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News and Commentary

An old kinase on a new path: Raf and apoptosis

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The cytosolic serine/threonine Raf kinases play a central role in evolutionary conserved signal transduction pathways. An impressive number of publications implicates these enzymes in a variety of biological processes, most prominently in regulated and deregulated proliferation. Because of this, they are considered attractive therapeutic targets. Gene ablation experiments have now revealed that in the context of the mouse embryo Raf-1 and B-Raf play essential anti-apoptotic roles that cannot be rescued by the other Raf isoforms. In the case of Raf-1 it is clear that its best-studied downstream target, the MEK/ERK pathway, does not mediate the anti-apoptotic function of the kinase. These experiments have shed new light on the biological role of the Raf kinases and have prompted a search for alternative substrates mediating it.

Activation of MEK/ERK: A family business

Raf is a family of three serine/threonine specific kinases (A-Raf, B-Raf, and Raf-1) ubiquitously expressed throughout embryonic development.[1] All three enzymes can be activated by the small G protein Ras and relay upstream input signals to the MEK/ERK module. Activated ERK controls an impressive roster of substrates, ranging from metabolic enzymes to specific transcription factors whose phosphorylation affects the pattern of gene expression. Within this signaling cascade, Raf interacts physically with MEK-1 via its kinase domain and with GTP-loaded Ras via its N-terminus. The high affinity binding to activated Ras mediates the translocation of Raf-1 from the cytosol to the plasma membrane, where activation occurs via a complex, yet incompletely defined mechanism involving phosphorylation and dephosphorylation.[2] Both Ras and Raf are proto-oncogenes, and deregulation of the ERK pathway is involved in the pathogenesis of severe diseases that affect large portions of the population. Specifically, the ERK pathway is deregulated in more than 30% of common cancers. Therefore, the Raf kinases are an attractive target for novel therapies aimed at interfering with its activation process and at eventually reversing its deregulated functions.

The three Raf isoforms differ in their ability to interact with Ras,[3,4] to activate the MAPK pathway,[5] and to transform NIH 3T3 cells.[5] In all three cases, the ranking is B-Raf $>>$ Raf-1 $>>$ A-Raf. *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.[6] 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.[7] c-raf-1-deficient embryos show increased apoptosis of embryonic tissues[8] or, more selectively, of the fetal liver,[9] depending on the genetic background. These divergent phenotypes show that Raf isoforms serve distinct essential functions in different tissues and that they cannot always compensate for each other.

The genetic experiments described above have yielded two rather unexpected results:

(1) both B-Raf and Raf-1 have individual, essential pro-survival functions that cannot be executed by the other Raf isoforms; and
(2) in the case of Raf-1, the essential anti-apoptotic function is not mediated via the MAPK cascade.[8,9]

The essentials: Raf kinases and apoptosis

These results are surprising given the overwhelming amount of published evidence indicating a role for Raf in cell cycle progression. However, the concept that Raf kinases play a prosurvival role is not entirely new. It has been put forward in a number of studies largely based on overexpression and, in some cases, forced mitochondrial localization of the constitutively active enzymes or of dominant negative, kinase-dead mutants.[10–20] The matter of the mechanism(s) and substrate(s) involved in this prosurvival function is still entirely open.

The classical Raf effector module MEK/ERKs has been reported to antagonize apoptosis,[18,21–25] and a role for these effectors has been proposed downstream of both B-Raf and Raf-1.[15,18] In the case of B-Raf, apoptosis inhibition occurs downstream of cytochrome *c* release, suggesting that caspase inhibitors (IAPs[26]) might be the relevant targets.[15] Indeed, sensory and motoneurons from B-Raf-deficient embryos in culture fail to express specific IAPs, and are unable to survive in response to neurotrophic factors.[27]

In contrast, a mitochondrial pool of Raf-1 molecules, which do not activate MEK, reportedly exert their prosurvival effects upstream of cytochrome *c* release by phosphorylating the pro-apoptotic Bcl-2 family member Bad.[13,14,20,28,29] Phosphorylation of Bad results in its translocation to the cytosol and ultimately in the inhibition of cytochrome *c* release. Anti-apoptotic Bad phosphorylation, however, can be brought about by other kinases, e.g. by the MEK/ERK module[30,31] but, most prominently, by the antiapoptotic kinase Akt/PKB.[32] The relationship between

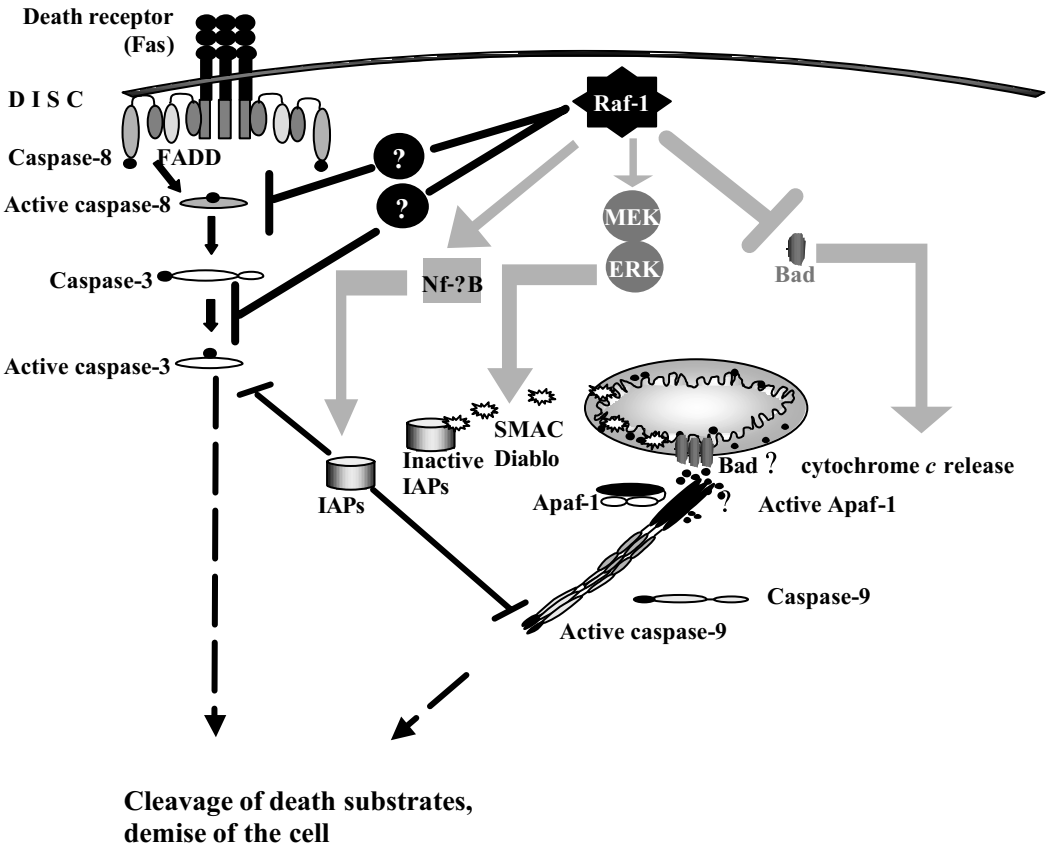

Figure 1 Interactions of Raf-1 with the apoptotic machinery. Raf-1 may function as a general inhibitor of apoptosis via its best-studied target MEK/ERK, which are reportedly able to antagonize activated caspases; via the activation of the anti-apoptotic factor NF-κB, which influences the expression of IAPs; and by phosphorylating Bad and causing its relocation from the mitochondria to the cytosol, thereby protecting the mitochondria. However, these pathways (gray arrows) are not affected by the lack of Raf-1 in knock-out cells, and therefore they do not contribute to the characteristic increase of apoptosis caused by the absence of the kinase. Instead, Raf-1 appears to antagonize apoptosis by restraining caspase activation (black arrows), either directly or via a yet unknown effector

Raf and Akt/PKB is presently unclear. Akt is able to phosphorylate Raf-1 on a negative regulatory residue, thereby reducing its activity toward the MEK/ERK module.[33] This might be a way of re-directing Raf-1 activity towards other anti-apoptotic substrates. Consistent with this idea, mitochondrial Raf-1 has been recently proposed to mediate the prosurvival function of Akt in hematopoetic cells.[13] On the other hand, Akt appears to mediate at least part of the prosurvival signal of oncogenic Raf.[18]

In addition to MEK/ERK and Bad, Raf-1 can regulate the anti-apoptotic transcription factor NF-κB,[34] which has been shown to participate in the transcriptional regulation of IAPs[35,36] as well as of c-FLIP, an inhibitor of caspase-8 activation.[37] Raf-1 apparently induces NF-κB activation by a mechanism that does not involve MEK/ERK but rather another MAPKKK, MEKK-1, upstream of the IκB kinase complex.[38]

The last MEK/ERK-independent prosurvival function of Raf-1 reported consists in antagonizing the activity of apoptotic signal-regulated kinase (ASK1). ASK-1 plays an essential role in TNF-α and H_2O_2-induced apoptosis,[39] while Raf-1 does not.[9] Thus, ASK-1 inhibition is unlikely to be the basis of the essential anti-apoptotic function of Raf-1.

Intriguingly, the investigation of the anti-apoptotic pathways that have been connected to Raf-1 in the literature gave only negative results: activation of the MEK/ERK and NF-κB is normal in Raf-1-deficient macrophages and fibroblasts, and cytochrome *c* release is not increased. However, caspase activation by selected apoptotic stimuli is enhanced in cells and mice lacking Raf-1[40] (Piazzolla *et al*., unpublished observations). The apoptotic stimuli affected by the lack of Raf-1 include pathogen-induced apoptosis of macrophages,[40] and, in fibroblasts, growth factor deprivation and Fas, but not TNF-α-mediated apoptosis.[9] Thus, Raf-1 does not perform a general prosurvival function, but is rather involved in counteracting specific apoptotic pathways by restraining caspase activation.

Although several pieces of the puzzle are missing, the analysis of knock-out animals has unequivocally established the essential anti-apoptotic role of Raf-1 and B-Raf, and, at least in the case of Raf-1, has shown that this function is effected by targets distinct from the ones described in the literature. We can look forward to future studies employing conditional knockouts to ascertain whether the anti-apoptotic function of the Raf kinases is still essential in the context of adult animals, and in which tissues; and whether the kinases have an irreplaceable function in the establishment or progression of tumors. The combination of phenotype analysis with the biochemical

investigations of their molecular basis in ablated cells will help defining the function(s) of Raf-1, B-Raf and their relevant biological targets. The information obtained will be instrumental in assessing the potential of these molecules as therapeutic targets and in directing the design and use of pharmacological kinase inhibitors.

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