Ras-Mediated Activation of the Raf Family Kinases

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The extracellular signal-regulated kinase (ERK) cascade comprised of the Raf, MEK, and ERK protein kinases constitutes a key effector cascade used by the Ras GTPases to relay signals regulating cell growth, survival, proliferation, and differentiation. Of the ERK cascade components, the regulation of the Raf kinases is by far the most complex, involving changes in subcellular localization, protein and lipid interactions, as well as alterations in the Raf phosphorylation state. The Raf kinases interact directly with active, membrane-localized Ras, and this interaction is often the first step in the Raf activation process, which ultimately results in ERK activation and the downstream phosphorylation of cellular targets that will specify a particular biological response. Here, we will examine our current understanding of how Ras promotes Raf activation, focusing on the molecular mechanisms that contribute to the Raf activation/inactivation cycle.

Members of the Raf serine/threonine kinase family are the initiating enzymes in the three-tiered extracellular signal-regulated kinase (ERK) cascade, and in mammalian cells, there are three Raf proteins, A-Raf, B-Raf, and C-Raf (also known as Raf-1) (Wellbrock et al. 2004). The Raf kinases were first discovered when the *raf-1* gene was identified as the cellular counterpart of the murine retroviral oncogene, *v-raf* (Rapp et al. 1983). Later, this kinase family rose to prominence when they were found to be direct effectors of activated Ras, mediating signal transmission from Ras to the downstream kinases MEK and ERK (Marshall 1996). Not surprisingly, given their central position in such a major signaling pathway, mutations in the Raf kinases also contribute to human disease states. In particular, somatic mutations in B-Raf are important drivers of human cancer (Dho-

men and Marais 2007), and germline mutations in either B-Raf or C-Raf can be causative for certain developmental disorders in the Rasopathies spectrum (Tartaglia et al. 2011).

Despite their critical function in cell signaling, determining how Raf activity is regulated has been a challenging task—resulting from the complexity of the Raf activation process. In this article, we will review the molecular mechanisms involved in Raf kinase regulation. Our discussion will include an examination of the presignaling inactive state of the Rafs as well as the events that contribute to Raf kinase activation, with a focus on the crucial role that the Ras GTPases play in promoting Raf activation under normal growth conditions as well as in human disease states. Whenever possible, we will also comment on the regulatory differences that have been identified for the individual Raf members,

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and we will conclude with a description of the mechanisms that attenuate Raf activity following signal transmission.

THE RAF PRESIGNALING INACTIVE STATE

All members of the Raf kinase family contain three conserved regions, CR1, CR2, and CR3, and each Raf protein can be divided into two functional domains—an amino-terminal regulatory domain and a carboxy-terminal catalytic domain (Fig. 1A) (Daum et al. 1994). Both the CR1, which consists of a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and the CR2, a region rich in serine/threonine residues, are found within the amino-terminal regulatory domain, whereas the CR3 comprises the carboxy-terminal kinase domain. The Raf proteins do not contain any intrinsic subcellular localization motifs, and in quiescent cells, they

localize to the cytosol as monomers (Nan et al. 2013). Before pathway activation, the Raf monomers are maintained in a stable, presignaling inactive state through multiple regulatory mechanisms, including autoinhibition, phosphorylation of negative regulatory sites, and binding of inhibitory proteins (Figs. 1B and 2).

Raf Autoinhibition

The first indication that the Raf amino-terminal domain might possess autoinhibitory activity came from the observation that this domain is absent in the oncogenic v-Raf protein (Rapp et al. 1983). Subsequently, it was shown that deletion of the amino-terminal domain could convert any of the mammalian Raf proteins into constitutively active kinases capable of inducing cellular transformation (Stanton et al. 1989; Heidecker et al. 1990). Further studies in

Figure 1. Raf domain structure and protein interactions. (A) The Raf kinases can be divided into two functional domains: an amino-terminal regulatory domain and a carboxy-terminal kinase domain and they contain three conserved regions (CRs): CR1, which contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD); CR2, an area rich in serine/threonine residues (S/T); and CR3, which contains the protein kinase domain. (B) Numerous Raf-binding partners have been identified.

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Figure 2. Regulation of the Raf kinases by phosphorylation. (Top) Raf is both positively and negatively regulated by phosphorylation on numerous sites. (Bottom) Shown are the amino acid sequence alignments of the sites of Raf phosphorylation, with the residues phosphorylated shown in red, and the relevant kinases indicated above the sequences. A glutamic acid residue that can act as a phosphomimetic to promote the active conformation of the activation segment (AS) is also shown in green.

which the amino- and carboxy-terminal domains of B-Raf or C-Raf were expressed as individual proteins revealed that the isolated amino-terminal domains of the Rafs could bind their respective kinase domains and block signal transmission to MEK and ERK (Cutler et al. 1998; Chong and Guan 2003; Tran and Frost 2003; Tran et al. 2005). Regions in the CR1, including the CRD and parts of the RBD, are critical for the amino-terminal autoinhibitory activity, and phosphorylation of sites in the carboxy-terminal kinase domain contribute prominently to the relief of autoinhibition, with phosphorylation of sites in the negative charge regulatory region (N-region) being particularly important (Cutler et al. 1998; Tran and Frost 2003; Tran et al. 2005).

In addition to the autoinhibition mediated by the amino-terminal domain, structural data indicate that the Raf kinase domain itself is held in an inactive conformation. Like all other protein kinases, the Raf kinase domain consists of

an amino-terminal lobe (N-lobe) and a carboxy-terminal lobe (C-lobe) that are connected to one another through a flexible hinge region (Shaw et al. 2014). The N-lobe contains five antiparallel β -strands and the regulatory α C-helix, whereas the C-lobe is comprised primarily of α -helices and contains the activation segment (AS). For all kinases to assume the active/ON state conformation, spatially conserved hydrophobic residues spanning both lobes of the kinase domain must align to form two structural entities called the regulatory and catalytic spines (McClendon et al. 2014). The regulatory spine (R-spine) consists of four residues, with each residue derived from a key catalytic element in the kinase domain—a residue from the β 4-strand, the α C-helix, the DFG motif, and the HRD sequence. In many inactive kinase structures, the R-spine is misconfigured and for the spine residues to align, both the α C-helix and the AS must change positions. Although the structure of an authentic OFF state Raf kinase has not been solved,

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the structure of a monomeric, inhibitor-bound B-Raf kinase domain has been determined that has features characteristic of other inactive protein kinases (Thevakumaran et al. 2015). In this monomeric B-Raf structure, the α C-helix is shifted to an outward position and residues adjacent to the DFG motif in the AS form a small helix called the AS-H1-helix (Fig. 3). Moreover, extensive interactions between the α C-helix, the AS-H1-helix and other residues in the active site, including residues in the conserved DFG and HRD motifs, stabilize the α C-helix in the inactive OUT position and inhibit the formation of an essential salt bridge that defines the active state (E501-K483 for B-Raf, E393-K375 for C-Raf, E354-K336 for A-Raf). A similarly configured AS-H1-helix has been observed in the inactive conformation of the epidermal growth factor receptor (EGFR) kinase (Zhang et al. 2006), and it is important to note that most activating B-Raf mutations identified in human cancer occur in residues comprising the AS-H1-helix (Lavoie and Therrien 2015). Thus, it is tempting to speculate that in the context of full-length Raf proteins, Raf monomers are

held in an inactive state through intramolecular interactions occurring within the kinase domain as well as between the kinase domain and the amino-terminal regulatory domain.

14-3-3 Binding to the CR2 Site

The autoinhibited Raf conformation is further stabilized by interactions with the 14-3-3 family of phosphoserine/phosphothreonine-binding proteins (Muslin et al. 1996). All Raf kinases contain two high-affinity, phosphorylation-dependent 14-3-3 binding sites, one in the CR2 (S365 of B-Raf, S259 of C-Raf, S214 of A-Raf) and one following the kinase domain (S729 of B-Raf, S621 of C-Raf, S582 of A-Raf), both of which are phosphorylated in the presignaling inactive state (Morrison et al. 1993; Dougherty et al. 2004; Ritt et al. 2010). 14-3-3 proteins form constitutive dimers with each protomer of the dimer containing an independent binding channel, and it has been proposed that a 14-3-3 dimer can interact with both the CR2 and carboxy-terminal sites simultaneously to stabilize the autoinhibited conformation (Tzivion et al.

Figure 3. Regulation of the Raf kinase domain by dimerization. Shown are the crystal structures of a monomeric, B-Raf kinase domain in an inactive conformation (PDB, 4WO5) and of dimerized B-Raf kinase domains in an active conformation (taken from the B-Raf–MEK complex PDB, 4MNE). In the monomeric, inactive state, the α C-helix is shifted outward and residues adjacent to the DFG motif in the activation segment (AS) form a small helix known as the AS-H1-helix. When Raf kinase domains dimerize in a side-to-side manner, the α C-helix shifts inward to the active position and the AS-H1-helix is disrupted, allowing residues in the R-spine to align. The conserved Raf dimer interface motif RKTR is shown in red, the R-spine residues in the α C-helix (L505) and in the DFG motif (D595) are indicated in yellow, and the sequences comprising the AS-H1-helix are shown in green.

1998). Moreover, binding of 14-3-3 to the CR2 site has a known negative regulatory function and appears to mask regions within the amino terminus, such as the CRD, which are required for Raf activation (Michaud et al. 1995; Clark et al. 1997; McPherson et al. 1999; Light et al. 2002). Mutation of the CR2 serine site or residues that comprise the 14-3-3 binding motif have been identified in tumors and Rasopathies as gain-of-function mutations (Lavoie and Therrien 2015), resulting in increased Ras/Raf binding, membrane recruitment, and Raf activation (Michaud et al. 1995; Dhillon et al. 2002a; Light et al. 2002; Pandit et al. 2007; Razzaque et al. 2007). Protein kinase A (PKA) and AKT have been identified as the primary cellular kinases that phosphorylate the CR2 site; however, the CR2 site can also be phosphorylated by LATS1 through signaling cross talk with the MST/Hippo pathway (Zimmermann and Moelling 1999; Dhillon et al. 2002b; Dumaz and Marais 2003; Romano et al. 2014).

Hsp90 Chaperone Complex

In their presignaling inactive state as well as in their active signaling state, all members of the Raf kinase family interact with Hsp90 complexes, which include Hsp90 proteins, the Cdc37 targeting subunit, and the peptidyl-prolyl *cis-trans* isomerase FKBP5 (Stancato et al. 1994; Eisenhardt et al. 2016; Diedrich et al. 2017). The Hsp90 complex binds to the Raf kinase domain and this interaction is required for Raf protein stability (Schulte et al. 1995).

B-Raf–MEK1 Complex

Another protein that has been found to associate with inactive B-Raf complexes is MEK1 (Haling et al. 2014; Eisenhardt et al. 2016). In experiments investigating whether preformed Raf/MEK complexes exist before Ras pathway activation, MEK1 was found to preferentially interact with B-Raf proteins found in the cytosol of quiescent cells or in cancer cell lines that contain low levels of active MEK (Haling et al. 2014). When cells were treated with growth factors to activate the pathway, the B-Raf–MEK complex

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was disrupted in a manner that correlated with the appearance of active phosphoMEK, but could reform when phosphoMEK levels returned to baseline, suggesting that phosphorylation and activation of MEK disrupts the interaction. Structural analysis of the B-Raf–MEK1 complex indicates that the two kinase domains interact in a face-to-face manner (Haling et al. 2014), with primary points of contact being the AS of both proteins and their respective α G-helices, a structural element implicated in kinase–substrate interactions. Although it is not clear why MEK1 preferentially binds inactive B-Raf, it is interesting to note that the RKIP inhibitory protein has been found to interact with the C-Raf kinase domain in a manner that is mutually exclusive with MEK binding (Yeung et al. 2000). Phosphorylation of RKIP on S153 by protein kinase C (PKC) disrupts the RKIP–C-Raf interaction and appears to account for the release of RKIP from C-Raf under signaling conditions (Corbit et al. 2003).

From the above findings, the following model has emerged: In quiescent cells, members of the Raf kinase family exist as autoinhibited monomers, with the inactive conformation stabilized by the phosphorylation-dependent binding of 14-3-3 dimers as well as by the interaction with Hsp90 complexes. A pool of B-Raf and MEK1 exists in preformed complexes, whereas MEK may be precluded from C-Raf complexes as a result of RKIP binding. The presignaling, inactive Raf complexes localize to the cytosol, poised ready for recruitment in response to upstream pathway activation (Fig. 4).

THE RAF ACTIVATION PROCESS

For the Rafs to become active enzymes, autoinhibition mediated by the amino-terminal domain must be relieved and the kinase domain must assume an active/ON state conformation. After more than 30 years of research, we now know that the cell accomplishes this task through an intricate series of events that include a change in Raf subcellular localization, protein and lipid interactions (Fig. 1B), as well as regulatory phosphorylation/dephosphorylation events (Fig. 2).

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Figure 4. The Raf activation–inactivation cycle. A model depicting the Raf kinases in a presignaling inactive state, a signaling active state, and a postsignaling inactive state is shown (see text for details). GDP, Guanosine diphosphate; GTP, guanosine triphosphate; RBD, Ras-binding domain; CRD, cysteine-rich domain; ERK, extracellular signal-regulated kinase.

Ras Binding

A major breakthrough in understanding Raf activation came with the discovery that all Raf kinases selectively interact with active guanosine triphosphate (GTP)-bound Ras (Van Aelst et al. 1993; Vojtek et al. 1993; Zhang et al. 1993). Binding to active Ras does not stimulate Raf enzymatic activity directly, but instead localizes the normally cytosolic Raf proteins to the plasma membrane. This change in localization is a critical step in the Raf activation process as artificially targeting Raf to the plasma membrane results in a kinase that is constitutively active in a Ras-independent manner (Leevers et al. 1994; Stokoe et al. 1994). The initial contact with Ras is made by the Raf-RBD, which adopts a conserved ubiquitin-like structure that binds with high affinity (18 nM) to the Ras effector domain in its GTP-bound state (Herrmann et al. 1995; Nassar et al. 1995). In addition to the RBD, the Raf CRD makes distinct contacts with lipid modified Ras. The Raf CRD is a zinc-coordinated structure of the atypical type (Mott et al. 1996), and as an isolated domain, the CRD shows constitutive low-affinity binding to Ras with no preference for its nucleotide state (Williams et al. 2000). However, in the context of the

RBD, the CRD can further facilitate the membrane recruitment of the Rafs by selectively binding the farnesyl groups of processed Ras (Luo et al. 1997; Williams et al. 2000). Ras binding also allows the CRD zinc finger motifs to directly contact phosphatidyl serine in the plasma membrane (Ghosh et al. 1996; Hekman et al. 2002). Full activation of Raf requires that both the RBD and the CRD are engaged, and CRD interactions with Ras and phosphatidyl serine are critical for relief of autoinhibition as well as stably linking Raf to the plasma membrane (Roy et al. 1997; Cutler et al. 1998; Bondeva et al. 2002). Notably, more than 40% of activating B-Raf mutations identified in the Rasopathy cardio-facio-cutaneous (CFC) syndrome occur in the CRD, further demonstrating the biological importance of this domain (Sarkozy et al. 2009).

Displacement of 14-3-3 and Dephosphorylation of the CR2 Site

In addition to changing the subcellular localization of the Rafs, experimental evidence suggests that the interaction with Ras and membrane phospholipids displaces 14-3-3 from the CR2 site, thereby exposing the site to phosphatase

activity (Rommel et al. 1996; McPherson et al. 1999). In response to epidermal growth factor signaling, a complex consisting of active M-Ras, Shoc2, and the catalytic subunit of protein phosphatase 1 (PP1) plays a prominent role in mediating the dephosphorylation of the CR2 site (Rodriguez-Viciano et al. 2006). However, the CR2 site is also a substrate for the heterotrimeric protein phosphatase PP2A (Abraham et al. 2000; Jaumot and Hancock 2001; Ory et al. 2003a). PP2A has been identified in active Raf complexes and may contribute to the dephosphorylation of the CR2 site in specific cell types or under certain signaling conditions (Ory et al. 2003a; Eisenhardt et al. 2016).

Raf Dimerization and Activating Phosphorylation

The interaction with Ras also serves a critical function in promoting the phosphorylation of the Raf kinase domain on activating sites and is often a prerequisite for Raf dimerization. Phosphorylation sites that are particularly important for Raf activation are found in the negative charge regulatory region (N-region). The N-region of all Raf proteins contains either negatively charged amino acids or residues that become negatively charged as a result of phosphorylation (Mason et al. 1999). Negative charges in this region contribute to the relief of autoinhibition (Cutler et al. 1998; Tran and Frost 2003; Tran et al. 2005) and are needed to form intermolecular contacts that stabilize the Raf dimer structure (Baljuls et al. 2011; Hu et al. 2013). The N-regions of both C-Raf and A-Raf are comprised of phosphoacceptor residues that only become phosphorylated at the cell surface in response to signaling stimuli (Mason et al. 1999). In contrast, the N-region of B-Raf maintains a constant charge because of the presence of two aspartic acid residues and a serine site (S445) that is constitutively phosphorylated by the CK2 kinase (Mason et al. 1999; Ritt et al. 2007). The tyrosine residues in the N-region of C-Raf and A-Raf are phosphorylated primarily by members of the SRC family kinases, whereas the N-region serine site can be phosphorylated by members of the PAK and PKC families, or by CK2 if the

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tyrosine sites are phosphorylated (Fabian et al. 1993; Marais et al. 1995; Diaz et al. 1997; King et al. 1998; Chaudhary et al. 2000; Chioleches et al. 2001; Hamilton et al. 2001; Ritt et al. 2007).

A second major breakthrough in understanding Raf activation came with the discovery that under most conditions, dimerization of the Raf kinases is required. Structural studies indicate that the Raf kinase domains form side-to-side dimers with interactions mediated by both the N- and C-lobes of the kinase domain (Rajakulendran et al. 2009). Although it is unclear whether any contacts are made between the Raf amino-terminal domains, binding to Ras, concurrent with the membrane clustering of active Ras proteins, is thought to increase the local concentration of Raf at the cell surface and induce conformational changes that promote Raf dimerization (Plowman et al. 2005; Terai and Matsuda 2005; Tian et al. 2007; Hibino et al. 2009). In addition to the interaction with Ras, the retained binding of 14-3-3 to the Raf carboxy-terminal site is also required for Raf dimerization in cells (Rushworth et al. 2006). Whether the bound 14-3-3 dimers facilitate contact between the Raf kinase domains or act to stabilize the dimerized structure is currently unknown; however, Raf proteins that contain mutations in the carboxy-terminal serine site fail to dimerize and cannot be activated (Garnett et al. 2005; Rajakulendran et al. 2009; Ritt et al. 2010). The Raf kinases can homodimerize or heterodimerize with any other Raf family member, and they can form side-to-side dimers with the closely related kinase suppressor of Ras (KSR) proteins (Hu et al. 2013). Although the factors that determine the dimerization preferences are poorly understood, it has been shown that B-Raf/C-Raf heterodimers predominate in Ras-mediated signaling and appear to have the highest catalytic activity (Weber et al. 2001; Rushworth et al. 2006; Freeman et al. 2013).

Structural studies have provided much insight regarding how dimerization promotes Raf activation. As stated above, for protein kinase domains to adopt an active/ON conformation, the hydrophobic residues comprising the regulatory R-spine must align, and for many

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kinases, this requires that the α C-helix in the N-lobe and the AS in the C-lobe shift to an active position. For the Raf kinases, the α C-helix and the dimer interface are allosterically linked in that the hydrophobic R-spine residue in the α C-helix (L505 for B-Raf, L397 for C-Raf, L358 for A-Raf) lies adjacent to the conserved RKTR motif in the dimer interface (Rajakulendran et al. 2009), and when dimerization occurs, contacts made at the dimer interface are thought to promote key conformational changes (Fig. 3). In particular, dimer interface contact is thought to shift the α C-helix into the active IN position and disrupt its interactions with the AS-H1-helix, which in turn may help destabilize the helix (Thevakumaran et al. 2015). Interestingly, a protomer does not need to possess catalytic activity to transactivate the second protomer as oncogenic mutations that abolish the kinase activity of B-Raf can promote tumorigenesis through the allosteric activation of C-Raf (Wan et al. 2004). B-Raf proteins that are inactivated by inhibitor treatment can also transactivate C-Raf in a Ras-dependent manner to promote paradoxical ERK cascade signaling (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010). Notably, the key determinant for whether a protomer in a dimer complex can function as an activator for the second protomer is the presence of charged and/or phosphorylated residues in the N-region (Hu et al. 2013).

Similar to many other kinases, it is also thought that phosphorylation of the AS is required for full Raf activity, functioning to expose the catalytic cleft and to promote the alignment of the R-spine residue in the DFG motif. Two conserved AS residues have been implicated, T598/S601 for B-Raf, T491/S494 for C-Raf, and T452/T455 for A-Raf, with substitution of phosphomimetic residues at these sites resulting in increased kinase activity (Zhang and Guan 2000; Chong et al. 2001). Given that these residues localize to the region of the AS that forms the AS-H1-helix, it has been proposed that phosphorylation of these sites may be required to disrupt the AS-H1-helix, which binds and stabilizes the α C-helix in an inactive OUT position (Thevakumaran et al. 2015). Through in

vitro kinase assays, one study has provided evidence that the phosphorylation of these sites occurs in *cis* after Raf dimerization (Hu et al. 2013). Strikingly, however, detecting these phosphorylation events in cells has been particularly difficult. For example, these phosphorylation sites have not been identified in any large-scale phosphoproteomic study despite the frequent detection of other phosphorylation sites, including the Raf N-region sites (Lavoie and Therrien 2015). Thus, it is possible that phosphorylation of the AS sites occurs at a very low level or is highly transient, or based on the B-Raf/MEK1 structure may not always be required. In most Raf crystals the AS is largely unstructured; however, in the B-Raf-MEK1 complex, residue E611 in the B-Raf AS acts as a phosphomimetic to form an intramolecular salt bridge with R575 in the conserved HRD motif, allowing the AS to assume an active conformation (Haling et al. 2014). The E611 residue aligns with AS phosphorylation sites known to be required for the activation of other kinases, including PKC α , and given that residues analogous to E611 and R575 are conserved in all Raf members, it is possible that the AS of the Raf kinases can adopt an “active” conformation in the absence of phosphorylation. Thus, although phosphorylation of the AS sites would promote the active conformation, their phosphorylation may not be essential under all signaling conditions or for all Raf proteins.

Nonetheless, it is clear that the conformation of the AS is a critical determinant in Raf kinase activation, as many oncogenic B-Raf mutations occur in this segment, with V600E being the most prevalent. In the case of V600E, not only would this substitution act as a phosphomimetic to disrupt the AS-H1-helix, structural studies indicate that the negatively charged side chain of the glutamic acid can form an intramolecular salt bridge with K507, a component of the RKTR dimer interface motif that lies at the tip of the α C-helix. As a result of this interaction, both the α C-helix and the AS stably adopt an active conformation in the absence of dimerization (Haling et al. 2014; Thevakumaran et al. 2015). Moreover, recent studies indicate that mutations to the B-Raf V600 residue are among

the few disease-associated Raf mutations that promote Raf activation in a dimerization-independent manner (Yao et al. 2015).

POSTSIGNALING INACTIVATION OF RAF

Once Raf has been activated and the signal transmitted to the downstream kinases MEK and ERK, it is critical that the Rafs return to an inactive state, as constitutive activation can result in tumorigenesis. Several mechanisms that contribute to Raf inactivation have been identified. As expected, activating phosphorylation sites must be dephosphorylated, and protein phosphatase 5 (PP5) has been reported to dephosphorylate the critical S338 N-region site of C-Raf (von Kriegsheim et al. 2006). Raf autophosphorylation has also been implicated as a mechanism for inactivating the Rafs. In particular, a report examining inhibitor-bound B-Raf dimers has identified autoinhibitory sites in the ATP-binding P-loop (Holderfield et al. 2013), and a phosphoproteomic study characterizing the V600E-B-Raf mutant has identified an autoinhibitory site in the AS (S614-B-Raf) (Dernayka et al. 2016). Further studies will be needed to determine the prevalence and contribution of these autophosphorylation events in the context of Ras-mediated signaling.

Another important mechanism that attenuates the activity of all Raf family members involves a negative feedback loop in which active ERK phosphorylates the Rafs on multiple S/TP sites (Brummer et al. 2003; Dougherty et al. 2004; Ritt et al. 2010). Phosphorylation of the S/TP sites disrupts the interaction with Ras as well as Raf dimerization, and results in Raf proteins that are signaling incompetent (Dougherty et al. 2004; Ritt et al. 2010). Of note, some of these S/TP sites are also phosphorylated by the c-Jun amino-terminal kinases (JNKs) to prevent Raf signaling in times of cellular stress, and this stress-mediated desensitization of the Rafs can be induced by certain cancer therapeutics, such as rigosertib and paclitaxel (Ritt et al. 2016).

Ultimately, the postsignaling hyperphosphorylated Raf proteins must be recycled to a presignaling inactive state that is competent to

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respond to a new round of activating signals. Dephosphorylation of the ERK target sites is mediated by PP2A in a manner that requires the Pin1 prolyl-isomerase (Dougherty et al. 2004). More specifically, the WW-domain of Pin1 binds to the pS/TP sites, allowing Pin1 to isomerize the pS/T-proline bond to a *trans* conformation that can then be dephosphorylated by PP2A. Whether PP2A also serves to dephosphorylate the sites of autophosphorylation is currently unknown; however, once dephosphorylated, the Raf proteins can then reestablish the intramolecular contacts that mediate the autoinhibited state. Finally, with the rephosphorylation of the CR2 site and the rebinding of 14-3-3 to this site, the Raf kinases are returned to their presignaling, inactive conformation.

OTHER DIMER PARTNERS: KSR

Members of the KSR family are close relatives of the Raf kinases, and like the mechanisms mediating Raf activation, the function of KSR in Ras signaling is complex. KSR was first discovered as a positive modulator of the Ras pathway through genetic studies performed in *Drosophila* and *Caenorhabditis elegans* (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995) and two KSR proteins (KSR1 and KSR2) are present in mammalian cells. The KSR family shares significant sequence homology with the Rafs, and like the Rafs, have a carboxy-terminal kinase domain and an amino-terminal domain that contains a CRD followed by a region rich in serine/threonine residues (Therrien et al. 1995). However, the KSRs do not have an RBD, but instead contain a coiled-coil fused to a sterile- α motif (CC-SAM) domain and a region rich in proline residues (Therrien et al. 1995; Koveal et al. 2012). Although it was initially thought that the KSRs would function as typical protein kinases, the mammalian KSR proteins were found to lack a lysine residue that is normally required for the phosphotransfer reaction (Therrien et al. 1995). Moreover, when residues known to be critical for enzyme catalysis were mutated in *C. elegans* or *Drosophila* KSR, little to no effect on the biological function of these KSR family members was observed (Stewart et al. 1999; Roy

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et al. 2002), raising the question of whether the KSRs indeed possess intrinsic kinase activity—a question that is still debated.

Subsequently, reports emerged indicating that the KSR proteins may have scaffolding activities, interacting with the three core components of the ERK cascade (Morrison 2001). In quiescent cells, KSR is found in a multiprotein complex containing MEK, 14-3-3, and components of the Hsp90 complex (Xing et al. 1997; Denouel-Galy et al. 1998; Yu et al. 1998; Cacace et al. 1999; Stewart et al. 1999). Similar to the Rafs, binding of 14-3-3 to sites in the KSR amino-terminal domain sequesters the inactive KSR complex in the cytosol (Müller et al. 2001; Ory et al. 2003b), and as with B-Raf, the kinase domains of KSR and MEK interact in a face-to-face manner (Brennan et al. 2011). On signal activation, KSR rapidly translocates to the plasma membrane coincident with the dephosphorylation of one of the 14-3-3 binding sites (Ory et al. 2003b). The CRD and the CC-SAM domain are critical for membrane recruitment of KSR (Zhou et al. 2002; Koveal et al. 2012), and at the membrane, KSR serves to increase the local pool of MEK, thus facilitating MEK activation. Moreover, KSR proteins can also form side-to-side dimers with the Rafs (Rajakulendran et al. 2009; Brennan et al. 2011), and if KSR is phosphorylated on residues analogous to the Raf N-region sites, it can function as an allosteric activator of Raf (Hu et al. 2013). Importantly, the KSR proteins also contain FxFP docking sites for activated ERK (Jacobs et al. 1999), and binding of ERK to the membrane-localized KSR complexes facilitates the phosphorylation of KSR and the Rafs on S/TP sites (McKay et al. 2009). Phosphorylation of these feedback sites disrupts the signaling complexes and promotes the release of KSR and the Rafs from the cell surface (McKay et al. 2009). Thus, KSR can modulate the dynamics of Ras pathway signaling by both potentiating and attenuating Raf activity and signal transmission to MEK and ERK.

CONCLUDING COMMENTS

The past decades have witnessed tremendous advances in our understanding of the Raf activation/inactivation process (Fig. 4).

The early years of research were focused on elucidating the biochemical properties of the Rafs, determining their subcellular localization, phosphorylation state, and binding partners. Subsequently, the pace of discovery accelerated with the characterization of disease-associated mutations and the analysis of Raf crystallographic structures. Although much has been learned, outstanding questions still remain. In particular, it is unclear what factors determine Raf dimerization preferences and whether these preferences change depending on the signaling environment. Moreover, the structural features of a full-length Raf protein are unknown as are the structural changes induced by Ras binding and membrane interactions. The ultimate goal is that with further insight regarding the complexities of Raf regulation, new strategies for therapeutic intervention will emerge for targeting Ras-mediated Raf activation in human disease states.

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