B*uCy* RAFs drive cells into MEK addiction

Ulf R. Rapp,[1,*] Rudolf Götz,[1] and Stefan Albert[1]

[1]Institut für Medizinische Strahlenkunde und Zellforschung (MSZ),Versbacher Strasse 5, 97078 Würzburg, Germany
*E-mail: rappur@mail.uni-wuerzburg.de

RAF research is booming since the discovery of mutant *B-RAF* in ~8% of human cancer. One reason for the excitement is the availability of RAF-targeted therapies. RAF inhibitors have been developed because RAF functions at a convergence point of signal transduction. Two recent papers by the groups of Rosen and Marais dramatically advance our understanding of *RAF* oncogenes in human tumors. The results confirm that the mitogenic cascade (RAF-MEK-ERK) is essential for *RAF* transformation, that RAF kinases work in concert, and that *RAF*-transformed cells are hooked on MEK, making them sensitive to growth inhibition by kinase inhibitors.

Melanoma is an insidious form of skin cancer with rising incidence and mortality rates. The discovery of *B-RAF* mutations in the majority of melanomas (Wellbrock et al., 2004) raised the hope for new therapies, as a clinically tested RAF inhibitor (Sorafenib) is available. However, preliminary results show lower than expected efficacy. Another concern stems from the finding that a small fraction of *B-RAF* mutations generates an enzyme that is impaired in its ability to activate the common RAF substrate MEK. Alternative routes of B-RAF signaling therefore had to be considered. Both concerns have now largely been alleviated by recent findings. A paper from the Marais group (Garnett et al., 2005) establishes that kinase-impaired mutants also work through the mitogenic cascade culminating in ERK activation. The mechanism is rescue of kinase-impaired mutant B-RAF by wild-type C-RAF through a process that involves 14-3-3-mediated heterooligomerization and transactivation. Limitations of available RAF inhibitors seem no longer disarming because another publication from the Rosen lab (Solit et al., 2005) teaches us that ^{V600E}*B-RAF*-transformed human tumor cells are differentially sensitive to MEK inhibition.

The RAF family of protein serine/threonine kinases in mammals consists of three members, A-, B-, and C-RAF. RAF kinases, like their major substrate MEK, have a restricted substrate range (Wellbrock et al., 2004). G proteins of the Ras family initiate RAF activation at the plasma membrane and act through binding to the Ras binding domain (RBD) in the N-terminal half of the RAF molecule. Ras-RAF coupling leads to conformational changes followed by phosphorylations (Figure 1). Although essential steps of this process are well established, numerous questions remain. RAF phosphorylation has been the most controversial aspect of RAF research since the discovery of growth factor-induced tyrosine phosphorylation of C-RAF (Morrison et al., 1988). The following picture has emerged. There are three classes of sites for regulatory phosphorylation: docking sites for 14-3-3 proteins (Muslin et al., 1996), targeting sites (Alavi et al., 2003), and conformation relevant sites (Wan et al., 2004). All RAFs have at least two 14-3-3 sites, a strong C-terminal site and weaker internal site that differ in binding affinities by a factor of 10 (C-RAF). Phosphorylations that affect the active conformation lie in the N-terminal regulatory loop (N region) and in the activation segment (Figure 1). B-RAF is special in that two N region tyrosines present in C-RAF are occupied by aspartic acid. One of the two serines in this region, S338, is a targeting site for mitochondria (Alavi et al., 2003).

Difficulties in establishing precise roles for the various phosphorylations stem from the fact that it is not a trivial task to purify homogeneous populations of phosphorylated RAF and to reconstitute RAF activation in vitro. Compounding this, RAF enzymes are lipophilic, and activation takes place at the membrane in concert with G and 14-3-3 proteins. Regulation of RAF by 14-3-3 differs between isozymes and is best understood for C-RAF. Phosphorylation of the internal S259 and C-terminal S621 sites is coupled, pS259 being a prerequisite for efficient S621 phosphorylation. In spite of high-affinity binding, both sites show turnover, probably because cytosolic 14-3-3 bound RAF is in equilibrium with 14-3-3-depleted RAF in membranes (Hekman et al., 2002, 2004). Thus, membrane binding strips 14-3-3 from RAF proteins, allowing access to phosphatases. Phosphatase regulation of the internal S259 has been described by several groups (Dhillon et al., 2002; Ory et al., 2003), although the details are not completely understood. A recent finding on the essential function of a membrane chaperone, Prohibitin, in the process of C-RAF activation and 14-3-3 displacement may shed further light on this process (Rajalingam et al., 2005). In any case, it is apparent that there are two populations of RAF in resting cells, a phosphorylated and 14-3-3 bound form in the cytosol and an unphosphorylated form(s) in the membrane. As growth factor stimulation recruits only a small percentage of RAF, >5% (Zhu et al., 2005), and the earliest phosphorylation event (in NGF-stimulated PC12 cells) targets the internal 14-3-3 binding site, it seems likely that the pool from which RAF is being recruited for activation is unphosphorylated RAF in the plasma membrane. A tentative model has been proposed for the subsequent steps in the choreography of C-RAF activation that includes Ras-driven B- and C-RAF heterooligomerization and *trans*-phosphorylation of C-RAF on S621 and perhaps other sites (Hekman et al., 2004). Variations of this theme might be exercised in homooligomerization of B-RAF.

While 14-3-3 binding to RAF provides perhaps the best example for regulation of RAF by phosphorylation, there is evidence that N region and activation segment phosphorylation determine the magnitude of the response (Wellbrock et al., 2004). The sites in the activation segment for which there is the largest consensus in the literature are T491 and S494 in C-RAF (Chong et al., 2001) and the corresponding positions (T599 and S602) in B-RAF. The finding that the most frequent activating mutation of B-RAF (V600E) potentially mimics phosphorylation at these sites supports this concept. Recently, this model was challenged by a study of EGF-stimulated C-RAF that used a combination of mass spectrometry and amino acid exchange

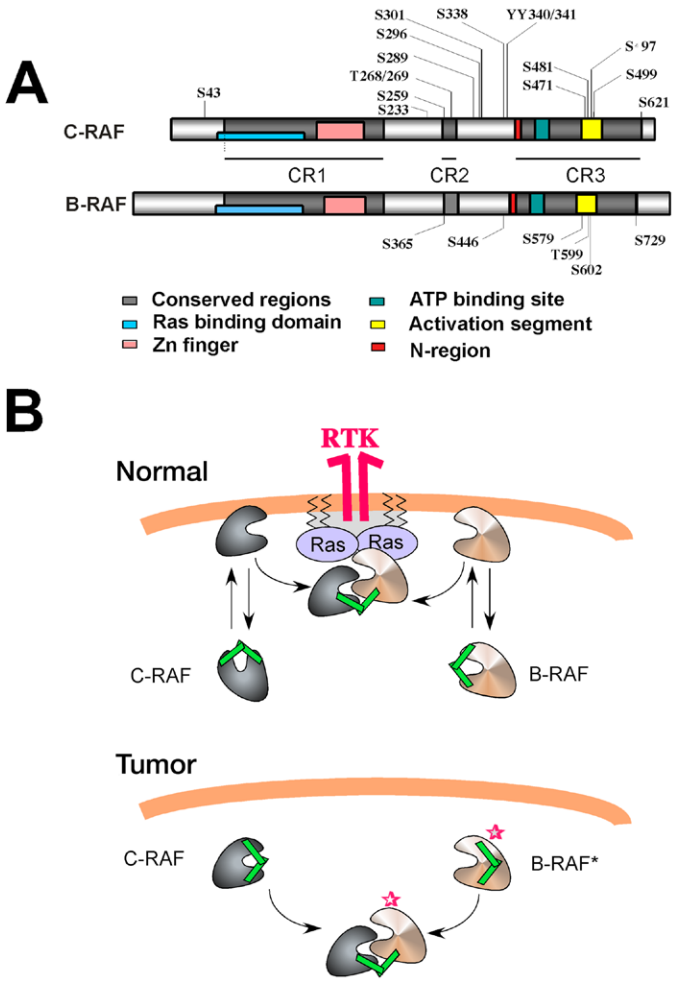

Figure 1. RAF activation in normal and B-RAF tumor cells

A: Schematic structure of B- and C-RAF with regulatory phosphorylation sites.
B: Heterooligomerization that is thought to be associated with *trans*-phosphorylation on C-RAF S621 and perhaps C-RAF S471/B-RAF S579. Under normal conditions, Ras induces B- and C-RAF complex formation. Mutant B-RAF forms heterooligomers in the cytosol independently of Ras. It is possible that a 14-3-3 dimer (green) is involved in crosslinking between C- and B-RAF.

at a novel site, S471 and S578, in the activation segment of C- and B-RAF that was essential for activation (Zhu et al., 2005). Perhaps one shortcoming of this study is the use of in vitro-coupled kinase activity assays, as S471 is critical for MEK1/2 binding. Direct testing by use of biophysical methods of S471 phosphorylation effects on conformation of the RAF kinase domain would have been desirable. As it stands, an alternative role for the function of pS471 can not be excluded where docking of MEK on RAF might be facilitated by pS471, and the binding might itself induce the active conformation. Such a model of substrate-assisted activation would explain why RAF kinases do not work well with substrate peptides and why the range of substrates is so small (Rapp et al., 2004). Whichever way that will sort out, it is clear by now that B-RAF can be activated by mutations in the N-terminal portion of the activation segment that do not mimic phosphorylation but introduce charge, and that there is a second cluster of mutations not in the N region or the internal 14-3-3 site but in the nucleotide binding loop (P loop). Atomic structure revealed that the P loop and N-terminal portion of the activation segment normally interact via hydrophobic forces, thereby stabilizing the inactive conformation (Wan et al., 2004). Most of the activating mutants disrupt this interaction and thereby activate the enzyme. However, at least three mutants (G466E, G466V, and G596R) have impaired MEK kinase activity in vivo (Houben et al., 2004; Wan et al., 2004). In their original publication describing three classes of activating B-RAF mutations (high activity, intermediate activity, and kinase impaired), Wan et al. had already demonstrated that the kinase-impaired group required C-RAF for cell transformation, and both groups speculated that heterooligomerization of B-RAF with C-RAF might be the mechanism of signal transduction. In their current paper, Garnett et al. not only verify this proposal for crosstalk between mutant B- and wild-type C-RAF but also confirm and extend the observation of Ras-driven B- and C-RAF heterooligomerization for wild-type enzymes (Garnett et al., 2005). Complex formation between kinase-impaired mutant B-RAF and C-RAF takes place in the cytosol in a Ras-independent manner that requires activation segment phosphorylation and binding of 14-3-3 to C-RAF. Wild-type B-RAF forms a complex with C-RAF, in a process that is Ras dependent and takes place at the membrane.

By use of alanine exchange of known phosphorylation sites and overexpression in COS cells, Garnett et al. (2005) defined the requirements for activation of C-RAF by B-RAF mutants or by ^{G12V}Ras. The C-terminal 14-3-3 binding site is essential, and phosphorylation sites in the activation segment T491 and S494 play an important role for both processes. In contrast, N region phosphorylation is not essential for C-RAF activation by mutant B-RAF. The authors go on to show that cross-activation of C-RAF by Ras-activated wild-type B-RAF or mutant B-RAF is a one-way street, as wild-type C-RAF or any of several activating C-RAF mutants do not activate B-RAF. These findings together with earlier data strengthen the view that heterooligomerizations between B- and C-RAF are important in growth factor regulation of normal cells in addition to providing a route to rescue transforming activity of MEK kinase-impaired B-RAF mutants present in human tumors.

Why do we have three *RAF* genes in most vertebrates? What is the gain of function relative to the situation in insects and nematodes that make do with just one *RAF* gene? *B-RAF* is most closely related to solitary *RAF*s and thus is the founder kinase gene; *A-RAF* most likely was spawned by *C-RAF*. There are too many gaps in the collection of sequenced genomes to allow us to correlate emergence of a new *RAF* gene with a new trait. Several possibilities exist, gain of substrates and/or tighter control of signal flow through the cascade. There is no evidence that more *RAF* genes would translate into more RAF substrates. At the moment, the best guess is that having three RAF enzymes with widely differing basal and inducible activity (Emuss et al., 2005) improves fine tuning of the mitogenic cascade. The principle is reminiscent of the EGF receptor family that has four members that differ strikingly in their regulation. The strongest pair is a combination of the "deaf and the dumb" (Citri et al., 2003) HER2 and HER3, where HER2 is ligand independent and HER3 has an inactive kinase domain but can be *trans*-phosphorylated by HER2. RAF kinase is a progenitor in the evolution of (receptor) protein tyrosine kinases and may share some of their tricks of the trade.

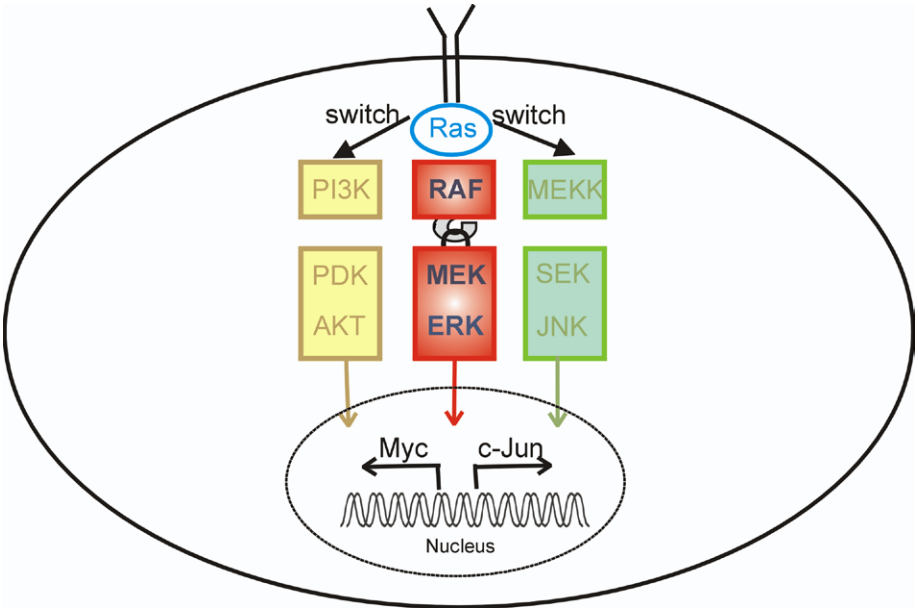

Figure 2. Coupling of Ras to the mitogenic and alternate cascades

Whereas Ras has a choice of different effectors in different cell contexts or depending on the mix of signals that enter the cell, RAF connects with the MEK-ERK module upon receptor stimulation. Mutant RAF is constitutively "hooked up" with MEK in the absence of receptor stimulation. The switch of Ras to alternate effectors may correlate with the position of the cell in a differentiating lineage. The coupling choice of Ras can be modified conditionally, by crosstalk with other signaling pathways (for example cAMP regulating) or stably as part of a cellular phenotype that determines the mix of signaling elements.

The strongest transforming RAF complex is probably a ^{V600E}B-RAF homodimer, although in some cell contexts this complex may be too strong for induction of proliferation and instead drive cells into terminal differentiation (Rapp et al., 1994a; Traverse et al., 1994), late G1 arrest (Kerkhoff and Rapp, 1998), or senescence (Serrano et al., 1997; Zhu et al., 1998). Perhaps as a consequence the presence of ^{V600E}*B-RAF* is not a prognostic factor for disease progression, whereas its occurrence in metastatic lesions predicts poor outcome (Houben et al., 2004). Besides the value of knowing the *B-RAF* status for prediction of disease progression, is it helpful for selection of treatment options? This question was addressed by Solit et al. (2005), who examined a panel of cancer cell lines for sensitivity toward MEK inhibitors. Three categories were included, one with ^{V600E}*B-RAF*, another with mutant *Ras*, and a third with both genes wild-type. Although the MEK inhibitor CI-1040 suppressed pERK in all lines, growth inhibition was selectively achieved in ^{V600E}*B-RAF*-positive lines and less efficiently in a fraction of ^{G12V}*Ras* lines. These findings are consistent with earlier data from a *RAF* transgenic mouse tumor model (Kramer et al., 2004) and a mouse xenograft metastasis model with a panel of ^{V600E}*B-RAF*-positive human melanoma lines (Collisson et al., 2003). The report by the Rosen group is the first to highlight selective sensitivity of human tumor cell lines. Extension of this study to the NCI 60 cell lines for which a large body of data from inhibitor screening assays could be interrogated yielded supportive information, as the top-ranking compounds that scored on ^{V600E}*B-RAF*-positive lines happen to represent predominantly MEK inhibitors with similar effectiveness as CI-1040. Sensitivity to MEK inhibition could also be demonstrated in vivo using xenograft models and was not restricted to melanomas but included colon and breast tumor lines. In fact, in the latter case it was the observed CI-1040 sensitivity that led to the discovery of ^{V600E}*B-RAF* mutation. It will be interesting to explore whether human *B-RAF* mutant cells also show selective sensitivity to growth inhibition by antagonists of downstream effectors of the mitogenic cascade as was observed for c-*jun* in mouse cells (Rapp et al., 1994b). Lack of responsiveness of ^{G12V}*Ras* tumor lines or lines with wild-type *Ras*/*RAF* is not a total surprise, as earlier reports have described cell contexts that uncoupled Ras from the RAF-MEK-ERK cascade (al-Alawi et al., 1995; Repasky et al., 2004). Such a bypass may be hard-wired as part of a cell lineage- or developmental stage-specific program. A bypass may also be reversibly induced by hormones or growth factors (Figure 2). An example are WRT thyroid cells, in which treatment with thyrotropin uncoupled RAF from Ras through a cAMP-dependent pathway (al-Alawi et al., 1995). Pathway switching is also known to occur in the course of progression of cells through a differentiating lineage; for example, in early progenitors Ras may preferentially use PI-3 kinase for proliferation (Takahashi et al., 2003). The CI-1040-resistant tumor cell lines that carry *Ras* mutations and the others that have wild-type alleles of *Ras* and *B-RAF* may correspond to such early stages.

As MEK inhibitors work so well against tumor cells carrying ^{V600E}*B-RAF*, it becomes all the more important to identify all tumors that are RAF dependent. Screening for the most frequent *B-RAF* mutations may not be sufficient, since *B-RAF* can also be activated by chromosomal rearrangements and copy number gains. (Ciampi et al., 2005). Moreover microRNAs exist for all three RAF genes, making it likely that mutant RAF alleles with deletions in target sequences will be found in some tumors (http://microrna.sanger.ac.uk/).

Attacking *RAF* tumors by aiming at its substrate MEK is one approach that now looks very promising, but RAF kinases themselves may have some advantages as targets. Luckily, RAF kinase inhibitors have been developed. The first generation is available as a drug for treatment of renal cancer (http://www.onyx-pharm.com/wt/page/index/). Others will surely follow. In fact, there are reports on inhibitors that specifically block ^{V600E}B-RAF (http://www.plexxikon.com/therapeutic-products.shtml).

It took 10 years to get from the discovery of RAF to delineation of the mitogenic RAF-MEK-ERK cascade and another 10 for development of RAF inhibitors. RAF's relevance to human cancer was unambiguously established 4 years ago by the discovery of frequent *B-RAF* mutations in >50% of melanoma and to a lesser extent in other malignancies. With the finding that cells transformed by *RAF* lose other options for growth control, we are now in the position to fight these cancers by turning the cascade against them. Addiction has multiple dimensions, including a genomic one. At the level of RAF, addiction is adduction. RAF is literally hooked on MEK, a situation that may also be exploited in

screens for interaction blockers. It seems reasonable to expect that additional druggable targets are buried in this signaling chain. By finding them, we may be able to overcome resistance of individual targets and perhaps eliminate disease.

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