be different. Formal proof of this hypothesis will require evidence that the protein and/or lipid composition of the plasma-membrane microdomains that are occupied by activated K-ras and H-ras are significantly different.

Insights from chimeric Ras studies. In the absence of such data, there is substantial circumstantial evidence showing that the carboxy-terminal membrane anchor regulates Ras signal output. For example, K-rasG12V is a more potent activator of Rac than H-rasG12V, and it induces greater membrane ruffling, pinocytosis and cell motility than H-rasG12V¹⁰. These phenotypes are completely dependent on the carboxy-terminal 25 amino

Box 3 | Imaging Ras activation

Ras activation has been assayed in live cells using fluorescence resonance energy transfer (FRET). Three basic FRET methods that rely on detecting a GTP-dependent interaction between a Raf-RBD (Ras-binding domain) construct and Ras are used.

Chimeric probes

The prototype chimeric probe Raichu-Ras (Raichu stands for Ras and interacting protein chimeric unit; see figure part a) — a chimaera of cyan fluorescent protein (CFP)-tagged Raf-RBD and yellow fluorescent protein (YFP)-tagged H-Ras — will emit intramolecular FRET only when the Ras module is GTP-loaded⁸⁰. Raichu-Ras is targeted to the plasma membrane using the anchor from K-ras. Raichu-Ras emits FRET (see figure part b) in response to stimulation by epidermal growth factor (EGF), but only at the plasma membrane⁸⁰.

Bystander FRET

The method relies on the swamping of endomembrane and plasma membrane with ectopically expressed CD8-YFP; it has the main advantage of detecting the activation of endogenous, rather than transfected, Ras. Bystander FRET is elicited when activated endogenous Ras in the vicinity of YFP-tagged CD8 recruits Raf-RBD-CFP⁸³ (see figure part c). EGF stimulation shows endogenous Ras activation (by the appearance of FRET) first at the plasma membrane, then at later time points in the Golgi⁸³ (as shown by the arrowheads; see figure part d). An important question that follows from a comparison of these first two approaches is why does Raichu-Ras not detect active Ras on the Golgi? The probable explanation is that Raichu-Ras is membrane targeted by the K-ras anchor, which will exclude the construct from the Golgi (FIG. 2). Raichu-Ras targeted by the H-ras anchor might give different results.

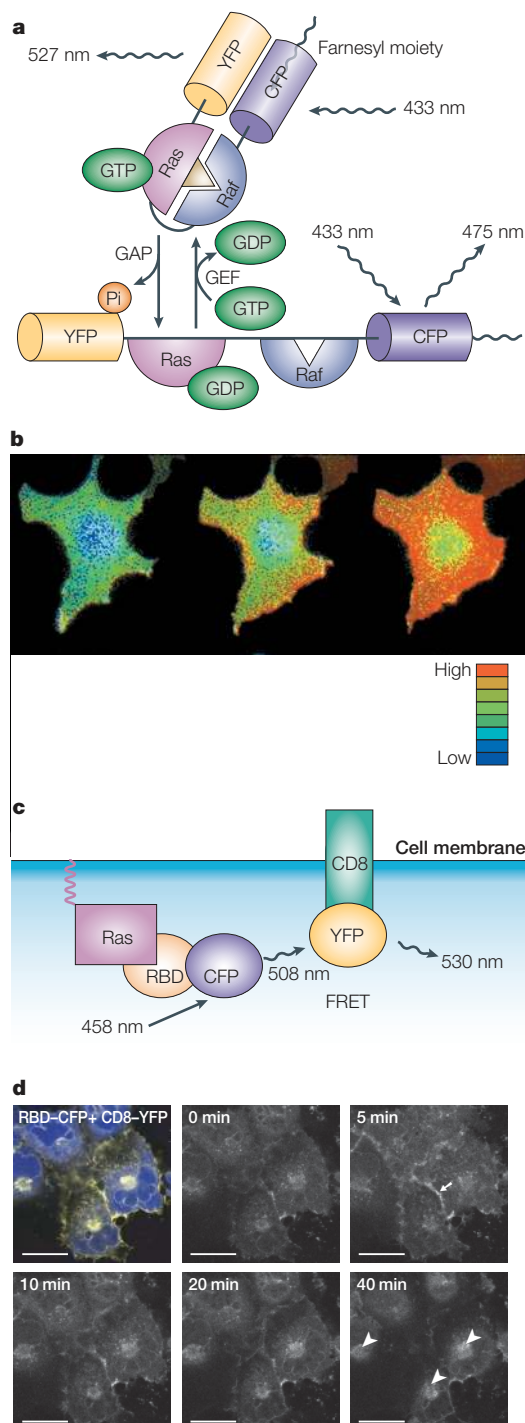
Separate expression constructs

CFP-H-Ras and YFP-Raf-RBD can be used separately to monitor Ras activation, but they are less sensitive. FRET is detectable at the plasma membrane in serum-starved cells, but EGF stimulation does not increase total FRET — rather, the distribution changes, highlighting membrane ruffles and endosomal-like intracellular vesicles as sites of Ras activation⁷⁷.

Non-FRET methods

GFP-Raf-RBD decorates membranes that ectopically express activated Ras. Cells expressing H-rasG12V or N-rasG12V recruit GFP-Raf-RBD from the cytosol to plasma membrane and Golgi, but cells expressing K-rasG12V only recruit GFP-Raf-RBD to plasma membrane⁸³. Longer Raf probes that include the cysteine rich domain might be more sensitive and allow monitoring of endogenous Ras activation¹²⁶.

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RAB PROTEINS

Rab proteins form the largest subfamily of small GTPases of the Ras superfamily. They regulate budding, tethering, fusion and motility at various sites within cells.

YEAST TWO-HYBRID APPROACH

A technique that is used to test if two proteins physically interact with each other. One protein is fused to the GAL4 activation domain and the other to the GAL4 DNA-binding domain, and both fusion proteins are introduced into yeast. Expression of a GAL4-regulated reporter gene indicates that the two proteins physically interact.

acids, because an H-ras1–164–K-ras165–188 chimera behaves exactly like K-ras¹⁰. If the CAAX motif of oncogenic mutant H-rasQ61L (where Q is glutamine) is replaced with a polybasic extension, the mutant — which is known as Ext61L — undergoes palmitoylation and traffics to the plasma membrane^{68–70}. Ext61L is a more potent activator of phosphatidylinositol 3-kinase (PI3K) and Rac, but a weaker activator of B-Raf than H-rasQ61L⁷¹. This alteration in the use of effectors could reflect either a different microlocalization of Ext61L and H-rasQ61L or an increased concentration of PtdIns(4,5)P₂ in the H-ras microdomain that is recruited by the Ext61L polybasic domain.

Insights from cholesterol depletion studies. The functional interactions between lipid rafts and Ras signalling are complex. Depleting cell-surface cholesterol — either biochemically with cyclodextrin, or biologically, by expression of dominant-negative caveolins — blocks H-ras-mediated, but not K-ras-mediated, Raf activation^{72,73}. So H-ras, but not K-ras, signalling requires intact lipid rafts; a similar dependence on intact rafts for signalling is shown by TC10, which has an almost identical membrane anchor to H-ras^{74,75}. Cyclodextrin treatment also activates ERK/MAPK^{48,52,76}, which indicates that intact lipid rafts might either be important for some aspects of negative regulation of ERK/MAPK signalling⁴⁸, or that the redistribution of cholesterol to intracellular stores triggers Ras-independent activation of the ERK/MAPK cascade⁵². Conversely, mutations in the H-ras HVR linker domain that confine activated H-ras to lipid rafts impair Raf-1 and PI3K activation and abolish the biological activity of H-ras, as shown by the differentiation of PC12 CELLS. Therefore, although Raf activation can proceed inside and outside lipid rafts^{47,51,52}, the specific activity of Raf activated by H-rasG12V that is mislocalized to lipid rafts is much lower than Raf that is activated by non-raft-localized H-rasG12V^{51,53}. Taking these results together, it seems probable that an early event in the multistep process of Raf activation requires H-rasG12V to transiently access rafts, even though Raf activation is then completed at other sites in the plasma membrane. One reason that K-ras is a more potent activator of Raf^{6,7} could be because activation can proceed in a single microdomain.

Where in cells is Ras activated?

In addition to the plasma membrane, N-ras and H-ras are expressed stably on the Golgi in transfected cells, and at least transiently on the ER; Ras has also been visualized on endosomes^{22,25,77–79}. Do these non-plasma-membrane pools contribute to Ras signal output? To tackle this question, several methods have been developed that can identify the location of active GTP-bound Ras in living cells (BOX 3). Matsuda and colleagues, using a chimeric FRET probe, have shown that epidermal growth factor (EGF)-stimulated Ras activation in COS CELLS is only visible at the plasma membrane and is particularly strong at sites of membrane ruffling⁸⁰ (BOX 3). By contrast, FRET emitted from a Rap1 probe shows that Rap activation in response to EGF occurs mainly in the centre of the cell⁸⁰.

Chemical inhibitors of endocytosis inhibit Rap, but not Ras, activation⁸⁰, which is consistent with earlier biochemical studies showing that nerve growth factor (NGF)-stimulated Rap, but not Ras, activation in PC12 cells is dependent on endocytosis of the NGF receptor TrkA⁸¹. This interesting spatial separation of Ras and Rap activation has been examined in more detail⁸². Using a combination of wild-type and GTPase-deficient Ras and Rap1 FRET probes, Ohba *et al.* have derived kinetic estimates of guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP) activity at various locations in COS cells⁸². They show that RasGAP activity is high in the vicinity of ER and Golgi and low at the plasma membrane, but that there is an exact mirror image of RapGAP activity⁸². They concluded that it is these contrasting GAP gradients that result in plasma-membrane-restricted Ras activation and ENDOMEMBRANE-restricted Rap activation⁸².

Ras activation on endomembranes? The activation of ectopically expressed H-ras, N-ras and K-ras can also be qualitatively assessed by colocalization with GFP-tagged Ras binding domain (RBD) of Raf (GFP-Raf-RBD; BOX 3). When cells expressing wild-type H-ras are stimulated with EGF, GFP-Raf-RBD is initially recruited to the plasma membrane and then progressively to the Golgi⁸³; this clearly indicates that ectopically expressed H-ras is activated on Golgi membranes. To investigate the more difficult question of whether this is also the case for endogenous Ras, Philips and colleagues⁸³ developed 'bystander FRET' (BOX 3). They have shown that EGF stimulates the rapid activation of endogenous Ras at the plasma membrane, which is then followed by a sustained increase in Ras activation on Golgi membranes⁸³ (BOX 3).

Taken together, these studies show that growth factors stimulate activation of endogenous and ectopically expressed Ras at the plasma membrane, and with a different time-course on the Golgi. The validity of this conclusion is strengthened further by the identification of a potential mechanism for Ras activation on the Golgi (REF. 83 and M. Philips, personal communication). Src-mediated activation of phospholipase C γ , which is triggered by growth-factor receptors, generates diacylglycerol and Ca²⁺ — second messengers that activate the Ras guanine nucleotide-releasing protein (Ras-GRP) family of GEFs and stimulate their translocation to endomembrane^{84–86} (FIG. 5). This mechanism is therefore quite distinct from the more classical activation of Ras at the plasma membrane that is mediated by Shc-Grb2-Sos (for Src-homology-2-containing, growth factor receptor-bound 2 and son-of-sevenless, respectively). Indeed, recent work indicates that, in fibroblasts, activation of Ras at the plasma membrane is suppressed by the calcium-activated GAP, CAPRI (for Ca²⁺-promoted Ras inactivator)⁸⁷, coincident with continued Ras activation on the Golgi by RasGRP1 (M. Philips, personal communication). Intriguingly, this signalling pathway also leads to selective activation of N-ras on the Golgi in response to low level T-cell-receptor activation in Jurkat cells (M. Philips and A. Pellicer, personal communication).

PC12 CELLS

A clonal line of rat adrenal pheochromocytoma cells that, much like sympathetic neurons, respond to nerve growth factor and can synthesize, store and secrete catecholamines. PC12 cells contain small, clear synaptic-like vesicles and larger dense core granules.

COS CELLS

Cells from the monkey CV1 cell line that have an integrated SV40 genome lacking an origin of replication. Plasmids with an SV40 origin of replication are replicated to a high copy number when transfected.

ENDOMEMBRANE SYSTEM

A hypothetical integrated membrane system of eukaryotic cells that represents a developmental and functional continuum. It comprises the endoplasmic reticulum, nuclear membrane, Golgi apparatus and vesicles.

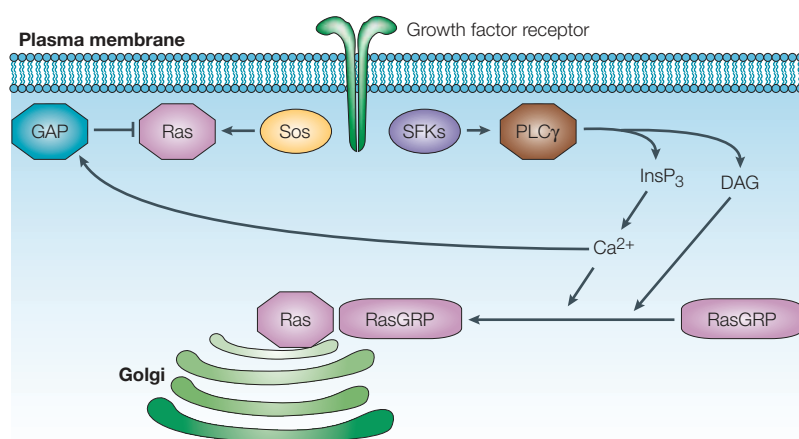


Figure 5 | Signalling to endomembrane-localized Ras. Growth-factor receptors at the plasma membrane activate phospholipase $C\gamma$ (PLC γ) through Src-family kinases (SFKs). PLC γ hydrolyses phosphatidylinositols to generate diacylglycerol (DAG) and inositol trisphosphate (InsP $_3$). InsP $_3$ in turn liberates calcium (Ca $^{2+}$) from intracellular stores. DAG and calcium activate the Ras guanine nucleotide-releasing protein (RasGRP) family of Ras exchange factors and stimulate translocation to endomembranes. Golgi-recruited RasGRP then activates Golgi-localized Ras. The calcium signal might also activate the calcium-sensitive Ras GTPase-activating protein (GAP), CAPRI (for calcium-promoted Ras inactivator), which would contribute to a simultaneous attenuation of signalling from plasma-membrane localized Ras. Sos, son of sevenless.

Ras signalling from endomembranes

If Ras is activated on endomembranes, then can it engage and activate effectors? The general answer seems to be yes, albeit with reduced efficiency in the ER compared with the plasma membrane. Non-palmitoylated, oncogenic mutant H-Ras activates Raf, PI3K and Jun amino-terminal kinase (JNK) less efficiently than palmitoylated H-Ras, but sufficiently well to retain 10–75% of its transforming activity in NIH3T3 FOCUS ASSAYS^{14,23,83,88}. More sensitive assays of biological activity in *Xenopus laevis* oocytes, however, indicate that non-palmitoylated H-ras is actually severely compromised for signalling⁸⁹. In NIH3T3 focus assays, H-rasQ61L that is targeted to the endomembrane, using the first transmembrane domain of avian infectious bronchitis virus M protein (M1-H-rasQ61L), has been reported to have either no⁹⁰ or significantly reduced⁸³ biological activity that remains dependent on a normally processed CAAX motif⁸³. M1-H-rasQ61L is a weak activator of Raf and PI3K, but activates JNK with equivalent potency to plasma-membrane localized H-rasQ61L⁸³. Consistent with this activation profile, targeting Raf-1 to the ER using an isolated CAAX motif also does not result in kinase activation^{91,92}. By contrast, H-rasQ61L that is targeted to the Golgi with a KDEL motif (where K is lysine, D is aspartic acid, E is glutamic acid and R is arginine) activates Raf and PI3K with the same potency as plasma-membrane-localized H-rasQ61L, but is a weak activator of JNK⁸³. Lipid rafts and possibly other microdomains form in the *trans* Golgi, but the extent to which Ras proteins interact with specific microdomains on this organelle has not been examined. If lateral segregation of Ras on Golgi membranes mirrors what occurs at the cell surface then a common set of mechanisms could contribute to the

NIH3T3 FOCUS ASSAY

This assay measures the transforming potency of an oncogene. The growth of NIH3T3 cells arrests as a continuous monolayer, but transformed cells do not arrest and grow over the adjacent monolayer as a focus of clonal cells.

EEA1

The antigen that is involved in a human autoimmune disease. It is a marker of the early endosome.

regulation of Ras signal output from endomembrane and plasma membrane.

Signalling from endosomal Ras

The evidence that Ras transits through the endosomal compartment after localization to the plasma membrane is strong. Ras has been identified in endosomes prepared from rat liver⁹³, clathrin-coated vesicles purified from NGF-stimulated PC12 and dorsal-root-ganglion neuronal cells⁷⁸, and early endosome-associated protein (EEA1)-positive endosomes from insulin-stimulated fibroblasts⁹⁴. EGF or insulin stimulates the rapid internalization of GFP-H-Ras from the plasma membrane into endosomes in cells that overexpress the appropriate cognate receptor^{77,79}. H-ras and activated EGF receptor colocalize in the same endosomes. This occurs by endocytosis of the activated EGF receptor and H-Ras from common sites at the plasma membrane, as well as the fusion of endosomes that contain EGF receptor with endosomes that contain H-ras⁷⁷. FRET analysis of A431 cells expressing YFP-RBD and CFP-Ras confirms that activated Ras is present on enlarged endosomes in EGF-stimulated cells⁷⁷, which is consistent with biochemical studies showing that activated EGF receptors continue to stimulate Ras-GTP loading from within endosomes^{95,96}.

Interestingly, pretreating cells with cyclodextrin blocks insulin-stimulated GFP-H-ras internalization⁷⁹. So GFP-H-Ras must either be associated with lipid rafts to undergo insulin-stimulated endocytosis, or lipid rafts must be intact for activation of the insulin receptor to trigger endocytosis, or both. Insulin-receptor-positive endosomes are resistant to extraction with 1% Triton, which also points to their enrichment in cholesterol-rich lipid rafts⁷⁹. GFP-Raf is present in Ras-positive endosomes in EGF-stimulated and insulin-stimulated cells^{77,79}. FRET occurs between YFP-RBD and CFP-H-ras in endosomes in EGF-stimulated cells, which shows the presence of GTP-loaded H-ras, but no FRET occurs between full-length YFP-Raf and CFP-H-ras⁷⁷. So Ras and Raf colocalize in endosomes, but they might not actually form a complex. If so, Raf might be tethered to endosomal membranes by an interaction with phosphatidic acid, an attachment mechanism that operates through its kinase domain and is independent of Ras binding^{94,97}.

Endocytosis of many activated growth-factor receptors is necessary for efficient activation of the Raf-MEK-ERK/MAPK cascade, which might in part reflect a need to internalize MEK that has been activated at the plasma membrane to efficiently activate ERK/MAPK^{98–100}. This has been the subject of a recent detailed review¹⁰¹ and so is not discussed further here. In the narrower context of oncogenic Ras signalling, dynamin-dependent endocytosis contributes to the activation of Raf-1 by H-rasG12V but not by K-rasG12V¹⁰². In addition, mutants of Rab5 that increase endocytosis but reduce endocytic recycling can trap activated H-ras, but not K-ras, in enlarged endosomes. This could reflect either greater uptake of activated H-ras than K-ras into endocytic vesicles, or a faster rate of recycling of K-ras back to the

plasma membrane, or both. A preferential enrichment of CFP-H-ras over CFP-K-ras in endosomes is also evident in live cells⁷⁷. Ras microlocalization is implicated in this differential endosomal sorting because the distribution of H-ras-K-ras chimaeras is influenced by the origin of the carboxy-terminal HVR¹⁰².

This interplay between Ras signalling and endocytosis is physiologically relevant because a recently identified Ras effector, **Rin1** (REF. 103), operates as an exchange factor for Rab5 (REF. 104). So activated Ras, through Rin1, stimulates GTP-loading of Rab5 and, in turn, stimulates endocytosis and early endosomal fusion¹⁰⁴. General conclusions from this work are that activated Ras is present in endosomes in growth-factor-stimulated cells and in cells that are transformed by oncogenic mutant Ras. Endosomal Ras is competent to engage Raf and, in the case of H-ras, endocytosis is required for maximal signal output.

A role for non-plasma-membrane-localized Ras?

The answer to whether non-plasma-membrane-localized Ras has a specific role remains uncertain. On the one hand, signal output from Golgi-localized and ER-localized H-ras is quantitatively different from H-ras that is localized to the plasma membrane. And, as H-ras and N-ras show some degree of Golgi localization, whereas K-ras does not, the different signal outputs from palmitoylated Ras versus K-ras could be explained not only in terms of localization to different microdomains of the plasma membrane, but also to different subcellular compartments. Furthermore, the kinetics of endogenous Ras activation on the Golgi are different from those of Ras that is activated at the plasma membrane. The duration, as well as the magnitude, of ERK/MAPK activation has a profound effect on the cellular response to Ras activation¹⁰⁵, and so the prolonged activation of Ras on the Golgi could be important in driving cell differentiation rather than proliferation. On the other hand, to sustain this argument, we need to have some idea of what fraction of Ras is normally present on endomembranes compared with the plasma membrane. If the endomembrane fraction is low, then the contribution to biological function of Ras could be small. Examination of the subcellular localization of oncogenic mutant H-ras in Madin-Derby canine kidney (MDCK) cells by EM showed that Ras was localized extensively to the inner surface of the plasma membrane, but was also present on endocytic vesicles immediately adjacent to the plasma membrane and the cytoplasmic surface of vesicles in the Golgi¹⁰⁶. Formal quantification of these different pools was not attempted. Similarly, cell fractionation and metabolic labelling experiments designed to estimate the relative amounts of endogenous Ras in the Golgi and plasma membrane have so far only shown that a substantial amount of newly synthesized Ras (presumably N-ras and/or H-ras) passes through the Golgi²². It seems safe to assume that endogenous K-ras is not Golgi-localized, because even when it is overexpressed, K-ras is almost exclusively associated with plasma membrane. However, the extent to which endogenous H-ras and N-ras are stably localized in the Golgi still remains to be determined.

An alternative view is that, even if there is only a small pool of endogenous Ras in the Golgi, it could be biologically important in living cells. The Golgi is a highly dynamic structure and what seems to be stable must nevertheless reflect a pool of Ras that is rapidly turning over, being replenished both from the plasma membrane and the ER as fast as exocytic transport returns it to the cell surface. Inherent in such a model are numerous possibilities for sequestering essential Ras signalling complexes away from negative regulatory mechanisms that operate at the plasma membrane. The only conclusion that can be safely drawn at this point is that more work is needed to discover the biological role of Golgi-localized Ras.

Conclusions

Exciting new developments have shown that Ras is able to signal from intracellular membranes in addition to the plasma membrane. Many of the data that underpin these observations are, however, based on the ectopic expression of mislocalized Ras mutants. So far, the best evidence that intracellular endogenous Ras can signal is the Golgi pool of endogenous Ras-GTP that is generated in response to growth-factor stimulation⁸³. To determine the biological relevance of intracellular Ras signalling, it will be important to establish not only the extent of endogenous Ras association with Golgi and endosomal membranes, but also to quantify the signal output at these sites compared with that at the plasma membrane. These are technically challenging problems.

Recent studies have also yielded new insights into what remains the main Ras signalling platform, the plasma membrane. The idea that different Ras isoforms associate dynamically with spatially distinct microdomains of the plasma membrane is an important conceptual advance in Ras signalling, which also has wider implications. These microdomains need to be characterized further, in terms of their lipid and protein composition, to test formally the underlying hypothesis that the precise microenvironment in which Ras engages a set of effector proteins governs the ultimate signal output. Data from recent biophysical studies also indicate that the lipid microstructure of the plasma membrane might be malleable, which adds even more complexity to our evolving understanding of how Ras signalling complexes might be assembled at the plasma membrane. Further work is also needed to elucidate how GTP loading in the amino-terminal catalytic domains of H-Ras influences carboxy-terminal membrane interactions. Is this through conformational changes that are transmitted through the protein to the membrane anchor, or by as-yet-uncharacterized interactions between Ras effector proteins and plasma-membrane proteins or lipids? Finally, Ras is just one example of a lipidated signalling protein that is localized in the plasma membrane. To what extent will the complex membrane interactions of the different Ras isoforms be recapitulated — not only by other Ras-related proteins, but also, for example, by the Src-family kinases, which have an array of amino-terminal membrane anchors that are similar to H-ras, N-ras and K-ras?

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