BRAF as a potential therapeutic target in melanoma and other malignancies

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Introduction

Activating somatic mutations in the *BRAF* protooncogene were recently discovered in a wide variety of malignancies, and most notably so in melanoma (~60%–70% of cases) (Brose et al., 2002; Davies et al., 2002; Satyamoorthy et al., 2003), papillary thyroid cancer (~35%–70%) (Cohen et al., 2003; Kimura et al., 2003), and colon cancer (~10%) (Davies et al., 2002; Rajagopalan et al., 2002; Yuen et al., 2002). Tumor-derived *BRAF* alleles encode oncoproteins with constitutive serine/threonine kinase activity, and when ectopically expressed in immortalized cell lines, they cause hyperstimulation of the MAP kinase cascade and cellular transformation (Davies et al., 2002). Preliminary studies suggest that B-Raf is a promising target for drug development in melanoma and other malignancies that depend upon B-Raf signaling.

The potential importance of mutant *BRAF* alleles in tumorigenesis becomes apparent upon examining the function of Raf kinases in normal cellular physiology (see Table 1 and Figure 1). *BRAF* is a member of the Raf family of protein kinases, which includes *CRAF*, *BRAF*, and *ARAF* (Chong et al., 2003; Mercer and Pritchard, 2003). Expression of all three *RAF* genes can be detected in most tissues, with prominent expression of *BRAF* in neuronal tissue and *ARAF* in urogenital tissue. The entire *RAF* gene family is necessary for normal murine development, with the expression of both *CRAF* and *BRAF* required to complete gestation (Chong et al., 2003; Mercer and Pritchard, 2003). A diverse number of stimuli such as mitogens, hormones, and neurotransmitters promote the activation of Raf kinases by first triggering increases in the levels of Ras-GTP in cells. The GTP-bound forms of Ras directly bind and thereby recruit cytosolic dimers of Raf kinases to the plasma membrane, where Raf is activated through phosphorylation by other kinases and potentially by autophosphorylation (Chong et al., 2003; Mercer and Pritchard, 2003). Activated and membrane-associated Raf assembles a MAP kinase signaling complex that consists of two classes of kinases, MEK and ERK, and scaffolding proteins, including KSR, CNK, and RKIP (Chong et al., 2003). The MAP kinase cascade initiates with the phosphorylation and activation of MEK by Raf, and the subsequent phosphorylation and activation of ERK by MEK. Active ERK

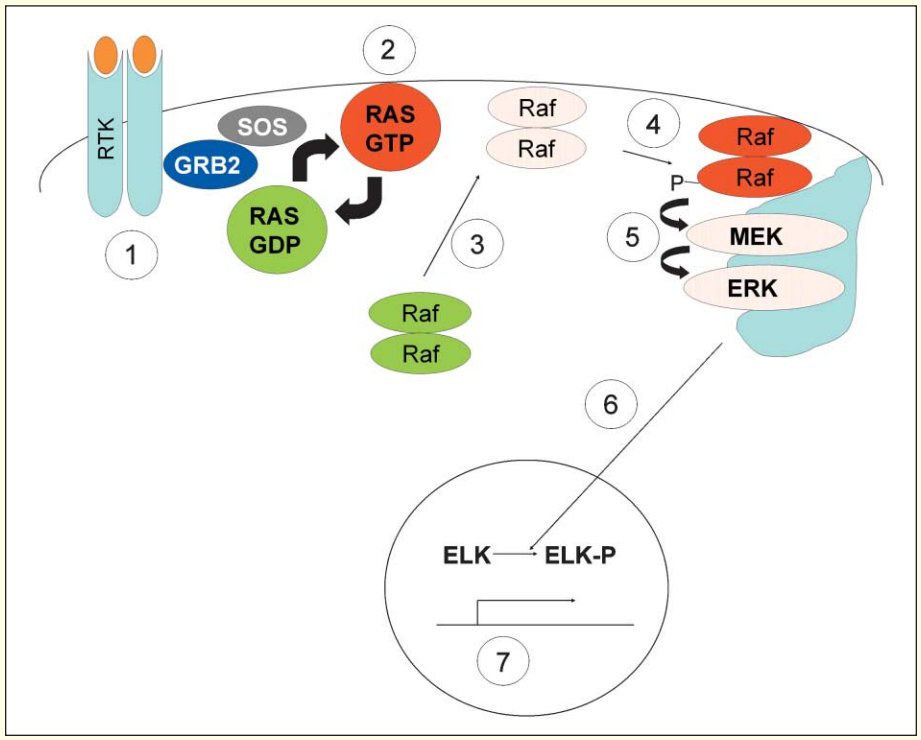

Figure 1. Activation of the Raf/MEK/ERK MAP kinase cascade

Step 1: mitogenic growth factors induce RTK dimerization and activation, including the recruitment of Grb2 and SOS to the plasma membrane.
Step 2: SOS activates Ras proteins by catalyzing GTP exchange for GDP.
Step 3: recruitment of inactive cytosolic Raf homodimers to the plasma membrane by Ras-GTP.
Step 4: Activation of membrane-associated Raf homodimers by phosphorylation.
Step 5: MAP kinase complex comprised of active Raf, scaffolding proteins such as KSR and CNK (light blue), and MEK and ERK. Sequential phosphorylation and activation of MEK, then ERK.
Step 6: translocation of active ERK to the nucleus and phosphorylation of multiple transcription factors such as ELK.
Step 7: transcriptional activation by phosphorylated transcription factors. Mutant B-Raf does not depend on plasma membrane-associated Ras-GTP and may be active in other cellular compartments (not shown).

Table 1. Characteristics of the *RAF* family members

Isoform	*CRAF*	*BRAF*	*ARAF*
Expression	broad	broad; high neurons	broad; high urogenital
Development	required	required	postnatal defects
Distant orthologs	–	+	–
Kinase activation	4 sites	2 sites	4 sites
MEK stimulation	++	+++	+
Oncogenic	++	+++	+
Somatic mutations	–	+	–

dissociates from the Raf/MEK/ERK complex and phosphorylates a number of cytoskeletal proteins, kinases, and transcription factors (Chong et al., 2003; Mercer and Pritchard, 2003) (see Figure 1). The functional consequences of substrate phosphorylation by ERK are dependent upon cellular context and include alterations in cellular motility and a multitude of gene expression changes that promote proliferation, differentiation, cellular survival, immortalization, and angiogenesis (Mercer and Pritchard, 2003).

Although oncogenic forms of all three *RAF* family members can be experimentally produced and several have been isolated from transforming retroviruses, the exclusive identification of somatic activating mutations in *BRAF* indicates unique properties for this paralog in cellular physiology and oncogenesis. B-Raf has substantially greater basal kinase activity toward MEK than does C-Raf or A-Raf (Chong et al., 2003; Mercer and Pritchard, 2003). These differences between C-Raf and B-Raf may relate to the finding that C-Raf contains four distinct Ras-GTP-dependent phosphorylation sites for maximal activation (S338, Y341, T491, and S494), whereas B-Raf possesses only two of these sites (S598 and T601) (Chong et al., 2003; Mercer and Pritchard, 2003). This provides the molecular shortcut for B-Raf to become activated by a single amino acid substitution. Indeed, the $BRAF^{V599E}$ missense mutation, which represents over 80% of the oncogenic *BRAF* alleles described to date, engenders constitutive and maximal activation of the B-Raf kinase activity, likely by mimicking the phosphorylation of S598/T601 in native B-Raf (Davies et al., 2002). The remaining oncogenic *BRAF* mutations cluster near the V599 site or in the G loop ATP binding region at residues 463–468.

Tissue-specific properties of oncogenic *BRAF*

The finding of *BRAF* mutations in a high percentage (~60%–70%) of cutaneous melanomas was somewhat surprising, as prior studies could attribute hyperactivation of the mitogenic receptor tyrosine kinase (RTK)-Ras-Raf-MAP kinase pathway in melanoma to the abundance of autocrine and paracrine growth factors (Lazar-Molnar et al., 2000), and to *N-ras* mutations (Herlyn and Satyamoorthy, 1996). Indeed, a recent report demonstrated that *BRAF* mutation is not a requisite event in a specific type of melanoma, with no *BRAF* mutations detected in 48 uveal melanomas (Edmunds et al., 2003). As uveal melanoma also differs from cutaneous melanoma in that the former has frequent chromosome 6 abnormalities (Metzelaar-Blok et al., 1999), it was predictable that distinct pathways for melanoma formation exist. Future studies will be needed to determine whether the prevalence of *BRAF* mutations in melanoma correlates with the site of the primary tumor and sun exposure/sunburn, a known risk factor for cutaneous melanoma. Also, it will be important to seek a molecular understanding of those melanomas that do not harbor *BRAF* or *RAS* mutations.

Perhaps even more unexpected was the demonstration that the $BRAF^{V599E}$ allele could be detected in as many as 80% of benign nevi, suggesting a role for oncogenic *BRAF* in nevus formation and melanoma initiation (Pollock et al., 2003). However, a proposed role for oncogenic $BRAF^{V599E}$ in tumor initiation conflicts with the finding that constitutive hyperactivation of Raf proteins causes premature senescence of primary human fibroblasts in culture (Zhu et al., 1998). There is currently no evidence that the benign nevi harboring $BRAF^{V599E}$ actually progress to malignancy. In fact, the majority may represent non-progressing terminally differentiated lesions that are analogous to nondysplastic colorectal aberrant crypt foci (ACF) (Takayama et al., 2001; Yamashita et al., 1995). Nondysplastic ACF harbor *KRAS* mutations in the absence of *APC* mutations and are generally considered to have a low malignant potential, whereas *KRAS* mutations that occur following *APC* mutation promote colorectal tumor progression (Takayama et al., 2001; Yamashita et al., 1995). Therefore, investigations into the function of $BRAF^{V599E}$ in benign and dysplastic nevi may yield important information about the type and timing of other genetic events necessary for melanoma genesis.

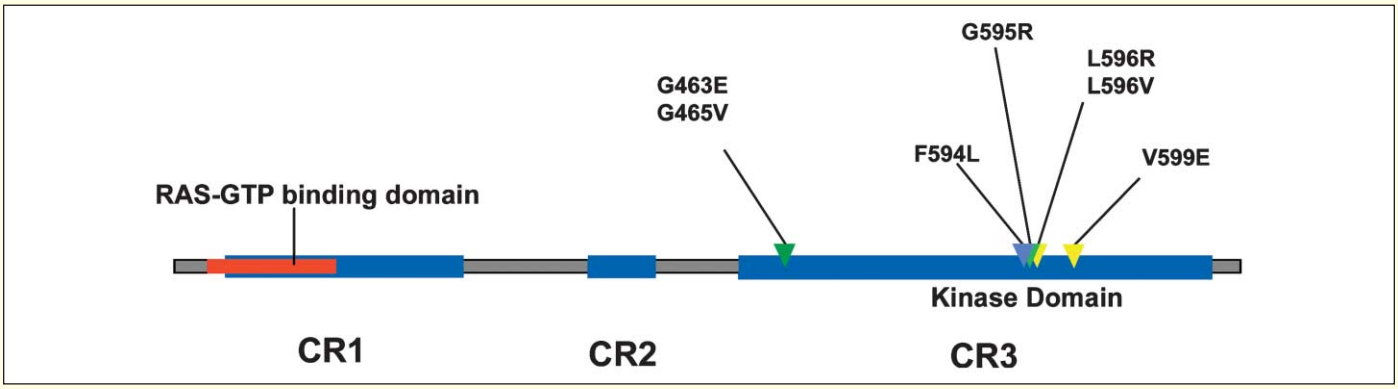

Figure 2. Structure of the *BRAF* gene, denoting the three conserved regions by thick blue bars

The Ras-GTP binding site is shown, as is the kinase domain. Common oncogenic mutations are denoted by triangles and corresponding amino acid changes.

Besides melanoma, several other tumor types are worthy of mention with regards to *BRAF* mutation. Colorectal cancers harbor mutant *BRAF* alleles in 4%–10% of the tumors (Rajagopalan et al., 2002; Yuen et al., 2002), with the majority of these mutations being $BRAF^{V599E}$. A strong association exists between mismatch repair deficiency and the presence of $BRAF^{V599E}$ in colorectal cancer, which is possibly explained by the underlying DNA repair defect (Rajagopalan et al., 2002). Additionally, there is a mutual exclusivity of $BRAF^{V599E}$ and *KRAS* mutations in tumor specimens, perhaps reflecting a redundant function of these two oncogenes and emphasizing the importance of the B-Raf pathway in oncogenic K-Ras signaling (Rajagopalan et al., 2002). In this regard, it will be important to evaluate the temporal sequence of *BRAF* mutation in colorectal tumorigenesis. For example, if *BRAF* and *KRAS* are truly interchangeable, then *BRAF* mutations should also be represented in colonic aberrant crypt foci that harbor wild-type *KRAS* alleles. Also reflecting the redundancy of the RTK-Ras-Raf-MAP kinase cascade, a substantial fraction of papillary thyroid cancer specimens harbor either $BRAF^{V599E}$, mutant *KRAS*, or mutant *RET* receptor tyrosine kinase (Cohen et al., 2003; Kimura et al., 2003), and a large fraction of low grade ovarian tumors harbor either $BRAF^{V599E}$ or mutant *KRAS* (Davies et al., 2002; Singer et al., 2003). Other tumors that harbor mutant *BRAF* alleles include cholangiocarcinoma (Tannapfel et al., 2003) and lung adenocarcinoma (Brose et al., 2002; Naoki et al., 2002). Of note, concomitant *RAS* mutations have been demonstrated in cancer specimens that harbor the uncommon G loop region *BRAF* mutations, suggesting differences in molecular pathway utilization by distinct mutant B-Raf proteins (Davies et al., 2002) (see Figure 2).

Oncogenic *BRAF* as a target for cancer therapeutics

Despite the large number of genetic alterations in cancer cells and their microenvironment, recent evidence demonstrates that the specific inhibition of a single critical pathway in tumor cells is sufficient to cause cell death and clinical response in several malignancies. For example, most patients with either chronic myelogenous leukemia (CML) or gastrointestinal stromal tumor (GIST) initially respond to monotherapy treatment with Imatinib mesylate (Gleevec, STI571), a small molecule inhibitor of the Abl and KIT tyrosine kinases (Demetri et al., 2002; Druker et al., 2001). As predicted, the responsiveness of CML and GIST patients to Gleevec directly correlates with the inhibition of the tyrosine kinase activity of Bcr-Abl and mutant KIT, respectively (Gorre et al., 2001; Tuveson et al., 2001). The development and effectiveness of Imatinib in CML and GIST patients suggests that therapies that specifically target other essential oncogenic pathways may have similar efficacy and minimal toxicity. Inhibition of the B-Raf kinase therefore represents a rational therapeutic strategy in melanoma that is analogous to Bcr-Abl and KIT inhibition by Imatinib in CML and GIST, respectively.

Indeed, an orally administered Raf kinase inhibitor, BAY 43-9006 (Lyons et al., 2001), is currently undergoing worldwide clinical evaluation in phase I and phase II trials in patients with a variety of malignancies, including melanoma. BAY 43-9006 inhibits both B-Raf and C-Raf (G. Bollag, personal communication), and therefore any effects of drug treatment may be attributable to effects on both kinases simultaneously. The early results from a phase I trial that combined BAY 43-9006 and the chemotherapeutic agents carboplatin and paclitaxel were presented recently (K.T. Flaherty et al., 2003, Proc. Am. Soc. Clin. Oncol., abstract #2854). Out of ten evaluable melanoma patients, three were described as having anatomic partial responses and six as having stable disease after at least two cycles of treatment for all ten patients. Notably, this level of responsiveness is superior to the results previously obtained from melanoma patients treated with these chemotherapeutic agents alone (Hodi et al., 2002). Correlative laboratory studies are currently investigating whether *BRAF* mutation status and MAP kinase pathway inhibition are predictive of clinical responsiveness to BAY 43-9006 in melanoma. Additionally, ongoing clinical trials are evaluating BAY 43-9006 as a single agent in melanoma patients.

Furthermore, recent preclinical findings in our own laboratories support the prediction that melanoma cells harboring mutant *BRAF* alleles are dependent upon continuous oncogene function. Following the knockdown of $BRAF^{V599E}$ levels with RNA interference methods, melanoma cells demonstrated profound inhibition of the MAP kinase cascade, diminished proliferative capacity, and the inability to support anchorage-independent growth. Significantly, these effects were not recapitulated following *CRAF* knockdown (S. Hingorani, M. Jacobetz, G. Robertson, M.H., and D.T., unpublished data). Currently, we are attempting to extend these observations to in vivo model systems.

Therefore, two approaches—kinase inhibition and protein depletion—are potential methods to target oncogenic B-Raf protein function. In addition to BAY 43-9006, Raf kinase inhibitors specific for B-Raf will be of great interest to evaluate when they become available. Furthermore, the existence of multiple Raf kinase inhibitors with different chemical structures and/or distinct modes of action is important because single agent Raf kinase inhibition will likely lead to the emergence of disease resistance in patients that initially respond, in analogy to patients with CML and GIST treated with Gleevec (Demetri et al., 2002; Druker et al., 2001; Hingorani and Tuveson, 2003). Finally, B-Raf protein depletion is worthy of pursuit as a therapeutic strategy. RNA interference is not yet a clinically viable approach, but may be in the near future. Alternatively, strategies that decrease the stability of B-Raf protein can be explored. For example, B-Raf is one of many proteins that binds to the molecular chaperone and heat shock protein Hsp90 (Jaiswal et al., 1996), and Hsp90 inhibitors have previously been shown to decrease the stability and thus the oncogenic phenotypes of various Bcr-Abl alleles in cell culture (Gorre et al., 2002).

In the year since the first description of activating *BRAF* mutations in cancer, laboratory investigations and clinical trials have provided an initial glimpse of this oncogene's role in cells and patients. This multidisciplinary translational research approach illuminates the great progress we have made as a community since the "War on Cancer" began some 30 years ago. Furthermore, the identification of mutant *BRAF* alleles may also serve as an example of the potential for large scale genomic screening efforts, long considered "fishing expeditions," to produce important, novel therapeutic targets that move rapidly into the clinic. Now is the time to clarify the role of this oncogene in tumorigenesis and to expeditiously identify the most efficacious therapies for patients afflicted with malignancies that harbor *BRAF* mutations.

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