# Visualizing Ras signalling in real-time

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## Summary

**Ras GTPases are universal molecular switches that act as kinetic timers of signal transduction events. They are post-translationally modified by the addition of lipid groups to their hypervariable carboxyl termini, which plug the proteins to membranes and influence their dynamic sorting and trafficking. For the past twenty years, the plasma membrane has been considered to be the predominant platform from which Ras operates. Recent work using live-cell imaging and novel probes to visualize where and when Ras is active has supported this long-held belief. However, an equally fascinating aspect of these imaging studies has been the discovery of dynamic Ras activity, as well as distinct signal output, from intracellular organelles. Activation of Ras on the Golgi exhibits kinetics different from Ras activation on the plasma membrane, and compartmentalized Ras signalling seems particularly prominent in lymphocytes. However, data on the spatial and temporal regulation of Ras activity has frequently differed depending on the nature of the probe, the cell type and the stimulus. Nevertheless, because Ras traffics through endomembranes en route to the plasma membrane, it seems likely that Ras can signal from such compartments. The burning question in this field concerns the significance of this observation for endogenous Ras signalling output.**

Key words: Ras, GTPase, Raf, Reporter, Probe, Biosensor, Imaging, Signalling, Membrane, Trafficking, Plasma membrane, Golgi, Endoplasmic reticulum, Endosomes, Prenylation, Palmitoylation

## Introduction

Three human *Ras* proto-oncogenes encode small GTPases (H-Ras, N-Ras, K-Ras4A and K-Ras4B) that operate as binary molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form at the membrane (Hancock, 2003). Each GTPase has the capacity to transduce signals from cell-surface receptors into the cytoplasm through specific effector pathways that regulate cell growth, differentiation and apoptosis. Ras exhibits slow rates of GDP/GTP exchange and GTP hydrolysis; thus, the fraction of cellular Ras in an active conformation depends on the rates of these reactions (Bourne et al., 1990). Guanine-nucleotide-exchange factors (GEFs) bind to Ras and markedly accelerate the rate of GDP dissociation. By contrast, deactivation requires the binding of GTPase-activating proteins (GAPs) that significantly enhance the intrinsic GTPase activity. A defective 'off' switch in this cycle has major implications for human disease. Mutant proteins that have specific point mutations that render the GTPase insensitive to GAP stimulation are locked in the GTP-bound state, causing aberrant downstream signalling (Downward, 2003). This can promote cell proliferation and protection from apoptosis; indeed, approximately 30% of human cancers contain oncogenic Ras mutants.

In 1980, pioneering work from Scolnick and colleagues (Willingham et al., 1980) demonstrated that H-Ras and K-Ras proteins are predominantly located on the inner leaflet of the plasma membrane in Harvey and Kirsten murine sarcoma virus (MSV)-transformed cells [see Malumbres and Barbacid (Malumbres and Barbacid, 2003) for a timeline detailing the identification of *Ras* oncogenes]. Immunocytochemistry indicated no specific localization of Ras in the nucleus or other intracellular sites. However, using electron microscopy, they observed that 'some p21 was seen on the cytoplasmic surface of what appeared to be uncoated endocytic vesicles near the plasma membrane, and small amounts were seen on the cytoplasmic face of a few vesicles in the Golgi apparatus'. With remarkable foresight, they speculated that MSV-induced transformation by Ras involves processes associated with guanine-nucleotide-binding proteins in the plasma membrane (Willingham et al., 1980). In hindsight, there was also evidence in many later studies that a significant amount of H-Ras is localized to intracellular structures such as the Golgi (Leevers et al., 1994; Marais et al., 1995; Thissen et al., 1997), but this was not further investigated at the time.

We now know far more about how post-translational lipid modifications direct Ras proteins to associate with multiple cell membranes, including the plasma membrane (Fig. 1), so that Ras can be activated (Apolloni et al., 2000; Choy et al., 1999; Dong et al., 2003; Lobo et al., 2002). Recently, significant advances have been made in understanding how post-translationally modified Ras isoforms traffic to the plasma membrane from the endoplasmic reticulum (ER) and Golgi (Apolloni et al., 2000; Choy et al., 1999; Dong et al., 2003; Lobo et al., 2002), are segregated in membrane domains (Hancock, 2003), and are differentially internalized and signal from endosomes (Roy et al., 2002). Perhaps one of the more provocative questions arising from this work concerns the signalling role of Ras-GTP on intracellular organelles such as the ER and Golgi (Bivona and Philips, 2003; Chiu et al., 2002). If these are important sites for interactions between Ras and its effectors, then their proximity to the nucleus has implications for pathways driving the control of gene transcription. In addition to endosomes, the ER and Golgi, another enigmatic location for Ras signalling are the mitochondria, which poses

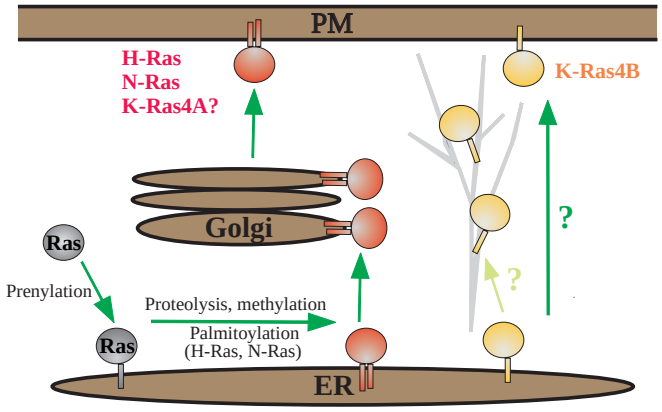



**Fig. 1.** A model for the subcellular localization of mammalian Ras proteins. A protein farnesyl transferase adds a farnesyl group (prenylation) to the cysteine residue of the Ras CAAX motif (A=aliphatic, X=any amino acid), which thereby anchors the protein to the endoplasmic reticulum (ER). Rce1 (Ras and a-factor-converting enzyme) and isoprenylcysteine carboxyl methyltransferase remove the AAX and methylate the farnesyl-cysteine residue. H-Ras and N-Ras (and possibly K-Ras4A) are palmitoylated on cysteine residues in their hypervariable domains and enter the classical secretory pathway en route to the plasma membrane (PM). Palmitoylation is an unstable modification and might be a means by which the rate of Ras trafficking can be regulated (Hancock, 2003). Studies indicate that the nucleotide status of Ras can also influence the stability of this modification (Baker et al., 2003). K-Ras has a polylysine sequence instead of cysteine residues and is not palmitoylated; instead, it bypasses the Golgi via a non-classical secretory pathway. This might depend on microtubules (Thissen et al., 1997), although there has been no evidence that fluorescently tagged K-Ras4B is delivered along microtubule tracks in live cells.

interesting questions about the balance between cell survival and apoptosis (and subversion by oncogenic Ras) in this compartment (Rebollo et al., 1999).

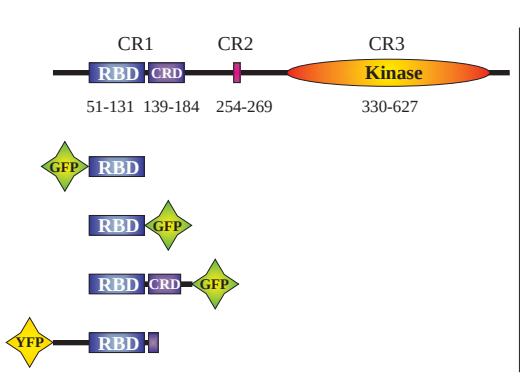
In the literature, a canonical view has developed for receptor tyrosine kinase (RTK) signalling cascades in which a Ras GEF (Sos) is recruited by adaptors to the receptor in order to activate Ras at the plasma membrane (Pawson, 2004). Ras is deactivated by the recruitment of GAPs such as p120 Ras GAP through Src-homology 2 (SH2) domains to phosphotyrosine residues on the activated receptor. The delineation of this pathway was a major advance in signal transduction research more than a decade ago (Malumbres and Barbacid, 2003; Pawson, 2004) and was soon followed by the demonstration that Ras-GTP recruits the serine/threonine kinase Raf to the plasma membrane to facilitate Raf activation (Leevers et al., 1994; Stokoe et al., 1994; Traverse et al., 1993). In addition, there are many dynamically regulated Ras GEFs and Ras GAPs that do not operate through phosphotyrosine-based recognition motifs at the RTK. For example, there is strong evidence that members of the GRP/CalDAG-GEF family and some members of the GAP1 family are specifically regulated by the second messengers $Ca^{2+}$ and/or diacylglycerol (DAG) (Cullen and Lockyer, 2002; Walker et al., 2003). The physiological significance of such modulation is not yet clear for many of these proteins, except for Ras GRP1, which has been shown to be a major target for DAG during T-cell receptor (TCR) signalling and is needed for proper thymocyte development in the mouse (Dower et al., 2000).

Since many RTKs are coupled to phospholipase C (PLC) signalling through PLCγ, perhaps it is of no surprise that there are GEFs and GAPs that are able to respond to the 'products' of PLC activity – $Ca^{2+}$ and DAG. What has been particularly exciting from this signalling perspective has been the recent discovery of a novel class of PLC, PLCε, as a candidate for an effector of Ras (Kelley et al., 2001; Lopez et al., 2001; Song et al., 2001). The most studied target of DAG is protein kinase C (PKC), and it has long been known that PKCs integrate DAG and $Ca^{2+}$ signals at the level of Ras and Raf through multiple mechanisms (Corbit et al., 2003; Kawakami et al., 2003; Marais et al., 1998), including potentially inhibiting GAP activity (Downward et al., 1990; Marais et al., 1998; Villalonga et al., 2002). Analysis of the spatio-temporal regulation of PKC isoforms by second messengers has offered great insight into the dynamic nature by which they are differentially recruited to membranes and scaffolds. The C2 and C1 domains of PKC are essential for these mechanisms and similar domains are built into members of the GRP and GAP1 families (Cullen and Lockyer, 2002; Walker et al., 2003), enabling them to respond dynamically to a given second messenger signal (Bivona et al., 2003; Caloca et al., 2003; Lockyer et al., 2001; Walker et al., 2004). Thus, it would seem that the study of Ras activity in space and real-time is of importance. Only with the development of cell-based assays to complement existing technologies will issues such as specificity, compartmentalization and effector output be truly refined.

Work in the past few years using Raf, or domains from Raf, as activity probes for Ras in live cells has highlighted unexpected mechanisms and locations for Ras signal output (Bivona and Philips, 2003; Hancock, 2003; Hingorani and Tuveson, 2003). Such methodologies use different domains from Raf fused to fluorescent proteins as probes, and can take advantage of additional fluorescence resonance energy transfer (FRET; Fig. 2). Here, we review the use of these biosensors, their advantages and their inherent limitations. Although we have concentrated here on Ras, activity probes for other small GTPases show equal promise (Pertz and Hahn, 2004).

## Use of Ras activity probes reporting membrane localization

Determining the activity of Ras following cell lysis is a sensitive method for analysing endogenous Ras-GTP levels (Gibbs, 1995; Satoh and Kaziro, 1995; Taylor et al., 2001; van Triest et al., 2001); however, there are some disadvantages. First, measurements are an average across the whole population, which is a problem if cells are behaving asynchronously after stimulation. Second, each time point is only a snap-shot record; therefore, resolution is limited by the delay between analyses. Third, determining spatial information about Ras signalling is difficult or impossible owing to the nature of cell lysis and the imprecision of cell fractionation. Because Ras needs to be attached to a membrane to be active, several groups have recently independently used the Ras-binding domain (RBD) from Raf fused to GFP (GFP-RBD) as a fluorescent reporter of Ras-GTP, following its membrane localization in real-time (Bondeva et al., 2002; Chiu et al., 2002) (Fig. 3).




| Construct | Reference |
|---|---|
| Raf-1 | |
| GFP-RBD(51-131) | Chui et al., 2002<br>Bivona et al., 2003<br>Caloca et al., 2003 |
| RBD(51-131)-GFP | Bondeva et al., 2002 |
| RBD(51-200)-GFP | Bondeva et al., 2002 |
| YFP-RBD(1-153) | Jiang and Sorkin, 2002 |

**Fig. 2.** Ras activity probes. These reporters are based on the Ras-binding domain (RBD) from Raf-1 fused to a fluorescent protein. Active Ras has been detected by the degree of membrane localization, in some cases with the additional sensitivity of a FRET indicator. Numbering applies to the human Raf-1 primary sequence. CR1 is the N-terminal conserved region of Raf-1 containing the RBD and cysteine-rich domain (CRD). CR2 is a serine- and threonine-rich region containing regulatory phosphorylation sites. CR3 is the catalytic domain of the kinase. Studies that have used various different domains as reporters are indicated and discussed in the text.

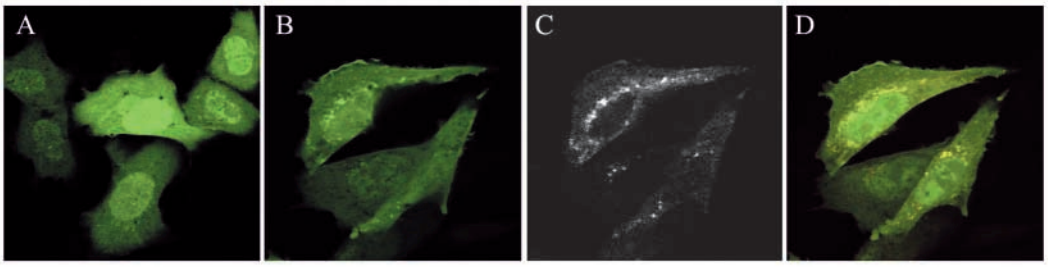



**Fig. 3.** Localization of ectopic H-Ras-GTP in non-starved CHO cells. CHO cells were transfected with GFP-RBD (51-131) in (A) or co-transfected with H-Ras (B-D) and fixed 24 h later. (A) The GFP-RBD is diffusely localized throughout the cytoplasm and is not clearly associated with any intracellular structure or feature at the plasma membrane. Although the GFP-RBD can be occasionally seen in small membrane ruffles, this is probably due to the nonspecific trapping of fluorescent protein since GFP alone can produce a similar localization. Single confocal z-section shown. (B) Co-transfection with H-Ras causes the recruitment of the GFP-RBD to perinuclear structures, and plasma membrane ruffles, in non-starved cells. Single confocal z-section shown. (C) Immunostaining for total Ras expression. (D) Maximum projection overlay of eight equally spaced z-sections from the dorsal to the ventral cell surface showing co-localization of GFP-RBD (green) and H-Ras (red).

Philips and co-workers have concentrated on over-expressing Ras isoforms along with a construct containing GFP fused to residues 51-131 of Raf-1 (Chiu et al., 2002). In their hands, a significant fraction of ectopic H-Ras appears to localize to the Golgi apparatus when co-expressed with GFP-RBD in non-starved COS-1 cells (Chiu et al., 2002). In the absence of serum, the GFP-RBD probe is entirely cytosolic, indicating that serum factors mediate the GTP loading of over-expressed Ras at the Golgi. In addition, constitutively active Ras co-expressed with GFP-RBD causes recruitment of the reporter to cell membranes, and no membrane-associated probe is detected by co-expression with dominant-negative Ras (Chiu et al., 2002). H-Ras mutants that cannot be palmitoylated, and therefore are unable to traffic through the secretory pathway to the plasma membrane, induce the translocation of GFP-RBD to the ER and Golgi. This indicates that, once farnesylated, over-expressed H-Ras can be GTP loaded on endomembranes, including the ER. Although no ER-resident Ras-GTP-interacting protein has been found in mammalian cells, recent data indicate that there are novel candidates in budding yeast (Sobering et al., 2003). Unlike H-Ras and N-Ras, K-Ras4B contains a polybasic sequence rather than sites for palmitoylation (K-Ras4A is palmitoylated but K-Ras4B is not). K-Ras4B exits the ER and bypasses the Golgi altogether en route to the plasma membrane (Fig. 1). Thus, when co-expressed with K-Ras4B, no GFP-RBD reporter localizes to the Golgi but it clearly associates with the plasma membrane (Chiu et al., 2002).

An exciting aspect to the study became apparent when Philips and co-workers monitored the behaviour of the probe during agonist stimulation (Fig. 4). They treated serum-starved COS-1 cells over-expressing H-Ras with epidermal growth factor (EGF) or insulin and observed that the plasma membrane and the Golgi exhibit different kinetics of probe recruitment (Bivona and Philips, 2003; Chiu et al., 2002). The translocation of the RBD to the Golgi was shown to be independent of endocytosis and was therefore due to active Ras in situ. In contrast to the recruitment of the RBD to the plasma membrane, translocation to the Golgi was dependent on Src kinases. To assay the Golgi response for endogenous Ras, Philips and co-workers used a novel ‘bystander’ FRET technique (Chiu et al., 2002): cyan fluorescent protein (CFP) was fused to the RBD and co-transfected with the transmembrane protein CD8 fused to yellow fluorescent protein (YFP-CD8). This labelled membranes with YFP-CD8, permitting FRET between CFP-RBD and YFP-CD8 when the RBD was bound to endogenous Ras-GTP in close proximity to over-expressed CD8. When starved cells expressing these constructs were stimulated by EGF or insulin, an increased FRET signal was detected on the plasma membrane and Golgi

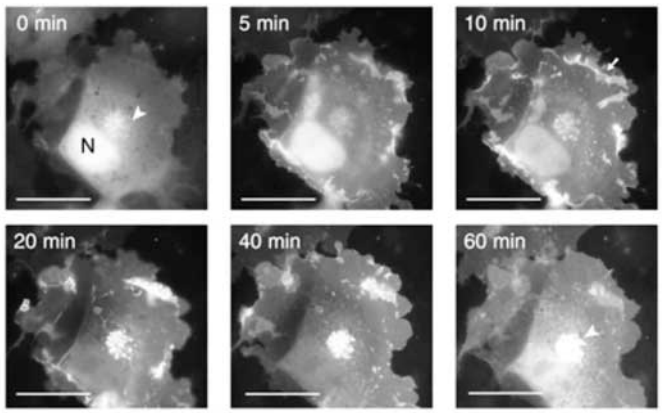



**Fig. 4.** Mitogen-stimulated activation of H-Ras on the PM and Golgi. COS-1 cells were transiently co-transfected with GFP-RBD (51-131) and H-Ras. 24 h after transfection, cells were serum-starved overnight and live-cell imaging was performed at 37°C. Digital images of a single cell were obtained before and after stimulation with EGF, as indicated, with identical acquisition settings. The nucleus (N) is marked. The arrow indicates activated regions of the PM and the arrowhead indicates the position of the Golgi. Figure reproduced with permission from the Nature Publishing Group (http://www.nature.com/ncb/) (Chiu et al., 2002).

(Chiu et al., 2002). Chiu et al. concluded that Src-dependent activation of a specific GEF(s) or inhibition of a specific GAP(s), or both, could be responsible for the spatial and temporal profiles of plasma membrane versus endomembrane Ras activity after mitogenic stimulation (Chiu et al., 2002). The implication of this work was the existence of specific pathways to Ras activation on alternative membrane compartments, and therefore potentially broader Ras effector signalling output.

Bondeva et al. used a different approach, avoiding co-transfection of GFP-RBD with Ras constructs (Bondeva et al., 2002). They compared the location of the probe in normal versus H-Ras-transformed NIH 3T3 cells (Bondeva et al., 2002). Their RBD constructs were fused at the C-terminus to GFP, unlike the Chiu et al. construct that had GFP at the N-terminus. This difference in orientation made no difference to reporter behaviour (T. Balla, personal communication). It is worth considering the physiological relevance, since Ras expression levels in virally transformed cells are likely to be substantially lower than those driven by a strong plasmid promoter in transiently transfected cells. RBD-GFP (residues 51-131) had little or no membrane localization in starved H-Ras-transformed NIH 3T3 cells; yet, incorporation of the Raf-1 cysteine-rich domain (CRD) into the probe (RBD-CRD residues 51-200; Fig. 2) was sufficient to induce a clear membrane translocation. This also correlated with in vitro analysis of the strength of the interactions between RBD (51-131) and Ras versus those between RBD-CRD (51-200) and Ras (Bondeva et al., 2002). Over-expression of the RBD-CRD reporter in the studies by Bondeva et al. appeared to saturate Ras-binding sites at the membrane, suggesting that there is a limit to the Ras-binding sites available even at moderate levels of RBD-CRD expression (Bondeva et al., 2002).

In common with Chiu et al., Bondeva et al. were able to use a GFP reporter to monitor the spatio-temporal kinetics of Ras activation, although they did not need a FRET method to detect endogenous Ras in normal NIH 3T3 cells stimulated by growth factor (Bondeva et al., 2002). However, unlike Chiu et al., they observed no localization of the reporters to the Golgi even in COS cells expressing constitutively active Ras (Bondeva et al., 2002). Their results thus argue against the idea that Ras is significantly active on endomembranes.

## Activation of Ras on the Golgi

Bivona et al. have provided an explanation for the observation that Ras might be activated on the Golgi after mitogenic stimulation (Fig. 4) by showing that Ras GEF GRP1 is a key exchange factor at this compartment (Bivona et al., 2003). In addition to a CDC25-homology GEF domain, GRP1 contains a pair of $Ca^{2+}$-binding EF hands and a DAG-binding C1 domain (Ebinu et al., 1998). The GRP1 transcript is detectable in several tissues but synthesis of the protein is particularly marked in primary mouse thymocytes and various mouse and human T-cell lines (Ebinu et al., 2000). *Grp*-null mice have marked defects in thymocyte differentiation, proliferation and DAG-dependent Ras signalling (Dower et al., 2000). Remarkably, in H-Ras-transfected Jurkat T cells, Bivona et al. demonstrated using the GFP-RBD reporter that Ras activation is restricted to the Golgi and is undetectable at the plasma membrane after TCR stimulation (Bivona et al., 2003). This coincides with PLCγ-dependent translocation of GRP1 to the Golgi, and knockdown of GRP1 by RNA interference (RNAi) inhibits Ras activation on the Golgi apparatus after TCR stimulation (Bivona et al., 2003). The lack of Ras activation at the plasma membrane could be attributable to the function of the $Ca^{2+}$-triggered Ras GAP CAPRI (Lockyer et al., 2001). By over-expressing GRP1 with GFP-RBD, Caloca et al. have independently concluded that ectopic GRP1 can activate endogenous Ras on the Golgi and endoplasmic reticulum in COS-1 cells (Caloca et al., 2003). Mounting evidence thus supports roles for novel signalling pathways regulating Ras activity on compartments other than just the inner leaflet of the plasma membrane.

## FRET-based Ras activity probes

FRET analysis using CFP as a fluorescent donor and YFP as an acceptor has become widespread in imaging applications and is rapidly becoming the method of choice for determining biochemical interactions over short distances in live cells (Miyawaki, 2003). However, the technique requires carefully controlled measurements to avoid artefacts. Because the spectral overlap between CFP and YFP can cause direct acceptor excitation by donor excitation, care must be taken when determining sensitized emission (Zimmermann et al., 2002). FRET efficiency depends on the concentration of donor and acceptor, which must be normalized for, unless an intramolecular reporter is used. Finally, motion artefacts can generate false FRET signals and should also be controlled for (Chamberlain et al., 2000).

Current FRET methods for Ras-GTP detection rely on over-expression of Ras and reporter constructs. In theory, the technique is sensitive and quantitative compared with analysis of the fluorescence intensities of GFP-RBD at the membrane versus the cytosol. Jiang and Sorkin (Jiang and Sorkin, 2002) have successfully used a corrected FRET method (Gordon et

al., 1998) to detect FRET between YFP-RBD (residues 1-153) and active CFP-Ras (Jiang and Sorkin, 2002). In experiments with starved cells, Jiang and Sorkin detected significant levels of ectopic, active H-Ras on cell membranes – predominantly perinuclear vesicular structures that could include the Golgi (Jiang and Sorkin, 2002). In contrast to Chiu et al., they concluded that over-expression of H-Ras led to activation of a substantial pool of small GTPase, regardless of serum starvation or even growth factor stimulation. This indicated that the method is more suitable for tracking the movement of active Ras, rather than where and how much activation is occurring per se (Jiang and Sorkin, 2002).

The Raichu-Ras probe is an innovative FRET reporter designed to assay active Ras in live cells by reporting the sum of GEF and GAP activities on the compartment it is targeted to (Mochizuki et al., 2001). This hybrid molecule (Fig. 5A) reports the intramolecular association of an RBD (51-131) with H-Ras, although the reporter actually includes the hypervariable region of K-Ras to ensure membrane localization. The probe has produced results in marked contrast to those of Chiu et al., despite use of the same cell type and equivalent stimulation (Miyawaki, 2003). In COS cells stimulated with EGF, the Raichu-Ras probe is clearly activated only at the plasma membrane (Mochizuki et al., 2001) (Fig. 5B). The explanation for the discrepancy between techniques is not clear, although this could be due to the nature of the K-Ras4B-specific post-translational modifications on the Raichu-Ras probe, which should direct it to the plasma membrane and bypass the Golgi altogether, despite the H-Ras coding sequence. However, merely replacing the H-Ras sequence with that of Rap1 (Raichu-Rap1) generates a reporter that appears to be selectively activated at perinuclear sites, and not the plasma membrane. This supports previous observations that Rap1 is predominantly localized to intracellular compartments and not to the plasma membrane. However, the functional significance of the Mochizuki et al. study has been disputed by others, who have measured mitogenic Rap1 activation exclusively at the plasma membrane using GFP reporters in the same cell lines (Bivona et al., 2004).

Matsuda and co-workers have taken a step further to offer a very different hypothesis for the localization of EGF-induced Ras signalling (Ohba et al., 2003). Provocatively, they have proposed that a gradient of cellular GAP activity, with the highest deactivation of Ras in the centre, radiates out to the plasma membrane. This idea was based on observing the uniform cytoplasmic expression of an artificial cAMP-responsive Ras GEF (e-GRF) but functional activation of Ras only at the peripheral plasma membrane (Ohba et al., 2003). In addition, Ras appeared to be uniformly activated in EGF-stimulated cells when Raichu-Ras probes with reduced sensitivity to GAPs were used (Ohba et al., 2003). Kinetic simulations of GEF and GAP activity were integrated into a virtual cell model to support the theory of a GAP gradient. At first glance, this is an eccentric proposition given that several potent Ras GAPs, including GAP1$^{m}$, which was used in the Raichu-Ras studies, are specific sensors of signalling events at the plasma membrane. This is because they dynamically translocate from the cytosol to this compartment in response to an agonist-evoked stimulus, such as the localized generation of a second messenger. For example, p120 Ras GAP is believed to terminate Ras signalling by being recruited to RTKs

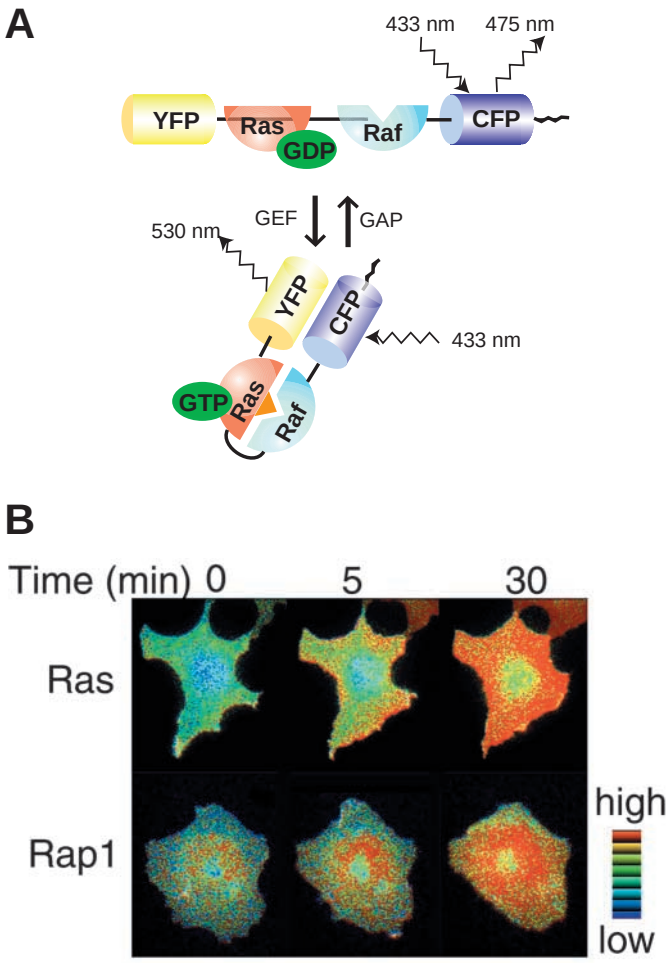



**Fig. 5.** The Raichu-Ras FRET probe. (A) Schematic representation of Raichu-Ras bound to GDP or GTP. (B) EGF activation of Ras. Intensity-modulated display mode (IMD) images of COS-1 cells expressing Raichu-Ras or Raichu-Rap1 and stimulated by EGF. Figure reproduced with permission from the Nature Publishing Group (http://www.nature.com/nature/) (Mochizuki et al., 2001).

(Pawson, 2004), GAP1$^{m}$ is a high-affinity receptor for phosphatidylinositol 3,4,5-trisphosphate at the plasma membrane (Lockyer et al., 1999) and CAPRI is a $Ca^{2+}$-activated Ras GAP when it translocates to the cell periphery (Bivona et al., 2003; Lockyer et al., 2001). It is of course possible that there are specific Ras GAPs that are active in the perinuclear region of the cell. Neurofibromin (NF-1) is just such a candidate and has been localized to both mitochondria (Roudebush et al., 1997) and microtubules (Gregory et al., 1993). However, the exceptional difficulty of working with NF-1 has precluded detailed study of its molecular regulation. Further experimental testing of the virtual cell theory of a Ras GAP gradient is required for it to gain credence (Ohba et al., 2003).

Full-length Raf-1 has also been tried as a FRET or membrane-localization reporter for active Ras with varying degrees of success (Bondeva et al., 2002; Hibino et al., 2003; Jiang and Sorkin, 2002). This might reflect the complex regulation of Raf-1 membrane recruitment (Bondeva et al.,

2002) or the unfavourable positioning of fluorescent protein partners in Ras and Raf for FRET analysis (Jiang and Sorkin, 2002). For detectable translocation of GFP-Raf-1 in EGF-stimulated cells, Hibino et al. had to over-express Ras (Hibino et al., 2003). They observed sustained recruitment of a small proportion of Raf to membrane ruffles for more than 60 minutes (Hibino et al., 2003). The physiological significance of this result is unclear and requires further investigation given that Ras activity returns to low levels within 30-60 minutes of mitogenic stimulation (Hibino et al., 2003). Moreover, they also observed perinuclear accumulation of Raf-1, suggesting active Ras is present on endomembranes (Hibino et al., 2003).

## Perspectives

The application of imaging techniques has begun to facilitate the analysis of Ras signalling events in real-time but there is plenty of scope for further development of what are currently relatively crude tools. The necessity of having to over-express Ras is the major disadvantage of the technique; in some studies, five times more Ras cDNA than reporter construct has been required, and it remains to be seen how far the interpretation of ectopic Ras signalling events can be applied to the endogenous situation. In theory, both the over-expression of Ras and the over-expression of the RBD could have unwanted side effects. For Ras, this is potentially a more serious problem given that the protein must be post-translationally processed by a series of enzymatic steps (Fig. 1). Exactly how much endogenous Ras resides on endomembranes in a given cell type at steady state is a burning issue. Several studies have suggested that a significant amount of Ras is present on intracellular membranes and/or the cytoskeleton, by immunocytochemistry (Choy et al., 1999; Lorenzo et al., 2001; Perez de Castro et al., 2004; Thissen et al., 1997). Whether the distribution of exogenous Ras matches endogenous and how the machinery for Ras post-translational modification handles the load are not clear. If Ras proteins are using the classical secretory pathway to reach the plasma membrane, then this is a minor concern. However, K-Ras4B does not, and little is known about the mechanisms for Ras trafficking and sorting from various compartments in mammalian cells; so the problem of handling large quantities of exogenous protein could apply to this machinery. It is also unclear whether any overload alters the activity of Ras in separate compartments, and this is a potential issue if endogenous GEF and GAP activities are differentially compartmentalized.

When considering the spatio-temporal analysis of Ras activity, it is worth noting some specific effects that over-expression might influence. There are interesting differences in palmitoylation between Ras isoforms – for example, H-Ras is probably twice as palmitoylated as N-Ras (Hancock, 2003). The degree of palmitoylation could vary between membrane compartments and might even depend on the activation status of the small GTPase (Baker et al., 2003). If there are differences in palmitoylation between proteins and between cellular compartments, then this could be a concern when one compares over-expression results obtained with different Ras isoforms. The post-translational enzymatic machinery might have to work much harder to modify H-Ras, and this could impact on differences in localization and activation status. Thus, validation of endogenous Ras activity is particularly important – for example, by the bystander FRET method (Chiu et al., 2002). Ras might also influence the dynamics of the very organelles that it traffics through in the secretory pathway; for example, inducible expression of oncogenic N-Ras causes the collapse of the Golgi complex and an increase in constitutive protein transport in NRK cells (Babia et al., 1999).

Experiments to determine the spatio-temporal pattern of activation of Ras by EGF stimulation have led to different conclusions: co-expression of H-Ras and GFP-RBD indicated a rapid activation at the plasma membrane followed by later activation on the Golgi (Chiu et al., 2002), whereas analysis with Raichu-Ras indicated activation at the plasma membrane but not at perinuclear sites, where Rap1-GTP loading was clearly enhanced (Mochizuki et al., 2001). The Raichu-Ras probe is an odd mixture of H-Ras and the RBD, membrane localization being provided by the hypervariable region of K-Ras4B (Mochizuki et al., 2001). It is becoming increasingly apparent that the nature of the hypervariable group on Ras proteins has significant influence on signalling specificity by determining membrane microlocalization and the efficiency of interaction with GEFs, effectors and even GAPs (Jaumot et al., 2002). Differences in post-translational modification between Ras isoforms and the possible influence on GEF specificity is a factor that should be considered when using chimeric Ras molecules such as Raichu-Ras. There is evidence that GEFs are sensitive to the prenylation status of Ras-family GTPases (Gotoh et al., 2001) and Sos1 requires prenylation of Ras proteins for efficient nucleotide exchange (Porfiri et al., 1994). If the Raichu-Ras probe is further developed, it would be interesting to couple H-, N- and K-Ras GTPases with their respective hypervariable domains and then determine the spatio-temporal profile of activation to see whether there are any differences.

Is the RBD sufficiently specific to report Ras activation over Rap? The answer so far is yes but only because so many studies have relied on the co-expression of a Ras isoform, and little detectable membrane localization has been seen in untransfected cells. The probe simply does not appear sensitive enough to report endogenous Ras-GTP with a great dynamic range – this conclusion is based purely on quantifying the amount of fluorescence at a given membrane – so interference from endogenous Rap has not been so much of an issue. There has been no evidence that the RBD is efficiently recruited to Rap1 in live cells even following over-expression of Rap-GTP (Chiu et al., 2002), although the RBD does work well in the Raichu-Rap1 intramolecular FRET reporter (Miyawaki, 2003).

Is the RBD useful if it inhibits effector and GAP interactions? In the case of Raichu-Ras, this does not appear to be a problem (Mochizuki et al., 2001); however, it might be when GFP-RBD is expressed alone. For example, Chiu et al. reported that insulin activates Ras on the plasma membrane and Golgi, using over-expressed H-Ras and GFP-RBD (Chiu et al., 2002). If the RBD inhibits coupling to Raf then this could have major consequences for GEF stimulation of Ras-GTP, since the mitogen-activated protein kinase kinase (MEK) can feedback on the Grb2-Sos complex to limit insulin-dependent Ras activation (Waters et al., 1995). However, such potential problems might not be as serious as they first appear. Although the RBD inhibits stimulation of the GTPase activity of Ras by p120 Ras GAP in vitro and blocks oncogenic-Ras-mediated

germinal vesicle breakdown (GVBD) in *Xenopus* oocytes, it has no effect on progesterone or insulin stimulation of GVBD through the endogenous Ras pathway (Scheffler et al., 1994). Similarly, RBD-GFP (51-131) inhibits ERK2 responses in cells in which RasG12V is over-expressed but has no effect on ERK2 activity in phorbol ester- or EGF-treated cells or in Ras-transformed fibroblasts (Bondeva et al., 2002). This strongly suggests that expression of the minimal RBD has minor effects on downstream Ras signalling and should not be viewed as a significant limitation of the method.

What is the significance and purpose of Ras signalling on the Golgi? This is a difficult question to answer given the difficulty of analysing endogenous Ras signalling on this compartment. H-Ras and N-Ras are on the Golgi, and they must traffic through the compartment on the way to the plasma membrane. So how much endogenous Ras is on the Golgi in a specific cellular context? If this pool is active then what Golgi-localized Ras effectors are engaged? And what are the consequences of compartmentalized Ras signalling? In support of the findings of Philips and co-workers, Perez de Castro et al. have recently shown that low-grade TCR stimulation of Jurkat T cells is specific to endogenous N-Ras and significant endogenous N-Ras resides on the Golgi as judged by immunocytochemistry (Perez de Castro et al., 2004). They detected active N-Ras only on the Golgi of Jurkat cells, using over-expression of N-Ras and YFP-RBD (Perez de Castro et al., 2004). Furthermore, Mitin et al. have evidence of the first Golgi-localized Ras effector, Rain (Mitin et al., 2004). Ectopic Rain is present at a perinuclear, juxta-Golgi region and is recruited to the trans-Golgi region following expression of activated Ras. This suggests that Rain can serve as an effector of Golgi-localized Ras because its localization is influenced by Ras-GTP. A caveat to these studies is that antibodies are not yet available to determine the endogenous location of Rain. Mitin et al. also discovered that Rain co-operates with Raf to cause synergistic transformation of NIH 3T3 cells; thus, Rain is a candidate for a Ras effector on the Golgi. These two studies have provided further evidence that compartmentalized Ras is likely to offer spatially and temporally restricted signalling output (Bivona and Philips, 2003), which should modify the view that Ras operates exclusively at the plasma membrane.

In summary, the use of real-time analysis of Ras signalling events has offered up some surprises and a few new controversies. There are limitations to the techniques, which should be considered when interpreting data, and the requirement for over-expression of Ras is currently an unwanted necessity. Despite these criticisms, many of the studies that have used real-time imaging have provided new insight into the kinetics of Ras activation and deactivation, and have raised the issue of compartmentalized Ras signalling. It will be fascinating to see how the study of the spatio-temporal regulation of Ras signalling on multiple cellular compartments inevitably develops.

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