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Raf and the road to cell survival: a tale of bad spells, ring bearers and detours

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Abstract

Research of the last years has demonstrated the absolute requirement of mitogenic signaling pathways for the control of cell survival. As reviewed here for the members of the Raf kinase family, apoptosis suppression proceeds through diverse mechanisms. They include the recruitment of novel effectors such as IAP and Bcl-2 proteins, key molecules in cell survival control, which interfere with the executions of the cell death at various levels, but also direct effects on metabolic events.

Keywords: Raf; Survival; Metabolism; Apoptosis; Mitochondria

1. Introduction

Cellular homeostasis depends critically upon the body's ability to coordinately replenish and eliminate cells. The latter is most often achieved by a process which is commonly referred to as apoptosis or programmed cell death, implying that it proceeds through a strictly ordered sequence of events leading to cellular demise, that is executed mainly by a family of proteolytic enzymes termed caspases [1]. Due to the inherent danger, which such a latent suicide system presents for the cell, complex mechanisms have evolved to prevent inadvertent activation. Work of the last years has shown that mitogenic signaling cascades impinge directly on this control and anti-apoptotic proteins of the Bcl-2 [2] or IAP [3] family may function as link. These pathways and proteins are frequently altered in tumors and resistance to induction of apoptosis is a major factor contributing to failed attempts to treat cancers.

In the past we have used molecular and genetic approaches to establish the critical role members of the Raf-family of protein serine/threonine kinases have in this process. This paper will review recent findings, which shed light on survival signaling by Raf proteins by identifying new targets and new pathways, which may link Raf proteins to cell survival control. Moreover, the survival activity of Raf and its ability to gear cells towards fuel production through glycolysis [4,5], a hallmark of transformed cells [6], underscore the importance of this protein as a critical determinant of cellular transformation [7].

2. How everything began

A role for cell survival signaling by C-Raf has been first demonstrated using the oncogenic version of the kinase [8]. The Raf oncogene cooperates with Myc in the naturally occurring avian sarcoma virus MH2 [9]. Expression of activated C-Raf (gag-v-raf) was able to significantly delay the onset of apoptotic cell death in IL-3-dependent promyeloid cell line 32D following growth factor removal [8], in contrast to c- and v-Myc that promoted cell cycle progression and cell death [10,11]. Increased apoptosis was seen in our analysis [12] of fibroblast cell lines derived from C-Raf-deficient animals [13] and has also been observed in tissues of C-Raf knockout animals [14,15]. This work [12,15] also suggested that Raf signaling may function in the prevention of cell death triggered via the death receptor and the mitochondrial pathway

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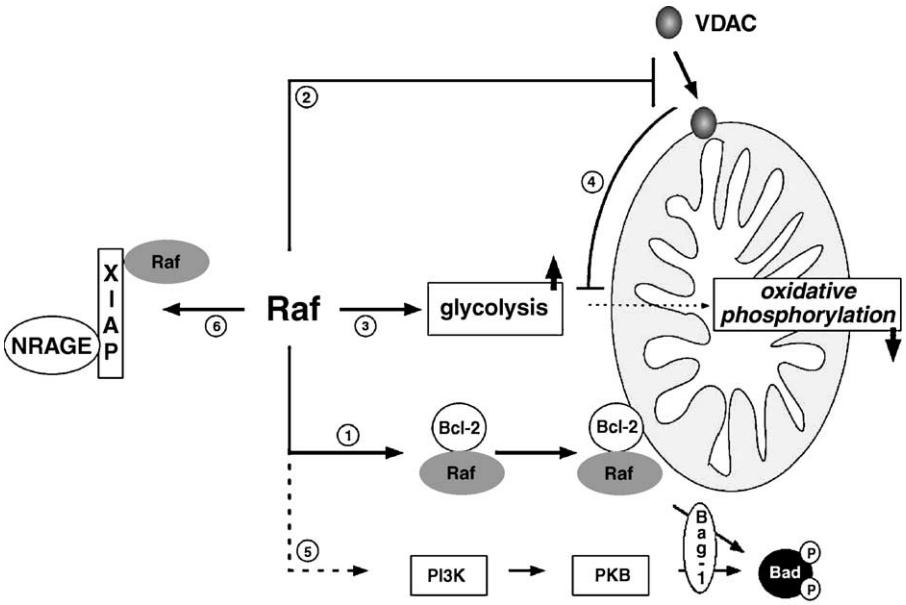

Fig. 1. Pathways involved in cell survival signaling by Raf proteins: apoptosis suppression by Raf involves mitochondrial targets, which are important for the maintenance of the integrity and the homeostasis of these organelles, and cytoplasmic proteins functioning in the post-mitochondrial control of the activation of the apoptosome.

[16]. Evidence for a critical role of mitochondria in Raf survival signaling has been supported by the demonstration of a mitochondrial Raf survival pathway, which involves Bcl-2 driven mitochondrial translocation of the kinase and apoptosis suppression by a mechanism, which leads to the phosphorylation and thereby inactivation of the pro-apoptotic Bcl-2 family protein Bad [17] (Fig. 1, pathway 1).

Our work also revealed that different members of the Raf family might have unique functions in survival control. *B-Raf*-deficient mice showed a very specific effect on endothelial cell survival [18]. Also sensory and motoneurons derived from *B-Raf*-deficient mice fail to respond to neurotrophic factors for survival. Rescue of these cells could only be achieved after transfection of exogenous B-Raf but not of C-Raf, suggesting singular properties for B-Raf in this process [19].

3. Mitochondrial gatekeeper VDAC links Raf signaling to the control of mitochondrial permeability

Mitochondria-dependent cell death is triggered by the release of apoptogenic factors, most importantly cytochrome *c* from the mitochondrial intermembrane space [20]. Cytochrome *c* is required for the formation of a functional apoptosome, which initiates the execution of the apoptotic cell death [21]. Expression of activated C-Raf residing in the outer mitochondrial membrane as a result of a fusion with targeting sequences derived from the yeast protein p70 Mas (Mas-BXB [17]) efficiently protected 32D cells by preventing cytochrome *c* release and subsequent activation of caspases 9 and 3 [4]. Similar results have been obtained with fibroblasts guarded against doxorubicin-induced cell death by Mas-BXB or gag-v-raf [12]. The work by Zhong *et al*. [12] showed that protection by mitochondrial Raf also occurred in cells lacking the expression of Bcl-2 or Bad, implying that Raf may maintain cell survival via Bcl-2/Bad independent pathways. A search for novel mitochondrial targets was undertaken, which lead to the identification of the voltage-dependent anion channel (VDAC), a mitochondrial porin involved in exchange of metabolites [22], as a novel C-Raf interacting protein [4] (Fig. 1, pathway 2). The kinase domain of C-Raf was sufficient for this interaction, but no phosphorylation of VDAC could be detected. Functional analysis of this binding suggested that C-Raf/VDAC interaction blocked the reconstitution of VDAC channels into artificial lipid bilayers [4]. Presence of active Raf in cells thus may have important consequences for the flow of metabolites in and out of mitochondria. It is a well established fact that tumor cells rely mainly on glycolysis for ATP production even under normoxic conditions (Warburg effect) [6]. This may prevent the generation of reactive oxygen species (ROS) implicated in DNA damage and apoptosis induction [5]. In part this glycolytic switch occurs as a result of HIF-dependent and HIF-independent transcriptional up regulation of glycolytic enzymes such as pyruvate kinase (PK) or lactate dehydrogenase (LDH) [6]. Raf kinases may also directly interact with PK and increase its catalytic activity [5,23] (Fig. 1, pathway 3), thereby further stabilizing tumor specific metabolic alterations. Decreased mitochondrial import, as a consequence of the reduction in the number of outer mitochondrial VDAC channels, may make the cellular commitment to gycolysis irreversible [5] (Fig. 1, pathway 4).

4. Teaming up at the mitochondria

Bag-1, a protein initially identified as Bcl-2 interacting protein [24] may play a critical role also in survival signaling by C-Raf. Bag-1 not only binds to, but also stimulates C-Raf kinase activity [25] and thus may play a role in the activation of C-Raf in a mitochondrial location. To analyze *bag-1* function in cell survival a knockout of this gene was performed [26]. *bag-1*-deficient mice exhibited massive apoptosis in the fetal liver and developing nervous system [26]. A potential mode of action for Bag-1 was suggested by our analysis of Bad phosphorylation in these cells. Reversion of the pro-apoptotic effect of Bad results from a cytoplasmic translocation of the protein triggered by phosphorylation and subsequent binding to cytosolic 14-3-3 proteins. Serine (S) 136, first identified as a target for protein kinase B (AKT) may be the most critical residue in this process, while phosphorylation on serine 112 may be less efficient in Bad inactivation [27] and other serines, 128 and 155, may serve different functions [28–30]. Intriguingly, the analysis of lysates derived from fetal livers failed to detect Bad S136 phosphorylation. One likely explanation for this was the failure of B-Raf and PKB to locate to the mitochondria in these cells suggesting a critical requirement of Bag-1 in coordinating the assembly of pro-survival kinases and substrates at the mitochondria [26]. Examination of Bad phosphorylation by purified Raf kinases revealed that B-Raf, like Pak1 kinase [31], targeted serines 112 and 136, whereas C-Raf was restricted to serine 112.[2] This finding may partly explain the differential ability of B-Raf to mediate the survival activity of neurotrophic factors GDNF, BDNF or CNTF in motoneurons and NGF in sensory neurons [19]. The spike activation of C-Raf in comparison with the sustained B-Raf activation by NGF, as first observed in PC12 cells [32] may be a second factor.

5. Detour to survival

The phosphorylation of Bad by C-Raf [17] suggested for the first time the existence of a substrate for C-Raf, which did not function in the conserved Ras-Raf-MEK-ERK signaling module essential for Raf-dependent control of proliferation, transformation, or differentiation [33]. However, genetic and biochemical data have pointed to a role for this pathway in apoptosis suppression [34,35]. Using a synthetic inhibitor of MEK activation we were able to show that MEK is essential for survival and growth in the presence of IL-3 in promyeloid 32D cells [36]. Moreover, expression of constitutively active MEK1 mutants significantly delays the onset of apoptosis upon growth factor withdrawal, whereas the presence of a dominant negative mutant accelerates cell death. Survival signaling by MEK most likely results from the activation of ERKs since expression of a constitutively active form of ERK2 was as effective in protecting NIH 3T3 fibroblasts against doxorubicin-induced cell death as oncogenic MEK. Surprisingly our experiments also demonstrated that apoptosis suppression by activated MEK as well as by oncogenic Raf implies MEK- and PI3K-dependent mechanisms and results in the activation of PI3K and in the phosphorylation of protein kinase B (PKB). Requirement for the PI3K effector PKB in this process is further demonstrated by the inhibitory effect of a dominant negative PKB mutant on C-Raf-induced cell survival whereas a constitutively active form of PKB synergizes with C-Raf [36]. We suspect that autocrine signals are required to connect the activation of Raf or MEK with the stimulation of PKB and thus the observed mechanism might be of particular importance to tumor cells that have activated the autocrine mode of self-stimulation and primarily rely on C-Raf (rather than B-Raf) for propagation of receptor signals (Fig. 1, pathway 5).

6. NRAGE: linking survival control to protein turnover

We have shown previously that inhibitor of apoptosis proteins (IAPs) block NGF-induced differentiation but also TNF-alpha induced death of PC12 cells by a mechanism which may involve direct binding of members of the Raf kinase family [37]. In order to gain insight into the underlying mechanisms a two-hybrid screen was performed with a PC12 library using the avian IAP ITA as a bait. The screen resulted in the identification of several overlapping fragments of a previously unknown gene [38], the analysis of which revealed a high homology with a large family of tumor antigens known as melanoma associated antigens (MAGE) [39]. This novel MAGE protein, which was later named NRAGE, also interacted with the human XIAP proteins, both in direct two hybrid tests as well as in co-immunoprecipitation experiments using mammalian cells. This interaction was dependent on an intact RING domain in the IAP proteins. Using IL-3-dependent 32D cells we were able to show that stable expression of NRAGE in these cells greatly enhanced cell death upon growth factor withdrawal concomitant with increased binding of NRAGE to XIAP [38]. Whereas the BIR domains of IAP proteins are essential for caspase inhibition the RING finger has been implicated in protein degradation by the ubiquitin proteasome system and may direct destruction either of the RING bearer itself, or of selected target proteins [3]. This raises the possibility that death stimuli induced interaction of NRAGE with the death receptor and XIAP may initiate destruction of XIAP protein, in a fashion that is sensitive to the regulation by C-Raf (Fig. 1, pathway 6).

Apoptosis induction by NRAGE also was resistant to the anti-apoptotic effects of Bcl-2. Furthermore, our experiments suggested that NRAGE may affect cell death by

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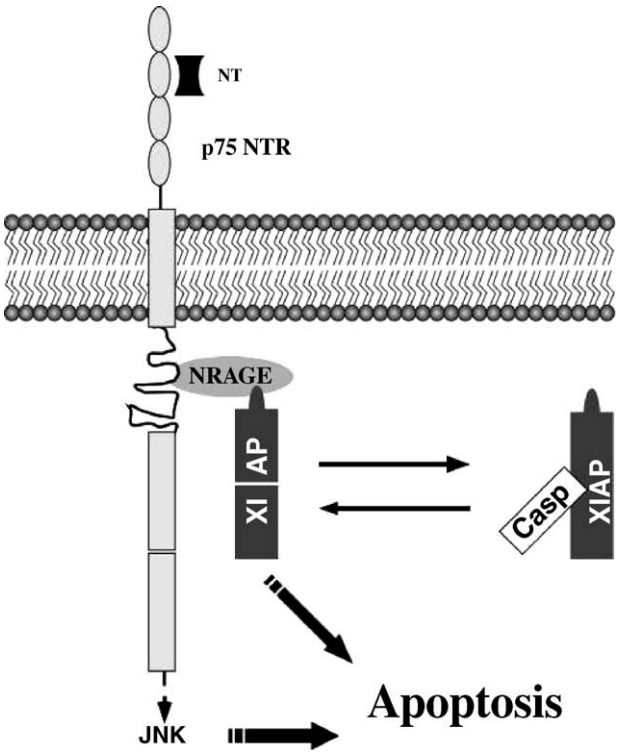

Fig. 2. NRAGE, a possible link between death receptor signaling and the cell survival machinery (modified from [43]).

additional mechanisms. The p75 neurotrophin receptor (p75NTR) causes Jun N-terminal kinase (JNK) activation and cell death [40]. Neuronal apoptosis can be efficiently triggered by expression of the mixed lineage kinase (MLK) 3 through the activation of JNKs [41]. Our experiments demonstrate that co-transfection of NRAGE with MLK3 greatly enhanced JNK activation [38], thus suggesting that NRAGE may control cell survival by at least two mechanisms: interference with IAP protein function and enhancement of signal flow through pro-apoptotic signaling cascades (Fig. 2).

7. Conclusions

Our work established the critical role of mitochondrial targets in the suppression of programmed cell death by Raf. Pro- and anti-apoptotic members of the Bcl-2 family comprise one set of Raf effectors in this cellular location and genetic anaylsis recently established the vital role Bcl-2 is playing in the transformation by C-Raf [42]. Additionally, the suggested link between C-Raf and VDAC function at the outer mitochondrial membrane may be critical for metabolism and survival of transformed cells, as it facilitates the cellular switch to glycolysis for the production of energy. The interaction of Raf with IAP family proteins, however, also suggests that protein kinases may target critical regulators of apoptosis downstream of mitochondrial events.

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