Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases

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Summary

Raf comprises a family of three kinases, A-Raf, B-Raf and Raf-1, which are best known as key regulators of the MEK–MAPK/ERK cascade. This module is often perceived as a linear pathway in which ERK is the effector. However, recent advances have unveiled a role for Raf outside this established signalling unit. Current evidence, including gene-knockout studies in mice, suggests that there are ERK-independent functions of Raf kinases. Regulation of apoptosis is one area in which Raf may function independently of ERK, although its substrates remain to be identified. Other studies have suggested that Raf has kinase-independent functions and may act as a scaffold protein.

Key words: Raf, Apoptosis, ERK/MAPK, Knockout mice, Signal transduction

Introduction

Fifteen years of intensive research has taught us much about cell signalling and kinase cascades, which connect extracellular signal molecules with the transcriptional machinery. The Raf-MEK-ERK cascade was the first signalling pathway to be entirely mapped from the cell membrane to the cell nucleus and its structure became the paradigm for MAP kinase modules in general. The Raf cascade is initiated by the small G protein Ras, which recruits Raf from the cytosol to the cell membrane for activation. Activated Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. ERK has many substrates both in the cytosol and the nucleus. It can affect gene expression directly by phosphorylating transcription factors, such as Ets, Elk and Myc, as well as indirectly by targeting other substrates, such as p90-RSK (ribosomal S6 kinase) family kinases, which can modify transcription factors and histones (Davie and Spencer, 2001; Lewis et al., 1998). Both *Ras* and *Raf* are proto-oncogenes, so it is not surprising to find that this pathway is primarily concerned with growth regulation, be it proliferation, transformation, differentiation or apoptosis.

Since the biological outcome is determined by the strength and duration of the activation of this pathway (Marshall, 1995), it is tightly regulated with the most intricate controls operating at the level of Raf (Avruch et al., 2001; Houslay and Kolch, 2000; Kolch, 2000). Briefly, Raf activation is a consequence of its binding to Ras and subsequent complex changes in phosphorylation and interaction with lipids. Although all three Raf isoforms can interact with Ras, there are important differences. For instance, Ras binding alone is sufficient to activate B-Raf, but not Raf-1 or A-Raf, which require secondary signals (Marais et al., 1997). Rap1, a Ras-related G protein, has been reported to activate B-Raf but to inhibit Raf-1 (Vossler et al., 1997). Rap1 and Ras are activated by an overlapping, but distinct set of stimuli (Bos et al., 2001), whose specific biological effects may be due to the selective activation of Raf isoforms. Therefore, it would be surprising if all three Raf isozymes were to rely on MEK alone as their only commonly accepted substrate. However, recent reports have produced indirect, but strong, evidence for a branching of signals at the level of Raf, in particular Raf-1, which is the focus of the majority of Raf research.

New Raf signalling pathways

Several studies have observed Raf-1-mediated effects in the absence of MEK and ERK activation (Fig. 1). For instance, Raf-1 can induce vimentin depolymerisation in vitro and in vivo independently of MEK (Janosch et al., 2000). This effect is stimulated by Raf-1 activation but is mediated indirectly by Raf-1-associated kinases. One was identified as the catalytic α subunit of CK2, which raises the possibility that CK2α can function as a novel Raf-1 effector. Also Raf-1 becomes highly activated in G2/M phase without concomitant MEK and ERK activation (Laird et al., 1999), although no target of mitotically activated Raf-1 has been identified.

Another candidate for a Raf effector is NF-κB, a transcription factor central to cell transformation and survival (Mayo and Baldwin, 2000). NF-κB is sequestered in the cytosol by its inhibitor protein IκB, which upon activation is phosphorylated and targeted for degradation (Karin and Delhase, 2000). It is unclear how exactly these pathways interact, and both ERK-dependent and ERK-independent mechanisms may be involved. The ERK substrate p90-RSK can phosphorylate IκB and induce its degradation (Ghoda et al., 1997). In addition, ERK can downregulate the expression of PAR-4, an inhibitor of NF-κB activation (Barradas et al.,

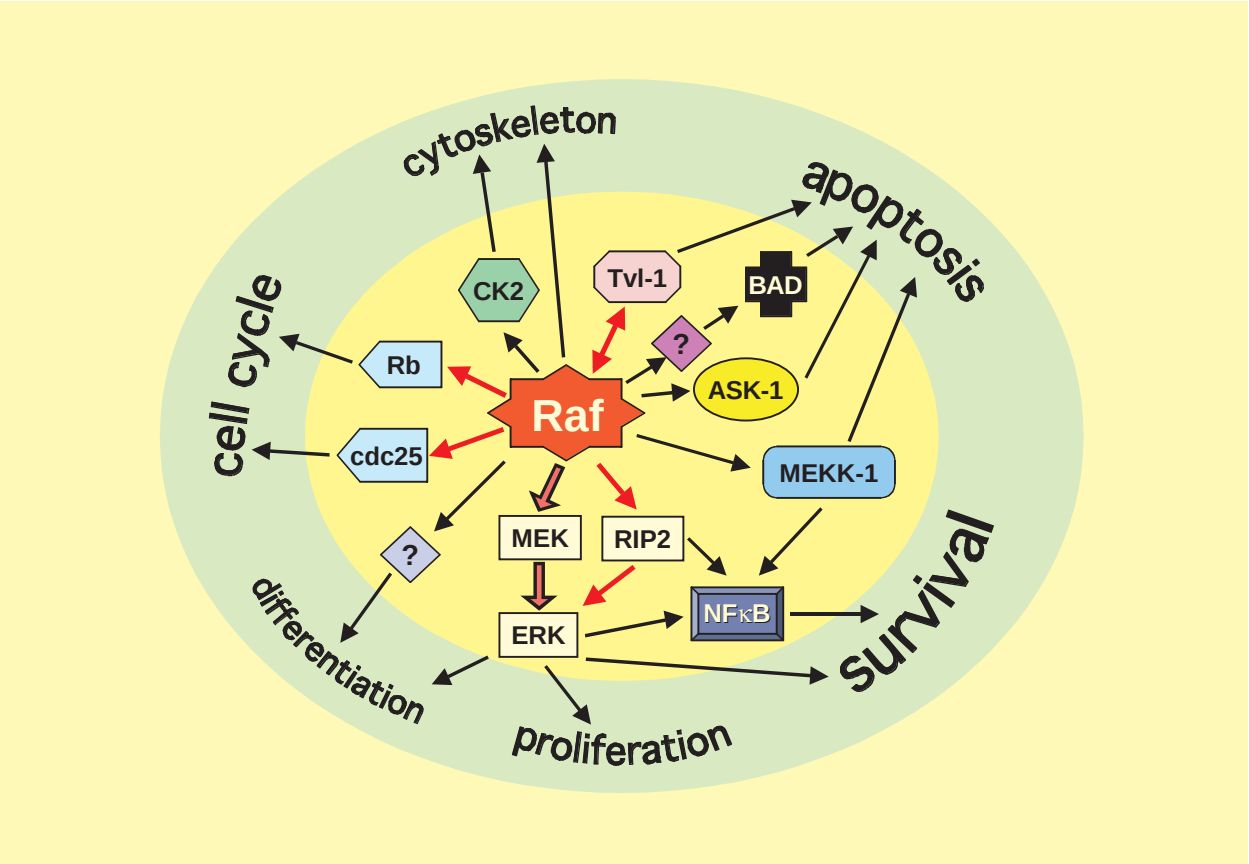

Fig. 1. Putative Raf signalling targets. All functional interactions shown have been described for Raf-1 only, with the exception of MEK phosphorylation which is common to all three Raf proteins. Red arrows with black frames indicate validated substrate phosphorylations, while red arrows indicate suggested substrates. Black arrows are used to describe functional interactions of uncharacterised nature, which may or may not include phosphorylation. See text for details.

1999; Qiu et al., 1999). An emerging theme is that Raf-1 induces IκB degradation via MEKK-1 (MEK kinase 1) independently of MEK and ERK and that this is essential for Raf-1-mediated transformation (Baumann et al., 2000). MEKK-1 was originally described as a kinase capable of activating MEK, but this seems to be an overexpression artefact; the physiological function of MEKK-1 is instead in the NF-κB and apoptosis pathways (Schlesinger et al., 1998). Regardless of the mechanism by which Raf-1 activates NF-κB, it does not seem to be essential, since NF-κB activation proceeds unimpaired in *Raf-1*$^{-/-}$ cells (Jesenberger et al., 2001; Mikula et al., 2001).

Other studies suggest that Raf-1 and ERK signalling can be distinct, or even oppose each other. For example, the transcription of atrial natriuretic factor (ANF) in cardiac myocytes is suppressed by ERK but activated by Raf (Jette and Thorburn, 2000). Likewise, in 3T3-L1 cells ERK impedes insulin-induced differentiation into adipocytes (Font de Mora et al., 1997), whereas v-Raf promotes differentiation. However, in contrast to insulin, v-Raf does not activate ERK, which suggests that v-Raf promotes differentiation independently of ERK. Further, a dominant negative Raf-1 mutant prevents insulin from stimulating differentiation but not from activating ERK (Porras and Santos, 1996). These findings suggest that during adipocytic differentiation ERK is activated independently of Raf and that Raf supports differentiation independently of ERK. Similarly, the neuronal differentiation of rat hippocampal cells can be driven by activated Raf but not by activated MEK (Kuo et al., 1996), which again suggests a bifurcation of Raf signalling and engagement of MEK-independent Raf effector(s). This hypothesis was borne out by an elegant study that generated a constitutively active Raf-1 point mutant that could not interact with MEK (Pearson et al., 2000). This mutant failed to transform fibroblasts and activate serum-response element (SRE)-mediated reporter gene expression. However, the activation of NF-κB-dependent gene expression, neuronal differentiation of PC12 cells and the activation of p90-RSK remained intact. Despite the caveat that this Raf-1 mutant still may produce a small but sufficient level of MEK activation, to date this is the clearest biochemical evidence for novel Raf-1 signalling targets.

All these experiments involved the forced expression of activated or dominant negative Raf-1 mutants, and hence are affected by the general problems of overexpression artefacts. Two issues are of particular concern in this context. First, constitutively active kinases may lose substrate specificity. The activated Raf-1 mutants used in these studies were generated by deletion of the regulatory domain, which not only suppresses kinase activity (Cutler et al., 1998) but also regulates the interaction with the activator Ras (Vojtek et al.,

1993) and the substrate MEK (Dhillon et al., 2002). The removal of such regulatory motifs may compromise substrate specificity as well as the dynamic regulation of activity. Second, the dominant negative Raf-1 mutants used were made either by removing the kinase domain or by disabling the kinase activity through a point mutation in the ATP-binding site. Both types of mutant can interact with Ras and hence can block the access of other effectors to Ras. In addition, the latter mutant is expected to compete for substrate(s) too. Therefore, as in the case of any other results obtained through overexpression, these findings need to be interpreted with caution. A definitive answer can be provided only by the identification of a bona fide Raf substrate other than MEK.

New substrates for Raf-1?

Whether there are non-MEK substrates of Raf-1 is a burning question that has few answers at present. Several new targets have been described, including cell cycle proteins pRb (retinoblastoma protein), p53 and cdc25, a novel substrate and regulator Tvl-1, and pro-apoptotic molecules such as RIP2 (receptor interacting protein 2) and BAD (Fig. 1). Intriguing as they may be, none of them has been confirmed. In addition, Raf-1 is associated with several known and unknown kinases (Janosch et al., 2000; Janosch et al., 1996), which makes it difficult to authenticate Raf-1 substrates.

One range of substrates pertains to cell cycle regulation. pRb plays a central role in the cell cycle, being the main repressive factor that holds cells in G1. In G1, pRb acts as a transcriptional repressor by binding to the transcription factor E2F and retaining it in the cytosol. To release E2F, pRb must be phosphorylated on multiple sites by cyclin-dependent kinases (CDKs) (Zheng and Lee, 2001). Raf-1 binds to pRb in growth-factor-stimulated cells, interacting only with the active (hypophosphorylated) form of pRb (Wang et al., 1998). Raf-1 kinase activity is required for the interaction, but direct phosphorylation of pRb by Raf-1 has been seen only in vitro. The authors propose that Raf-1 could be part of the cell cycle machinery that together with CDKs inactivates pRb by phosphorylation. This would link Raf-1 directly to cell cycle regulation. However, these observations are at odds with the results of timed microinjection studies. These show that, at least for cell cycle progression in response to insulin, Raf-1 activity is required only in the first 20 minutes after stimulation (Rose et al., 1998), whereas pRb phosphorylation occurs much later in G1. Another cell cycle protein postulated to be a Raf-1 substrate is Cdc25, a phosphatase that triggers CDK activation by removing inhibitory phosphates (Nilsson and Hoffmann, 2000). Active forms of Raf-1 interact with Cdc25, predominately the Cdc25A isoform, which is involved in the G1/S phase transition. At least in vitro, Raf-1 can activate Cdc25A by phosphorylation (Galaktionov et al., 1995). Another report that was never followed up found that Raf-1 can phosphorylate p53 in vitro and enhance its transcriptional activity (Jamal and Ziff, 1995).

The other group of proposed Raf-1 targets are proteins involved in apoptosis regulation, most notably BAD and ASK-1 (for discussion, see below). The ankyrin-repeat protein Tvl-1 is one such potential target. Tvl-1 has been reported to promote the assembly of pro-apoptotic Apaf-1 complexes, caspase activation and apoptosis (Patriotis et al., 2001), and bound to Raf-1 in a yeast two-hybrid screen with a murine T-cell cDNA library (Lin et al., 1999). Tvl-1 is both an in vitro substrate for Raf-1 and a novel regulator that enhances Raf-1 activation by EGF and Src plus Ras. At present, the functional relationships between these diverse Tvl-1 functions are unclear. Raf-1 may inhibit the pro-apoptotic function of Tvl-1 given that *Raf-1*$^{-/-}$ macrophages are hypersensitive to caspase-1 activation and apoptosis (Jesenberger et al., 2001). Another suggested Raf-1 substrate, RIP2 (also called CARDIAK or RICK) was originally described as a pro-apoptotic serine/threonine kinase involved in caspase activation and tumour necrosis factor (TNF) receptor and Fas signalling (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). Recently, RIP2 was reported to activate ERK and be activated by Raf-1 in response to TNF stimulation (Navas et al., 1999). Thus, in the TNF signalling pathway, RIP2 would take the place of MEK to couple Raf-1 to ERK. The physiological purpose of such a change in ERK activation is obscure but intriguing, because RIP2 can activate NF-κB (McCarthy et al., 1998; Thome et al., 1998). It thus may provide another link between Raf-1 and NF-κB.

Raf-knockout mice

Arguably the strongest evidence for MEK-independent Raf effectors comes from mouse genetics. A-*Raf*-knockout mice are born alive, can survive up to 21 days post-partum and display neurological and intestinal abnormalities (Pritchard et al., 1996). The B-*Raf* knockout is embryonic lethal and, in mice, embryos show overall growth retardation and increased apoptosis in endothelial tissues, which leads to vascular defects and death from vascular haemorrhage (Wojnowski et al., 1997). The first description of *Raf-1*-knockout mice showed embryonic lethality, growth retardation and complex developmental defects in placenta, lungs and skin (Wojnowski et al., 1998). However, the interpretation of this phenotype is confounded by the residual expression of a truncated Raf-1 protein that has severely reduced kinase activity, but may retain some functions or behave as a dominant negative mutant.

Last year saw the back-to-back publication of two *Raf-1*-knockout mice studies (Huser et al., 2001; Mikula et al., 2001) in which Raf-1 protein expression was abolished completely. Both groups observed embryonic lethality and poor development of the placenta and embryonic organs, in particular of the liver and the haematopoietic compartment. As in most knockout mice, the phenotypes were modified by genetic background and ameliorated in outbred strains. However, two common themes emerged: a normal proliferative capacity and an increased propensity towards apoptosis. Surprisingly, both studies found ERK activation by mitogens unimpaired in *Raf-1*$^{-/-}$ fibroblasts. This could reflect compensation by the other Raf isozymes, probably B-Raf, which was previously reported to be the main MEK activator in fibroblasts (Reuter et al., 1995). B-Raf has been shown to protect established fibroblasts against apoptosis through ERK-dependent interference with caspase activation (Erhardt et al., 1999). However, this study employed overexpressed B-Raf, which activated ERK constitutively. Thus, it is difficult to compare these results with those obtained from the *Raf-1*$^{-/-}$ fibroblasts, in which ERK was activated transiently by growth factors, but they indicate a significant anti-apoptotic role for Raf-1 that is independent of ERK.

All *Raf-1* gene-knockout studies observed similar defects in the placenta: hypovascularisation and a severe reduction of the spongiotrophoblast and labyrinth layers (Murakami and Morrison, 2001). This placenta phenotype also occurs in *MEK-1*$^{-/-}$ mice (Giroux et al., 1999), which suggests that the Raf-1–MEK axis is required for proper placenta development. However, despite *MEK-1*$^{-/-}$ embryos dying in midgestation, no increase in apoptosis was noted. *MEK-1*$^{-/-}$ fibroblasts fail to migrate on fibronectin (Giroux et al., 1999), a feature that unfortunately was not tested in the *Raf-1*-knockout studies. Thus, a comparison of all the knockout data argues for MEK-dependent as well as MEK-independent roles of Raf-1.

Huser and colleagues also made *Raf-1FF*-knock-in mice (Huser et al., 2001). Raf-1FF is a point mutant which, at least in cultured cells, cannot be efficiently activated by mitogens owing to the mutation of tyrosine residues 340 and 341 to phenylalanine. Astonishingly, the phenotype of these mice is normal. They survive to adulthood, are fertile and display none of the abnormalities associated with any of the *Raf* knockouts. The *Raf*-1FF mutant retains a low level of kinase activity, and it is unknown whether and how this activity is regulated in tissues. Thus, this mutant still could signal through MEK, and a conclusive answer has to await the knock-in of a true kinase-negative Raf-1 mutant.

At present it is unclear whether the strikingly different phenotypes of the *Raf*-knockout mice reflect the existence of isoform-specific substrates, tissue-specific expression or different potencies of activation of the ERK pathway. A-Raf and B-Raf expression appears more restricted, whereas Raf-1 is ubiquitously expressed (Storm et al., 1990). However, the knockout mice clearly show that Raf-1 cannot compensate for the loss of another Raf isoform. Indeed, Wiese and colleagues have shown that neurotrophic factors are unable to sustain the viability of sensory and motor neurons explanted from *B-Raf*$^{-/-}$ mice, whereas neurons from *Raf-1*$^{-/-}$ mice survive with the same efficiency as wild-type cells (Wiese et al., 2001). The basis for this difference is unknown at present. B-Raf is a more potent activator of MEK than Raf-1, and Raf-1 is better than A-Raf (Marais et al., 1997). The strength and duration of ERK activity can dictate diverse biological outcomes. For instance, in PC12 cells the sustained activation of ERK promotes differentiation, whereas a transient activation is associated with proliferation (Marshall, 1995). However, the observation that the regulation of ERK by mitogens is indistinguishable between *Raf*-1$^{-/-}$ and wild-type cells (Huser et al., 2001; Mikula et al., 2001) strongly suggests that the phenotype of the *Raf-1* knockout cells is due to a deficiency in another as yet unknown Raf-1-dependent signalling pathway.

In summary, the knockout studies yield three important and provocative conclusions. First, Raf isozymes are not functionally redundant. Second, the phenotype of the *Raf-1*-knockout mice cannot be explained by alterations in ERK regulation alone. Third, the kinase activity of Raf-1 may not be required for its biological functions. This latter point is especially intriguing in light of the fact that KSR (kinase suppressor of Ras), the closest relative of Raf kinases, seems to function more as a scaffolding protein than as a kinase (Morrison, 2001).

Raf and apoptosis

The *Raf* knockouts and biochemical studies together leave apoptosis as the most tangible candidate for a MEK/ERK-independent function of Raf kinases. Several studies have linked the anti-apoptotic function of Raf-1 to a re-localisation of Raf-1 to mitochondria (Fig. 2). The first evidence was provided by reports that Raf-1 can bind to Bcl-2 (Wang et al., 1994), the prototype of anti-apoptotic factors, thought to prevent apoptosis by preserving the integrity of mitochondria (Adams and Cory, 2001). The interaction involves the Raf-1 kinase domain and the Bcl-2 BH4 domain, which is essential for its anti-apoptotic function. This interaction resulted in Bcl-2-mediated translocation of Raf-1 to the mitochondrial membrane. The isolated Raf-1 kinase domain fused to the transmembrane region of the Mas70 protein (MasRaf-1) can protect cells from apoptosis, whereas a plasma-membrane-targeted Raf-1 failed despite efficiently activating ERK. By contrast, the MasRaf-1 kinase domain did not activate ERK but induced the hyperphosphorylation of BAD (Wang et al., 1996a). BAD is a proapoptotic relative of Bcl-2 that can be neutralised by phosphorylation-induced 14-3-3 binding and sequestration into the cytosol (Zha et al., 1996). These studies relied on overexpression, and interactions between endogenous Raf-1 and Bcl-2 proteins are yet to be shown. In fact, a follow-up study in which MasRaf-1 was expressed in *Bcl-2*$^{-/-}$ fibroblasts, and Bcl-2 was overexpressed in *Raf-1*$^{-/-}$ fibroblasts, demonstrated that either protein can protect against apoptosis independently of the other (Zhong et al., 2001). It also emerged that BAD is only a very poor in vitro substrate for Raf-1 (von Gise et al., 2001) and unlikely to be a direct substrate in vivo as originally proposed (Wang et al., 1996a). Despite these limitations, these reports have stimulated an exciting new field in Raf research.

Salomoni and colleagues showed that the transformation of 32D haematopoietic cells by the Bcr-Abl oncogene is crucially dependent on Raf-1 to prevent apoptosis (Salomoni et al., 1998). Bcr-Abl induces the translocation of a fraction of endogenous Raf-1 to mitochondria, and a transformation-

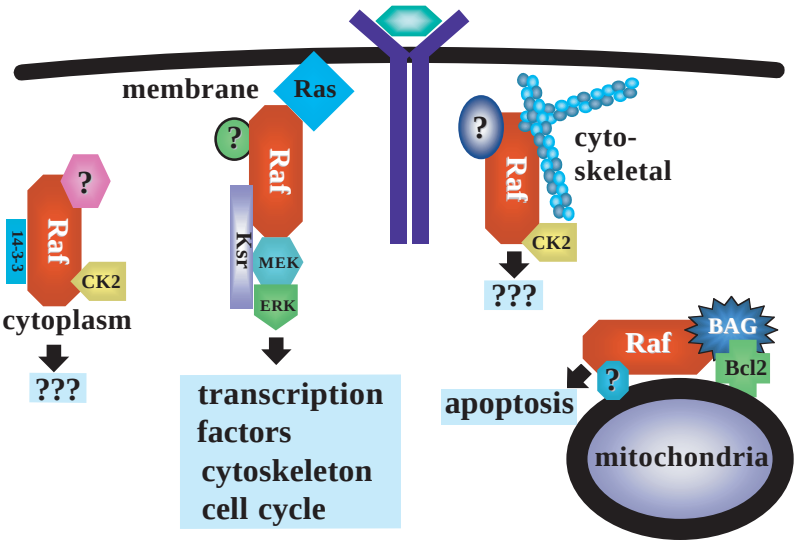

Fig. 2. Subcellular compartmentalization and protein interactions may determine the specificity of Raf activation and signalling. See text for details.

defective Bcr-Abl mutant can be complemented by the MasRaf-1 kinase domain. Again MasRaf-1 induced phosphorylation of BAD and its sequestration in the cytosol, confirming that Raf-1 can stimulate BAD phosphorylation. A more detailed dissection of the mechanism suggested that the anti-apoptotic function of Bcr-Abl is executed through BAD-independent and BAD-dependent pathways (Neshat et al., 2000). Apart from a requirement for phosphinositide 3-kinase kinase (PI-3K), the BAD-independent pathway remains elusive. The BAD-dependent pathway also needs PI-3K activity, but depends on the mitochondrial targeting of Raf-1 and is ERK independent. It can be completely blocked by a kinase-negative MasRaf-1 mutant. These findings imply that in the anti-apoptotic pathway involving mitochondrial Raf-1, Raf-1 functions downstream of PI-3K. A main executor of PI-3K in the survival pathway is Akt, a kinase that can phosphorylate and inactivate several inducers of apoptosis, including BAD (Datta et al., 1999). Curiously, the anti-apoptotic effect of Akt requires mitochondrial localisation and PKC-dependent activation of Raf-1, whereas ERK activation is dispensable (Majewski et al., 1999). Given that BAD is a direct substrate for Akt, but not Raf-1, these findings hint at an intricate relationship between the Raf and Akt pathways in apoptosis regulation.

There is also evidence that mitochondrion-located Raf-1 interacts with Grb10 (Nantel et al., 1999), an adapter protein that interacts with a variety of signalling molecules (including IGF-1, insulin and EGF), Eph family and growth hormone receptors, and kinases (including Bcr-Abl and JAK2). In Cos-7 and HeLa cells, Grb10 is located mostly at mitochondria. Nantel et al. have suggested that a Raf-1-Grb10 interaction at mitochondria could modify the activity of Raf-1. Two recent studies provide a clue to the physiological significance of such an interaction, showing that apoptosis protection by insulin-like growth factor 1 (IGF-1) is mediated by three pathways, all of which culminate in BAD phosphorylation (Peruzzi et al., 1999). One pathway induces the mitochondrial translocation of Raf-1 and Nedd4 (Peruzzi et al., 2001). Nedd4 is a target of caspases in apoptosis and also interacts with Grb10. Nedd4 is also a ubiquitin ligase, and Peruzzi et al. suggest that Raf-1-mediated translocation of BAD away from the mitochondria may stimulate ubiquitination and consequent degradation of BAD by the proteasome.

Another interesting anti-apoptotic function of Raf-1 was described by Chen and colleagues (Chen et al., 2001), who observed that Raf-1 can bind and inhibit apoptosis signal-regulated kinase 1 (ASK-1). ASK-1 is an important mediator of apoptotic signalling initiated by a variety of apoptotic stimuli; indeed, suppression of ASK-1 signalling is likely to be a general mechanism of cell survival (Tobiume et al., 2001). Curiously, a kinase-negative Raf-1 inhibits ASK-1-induced apoptosis with the same efficiency as wild-type Raf-1. Such a kinase-independent mechanism to counteract apoptosis could explain why the non-mitogen responsive Raf-1FF mutant can rescue the *Raf-1*-knockout mice.

Open questions and future prospects

Raf-1 is often regarded as a prototypic monogamous kinase that has only one substrate, MEK. However, the evidence for MEK- and ERK-independent functions of Raf-1 is accumulating and focusing on apoptosis. BAD has emerged as an important target but is unlikely to be a direct substrate. How Raf-1 inactivates BAD remains elusive. The anti-apoptotic function of Raf-1 seems to depend on its mitochondrial localisation. However, most pertinent studies have used overexpression and/or tagging with translocation epitopes. How Raf-1 is normally lured to the mitochondria and activated is not resolved. BAG-1 is one candidate. Originally isolated as Bcl-2-binding partner, it also interacts with and activates Raf-1 (Wang et al., 1996b). However, no Raf-1–BAG-1–Bcl-2 ternary complex has been observed, and BAG-1 turns out to be a Hsp70 co-chaperone that modulates Raf-1 activity in response to heat shock (Song et al., 2001). Interestingly, Rebollo et al. have suggested that, under certain conditions, Ras family proteins can be found at mitochondria (Rebollo et al., 1999). They would be obvious candidates for mediating both translocation and activation. Alternatively, a pool of Raf-1 might permanently reside at mitochondria. A recent study has placed A-Raf inside the mitochondria (Yuryev et al., 2000). A corollary of these studies is that the targets of Raf signalling may be determined by subcellular compartmentalization (Fig. 2). The importance of the spatial arrangement of signalling pathways for function is becoming increasingly appreciated. An attractive model would be that Raf kinases couple to the ERK pathway at the plasma membrane, to anti-apoptotic pathways at the mitochondrion, to cytoskeletal substrates in the cytosol and to cell cycle substrates in the nucleus.

The challenge will be to identify such substrates and prove that they are phosphorylated by Raf-1 in cells. This endeavour is hampered by the low specific activity of Raf-1 and the lack of a recognizable consensus motif for Raf-1 phosphorylation (Force et al., 1994). A combination of innovative genetics and biochemistry is needed. For instance, src family kinases have been engineered by point mutation to use substitute ATP analogues that are not accepted by other kinases as phosphate donors (Shah et al., 1997). These ATP analogues do not change the substrate specificity (Shah et al., 1997), but allow selective visualisation and identification of the direct substrates of the engineered kinase. Clues are also expected from the proteomic analysis of the composition of Raf-1 multiprotein signalling complexes. Because it has been demonstrated that physical interaction between Raf-1 and MEK is essential for MEK phosphorylation (Yeung et al., 2000), other substrates are likely to be found in association with Raf-1. The availability of murine *Raf-1*-knockout cells in conjunction with efficient gene transduction techniques allows us to employ cDNA libraries in functional screens for suppressors of the apoptosis susceptibility of *Raf-1*$^{-/-}$ cells. Such suppressors are strong candidates for Raf-1 effectors in the apoptosis pathway. The *Raf-1*$^{-/-}$ cells also provide an excellent system to validate potential effectors found biochemically. The classical genetic model organism *D. melanogaster* expresses a single Raf (D-Raf), which resembles B-Raf. In the widely studied eye-development pathway, D-Raf is fully dependent on MEK. However, D-Raf has a much broader function in embryonic pattern formation (Radke et al., 2001) and the development of imaginal disks (Li et al., 2000). It will be interesting to find out whether all these functions are mediated by MEK and ERK.

As an additional level of complexity, both genetic (Huser et al., 2001) and biochemical (Chen et al., 2001) studies raise the provocative possibility that Raf-1 may be able to signal

independently of its kinase activity. In this manner, Raf-1 could act as a scaffold or regulatory subunit. Such a function is even more difficult to prove. Genetic rescue studies in *D. melanogaster* indicate that kinase activity is essential for D-Raf to function (Radke et al., 2001). This may be different in mammalian cells, but clearly a kinase-negative *Raf-1*-knock-in is needed to prove the point. The embryonic lethality and the placenta defects of *Raf-1*$^{-/-}$ mice may obscure such effects. Thus, meaningful answers will require conditional gene-knockout technology whereby the *Raf-1* gene can be removed at will in selected tissues and at a chosen point in time. In any case the Raf field, thought to be mature, may be about to watch an old dog learn new tricks.

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