Minireview

Second nature: Biological functions of the Raf-1 "kinase"

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Abstract More than 20 years ago, Raf was discovered as a cellular oncogene transduced by transforming retroviruses. Since then, the three Raf isoforms have been intensively studied, mainly as the kinases linking Ras to the MEK/ERK signaling module. As this pathway is activated in human cancer, the Raf kinases are considered promising therapeutic targets, and we have learned a lot about their regulation, targets, and functions. Do they still hold surprises? Recent gene targeting studies indicate that they do. This review focuses on the regulation and biology of the best-studied Raf isoform, Raf-1, in the context of its kinase-independent functions.

Keywords: Raf; Kinase; Knock-out; Survival; Motility

1. History of Raf

In 1983, Ulf Rapp reported the cloning of an acutely transforming murine sarcoma virus (3611-MSV) and the characterization of its acquired oncogene, named *v-raf* (for *ra*pidly growing *f*ibrosarcoma) [1]. A similar sequence, named *v-mil*, was identified by Klaus Bister and coworkers as the transforming principle of a naturally occurring avian retrovirus MH2 [2]. Bister and Rapp then went on to show that 3611-MSV and MH2 had incorporated orthologues of the same cellular protein [3], which Karin Moelling and Ulf Rapp proved to be the first oncoprotein with serine/threonine kinase activity [4]. These seminal papers set the scene for the next 20 years of research on the cellular counterparts of *v-raf* and *v-mil*. The Raf kinase family comprises three isoforms, which differ in their expression profile, regulation, and ability to function in the context of the Ras–MEK–extracellularly regulated kinase (ERK) cascade. Although A-Raf and B-Raf transcripts can be detected, at different levels, in most embryonic and adult mouse tissues [5], Raf-1 was first reported to be the only isoform expressed ubiquitously [6]. Because of this and of reagent availability, Raf-1 has been the most intensively studied member of the family, and most of the groundbreaking contributions describing the role of Raf in signal transduction actually deal with Raf-1. Thus, it was Raf-1 which was first reported, in the late 1980s–early 1990s, to be phosphorylated and activated in response to growth factor stimulation [7–12]. Activated Raf-1 was then linked to one of the rising stars in the signal transduction sky of those years, the ERK/MAP kinase cascade. ERKs had just been discovered as proteins phosphorylated on tyrosine and threonine upon stimulation of receptor tyrosine kinases [13–16]. Soon after the description of the dual specificity kinase MEK as the upstream activator of ERK [17–19], Raf-1 was shown to activate MEK [20–22] and to physically associate with it [23]. Finally, in 1993, a number of groups demonstrated the recruitment of Raf-1 by activated Ras [24–27], instating Raf-1 as the link between Ras and ERK and completing the picture of the first MAPK pathway we all know from the textbooks (Fig. 1). The MEK/ERK module, with its impressive array of membrane, cytosolic, and nuclear substrates [28], is an excellent candidate as the downstream effector carrying out all the functions attributed to activated Raf in proliferation, differentiation, and survival by a variety of overexpression studies. Indeed, for almost a decade, MEK was the only commonly recognized substrate of the three Raf isoforms. As we will see below, recent gene ablation studies are changing this view radically, particularly in the case of Raf-1.

2. Regulation of Raf kinase activity

Despite intensive efforts, Raf regulation is far from completely understood. Again, most of the work in this area focused on Raf-1, and has revealed a complex process involving membrane recruitment, intra- and intermolecular interactions, and phosphorylation/dephosphorylation events resulting in kinase activation/release from repression. Raf-1 regulation has been the subject of recent reviews [29–31], and we will limit ourselves to an outline with an angle on repression/derepression.

All three Raf kinases share a common structure comprising three conserved regions (CR; see Fig. 2): CR1, containing the two Ras-binding sites Ras-binding domain (RBD), and cysteine-rich domain (CRD); CR2, rich in Ser/Thr residues; and CR3, representing the business end of the molecule, the kinase domain. The carboxy-terminal half of Raf-1 contains all the phosphorylation sites which stimulate activity, including the conserved Thr and a Ser residue in the so-called "activation segment" necessary for the activation of Raf-1 and B-Raf, and in all likelihood, of A-Raf [31]; and Ser and Tyr residues relevant for the activation of Raf-1 and A-Raf (S338 and Tyr341 in Raf-1). The kinases phosphorylating these residues in a physiological growth factor response have been searched

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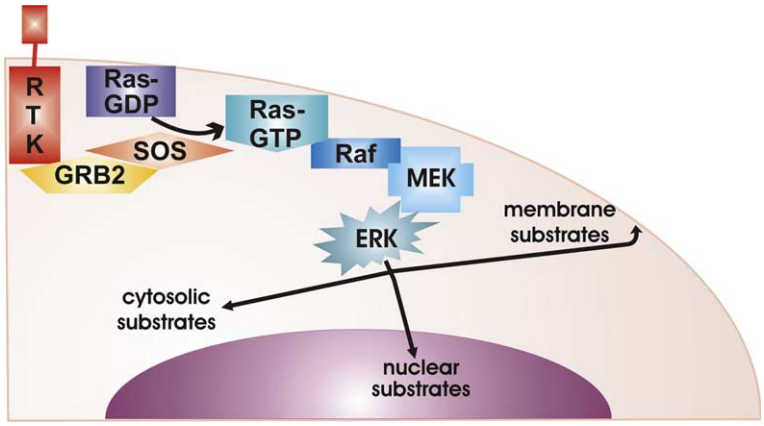

Fig. 1. General scheme of the ERK pathway. An activated receptor tyrosine kinase (RTK) causes the relocalization of the GRB2–SOS complex. SOS is a guanine nucleotide exchange factor (GEF) for Ras and causes the conversion of Ras-GDP into Ras-GTP. Ras-GTP recruits Raf to the membrane, thereby promoting its activation. Activated Raf phosphorylates and activates MEK, which, in turn, stimulates ERK activity. ERK has an impressive roster of substrates which have been implicated in the regulation of proliferation, survival, and differentiation.

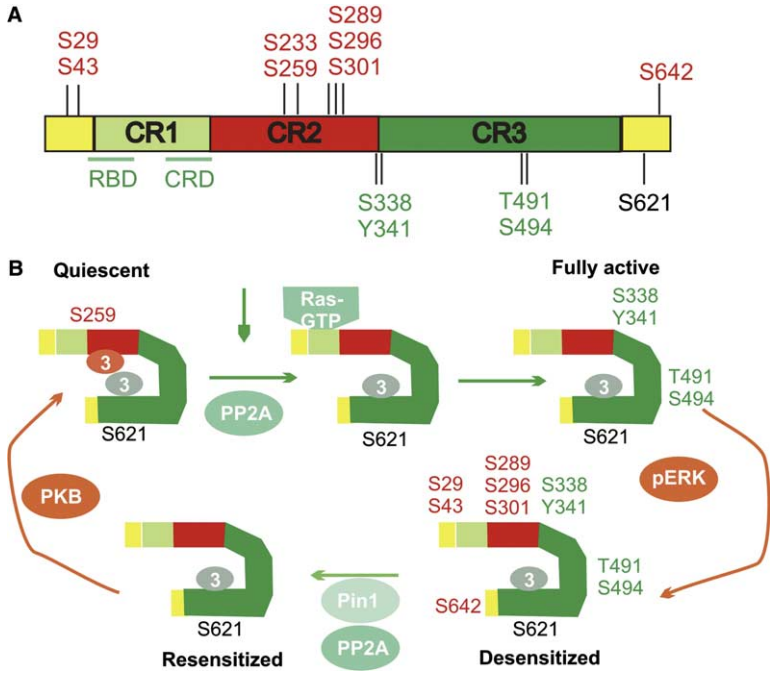

Fig. 2. Schematic view of Raf-1 (A) and its activation mechanism (B). Aminoacid residues and protein regions that participate in the positive regulation of Raf-1 MEK Kinase activity are indicated in different shades of green, whereas aminoacid residues and protein domains participating in the negative regulation of Raf-1 kinase activity are indicated in red. The CR1 region contains the RBD and the CRD involved in the relief from autoinhibition by Ras. The CR2 region is rich in serine residues which participate in the negative regulation of kinase activity. The CR3 region contains the kinase domain and the serine, threonine, and tyrosine residues involved in kinase activation (see text for details). (B) Raf-1 is regulated by intramolecular autoinhibition. In the quiescent state, phosphorylation of the S259 and S621 residues creates binding sites for the 14-3-3 proteins, which stabilizes the inactive conformation. Upon mitogenic stimulation, Ras and PP2A cooperate to release autoinhibition; the subsequent phosphorylation of activating sites by as yet incompletely identified upstream kinases yields a fully active MEK1 kinase. Following ERK activation, Raf-1 is phosphorylated on 6 negative regulatory sites, yielding a desensitized kinase. The prolyl isomerase Pin1 co-operates with PP2A to dephosphorylate the ERK-dependent negative regulatory residues. Finally, S259 becomes phosphorylated again, and Raf-1 reassumes its quiescent conformation, stabilized by a 14-3-3 dimer.

for by a number of different approaches, but their identity is still controversial (for review, see [29]). In addition, the carboxy-terminus contains a conserved constitutively phosphorylated Ser residue (S621 in Raf-1), which mediates the binding of Raf to the 14-3-3 and has been shown by mutational analysis to be necessary for Raf activity in vivo. Phosphorylation of the amino-terminal half of Raf-1, in contrast, mediates repression of kinase activity. Indeed, this region contains 7 out the 8 negative phosphorylation sites present in Raf-1: the inhibitory sites phosphorylated by PKA (S43, S233, and 259) or PKB (S259) which create additional binding sites for the 14-3-3 proteins; and 5 out of 6 newly discovered ERK phosphorylation sites (S29, S43, S289, and 296, and 301) [32]. A further ERK-dependent, inhibitory phosphorylation site (S624) is found in the carboxy-terminus. The discovery of these sites suggests the existence of a negative regulatory feedback

loop linking ERK stimulation to Raf-1 deactivation and therefore possibly limiting ERK activation.

The current model of Raf-1 regulation postulates that the N-terminal domain of Raf-1 binds to the kinase domain and suppresses the catalytic activity of the enzyme. This intramolecular autoinhibition is favored by 14-3-3 proteins binding to phosphorylation sites at the N- and C-terminus of Raf-1, and must be disrupted to permit Raf-1 activation. We and others have shown that this is accomplished via dephosphorylation of the inhibitory PKA/PKB site S259 by the phosphatase PP2A, which interacts with Raf-1 in mitogen-stimulated cells. Upon S259 dephosphorylation, Raf-1 is recruited to the membrane and binds to Ras [33–35] via the RBD and the CRD contained in the CR1 region. Ras binding is then followed by the phosphorylation of the activating residues in the CR3 region, which stabilizes an activated conformation. Activation is terminated by a negative feedback loop in which ERK and PKB phosphorylate Raf-1 on inhibitory sites. The activation-competent conformation of Raf-1 is finally re-established by the co-ordinated action of Pin1, a prolyl isomerase that converts pSer and pThr residues from the *cis* to the *trans* conformation, which is preferentially recognized and dephosphorylated by PP2A. Thus, at least two distinct Ras effectors, PKB and ERK, contribute to the negative regulation of Raf-1, and dephosphorylation of inhibitory sites is as important as activating phosphorylation for the stimulation of Raf-1 kinase activity.

In contrast to this complicated process, B-Raf activation seems to be much more direct, requiring basically only Ras binding [36] and phosphorylation of the activation segment [31] to disrupt intramolecular autoinhibition. In B-Raf, the aminoacids involved in Raf-1 activation are either constitutively phosphorylated (Ser 445, corresponding to Raf-1 Ser338) or negatively charged (Asp448, corresponding to Raf-1 Tyr341). The constitutive presence of negative charges in this region of B-Raf likely reduces the threshold for mitogen-induced kinase stimulation. In addition, it is unclear whether a 14-3-3 dimer stabilizes intramolecular autoinhibition in the case of B-Raf. 14-3-3 bind to B-Raf pS728 and a further potential 14-3-3 binding site in the CR2 (S364) can be generated by PKB [37]. Although phosphorylation of this site inhibits B-Raf activity, it has not been tested whether endogenous B-Raf is phosphorylated on pS364 in quiescent cells, and whether dephosphorylation of this site is necessary for B-Raf activation. Finally, only two of the six residues phosphorylated by ERK in Raf-1 are conserved in the other Raf proteins, and it is not know whether phosphorylation of these two residues only is sufficient for deactivation. Thus, comparison of Raf-1 and B-Raf regulation reveals that the latter kinase is ''primed'' for Ras-induced activation.

3. Raf and the MEK/ERK module

Although most of the work published on the activation of the MEK/ERK module was performed with Raf-1, evidence has been accumulating that B-Raf is the main MEK kinase in vivo. Cell fractionation and immunodepletion studies have shown that B-Raf is the main MEK kinase found in cell and brain lysates [38–40]. Furthermore, comparison of the three Raf kinases has shown that B-Raf binds best to MEK [41] and has the highest basal MEK kinase activity both in vitro [42] and in fibroblasts, when expressed as a conditionally oncogenic form [43]. Finally, growth factor-stimulated ERK activation is reduced in B-Raf-deficient, but not in A-Raf- or Raf-1-deficient cells [5,44–47]. These experimental facts correlate well with the observation that the Raf kinases from lower organisms, like *C. elegans*' *lin-45* or *Drosophila*'s *D-Raf*, are more similar to B-Raf than to the other two mammalian Raf kinases. Thus, B-Raf is likely to be the archetypal MEK kinase, whereas Raf-1 and A-Raf have likely diverged to perform other functions. Although at present the only confirmed substrate of B-Raf is MEK, recent work on B-Raf mutations found in human tumors has revealed an unexpected twist in the story: B-Raf mutants unable to phosphorylated MEK in vitro can still activate the MEK/ERK cascade in vivo, and they do so by binding to, and activating, Raf-1 [48]. It is yet completely unclear whether the mutations abrogate B-Raf kinase activity completely or whether they shift substrate specificity, whether kinase activity is required for the effect on Raf-1, or whether heterodimerization between Raf-1 and mutant B-Raf causes a conformational change promoting Raf-1 MEK kinase activity. In this context, it is noteworthy that wild-type Raf-1 and B-Raf can heterodimerize [49] and, more specifically, that the isolated autoinhibitory domain of Raf-1 can interact with, and inhibit, the catalytic domain of B-Raf [50]. The relevance of these data for the regulation of the wild-type enzymes during physiological responses has not yet been tested; however, they raise the interesting possibility that Raf-1 and B-Raf may cross-regulate each other in this context as well.

Complex formation is a recurring theme in Ras-ERK signaling, and a number of scaffold proteins have been described that, by recruiting selected signaling components, help maintaining signal fidelity and favor signal propagation through the cascade. KSR, for instance, is a Raf-related pseudokinase which binds to MEK, ERK, and Raf [51]; CNK interacts both with Raf and with components of the Ral signaling pathway [52]; and Sur-8 facilitates the interaction between Ras and Raf [53]. On the other hand, proteins have been identified that disrupt interactions in the cascade: RKIP, which decreases interaction between Raf and MEK and may regulate Raf activation [54]; Sprouty and Spred, which suppress Raf activation [55,56]; and IMP, which inactivates KSR [57]. Most of these proteins have been first identified in *Drosophila* or *C. elegans*; therefore, the prediction would be that they interact both with Raf-1 and B-Raf. Indeed, whenever tested, this was the case. In several cases, interaction of the scaffold with their target proteins or correct localization of the scaffold are modulated by phosphorylation/dephosphorylation events [58]; these multiple levels of regulation provide a high degree of plasticity, allowing the cell to redirect the signals towards, or away from, the ERK signaling pathway, and thereby to fine-tune its output.

4. Lessons from knock-out mice – Novel targets and novel functions for Raf-1

A-raf, *B-raf* and *c-raf-1*-deficient mice have been generated. *A-raf*-deficient mice are born alive and show neurological and intestinal defects, depending on the genetic background [59]. In contrast, *B-raf* and *c-raf-1*-deficient embryos both die around

midgestation. The former succumb to vascular hemorrhage due to apoptotic death of differentiated endothelial cells [60], whereas *c-raf-1*-deficient embryos show increased apoptosis of embryonic tissues [45] or, more selectively, of the fetal liver [46], depending on the genetic background. Ablation of the common Raf kinase target, MEK-1, results in embryonic lethality due to a placentation defect correlating with reduced cell motility [61]. These divergent phenotypes show that Raf-1, B-Raf, and MEK-1 serve distinct essential functions in embryonic development.

While little follow-up work has been done on the B-Raf and MEK-1 knock-out, a number of papers have advanced our understanding of the biological role of Raf-1. It has quickly become clear that one of the main functions of this protein is to restrict caspase activation in response to selected stimuli, notably Fas stimulation [45,46], pathogen-mediated macrophage apoptosis [62], and erythroid differentiation [63]. The MEK/ERK module is in principle capable of antagonizing apoptosis in a number of ways, including the expression of caspase inhibitors and the neutralization of pro-apoptotic Bcl-2 family members; a further prominent prosurvival molecule, the transcription factor NF-κB, has been proposed as a downstream target of Raf-1 (reviewed in [64]). However, neither MEK/ERK nor NF-κB activation are altered in Raf-1-deficient cells and embryos [45,46,62], indicating that the prosurvival role of Raf-1 does not depend on these functions. What, then, are the essential downstream targets of Raf-1 in apoptosis?

Recently, conditional mutagenesis has confirmed apoptosis signal-regulated kinase 1 (ASK1) as a pro-apoptotic molecule inhibited by Raf-1 in vivo [65]. ASK1 is a protein kinase which works upstream of JNK and p38 to promote apoptosis induced by stress or by death receptors, like the TNF-αR or Fas. A few years ago, Hanan Fu reported that Raf-1 forms complexes with, and antagonizes, ASK1, and that Raf-1 does so independently of its kinase activity [66]. More recently, ASK1 binding to Ha-Ras has been shown to inhibit the proapoptotic activity of the kinase [67]. Last year, elegant work by Kinyia Otsu has shown that cardiac-specific Raf-1 ablation induces cardiomyocyte apoptosis in vivo. This defect is accompanied by the transient activation of ASK1 and its downstream targets p38 and JNK, and could be rescued by inactivation of the ASK1 gene [65]. This work has firmly established that Raf-1 antagonizes ASK1 in vivo, at least in cardiomyocyte survival. Whether Raf-1 modulates ASK1 activity by direct binding or by competing for a common binding partner responsible for the inhibition of ASK1-induced apoptosis is at present unclear.

Hyperactivation of ASK1, however, does not explain the selective hypersensitivity of Raf-1-deficient fibroblasts towards FasL, but not TNF-α-induced apoptosis. In particular, conventional ablation of ASK1 reveals that this kinase is essential for TNF-α, but not Fas-induced apoptosis, making it unlikely that ASK1 is the Raf-1 target in this context. A protein with all the right credentials has been recently identified as a result of a proteomic effort combined with the analysis of knock-out cells and RNA interference. As in the case of ASK1, the protein in question is a kinase, MST2, and it is hyperactive in Raf-1 knock-out/knock-down cells [68]. Raf-1 binds to MST2 via its N-terminus, and specifically via the CR2 region that is not conserved in B-Raf; therefore, MST2 qualifies as "Raf-1-only" target. MST2 is activated selectively by Fas in Raf-1-deficient cells, indicating that MST2 inhibition is an essential function of Raf-1 in the context of Fas-induced apoptosis. Mechanistically, Raf-1 appears to prevent MST2 homodimerization, which leads to activation of this kinase, and additionally to recruit a phosphatase, (PP2A?), which dephosphorylates, and therefore inactivates, MST2 (Fig. 3). Kinase-dead Raf-1 is as efficient as wild-type Raf-1 in binding to, and antagonizing, MST2, proving that the kinase activity of Raf-1 is dispensable for this prosurvival function. Although the significance of MST2 inhibition in the context of the whole organism has not been assessed, these data identify a novel, kinase-independent target of Raf-1 in apoptosis.

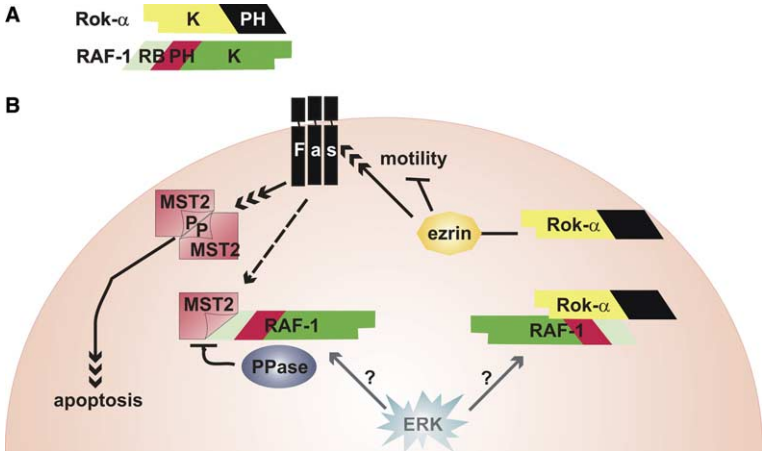

Fig. 3. Novel targets and functions of Raf-1. (A) Schematic view of Raf-1 and Rok-a, highlighting the similarities between the two molecules. RB, Ras-binding domain; PH, pleckstrin homology domain; K, kinase domain. (B) In wild-type cells, the presence of Raf-1 regulates the level of activity of Rok-α (right side), possibly by cross-inhibition via its pleckstrin homology domain; and of MST2 (left side), by inhibiting the formation of MST2 dimers and by recruiting a phosphatase (PPase) that dephosphorylates and inactivates MST2. In Raf-1 knock-out cells, hyperactivation of Rok-a leads to defects in actin remodeling and to impaired migration via the hyperphosphorylation of ezrin; phosphorylated ezrin further appears to impinge on apoptosis by reducing Fas internalization and enhancing the Fas signal. in addition, in the absence of Raf-1 Fas stimulation causes the dimerization and activation of MST2, which contributes to the increased apoptosis observed in Raf-1 knock-out cells. The possibility that ERK may redirect Raf-1 towards kinase-independent targets by phosphorylating inhibitory residues on Raf-1 is indicated.

Protection from apoptosis is not the only physiologically relevant function of Raf-1. Using conditional mutagenesis, we have recently demonstrated that Raf-1 is required for normal wound healing in vivo and for the migration of keratinocytes and fibroblasts in vitro. Strikingly, this novel function of Raf-1 can also be carried out by a kinase-dead mutant, and, just like prosurvival, it involves the inhibition of another kinase. The target of Raf-1 in motility is the Rho effector Rok-α, which is hyperactive and mislocalizes to the membrane of Raf-1-deficient cells. As a consequence of Rok-α hyperactivation, Raf-1 knock-out fibroblast and keratinocytes have a contracted appearance, a defective cytoskeleton characterized by tight cortical actin bundles, and fail to migrate. Chemical inhibition of Rok-α or expression of a dominant-negative Rok-α mutant rescue all defects of the Raf-1-deficient cells, indicating that Rok-α is the only target of Raf-1 in motility [69]. But how does Raf-1 regulate Rok-α? We know that inhibition is mediated by the Raf-1 autoregulatory region, which contains a cystein-rich pleckstrin homology (PH) domain (aa 100–144). Rok-α, like Raf, is regulated by autoinhibition, and its carboxy-terminal autoregulatory region features a PH highly homologous to the one found in Raf-1 (Fig. 3A). This leads to the hypothesis that Raf-1 may keep activated Rok-α in check by binding to the Rok-α kinase domain and repressing its function (Fig. 3B).

5. Conclusions and future perspectives

Two surprising lessons emerge from the data summarized above: first, the MEK kinase activity of Raf-1 is not required for the essential functions of this protein in survival and motility; and second, the autoinhibitory N-terminus of Raf-1, which is deleted in the retroviral oncogene, is used by the cell as a negative regulator of at least two other kinases, one promoting apoptosis, the other controlling cell shape and motility. Naturally, these insights raise a whole host of new questions. For instance, if MEK kinase activity is not the main function of Raf-1, why is it so tightly regulated? One possibility is that the negative regulatory mechanisms targeting Raf-1 kinase activity have evolved to separate Raf-1 from the MEK/ERK module, or even to redirect it towards other targets, which do not require kinase activity. Both MST2 and Rok-α bind to the N-terminal region of Raf-1, which should not be accessible in the ''quiescent'' state of the protein. Do they bind better to the ''desensitized'' Raf-1 produced as a consequence of ERK activation? We are currently performing structure-function studies with the Raf-1/Rok-α pair to answer these questions.

On a different note, are the functions of Raf-1 in apoptosis and motility completely separated, or do they intersect? Recent studies in the lab indicate that the latter may be the case. Rok-α and its target ezrin, hyperphosphorylated in Raf-1-deficient fibroblasts and responsible for the bundling of cortical actin in these cells, appear to mediate Fas clustering and inhibit Fas internalization, thereby rendering the cells selectively hypersensitive to Fas-induced apoptosis.

Finally, are the kinase-independent function all there is to Raf-1 physiology, or are there other, tissue-specific functions of Raf-1 as a (MEK1) kinase? What is the relative significance of the prosurvival function of Raf-1 and of its role in motility for embryonic development? And, possibly the most burning question, may one of these kinase-independent functions be of importance in tumor development or maintenance? Conditional mutagenesis coupled with the use of mouse tumor models will enable us to address these issue. And Raf biology will keep us intrigued for the next 20 years.

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