Identification of a Ras GTPase-activating protein regulated by receptor-mediated Ca^{2+} oscillations

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Receptor-mediated increases in the concentration of intracellular free calcium ($[\text{Ca}^{2+}]_i$) are responsible for controlling a plethora of physiological processes including gene expression, secretion, contraction, proliferation, neural signalling, and learning. Increases in $[\text{Ca}^{2+}]_i$ often occur as repetitive Ca^{2+} spikes or oscillations. Induced by electrical or receptor stimuli, these repetitive Ca^{2+} spikes increase their frequency with the amplitude of the receptor stimuli, a phenomenon that appears critical for the induction of selective cellular functions. Here we report the characterisation of RASAL, a Ras GTPase-activating protein that senses the frequency of repetitive Ca^{2+} spikes by undergoing synchronous oscillatory associations with the plasma membrane. Importantly, we show that only during periods of plasma membrane association does RASAL inactivate Ras signalling. Thus, RASAL senses the frequency of complex Ca^{2+} signals, decoding them through a regulation of the activation state of Ras. Our data provide a hitherto unrecognised link between complex Ca^{2+} signals and the regulation of Ras.

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Introduction

Many receptor tyrosine kinases and G-protein-coupled receptors are linked to the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) through the phosphoinositide-specific phospholipase C (PLC)-induced

hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). Once generated, IP_3 stimulates the release of calcium (Ca^{2+}) from internal stores thereby giving rise to an increase in the concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$). The increase in $[\text{Ca}^{2+}]_i$ is responsible for controlling a plethora of cellular processes such as fertilisation, secretion, contraction, proliferation, neural signalling, and learning (Bootman *et al*, 2001; Berridge *et al*, 2003). Understanding how such a simple ion can regulate so many diverse cellular processes is a key goal of Ca^{2+} and cell biologists. The answer seems to lie in the coupling of the Ca^{2+} signal, in terms of speed, amplitude, and spatiotemporal patterning, to an extensive molecular repertoire of Ca^{2+} sensing pathways that regulate cell physiology (Berridge *et al*, 2003).

Receptor-mediated increases in $[\text{Ca}^{2+}]_i$ are often observed as repetitive Ca^{2+} spikes or oscillations that increase their frequency with the amplitude of the receptor stimuli, a phenomenon that appears critical for the induction of selective cellular functions (Bootman *et al*, 2001; Berridge *et al*, 2003). Indeed, the frequency of Ca^{2+} oscillations determines the efficiency of mitochondrial ATP production (Hajnoczky *et al*, 1995), and gene expression driven by the transcription factors NF-AT, OAP, and NF- κ B (Dolmetsch *et al*, 1998; Li *et al*, 1998). To use such 'frequency modulation', cells have developed decoders that respond to the frequency and duration of the Ca^{2+} signal. At present, however, the only known examples of such decoders are calmodulin (Craske *et al*, 1999), protein kinase C (Oancea and Meyer, 1998; Mogami *et al*, 2003; Violin *et al*, 2003) and calmodulin-dependent protein kinase II (De Koninck and Schulman, 1998; Bayer *et al*, 2002). Here we describe the characterisation of RASAL, a Ras GTPase-activating protein, as a decoder of complex Ca^{2+} signals.

Ras proteins are binary molecular switches that, by cycling between inactive GDP-bound and active GTP-bound forms, regulate multiple cellular signalling pathways including those that control growth and differentiation (Bivona and Philips, 2003; Downward, 2003; Hancock, 2003; Hingorani and Tuveson, 2003). The extent and duration of Ras activation are governed by the interplay between guanine nucleotide exchange factors (GEFs), which induce the dissociation of GDP to allow association of the more abundant GTP, and GTPase-activating proteins (GAPs), which bind to the GTP-bound form and enhance the intrinsic Ras GTPase activity (Bivona and Philips, 2003; Downward, 2003; Hancock, 2003; Hingorani and Tuveson, 2003). The best-characterised upstream signals known to activate Ras emanate from tyrosine kinase-linked receptors. Stimulation of these receptors induces the docking of specific RasGEFs such as mSOS and RasGAPs like p120^{GAP} to the activated receptor, where they coordinate the overall activation of Ras (Bivona and Philips, 2003; Downward, 2003; Hancock, 2003; Hingorani and Tuveson, 2003). However, many other receptor types, including G-protein-coupled receptors, can also activate Ras. In some cases, this has been shown to involve transactivation of growth factor receptor tyrosine kinases, and in others a

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role for $[Ca^{2+}]_i$ has been proposed (reviewed in Cullen and Lockyer, 2002).

Direct evidence for a role of Ca^{2+} in the regulation of Ras signalling has come with the identification of Ca^{2+} -regulated RasGEFs and -GAPs (Cullen and Lockyer, 2002). Ras-GRF1 (Martegani *et al*, 1992; Shou *et al*, 1992; Farnsworth *et al*, 1995) and a closely related protein Ras-GRF2 (Fam *et al*, 1997) function as Ca^{2+} /calmodulin-dependent RasGEFs. A distinct family of GEFs that act on Ras and the related protein Rap1, the GRP/CalDAG-GEF family (Cullen and Lockyer, 2002), is regulated not only by Ca^{2+} but also DAG. This is a four-gene family encoding five GEFs: Ras-GRP1/CalDAG-GEFII, CalDAG-GEFI, Ras-GRP2, Ras-GRP3/CalDAG-GEFIII, and Ras-GRP4 (Ebinu *et al*, 1998; Kawasaki *et al*, 1998; Tognon *et al*, 1998; Clyde-Smith *et al*, 2000; Ohba *et al*, 2000; Yamashita *et al*, 2000; Lorenzo *et al*, 2001). Some of these proteins display sensitivity to Ca^{2+} through direct binding of Ca^{2+} to a pair of carboxy-terminal atypical EF hands, that is, GRP1, CalDAG-GEFI, and GRP2 (Ebinu *et al*, 1998; Kawasaki *et al*, 1998).

A great deal of evidence has also accumulated for the existence of Ca^{2+} -stimulated RasGAPs. For example, p120^{GAP} has been reported to be regulated by Ca^{2+} (Filvaroff *et al*, 1992; Gawler *et al*, 1995a,b), although others have questioned this conclusion (Clark *et al*, 1995). The prototypical Ca^{2+} -triggered RasGAP is CAPRI (Lockyer *et al*, 2001), a member of the GAP1 family (Maekawa *et al*, 1993, 1994; Baba *et al*, 1995; Cullen *et al*, 1995; Yamamoto *et al*, 1995; Lockyer *et al*, 1997, 1999; Allen *et al*, 1998; Minagawa *et al*, 2001; Walker *et al*, 2002). Members of this family, which includes RASAL (Allen *et al*, 1998), share a common molecular architecture of N-terminal tandem C_2 domains, a C-terminal pleckstrin homology domain adjacent to a Bruton's tyrosine kinase motif, and a central catalytic RasGAP-related domain. In unstimulated cells, CAPRI is a cytosolic, inactive RasGAP (Lockyer *et al*, 2001). However, upon agonist-evoked elevation in $[Ca^{2+}]_i$, CAPRI undergoes a rapid, C_2 domain-dependent association with the plasma membrane (Lockyer *et al*, 2001). Importantly, this membrane association activates the RasGAP activity of CAPRI (Lockyer *et al*, 2001).

In the present study, we show that like CAPRI, RASAL is a cytosolic protein that undergoes a rapid translocation to the plasma membrane in response to receptor-mediated elevation in $[Ca^{2+}]_i$, a translocation that activates its ability to function as a RasGAP. However, unlike CAPRI, which undergoes a transient plasma membrane association upon receptor stimulation (Lockyer *et al*, 2001), and does not sense oscillations in $[Ca^{2+}]_i$ (P Lockyer, personal communication, 2003), we show that RASAL oscillates between the plasma membrane and the cytosol in synchrony with simultaneously measured repetitive Ca^{2+} spikes. We propose therefore that RASAL constitutes a molecular machine that can sense the frequency of complex Ca^{2+} oscillations decoding into a dynamic regulation in the activation of Ras.

Results

Tandem C_2 domains of RASAL bind phosphatidylserine- and phosphatidylcholine-enriched liposomes in a Ca^{2+} -dependent manner

A feature of the RASAL tandem C_2 domains is their similarity to the well-characterised, high-affinity Ca^{2+} -dependent,

phospholipid-binding C_2 domains from synaptotagmin III and protein kinase C β II (Figure 1A). Indeed, each RASAL C_2 domain has a fully conserved consensus sequence necessary for C_2 domains to bind phospholipids in a Ca^{2+} -dependent manner (Cho, 2001). To examine whether the C_2 domains of RASAL were capable of Ca^{2+} -dependent phospholipid binding, we investigated the ability of RASAL to associate with sucrose-loaded liposomes composed either of phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, or phosphatidylcholine. Although no binding of a recombinant RASAL glutathione-S-transferase (GST) fusion protein was detected to liposomes in the absence of Ca^{2+} , addition of sufficient total Ca^{2+} to elevate the free Ca^{2+} content to 100 μ M resulted in the association of RASAL with phosphatidylcholine- and phosphatidylserine-containing liposomes (Figure 1B). To examine the C_2 domain dependency of the Ca^{2+} -induced association, we generated a series of RASAL deletion mutants. GST- ΔC_2A -RASAL, GST- ΔC_2B -RASAL, and GST- ΔC_2 -RASAL all failed to undergo Ca^{2+} -dependent binding to phosphatidylcholine- or phosphatidylserine-containing liposomes (Figure 1C). Consistent with both C_2 domains being required for efficient Ca^{2+} -induced association were the observations that site-directed mutagenesis of key aspartate residues either within the C_2A , namely D21A, or the C_2B domain, D202A, inhibited the Ca^{2+} -dependent association of the corresponding full-length RASAL (Figure 1C).

RASAL undergoes a C_2 domain-dependent association with the plasma membrane following a receptor-induced elevation in $[Ca^{2+}]_i$

To determine the physiological significance of the ability of the RASAL tandem C_2 domains to undergo Ca^{2+} -dependent phospholipid association, we observed the subcellular localisation of RASAL prior to and during stimulation with agonists that elevated the $[Ca^{2+}]_i$ concentration. Figure 2A shows images of a HeLa cell expressing a green fluorescent protein (GFP)-tagged RASAL chimera prior to and during stimulation with the muscarinic agonist carbachol. The four images, taken immediately before and 1, 2, and 3 s after stimulation, clearly show a receptor-induced plasma membrane translocation of GFP-RASAL. To analyse the kinetics of the translocation process in more detail, the relative increase in plasma membrane over cytosolic fluorescence intensity (R) was calculated for a series of 300 images captured at one frame a second (Figure 2B). This relative plasma membrane translocation parameter was plotted as a function of time for each cell analysed.

For maximal receptor stimuli using 100 μ M of carbachol, translocation was observed to be transient in nature (Figure 2C). Translocation required an elevation in $[Ca^{2+}]_i$ since it could be mimicked by addition of the Ca^{2+} ionophore ionomycin (data not shown), and was blocked by pretreatment with the intracellular Ca^{2+} chelator BAPTA-AM (data not shown). In addition, when the transfected cells were stimulated in the absence of extracellular Ca^{2+} , the plasma membrane translocation of GFP-RASAL could still be observed, although association reversed typically after a period of 30 s or less (Figure 2C). Thus, Ca^{2+} release from internal stores is capable of inducing the plasma membrane association of RASAL. The receptor-mediated plasma membrane association of RASAL was also observed in a variety of

Figure 1 The tandem C₂ domains of RASAL bind phosphatidylcholine-containing liposomes in a Ca²⁺-dependent manner. **(A)** The alignment of RASAL C₂A (top) and (bottom) C₂B domains with the corresponding domains from CAPRI, synaptotagmin III (Syt III), and protein kinase CβII (PKCβII). Predicted β strands are indicated. The arrows show the positions of aspartate side chains that form the three Ca²⁺-binding sites in the synaptotagmin I C2A domain. **(B)** Association of full-length recombinant RASAL with either phosphatidylcholine (PC)-, phosphatidylethanolamine (PE)-, phosphatidylinositol (PI)-, or phosphatidylserine (PS)-containing liposomes when assayed with a free Ca²⁺ concentration of either < 1 nM or 100 μM. Formation of liposomes and the resolution of the resultant RASAL/lipid complex were achieved as described in Materials and methods. **(C)** Quantification of the Ca²⁺-dependent association of RASAL with PC-containing liposomes highlighting the important role of the tandem C₂ domains. Binding was analysed using recombinant RASAL with either the individual C₂A (ΔC₂ARASAL) or C₂B (ΔC₂BRASAL) domains, both C₂A and C₂B domains (ΔC₂RASAL), or point mutants targeting key aspartate residues in either the C₂A (RASAL(D21A)) or C₂B (RASAL(D202A)) domains. Similar data were obtained using PS-containing liposomes.

other cell types stimulated with a range of agonists (Figure 2D). Consistent with a requirement for the C₂ domains in the Ca²⁺-induced association were the observations that neither ΔC₂RASAL nor the D21A or D202A mutants underwent significant plasma membrane association on stimulation with 100 μM of carbachol (Figure 2E).

These data clearly demonstrate that the tandem C₂ domains confer upon RASAL an ability to undergo plasma membrane association following an elevation in [Ca²⁺]_i. It should be noted that in contrast with the pleckstrin homology domain-dependent spatial regulation of GAP1^{IP4BP} and GAP1m (Lockyer *et al*, 1997, 1999; Cozier *et al*, 2000a, 2003), RASAL failed to associate with the plasma membrane following epidermal growth factor stimulation of PC12 cells or after cotransfection of HeLa cells with a constitutively active phosphoinositide 3-kinase (data not shown). This indicates that human RASAL is unlikely to have a high affinity for phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) or phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃). These data are entirely consistent with the human RASAL pleckstrin homology domain missing several of the key residues known to be required for binding to these phosphoinositides (Cozier *et al*, 2000a, b, 2003).

RASAL undergoes an oscillatory translocation to the plasma membrane that occurs in synchrony with repetitive Ca²⁺ spikes

Strikingly, in 21 out of the 31 HeLa cells imaged, stimulation with carbachol resulted in an oscillatory plasma membrane association of RASAL (Figure 3A, Supplementary data). Both sinusoidal-like and baseline oscillation were observed (Figure 3B). Furthermore, oscillatory membrane association was frequency encoding as submaximal agonist doses induced slower oscillations in membrane association (Figure 3B). Such an oscillatory plasma membrane association is reminiscent of receptor-triggered Ca²⁺ spikes (Bootman *et al*, 2001; Berridge *et al*, 2003). In order to investigate this phenomenon further, we measured changes in [Ca²⁺]_i simultaneously with the subcellular localisation of RASAL. To achieve this, HeLa cells expressing GFP-RASAL were loaded with the Ca²⁺-sensitive indicator Fura-2, and the receptor-induced change in the fluorescence of the Ca²⁺ indicator (measured by excitation at 380 nm) was imaged simultaneously with the membrane association of GFP-RASAL (excitation at 488 nm). Using a wide-field imaging system, we could clearly observe the receptor-induced association of RASAL with the plasma membrane (Figure 4A).

Figure 2 RasAL undergoes receptor-induced plasma membrane translocation. (A) Receptor activation induces the translocation of GFP-RasAL to the plasma membrane. The images shown were recorded from a HeLa cell after the addition of carbachol (100 μ M). Numbers indicate time in seconds, with the initial frame arbitrarily labelled 0, prior to translocation. (B) Definition of the relative plasma membrane translocation parameter (R). (C) Release of calcium from internal stores is sufficient for the receptor-induced translocation of GFP-RasAL to the plasma membrane. Traces are taken from two individual HeLa cells stimulated with carbachol (100 μ M) either in the presence (solid line) or absence (dashed line) of extracellular calcium, and are representative of responses seen in 10 of 31 and 15 of 24 cells imaged, respectively. (D) Examples of GFP-RasAL plasma membrane translocation in a variety of cell lines stimulated with different agonists in the presence of extracellular calcium. (i) CHO cells stimulated with ATP (50 μ M); (ii) COS cells stimulated with histamine (100 μ M); (iii) COS cells stimulated with ATP (50 μ M). (E) Receptor-induced translocation requires the tandem C₂ domains of RasAL. Traces are taken from individual HeLa cells transiently transfected with GFP chimaeras of the different RasAL mutants and are representative of the typical responses seen ($n \geq 10$ in each case).

Capturing simultaneous pairs of images at 380 and 488 nm allowed oscillatory increases in $[Ca^{2+}]_i$ to be imaged with translocation of GFP-RasAL. In Figure 4, a correlation can be observed between the Ca^{2+} spikes elicited by addition of 100 μ M carbachol and the oscillatory association of RasAL

with the plasma membrane. RasAL is therefore capable of sensing the dynamic receptor-mediated oscillations in $[Ca^{2+}]_i$ through a synchronous oscillatory association with the plasma membrane.

Ca²⁺-induced plasma membrane translocation of RasAL occurs in the form of translocation waves

Besides the temporal aspect of complex Ca^{2+} signalling that is manifested as repetitive Ca^{2+} spikes, each individual Ca^{2+} spike has a spatial component that is observed as an elevated wave of Ca^{2+} that originates from a localised region prior to spreading throughout the cell (Bootman *et al*, 2001; Berridge *et al*, 2003). To examine the spatial aspects of the receptor-induced plasma membrane association of RasAL, we employed total internal reflection fluorescence (TIRF) microscopy. In TIRF imaging, a laser beam is totally internally reflected from the glass–water interface and generates an exponentially decaying excitation field (an evanescent wave) with a penetration depth of approximately 70 nm. This method selectively excites fluorescent molecules at or near the plasma membrane that is attached to the coverslip, with only a minimal excitation of the cytosolic molecules. Translocation of GFP-RasAL from the cytosol to the plasma membrane therefore results in an increase in fluorescence intensity since the protein comes from the dark cytosol into the evanescent wave field near the plasma membrane. Compared to confocal microscopy, TIRF imaging significantly increases the signal-to-noise for plasma membrane translocation such that greater spatial and temporal information on translocation to the plasma membrane can be obtained.

In HeLa cells expressing GFP-RasAL, the addition of 50 μ M ATP resulted in a significant increase in fluorescence intensity at the plasma membrane, and in most of the series of TIRF images analysed ($n = 3$ out of 4), the increase was repetitive (Figure 5A). These data are consistent with the ability of RasAL to sense the dynamic receptor-mediated oscillations in $[Ca^{2+}]_i$ described above. More detailed analysis of a series of TIRF images revealed that the repetitive increase in GFP-RasAL fluorescence observed upon receptor stimulation occurred in the form of fluorescent waves that originated from discrete fluorescent ‘hot’ spots and propagated across the cell (Figure 5B). Thus the receptor-induced plasma membrane association of RasAL has both complex temporal and spatial properties that are driven by the intricate spatiotemporal patterning of intracellular Ca^{2+} signals.

Ca²⁺-induced plasma membrane translocation of RasAL enhances its ability to function as a RasGAP

Given that RasAL contains a conserved RasGAP-related domain (Allen *et al*, 1998), we addressed the physiological significance of the Ca^{2+} -induced plasma membrane association by investigating the ability of RasAL to function as a RasGAP. Using an *in vitro* assay, we failed to detect any RasGAP activity for recombinant RasAL (Figure 6A). Given the precedent set by the demonstration that CAPRI only functions as a RasGAP following its Ca^{2+} -induced association with the plasma membrane (Lockyer *et al*, 2001), we assayed the effect of the Ca^{2+} -dependent membrane association on the ability of RasAL to function as an *in vivo* RasGAP. To achieve this, we transiently transfected HeLa cells with RasAL and H-Ras and assayed the level of Ras-GTP using a GST fusion of the Ras-GTP-binding domain from c-Raf-1

A

B

Figure 3 Receptor-induced translocation of RASAL occurs in an oscillatory manner. **(A)** Selected individual frames taken from HeLa cells showing oscillatory plasma membrane association of GFP-RASAL during stimulation with 100 μ M of carbachol. Numbers indicate time in seconds, with the initial frame arbitrarily labelled 0, prior to the first translocation. A movie of this oscillatory translocation is available as Supplementary Material. **(B)** GFP-RASAL translocations in HeLa cells, showing (i) sinusoidal and (ii, iii) baseline oscillatory responses after addition of 100 or 1 μ M of carbachol.

(GST-RBD). In serum-starved cells, a small quantity of over-expressed H-Ras was in the GTP-bound state. Under these conditions, there was no enhancement of GTPase activity, demonstrating that cytosolic RASAL was incapable of functioning as an efficient RasGAP (Figure 6B). To test whether RASAL may display increased RasGAP activity following its Ca^{2+} -induced plasma membrane association, we examined the activity of RASAL after addition of an agonist that induced an elevation in $[\text{Ca}^{2+}]_i$, thus driving RASAL to the plasma membrane. In the absence of RASAL, ATP induced an increase in Ras-GTP levels (Figure 6C). In cells expressing

RASAL, however, the ATP-induced elevation in active Ras was absent; indeed, the level was significantly reduced (Figure 6C). Consistent with the increase in GTPase activity resulting from the Ca^{2+} -induced membrane association of RASAL was the observations that the RASAL(D202A) mutant was incapable of reducing the ATP-stimulated increase in active Ras (Figure 6C). Finally, the RASAL GAP-related domain mutant, RASAL(Q507N), was also incapable of reducing the ATP-stimulated increase in active Ras (Figure 6C). Together, these data show that the C_2 domain-dependent, Ca^{2+} -induced translocation of RASAL to the plasma mem-

Figure 4 Oscillations in the translocation of RASAL are synchronous with repetitive Ca^{2+} spikes. (A) Parallel measurements of intracellular Ca^{2+} and GFP-RASAL plasma membrane translocation. HeLa cells expressing GFP-RASAL were loaded with Fura-2 AM and stimulated with carbachol (100 μM) in the presence of extracellular Ca^{2+} . Fluorescence intensities were recorded sequentially at excitation wavelengths of 380 nm for Fura-2 (a decrease in fluorescence intensity indicating an increase in intracellular Ca^{2+} concentration) and 488 nm for GFP-RASAL. The first frames of the two wavelengths are labelled 0 s just after the addition of agonist. (B) Time course comparing intracellular Ca^{2+} with plasma membrane translocation of GFP-RASAL. Images recorded were of insufficient quality to determine R values, hence GFP-RASAL translocation was measured by averaging pixel intensities from a cytoplasmic region of interest. Similarly, Fura-2 fluorescence was measured by averaging pixel intensities from a region of interest. Average pixel intensities for the two excitation wavelengths are shown as inverse changes against time, that is, the average pixel intensity for the region of interest from each frame was compared to the first imaged and the relationship plotted as an inverse against time to represent increases in intracellular Ca^{2+} and increases in GFP-RASAL translocation.

brane results in an activation of its ability to function as a RasGAP.

Live cell imaging confirms the activation of RASAL upon its Ca^{2+} -induced plasma membrane association

To extend the observation that the Ca^{2+} -induced plasma membrane association of RASAL leads to an activation in its ability to function as a RasGAP, we analysed the importance of the subcellular localisation of RASAL in its ability to regulate the activation state of Ras in single living cells. To achieve this, we made use of an amino-terminally tagged chimera of the Ras-GTP-binding domain (RBD) of c-Raf-1 (amino acids 51–131) and GFP (GFP-RBD) (Chiu *et al*, 2002; Bivona *et al*, 2003). This fluorescent probe has previously been shown to report the localisation of active, GTP-bound Ras in living cells (Chiu *et al*, 2002; Bivona *et al*, 2003). When expressed alone, GFP-RBD homogeneously localised in the cytosol and nucleoplasm, without accumulating on any membrane structure (data not shown). However, when GFP-RBD was coexpressed with wild-type H-Ras in HeLa cells grown in full serum, a significant amount of the fluorescent reporter accumulated at the plasma membrane (Figure 7A). Under these steady-state conditions of growth in serum, the GFP-RBD fluorescent probe is therefore detecting the cellular Ras that is activated at the plasma membrane (Chiu *et al*, 2002; Bivona *et al*, 2003). To examine the effect of RASAL expression on the steady-state level of Ras-GTP, we transiently transfected HeLa cells

with expression vectors encoding for RASAL, wild-type H-Ras, and GFP-RBD in a ratio such that cells expressing GFP-RBD were also expressing detectable H-Ras and RASAL. Under these conditions, a significant amount of the fluorescent reporter accumulated at the plasma membrane when RASAL was present in the cytosol (Figure 7B). This is entirely consistent with the conclusion that, even when overexpressed, cytosolic RASAL is inefficient as a RasGAP. To confirm whether the Ca^{2+} -induced plasma membrane association of RASAL leads to the activation in its ability to function as a RasGAP, we examined the plasma membrane accumulation of the GFP-RBD probe, prior to and during the Ca^{2+} -induced plasma membrane association of RASAL. Here, HeLa cells transiently transfected with H-Ras, GFP-RBD, and RASAL were imaged prior to and during stimulation with 50 μM ATP. As shown in Figure 7B, the addition of ATP induced the dissociation of the GFP-RBD probe. The receptor-mediated dissociation of the probe required the ability of RASAL to undergo Ca^{2+} -induced plasma membrane association, since in cells expressing RASAL(D202A) no dissociation of the GFP-RBD was observed (Figure 7C). Furthermore, a functional RasGAP domain was also required, as cells expressing RASAL(Q507N) also failed to induce dissociation of the probe following receptor activation (Figure 7C). These observations are entirely consistent with the conclusion that the Ca^{2+} -induced, C₂ domain-dependent plasma membrane association of RASAL results in an activation in its ability to function as a RasGAP.

Figure 5 TIRF microscopy reveals spatial aspects of GFP-RASAL plasma membrane recruitment. **(A)** An evanescent wave was used to excite GFP-RASAL at the plasma membrane of a HeLa cell (within approximately 70 nm of the coverslip surface) during stimulation with 50 μ M of ATP. Time course shows oscillations in GFP-RASAL plasma membrane recruitment. The inset shows the cell imaged and the region of interest from which the pixel intensities were taken for the trace. **(B)** An evanescent wave was used to excite GFP-RASAL at the plasma membrane of a HeLa cell. The cell was stimulated by addition of ATP (50 μ M), with the first image shown taken immediately before GFP-RASAL translocation (arbitrarily labelled as 0). Subsequent frames were taken every 0.2 s. In the pseudocolour images, the association of GFP-RASAL can be seen to recruit from a 'hot' spot centre right of the cell and rapidly propagate out.

To compare the kinetics of these translocations, we performed a more detailed analysis. Using the analysis described in Figure 2B, we obtained values for the half-maximal plasma membrane association and dissociation parameters ($t_{1/2}$ values) for GFP-RASAL and the GFP-RBD probe of 3.2 ± 1.2 and 7.2 ± 3.0 s, respectively ($n \geq 10$). Thus the receptor-induced plasma membrane dissociation of the GFP-RBD probe occurs with a similar time constant as the association of RASAL.

siRNA suppression of RASAL leads to an enhanced activation of Ras following hormonal stimulation of HeLa cells

The data present above clearly demonstrate that RASAL displays an ability to function as a Ca^{2+} -regulated RasGAP. However, these data do not address the importance of endogenous RASAL in the regulation of Ras activation. We addressed this by using a specific 21-nucleotide small

Figure 6 The C₂ domain-dependent, Ca²⁺-induced plasma membrane translocation of RASAL leads to a stimulation in its ability to function as a RasGAP. (A) *In vitro* full-length recombinant RASAL does not function as a RasGAP. Assays were performed through the analysis of liberated [³²P]P_i as described in Materials and methods. GAP1^{IP4BP} was used as a positive control. Data are the means $\pm$ s.e.m. from three individual determinations using H-Ras as substrate. (B) ATP stimulation activates the ability of RASAL to function as a RasGAP. Under serum-starved conditions, a significant quantity of expressed H-Ras is GTP-bound in HeLa cells transiently transfected with H-Ras. After 60 s of stimulation with 50 μ M ATP, the quantity of H-Ras-GTP increases approximately two-fold. In cells expressing H-Ras and wild-type RASAL, there is a detectable decrease in the quantity of H-Ras-GTP after 60 s of stimulation with ATP (cf. RASAL-ATP with RASAL + ATP). The ability of RASAL to function as a RasGAP requires the ability to undergo Ca²⁺-induced association with the plasma membrane and a functional GAP-related domain (cf. RASAL + ATP with RASAL(D202A) + ATP and RASAL(Q507N) + ATP). (C) Quantification of H-Ras-GTP levels from HeLa cells before and 60 s after stimulation with 50 μ M ATP expressed as a percentage of the pull-down in control, serum-starved cells prior to ATP stimulation (average of three separate experiments $\pm$ s.e.m.).

interfering RNA (siRNA) duplex to suppress expression of endogenous RASAL in HeLa cells (Elbashir *et al*, 2001). In control cells, stimulation with 50 μ M ATP induced a 2.5-fold elevation in the level of Ras-GTP (Figure 8A). In contrast, in cells treated with the RASAL-specific siRNA, which induced a

>80% reduction in the level of RASAL mRNA (Figure 8B), ATP stimulation induced an approximate 5.2-fold elevation in Ras-GTP (Figure 8A). This enhanced ATP-dependent activation of Ras was not observed in control cells treated with a random siRNA (Figure 8A). Thus, suppression of endogenous RASAL results in an elevation in Ras activation following stimulation. Such data are entirely consistent with a role for endogenous RASAL in the regulation of the activation state of this GTPase following ATP stimulation of HeLa cells.

Discussion

In the current study, we have documented that like the tandem C₂ domains of CAPRI, the corresponding domains of RASAL contain conserved C₂ motifs necessary for C₂ domains to bind phospholipids in a Ca²⁺-dependent manner. Consistent with this, we have shown that the tandem RASAL C₂ domains bind phospholipids in a Ca²⁺-dependent manner, and that this activity is translated into a dynamic Ca²⁺-induced plasma membrane association of RASAL following receptor-induced elevation in [Ca²⁺]_i. RASAL therefore joins the small family of Ca²⁺-regulated C₂ domain-containing proteins, including Nedd4 (Plant *et al*, 1997), PKC (Oancea and Meyer, 1998; Codazzi *et al*, 2001), cPLA₂ (Evans *et al*, 2001), and CAPRI (Lockyer *et al*, 2001), that have been shown to bind cellular membranes *in vivo* following changes in [Ca²⁺]_i.

Functionally, we have established that the plasma membrane association of RASAL enhances its ability to function as a RasGAP. Using pull-down assays coupled with a method for the analysis of Ras activation in single living cells, we have shown that although cytosolic RASAL is incapable of functioning as an efficient RasGAP, plasma membrane association leads to an elevation in its RasGAP activity. These data establish therefore that like CAPRI (Lockyer *et al*, 2001) cytosolic RASAL is an inactive RasGAP that upon a receptor-mediated elevation in [Ca²⁺]_i undergoes a rapid, C₂ domain-dependent association with the plasma membrane, an association that leads to an increase in its ability to function as a RasGAP. Thus, as with CAPRI, RASAL constitutes a molecular entity that couples Ca²⁺ to the regulation of Ras deactivation.

Unlike CAPRI however, which undergoes a transient plasma membrane association upon receptor stimulation

Figure 7 The GFP c-Raf-1 Ras-binding domain fusion protein can be used to demonstrate RASAL-dependent Ras inactivation in live cells. (A) In HeLa cells grown in full serum, a significant amount of the GFP-RBD fluorescent reporter accumulates at the plasma membrane in cells cotransfected with the wild type (a). This is not observed in transfected cells serum starved for 12 h (b). Similar results were obtained in a further 30 cells imaged. (B) Agonist-induced dissociation of GFP-RBD from the plasma membrane. HeLa cells transfected with H-Ras, GFP-RBD, and RASAL were placed in Ca²⁺-containing extracellular medium and imaged using confocal microscopy. ATP (50 μM) was used to elevate [Ca²⁺]_i. The two images (a and b) of the cells were taken at the time points indicated on the corresponding trace. Similar results were obtained in a further five images of the cells. (C) HeLa cells transfected with H-Ras, GFP-RBD, and either RASAL(D202A) (a and b) or RASAL(Q507N) (c and d) were placed in Ca²⁺-containing extracellular media and imaged using confocal microscopy prior to (images a and c) or 30 s after (images b and d) the addition of 50 μM ATP. In each case, similar results were obtained in a further 10 images of the cells.

Figure 8 siRNA suppression of endogenous RASAL leads to an enhanced receptor-mediated activation of Ras. (A) HeLa cells were transiently transfected with either RASAL-specific siRNA or random siRNA. After 48 h, cells were stimulated for 2 min with 50 μM ATP, and the amount of active Ras-GTP quantified as described above. Data are expressed as a percent of control cells (average of three separate experiments ± s.e.m.). (B) By RT-PCR, HeLa cells express endogenous RASAL, which, unlike GAPDH, can be suppressed following a 48 h treatment with a RASAL-specific siRNA.

(Lockyer *et al*, 2001), and does not sense underlying Ca²⁺ oscillations (P Lockyer, personal communication, 2003), we have established that in cells in which the receptor-induced elevation in [Ca²⁺]_i occurs as a series of repetitive Ca²⁺ spikes, the plasma membrane association of RASAL also occurs in an oscillatory manner, which mirrors the underlying oscillatory nature of the Ca²⁺ signal. Furthermore, we have established that each repetitive plasma membrane association of RASAL occurs in the form of an association wave that originates from discrete 'hot' spots prior to propagating across the entire cell. Finally, and perhaps most importantly, we have established that the oscillatory membrane association of RASAL is frequency modulated, such that upon increasing the amplitude of receptor stimuli, the frequency of membrane association is enhanced. As RASAL can only efficiently function as a RasGAP when membrane associated, this protein is therefore capable of sensing the frequency of [Ca²⁺]_i oscillations, decoding the contained information through a dynamic regulation in the extent and duration of Ras activation.

With the exception of the work described here, we know very little about how, and indeed whether, the frequency of Ca^{2+} oscillations may be optimised for the Ca^{2+} -mediated regulation of Ras signalling. Although it is clear that the release of internally stored Ca^{2+} can lead to Ras activation through Ras-GRF and Ras-GRP family members (Cullen and Lockyer, 2002), in most cases this has been achieved through the addition of thapsigargin and ionomycin, reagents that do not release Ca^{2+} with the same spatiotemporal patterning as physiological stimuli. In none of these studies has the ability of these RasGEFs to couple complex Ca^{2+} signals to the activation of Ras been examined. In the light of the data presented here, an intriguing issue is whether the frequency and amplitude of Ca^{2+} signals are also capable of coordinating Ras activation as well as deactivation.

In conclusion, our characterisation of RASAL has provided evidence not only for a novel Ca^{2+} sensor but also for a hitherto unrecognised link between the frequency of receptor-mediated Ca^{2+} oscillations and the regulation of the activation state of plasma membrane-associated Ras. Given that receptor-mediated increases in $[\text{Ca}^{2+}]_i$ also act as the signal for the Ras-GRP1-dependent activation of Ras on the Golgi complex (Bivona *et al*, 2003; Caloca *et al*, 2003), it would appear that receptor-mediated increases in $[\text{Ca}^{2+}]_i$ may be a universal mechanism for achieving a dynamic spatial and temporal modulation of Ras signalling (Hancock, 2003).

Materials and methods

Expression and purification of GST fusion proteins in *Escherichia coli*

The pGEX plasmids containing the coding sequences for the various proteins were transformed individually into the *E. coli* strain BL21(DE3) to express and purify as GST fusion proteins as previously described (Cozier *et al*, 2000a). Isolation of deletion and site-direct mutants of RASAL was performed using the Transformer Kit (Clontech) as previously described (Cozier *et al*, 2000a).

Sucrose-loaded liposome assays

Phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol (all in CHCl_3) were dried down to form a thin film in a 0.5 ml minifuge tube (Beckmann) and then bath sonicated in 0.2 M sucrose, 20 mM KCl, 20 mM Hepes (pH 7.4), 0.01% azide to yield a $10 \times$ lipid stock (Cozier *et al*, 2003). This was diluted 10-fold in reaction buffer (0.12 mM NaCl, 1 mM EGTA, 0.2 mM CaCl_2 (free Ca^{2+} concentration of approximately 50 nM), 1.5 mM MgCl_2 , 1 mM dithiothreitol, 5 mM KCl, 20 mM Hepes (pH 7.4), 1 mg ml⁻¹ bovine serum albumin) containing 250–500 ng of recombinant protein. Protein–lipid complexes were allowed to form by incubation at 30°C for 4 min prior to centrifugation (100 000 g for 30 min). After spinning, the supernatants were carefully removed and the pellets retrieved by the addition of an equal volume of 60°C SDS sample buffer and subsequent bath sonication. Proteins present in both the supernatant and lipid vesicles were separated by SDS-PAGE and visualised by Western blotting. Detection was performed using the ECL Western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Developed films were analysed by volume integration using ImageQuant software (version 3.3, Molecular Dynamics Inc.) in a similar manner to that described previously (Lockyer *et al*, 2001; Cozier *et al*, 2003).

In vitro RasGAP assays

These were performed under first-order kinetics as described in Cullen *et al* (1995). Briefly, H-Ras was loaded with [γ -³²P]GTP (3000 Ci mmol⁻¹, Amersham) for 5 min at 25°C. GTPase activity was assayed at 25°C by addition of the various GTPase-activating

proteins to the loaded GTP-binding protein. At the required time points, activity was stopped by addition of 5 mM silicotungstate and 1 mM H_2SO_4 , with the liberated [³²P]P_i being extracted with isobutanol/toluene (1/1 v/v), 5% (w/v) ammonium molybdate, and 2 M H_2SO_4 . The upper phase was removed for scintillation counting.

Ras pull-down assays

GST fusion of the Ras-GTP-binding domain from Raf-1 (GST-RBD) was purified as previously described (Lockyer *et al*, 1999, 2001). Dishes (100 mm) of HeLa cells (8×10^5 cells) were transiently transfected by lipofection (Fugene 6, Roche) with 2.5 µg H-Ras DNA. The cells were serum starved for 2 h at 37°C in KH buffer (5 mM Hepes, 10 mM glucose, 25 mM NaHCO_3 , 1.2 mM K_2HPO_4 , 118 mM NaCl, 1.2 mM MgSO_4 , 1.3 mM CaCl_2 (pH 7.4)) 24 h post-transfection, prior to stimulation with 50 µM ATP (Sigma). Cell lysis, H-Ras-GTP pull-down, and detection of immobilised H-Ras were carried out as previously described (Lockyer *et al*, 1999). Blots were analysed by volume integration using ImageQuant software (Molecular Dynamics). These were performed and quantified as previously described (Lockyer *et al*, 2001; Cozier *et al*, 2003).

Cell culture and transient transfection

COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) foetal calf serum, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. CHO cells were cultured in F-12 Nutrient Mixture (Invitrogen) containing 5% (v/v) foetal calf serum (Invitrogen), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Cell cultures were maintained at 37°C in a 95% air–5% CO_2 humidified incubator. All cell types were transfected using GeneJuice transfection reagent (Novagen) according to the manufacturer's instructions. For triple-transfection experiments requiring cotransfection of c-Raf-1 RBD, RASAL, and H-Ras, plasmids were cotransfected using the molar ratio 1:2:2. This ensured that cells expressing the c-Raf-1 RBD would also be expressing RASAL and H-Ras.

Live cell laser scanning confocal microscopy

At 24 h post-transfection, serum-starved cells expressing EGFP-tagged proteins were analysed at 37°C in Krebs–Ringer phosphate buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO_4 , 1.25 mM CaCl_2 , 5 mM sodium phosphate) containing 2 mM NaHCO_3 and 25 mM Hepes (pH 7.4). Fluorescence imaging was performed with an UltraView™ LCI confocal optical scanner (Perkin Elmer Wallac).

TIRF microscopy

To assess translocation of GFP-RASAL, we employed a TIRF microscope similar to that previously described (Pinton *et al*, 2002). The incident light for total internal reflection illumination was introduced from the objective lens (Olympus, numerical aperture = 1.65, $\times 100$ magnification) through a single-mode optical fibre and two illumination lenses. To observe the EGFP fluorescence image, we used a 488 nm laser (argon ion laser, 30 mW, Spectra Physics) for total internal fluorescence illumination and a long-pass filter (515 nm) for barrier. The laser beam was passed through an electromagnetically driven shutter (Till Photonics). The shutter was opened synchronously with camera exposure under control by TillvisiON software (Till Photonics). Images were acquired at five frames per second. To analyse the data, translocation events were manually selected and the average fluorescence intensity of individual plasma membrane regions was calculated.

RASAL suppression using siRNA

HeLa cells were seeded in 35 mm dishes for approximately 30% confluency on the day of transfection. OligoFectamine (Invitrogen) was used to transfect duplex siRNA (Dharmacon) into cells according to the manufacturer's guidelines. Cells were used 48 h after transfection. Duplex siRNA sequence targeted the human RASAL mRNA sequence 5'-AAGAAGACUCGCUUCCGCAC-3'. A scrambled siRNA, targeting no known sequence in the human genome, was used as a control: 5'-AAGACAAGAACGACGACCA-3'.

RT-PCR

For siRNA treatment, 35 mm dishes of HeLa cells were transfected with an siRNA duplex. At 48 h after transfection, cells were scraped off in 200 µl PBS, spun down at 4°C and the pellet resuspended in

1 ml TRI reagent (Sigma). mRNA was isolated according to the manufacturer's guidelines. In all, 2 µg total RNA was used in a 50 µl reaction with 200 U M-MLV reverse transcriptase (Promega) and oligo-dT₃₀ primer. A measure of 15 µl of reaction was used in a 50 µl PCR reaction with a primer combination (RASAL: sense 5'-CTGGCCTTCTACGTGCTGGAT-3', antisense 5'-ATTCTTGCCACCAT GTCCCA-3'; GAPDH: sense 5'-GAACGGGAAGCTCACTGGCATG-3', antisense 5'-GTCCACCACCTGTTGCTGTAG-3').

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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