Ras binding opens c-Raf to expose the docking site for mitogen-activated protein kinase kinase

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A key signalling molecule, c-Raf, is situated downstream from Ras and upstream from the mitogen-activated protein kinase kinase (MEK). We studied the mechanism underlying the signal transduction from Ras to MEK by using probes based on the principle of fluorescence resonance energy transfer. In agreement with previous models, it was found that c-Raf adopted two conformations: open active and closed inactive. Ras binding induced the c-Raf transition from closed to open conformation, which enabled c-Raf to bind to MEK. In the presence of a cytosolic Ras mutant, c-Raf bound to, but failed to phosphorylate, MEK in the cytoplasm. In contrast, the cytosolic Ras mutant significantly enhanced MEK phosphorylation by a membrane-targeted c-Raf. These results demonstrated the essential role of Ras-induced conformational change in MEK activation by c-Raf. Keywords: fluorescence resonance energy transfer (FRET); Raf; Ras; mitogen-activated protein kinase kinase (MEK); green fluorescent protein (GFP)

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INTRODUCTION

c-Raf serine-threonine kinase regulates various aspects of cellular function, including the proliferation, differentiation and oncogenic transformation of higher eukaryotic cells (Avruch *et al*, 1994; Daum *et al*, 1994). In addition to Ras, several serine-threonine kinases including Akt, PAK, PKC and PKA regulate Raf either positively or negatively (Kolch, 2000). Furthermore, scaffold proteins such as 14-3-3, spred, KSR and RKIP also have critical roles in the transmission of signals from Raf to the downstream mitogen-activated protein kinase (MAPK) cascades (Tzivion *et al*, 1998; Yeung *et al*, 1999; Morrison, 2001; Wakioka *et al*, 2001; Corbit *et al*, 2003). Despite the large amount of data on c-Raf regulation, many questions remain unsolved. For example, it is still under debate as to whether the recruitment of c-Raf to the membrane compartment is the only role of Ras: that is, it remains

unclear whether the conformational change induced by Ras binding is required for c-Raf activation.

The recent development of a technology based on green fluorescent protein (GFP) and fluorescence resonance energy transfer (FRET) has enabled us to monitor conformational changes in signalling molecules as they occur in living cells (Miyawaki, 2003). Here, we have developed a FRET-based probe for c-Raf, designated as photometric Raf indicator (Prin), which was used to examine the effect of Ras binding on the conformational changes of c-Raf.

RESULTS AND DISCUSSION

A probe for the conformational change of c-Raf

We developed a probe, designated as Prin–c-Raf, for the monitoring of conformational changes in c-Raf in living cells by fusing yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) at the amino and carboxyl termini of c-Raf, respectively (Fig 1A). A flexible linker was placed between c-Raf and CFP to minimize the effect of relative orientation of the transition dipoles of fluorophores on FRET efficiency. Using this probe design, the efficiency of FRET increases when c-Raf adopts an inactive closed conformation in the cytosol, and the efficiency decreases when c-Raf assumes an active open conformation at the plasma membrane. Basic characterization of this probe is described in supplementary information online (including supplementary Fig 1 online).

Previous biochemical analyses have identified several amino-acid residues that are critical in the regulation of c-Raf kinase (reviewed in Morrison & Cutler, 1997; Avruch *et al*, 2001; Chong *et al*, 2003; Fig 1B,C). First, we examined the roles of Ser 259 and Ser 621, which, on phosphorylation, serve as 14-3-3 binding sites. Substitution of these residues by alanine caused Prin–c-Raf to adopt an open conformation and rendered the probe refractory to both RasV12 and Akt-active. This observation supports a previously reported proposal that 14-3-3 negatively regulates c-Raf by holding it in the closed conformation and that Akt downregulates c-Raf by phosphorylating Ser 259 (Zimmermann & Moelling, 1999; Kolch, 2000; Avruch *et al*, 2001). A second, Cys168Ser mutation in the cysteine-rich domain of conserved region (CR) 1 was found to lead to the open conformation of Prin–c-Raf. This observation again was in agreement with a previous report stating that Cys168Ser mutation disturbs the intramolecular

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Fig 1 | Effects of amino-acid substitutions on the conformation and activity of c-Raf. (A) Schematic representation of Prin-c-Raf. FRET efficiency increases when Prin-c-Raf adopts the closed conformation in the cytosol. Ras binding at the plasma membrane changes the conformation to the open state, a condition under which FRET efficiency is reduced. (B) Schematic representation of amino-acid residues analysed in this study. (C) Conformation index (CFP/YFP) of Cos-1 cells expressing Prin-c-Raf mutants in the presence or absence of H-RasV12 or Akt-active. The bars and lines represent the average and s.d., respectively ($n = 20$). (D) Cos-1 cells expressing Prin-c-Raf mutants in the presence or absence of H-RasV12 or Akt-active were analysed by immunoblotting with anti-c-Raf-phospho-Ser 259 or anti-c-Raf antibody.

binding of CR1 to the CR3 kinase domain and raises the basal activity of c-Raf (Morrison *et al*, 1993). Third, an Arg89Leu mutation in the Ras-binding domain of Prin-c-Raf not only abolished the Ras-dependent induction of the open conformation, but also increased the levels of Prin-c-Raf in the closed conformation in the presence of RasV12. This observation could be interpreted as reflective of the Ras-induced activation of Akt that results in an increase in the level of Ser 259 phosphorylation (Dhillon *et al*, 2002a). Finally, we evaluated the role of two clusters of activation-related phosphorylation sites in c-Raf (Mason *et al*, 1999; Chong *et al*, 2001; Wan *et al*, 2004): Ser 338/Tyr 341 and Thr 491/Ser 494. Mutation of these residues in S338A/Y341A and T491A/S494A mutants did not alter the overall response of Prin-c-Raf to RasV12 and Akt-active, negating the involvement of their phosphorylation in the conformational changes detectable by our probe.

The inhibitory role of Ser 259 phosphorylation in c-Raf activity is widely accepted (Tzivion *et al*, 1998; Jaumot & Hancock, 2001;

Fig 2 | MEK binding to c-Raf in the open conformation.

(A) Conformation of Prin-c-Raf in the presence or absence of H-RasV12 or H-RasV12ΔC in Cos-1 cells ($n = 20$). (B) The binding of CFP-c-Raf, CFP-c-RafR89L and c-Raf-CFP to YFP or MEK-YFP was analysed in the presence or absence of H-RasV12ΔC or Rap1V12ΔC in Cos-7 cells by intermolecular FRET. (C) HeLa cells coexpressing H-Ras, CFP-c-Raf and MEK-YFP were stimulated with 25 ng/ml epidermal growth factor and imaged using time-lapse (supplementary video 4 online). Representative images of CFP and corrected FRET (lower panel) are shown. Scale bar, 10 μ m. (D) Conformation of membrane-anchored Prin-c-Raf-pm in the presence or absence of H-RasV12 or H-Ras-V12ΔC in Cos-1 cells ($n = 20$). (E) Binding of CFP-pm, CFP-c-Raf-pm, CFP-c-RafR89L-pm, c-Raf-CFP-pm and CFP-c-RafS259A-pm to YFP or MEK-YFP was quantified in the presence or absence of H-RasV12ΔC and Akt-active in HeLa cells by intermolecular FRET. Asterisks indicate the significance of t -test analysis: * $P < 0.01$; ** $P < 0.001$.

Dhillon *et al*, 2002b; Kubicek *et al*, 2002; Moelling *et al*, 2002; Dumaz & Marais, 2003). However, it has been reported that the fraction of c-Raf phosphorylated on Ser 259 is small in NIH3T3 and COS cells (Morrison *et al*, 1993; Dhillon *et al*, 2002b). Indeed, we found that, in Cos-1 cells, less than 10% of Prin-c-Raf was phosphorylated on Ser 259 compared with Prin-c-Raf in the

presence of RasV12 or Akt-active (Fig 2D). From the observation that Ser 621 is phosphorylated constitutively (Morrison *et al*, 1993) and that 14-3-3 binds to CRD in a phosphorylation-independent manner (Kolch, 2000; Avruch *et al*, 2001), it is possible to speculate that the phosphorylation of Ser 259 is required to induce 14-3-3 binding to CRD, but that it is not necessary for persistence of the closed conformation.

Binding of MEK to c-Raf in the open conformation

A question that we approached by using a Prin-c-Raf probe is whether recruitment to the plasma membrane is sufficient for activation (Leevers *et al*, 1994; Stokoe *et al*, 1994; Jaitner *et al*, 1997) or whether both plasma membrane recruitment and Ras-induced conformational changes are required for c-Raf activation (Drugan *et al*, 1996; Mineo *et al*, 1997; Li *et al*, 1998). First, to distinguish between the effects of the Ras-induced conformational change of c-Raf and the Ras-dependent recruitment of c-Raf to the plasma membrane, we prepared a Ras mutant, RasV12ΔC, that lacked the C-terminus and thereby localized mostly in the cytoplasm. RasV12ΔC was found to induce a conformational change in Prin-c-Raf as efficiently as did the authentic RasV12 (Fig 2A). Neither membrane recruitment nor the phosphorylation of Ser 338 of c-Raf was induced by the expression of RasV12ΔC, indicating that Ras binding was sufficient to induce the open conformation.

Second, we speculated that such a conformational change in c-Raf might induce its binding to MAPK kinase (MEK). We tested this possibility using an intermolecular FRET method with CFP-c-Raf (c-Raf fused to the C-terminus of CFP), c-Raf-CFP (c-Raf fused to the N-terminus of CFP) and MEK-YFP (MEK fused to the N-terminus of YFP) as probes (Fig 2B). To reduce any of the secondary effects of MEK expression, a kinase-absent MEK mutant was used in this assay. In Cos-7 cells, RasV12ΔC, but not Rap1V12ΔC, remarkably increased the extent of CFP-c-Raf binding to MEK, thus supporting our hypothesis. Arg89Leu mutation in the Ras-binding domain of CFP-c-Raf cancelled this RasV12ΔC-dependent increase when binding to MEK. These observations were in agreement with previous reports showing the presence of the Ras-c-Raf-MEK complex in a Ras-GTP-dependent manner (Moodie *et al*, 1993; Jelinek *et al*, 1994; Catling *et al*, 1995; Xiang *et al*, 2002). Notably, FRET was not detected between c-Raf-CFP and MEK-YFP, irrespective of the presence of RasV12ΔC. This observation suggested that the C-terminus of c-Raf was not sufficiently close to the N-terminus of MEK for the detection of FRET in the c-Raf-MEK complex.

Third, we examined whether or not epidermal growth factor (EGF)-dependent recruitment of MEK to c-Raf could be visualized *in vivo*. On EGF stimulation, c-Raf translocated rapidly to the plasma membrane, where the corrected FRET value, FRET^C, was markedly increased (Fig 2C; supplementary video 4 online). After 40 min, c-Raf returned to the cytosol with a concomitant decrease in FRET^C. This observation strongly suggested that, in EGF-stimulated cells, c-Raf bound to MEK only in association with Ras. To exclude the possibility that translocation of both c-Raf and MEK to the plasma membrane was sufficient for the occurrence of FRET, CFP-c-Raf was replaced with c-Raf-CFP in a similar experimental condition. On EGF stimulation, both c-Raf-CFP and MEK-YFP were translocated to the plasma membrane; however, the increase in FRET^C was markedly lower than that with CFP-

Fig 3 | MEK activation by c-Raf in the open conformation. (A) *In vitro* MEK kinase activity of Prin-c-Raf and Prin-c-Raf-pm that were immunoprecipitated from HeLa cells in the presence or absence of RasV12 or H-RasV12ΔC ($n = 3$). (B) HeLa cell lysates were immunoprecipitated for the *in vitro* MEK kinase assay (IP) or were directly used (TCL) for immunoblotting with anti-GFP (c-Raf), anti-phospho-MEK (pMEK) and anti-Flag antibodies (Ras).

c-Raf (supplementary video 5 online), negating the aforementioned possibility.

Fourth, we examined whether the translocation of c-Raf to the plasma membrane might affect its conformation by using a Prin-c-Raf mutant that was fused to the C-terminal region of K-Ras4B (Fig 2D). The FRET efficiency of this probe, Prin-c-Raf-pm, was similar to that of the authentic Prin-c-Raf probe, demonstrating that membrane translocation alone did not induce the open conformation. Both RasV12 and RasV12ΔC induced the open conformation of Prin-c-Raf-pm as efficiently as Prin-c-Raf.

Finally, we confirmed that the expression of RasV12ΔC induced the binding of c-Raf-pm to MEK by the intermolecular FRET method described above (Fig 2E). Again, RasV12ΔC induced the binding of CFP-c-Raf-pm to MEK-YFP. CFP-c-RafR89L-pm did not show this RasV12ΔC-dependent increase in binding to MEK. It was also shown that Akt-active suppressed the binding of c-Raf-pm to MEK and that mutations of 14-3-3 binding sites, Ser259Ala and Ser621Ala, increased c-Raf-pm binding to MEK. These results were in agreement with those obtained using our model; namely, it was found that Ras binding opens c-Raf to expose the docking site for MEK.

Essential role of Ras-induced conformational change

Next, we evaluated the role of Ras-induced c-Raf binding to MEK in the activation of MEK. First, Prin-c-Raf and Prin-c-Raf-pm were immunoprecipitated from cell lysates and were examined for their *in vitro* kinase activity by using GST-MEK as a substrate (Fig 3A). Coexpression of RasV12, but not RasV12ΔC, markedly increased the MEK kinase activity of Prin-c-Raf. Prin-c-Raf-pm phosphorylated MEK as efficiently as did Prin-c-Raf purified from the RasV12-expressing cells. Coexpression of either RasV12 or RasV12ΔC increased this *in vitro* kinase activity, albeit only slightly. This observation seems to support a previous proposal that plasma membrane recruitment is sufficient for the activation of c-Raf. However, the stoichiometry of Prin-c-Raf binding to RasV12 *in vitro* was found to be very low under this condition (K.T., unpublished data), a finding that has been observed in previous studies (Leevers *et al*, 1994; Marais *et al*, 1998). Hence,

Fig 4 | Four states of c-Raf during the signalling process. (A) Semiclosed inactive state: in resting cells, a major fraction of c-Raf adopts semiclosed conformation. (B) Open inactive state: on stimulation, Ras-GTP binds to c-Raf and induces MEK binding to c-Raf. (C) Open active state: modifications at plasma membrane such as phosphorylation and lipid binding allow c-Raf to phosphorylate MEK. (D) Closed inactive state: c-Raf phosphorylated on Ser 259 adopts closed conformation by means of 14-3-3 binding and returns to the cytosol. Filled circles represent phospho-tyrosine, phospho-serine and phospho-threonine residues.

we speculated that the *in vitro* kinase assay adopted in previous studies primarily monitored c-Raf activation by a mechanism dependent solely on membrane translocation, but that this assay did not assess the effects of Ras-induced conformational changes on the MEK *in vivo* kinase activity of c-Raf. To overcome this methodological problem, we assessed the activity of Prin-c-Raf-pm by measuring the phosphorylation of Ser 217/Ser 221 of endogenous MEK (Fig 3B). A portion of the cell lysates was immunoprecipitated and used for the *in vitro* kinase assay and, in a parallel assay, a portion of the cell lysates was analysed for the phosphorylation of MEK. In contrast to the *in vitro* kinase experiment, RasV12ΔC remarkably activated Prin-c-Raf-pm in the cells, supporting our hypothesis that Ras binding is able to elevate the kinase activity of c-Raf in the context of living cells.

Altogether, these observations have delineated two essential steps for c-Raf activation. First, Ras binding unfolds c-Raf to expose the docking site for MEK. Second, translocation of c-Raf to the plasma membrane was additionally required for the phosphorylation of MEK. Phosphorylation of c-Raf at Ser 338 and/or Tyr 341, or lipid binding to the regulatory region seems to have an essential role in the latter process (Mason *et al*, 1999; Andresen *et al*, 2002).

Speculation

We propose to modify the models of the Ras-Raf-MEK signalling mechanism as follows (Fig 4). Semiclosed inactive state (Fig 4A): in resting cells, a major fraction of c-Raf is not phosphorylated on Ser 259, but remains in a semiclosed inactive state, probably by means of CRD interaction with the catalytic domain (Cutler *et al*, 1998). It is to be noted that the semiclosed state seems to represent the dynamic equilibrium between a small fraction of open conformation and a large fraction of closed conformation. This idea seems to fit our finding that the significant level of association between MEK and c-Raf was detected in the absence of Ras

(Fig 2B) and the earlier observation that expression of membrane targeting of c-Raf is sufficient to activate ERK (Leevers *et al*, 1994; Stokoe *et al*, 1994). Open inactive state (Fig 4B): on stimulation, Ras-GTP binds to c-Raf and exposes its MEK docking site. Importantly, however, the ensuing binding of c-Raf to MEK is not sufficient for MEK phosphorylation. Open active state (Fig 4C): previous studies have shown that phosphorylation of several amino-acid residues including Ser 338, Tyr 341, Thr 491 and Ser 494 and/or lipid binding to c-Raf should occur at the plasma membrane to enable the phosphorylation of MEK, probably because these modifications are required for the activation of the catalytic domain (Mason *et al*, 1999; Chong *et al*, 2001; Wan *et al*, 2004) or for binding to MEK (Xiang *et al*, 2002). In our assay system, membrane translocation of c-Raf seems to be sufficient for the induction of such a modification. However, such a modification of c-Raf, and thereby the subsequent activation of MEK, may be regulated depending on the cellular context or on the subcellular compartments. Closed inactive state (Fig 4D): phosphorylation of Ser 259 by Akt renders c-Raf able to adopt a closed conformation, although further research is required to understand the extent this phosphorylation of Ser 259 contributes to the termination of Raf activation. Finally, the transition from the closed inactive state to the semiclosed inactive state by dephosphorylation of Ser 259 should also be subject to regulation, and the level of Ser 259 phosphorylation probably varies among different cell types. It is possible that the level of Ser 259 phosphorylation may be lower in transformed cells, such as the HeLa and Cos-1 cells used in our study, than in the nontransformed cells.

An increasing number of scaffold proteins have been shown to regulate Ras-Raf-MEK signalling (Morrison & Davis, 2003). In most of our experimental conditions, the number of Prin-c-Raf probes was about 10^6 per cell (data not shown). Apparently, 14-3-3 exceeds this number, but contribution of the other scaffold proteins in our assay system should be examined and integrated into the model in future studies.

METHODS

A FRET probe for c-Raf. Prin-c-Raf and its mutants were constructed as described previously (Mochizuki *et al*, 2001). From the N-terminus, Prin-c-Raf consists of the following: YFP (amino acids (aa) 1–239), a spacer (Leu-Asp), c-Raf, a spacer (Gly-Gly-Arg) and CFP (aa 1–237). Here, we used modified YFP and CFP, as described previously (Itoh *et al*, 2002). Full-length and mutant complementary DNAs of human c-Raf (aa 1–648) were all amplified by PCR-based methods.

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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