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REVIEW

Signal control through Raf: in sickness and in health

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The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade is the prototype mammalian mitogen-activated protein kinase (MAPK) signaling cascade that regulates a number of processes, including proliferation, differentiation, survival, migration, stress responses and apoptosis. How this seemingly linear cascade is modulated to achieve a specific cellular function has been a main focus of the field. In this review, we describe new as well as old findings in the regulation of the ERK1/2 pathway in normal and disease states via MAP3Ks.

***Keywords*:** ERK1/2; MAPK; Raf

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Introduction

Since we first cloned the extracellular signal-regulated kinases 1/2 (ERK1/2) over twenty-one years ago, a myriad of growth factors and cytokines have been identified as their activators [1]. Originally described as microtubule-associated protein kinases activated by insulin, and now known as mitogen-activated protein kinases (MAPK), these enzymes are regulated through a protein kinase cascade (Figure 1). The cascade is usually initiated by ligands that bind a variety of membrane receptors, which engage adaptor proteins and exchange factors to induce activation of the small G protein, Ras [2-7]. GTP-bound active Ras then recruits and activates the first protein kinase in the cascade, one or more of the Raf proteins, Raf-1 (C-Raf), B-Raf, and/or A-Raf [8, 9]. Raf kinases phosphorylate and activate the MAP2K protein kinases, MEK1/2, that eventually phosphorylate and activate ERK1/2 [10-13]. While this seemingly simple, linear cascade involves progressive signal amplification initiated by ligands and governed by phosphorylation, proteins associated with other pathways and an array of modifications fine tune the signal. Integration of these other inputs directs the MAPK cascade to couple signals from specific stimuli to generate subsequent cellular activities that may lead to differentiation, proliferation, and motility, for example, as appropriate [14]. Understanding how cells interpret and process abundant and diverse inputs remains challenging. Cells use scaffolding proteins, phosphatases, and second messengers to refine complex signaling programs connecting extracellular stimuli to intracellular responses. This review will focus on understanding how such proteins control signaling at the MAP3K level to regulate attenuation or augmentation of the ERK1/2 pathway.

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B-Raf in cancer

Homologs of all three Raf serine/threonine protein kinase family members are found in vertebrates, while a single Raf equivalent exists in invertebrates [15]. The Raf proteins contain three conserved regions, CR1, CR2, and CR3. CR1 includes the Ras-binding domain; CR3 comprises the kinase domain, which is typical of the protein kinase family, containing two folding domains with adenosine triphosphate-binding site at their interface and a regulatory activation loop at the mouth of the active site. B-Raf is the most commonly mutated protein kinase in the human genome; most of the mutations occur in the kinase domain, either in the glycine-rich loop or the activation loop [16, 17] (Table 1). A substitution of glutamate for valine at residue 600 accounts for 90 % of the B-Raf mutations observed in human cancers including, malignant melanoma, colorectal cancer, ovarian, and papillary thyroid carcinomas. In addition to V600E, > 30 other mutations of the *BRAF* gene have been found in human cancers, the most frequent of these mutations

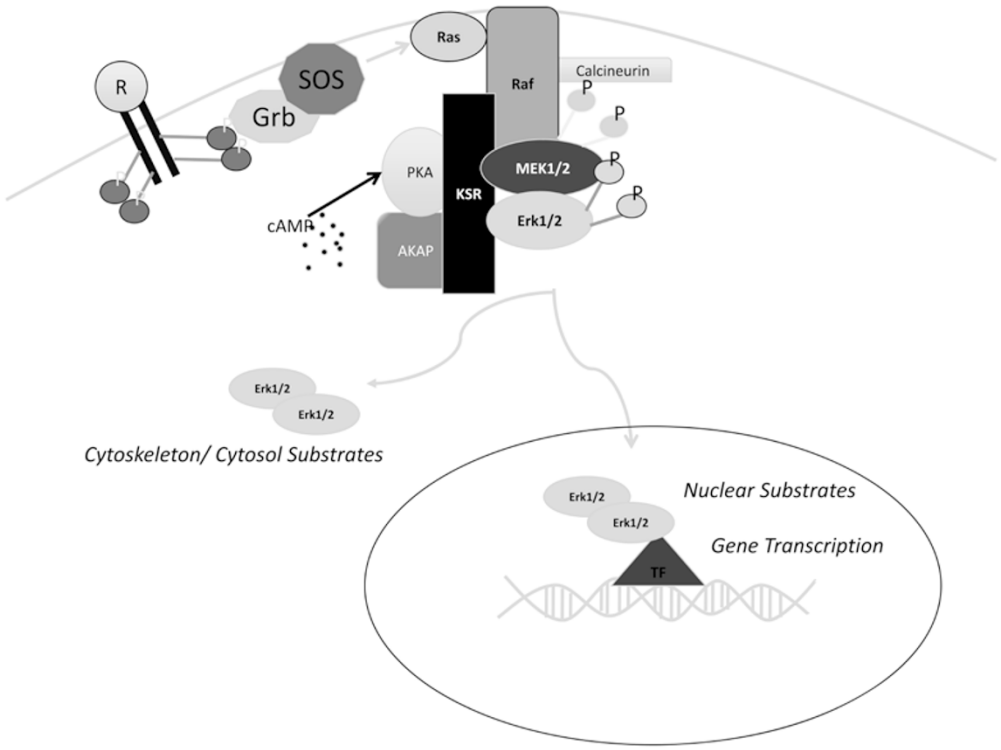

Figure 1 Activity of the ERK1/2 MAPK cascade can be modulated by inputs at different sites in the pathway. The best studied mechanism of ERK1/2 activation is through receptor tyrosine kinases. Signaling events at the plasma membrane initiate the cascade through ligand/receptor binding. The signal is transmitted by recruiting adaptor proteins (e.g., Grb2) and exchange factors (e.g., SOS) that induce the activation of Ras at the plasma membrane. The activated, GTP-bound Ras then transmits the signal by activating Raf protein kinases (MAP3Ks). Rafs activate MEK1/2 through phosphorylation, which in turn phosphorylate ERK1/2. Once activated, ERK1/2 propagate the signal by affecting a variety of downstream signaling pathways. Additional components that can provide inputs to the cascade are shown including a KSR-associated AKAP and the calcium-dependent phosphatase calcineurin.

Table 1 Most common Raf mutations identified in tumors

Gene	Most Common Mutation Primary Tissue	% Mutated	AA Mutation
BRAF	Skin	40%	V600R/K/E; L597R/Q/L; K601E/I/K/N; D594G/N/V; G469R/V/E/S; I592M
	Thyroid	38%	V600E
	Ovary	12%	V600E/G; L597R/Q
	Large Intestine	12%	V600R/ P453T; G469E/V
ARAF	Skin	14%	A345G
CRAF	Liver	100%*	N115S
	Stomach	3%	R495H

The mutation data were obtained from the Sanger Institute Catalogue Of Somatic Mutations In Cancer website, http://www.sanger.ac.uk/cosmic [94].
*Mutation was found in one sample.

in melanoma [17, 18]. The frequency of mutations in B-Raf and not A- or C-Raf may be attributed to melanocyte biology as well as to details of its constitutive activation and regulation.

B-Raf/C-Raf interactions in cancer and cancer therapeutics

Phosphorylation of the activation loop is required for

the activity of all three Raf family members. In addition to having a higher basal kinase activity than C-Raf or A-Raf, B-Raf also lacks the need for tyrosine or multiple serine phosphorylations that are required for the activity of A-Raf and C-Raf [19]. Higher B-Raf activity may be due to Ras-independent constitutive phosphorylation of S445, in a region preceding the catalytic domain. Activation of the pathway can also be attributed to the formation of Ras-dependent homo- and heterodimers, between B-Raf and C-Raf [9, 20]. A much greater understanding of the relevance of B-Raf/C-Raf interactions has arisen from the analysis of the mechanisms of action of second-generation selective oncogenic B-Raf inhibitors. Several of these compounds, while initially inhibiting ERK1/2 phosphorylation, eventually promote C-Raf/ERK1/2 activation, leading to an enhanced cancer cell proliferation or drug refractory tumors [21-24]. Insights into how several of these inhibitors become activators of the MAPK pathway ultimately revealed that pathway activation occurred from drug-induced dimerization of C-Raf with wild type or mutant B-Raf. Even kinase dead B-Raf (a mimic of inhibited B-Raf) promotes signaling [22, 23, 25]. The strategy underlying the development of second-generation inhibitors was to target mutant B-Raf selectively. Thus, such inhibitors had no inhibitory effect on cells with other activated oncogenes, such as mutant Ras, if they have wild-type B-Raf [22, 23, 25]. In addition to mechanisms involving C-Raf, several of the Raf inhibitors also induced B-Raf interaction with the kinase suppressor of Ras 1 (KSR1, discussed further below) that competed with C-Raf to attenuate the signaling pathway [26]. B-Raf complexes formed with KSR1 differ greatly from the complexes that induce C-Raf binding. In addition to altering the activating effects observed during signaling to ERK1/2, the KSR/B-Raf complexes are also Ras-independent, suggesting that KSR1 expression may be important for inhibitor efficacy.

Kinase suppressor of Ras

Originally discovered in model organisms as a protein required for activated Ras to accelerate MAPK signaling, KSR isoforms are members of the protein kinase superfamily structurally related to Raf. The relevance of the catalytic activity of KSR proteins is in debate [27-31]. KSRs contain arginine instead of the invariant catalytic lysine in the N-terminal region of the kinase domain present in other protein kinases. KSR1 may be most important as a scaffold due to its ability to bind simultaneously to components at each level of the MAPK cascade (MAP3K, MAP2K, and MAPK). Because signaling is diminished if it is expressed in excess of its binding partners, KSR1 fits the characteristics of a scaffold [27, 30, 32]. A functional catalytic lysine appears to be absent from KSR, unlike what we found in WNK protein kinases; in WNKs the catalytic lysine is not in the canonical position, but it is nearby in a distinct structural element [33]. Nevertheless, KSR1 purified from *E. coli* can directly phosphorylate MEK1 *in vitro*, albeit at a rate that may be representative of a catalytically impaired Raf mutant [29]. Regardless, KSR is a critical component of the MAPK pathway and thus subject to modulation just as are the other proteins in the pathway. The E3 ligase and Ras effector protein, impedes mitogenic signal propagation (IMP), attenuates Raf-MEK association via inactivation of KSR homo- and heterodimerization with RAF proteins [34]. The association between IMP and KSR1 also blocks KSR1 interaction with MEK1, preventing activation of ERK1/2 [35].

While *Drosophila* and *C. elegans* have only one KSR protein, mammals have two KSR family members [27]. KSR1 knockout mice have been generated and show no extreme abnormalities other than modestly impaired immune and neuronal signaling, suggesting a functional redundancy between KSR1 and KSR2 [36, 37]. KSR2 shares 43% sequence identity with KSR1, which includes the conserved regions required to bind to the three members of the ERK1/2 cascade, and maintains the ability to regulate pathway activation in a concentration-dependent manner [32]. However, novel roles for KSR2 have been reported in metabolic pathways. KSR2 has been shown to regulate AMPK to promote glucose and fatty acid metabolism, and its loss in mouse models leads to obesity and insulin resistance [38].

Glucose metabolism and MAPK activation

Pancreatic β cells are specialized endocrine cells that secrete insulin in response to elevated blood glucose in order to facilitate proper glucose utilization. Glucose metabolism in β cells causes an increase in the adenosine triphosphate/adenosine diphosphate ratio, which leads to closure of the adenosine triphosphate-dependent potassium channel, ultimately resulting in calcium entry into the cells and release of insulin. Thus, fluctuations in intracellular calcium are integral to β-cell function. ERK1 and ERK2 have significant roles in maintaining β cell function. We find that the nutrient-dependent regulation of ERK1/2 and their nutrient-dependent actions are also controlled by calcium [39]. In β cells, ERK1/2 are activated by both glucose and other nutrients, as well as other factors that potentiate insulin secretion such as glucagon-like peptide, which acts through the second messenger cAMP [39].

Glucose-induced insulin gene transcription has been shown to require a set of core transcription factors, including BETA2 (also known as NeuroD1), PDX1, MafA, and NFAT [29, 40-46]. We showed that these transcription factors act synergistically to promote insulin gene transcription in an ERK1/2-dependent manner, as phosphorylation of both Beta2 and PDX-1 is direct [47]. Moreover, transactivation of NFAT and MafA is also ERK1/2-sensitive, as inhibition of the pathway via the MEK1/2 inhibitor U0126 dissociates both factors from the insulin promoter [40].

Previous work in the immune system showed that the calcium- and calmodulin-dependent phosphatase calcineurin is required for NFAT function [48]. Consistent with their actions in the immune system, calcineurin inhibitors, such as FK506 and cyclosporin A, are immunosuppressants that decrease the rejection of transplanted tissues, including pancreatic islets. These inhibitors, however, reduce insulin gene transcription and secretion, which may further exacerbate complications in diabetic patients following islet transplantation [40, 49].

Calcium-dependent phosphatases

In addition to phosphoprotein phosphatases 1 and 2A and the canonical dual-specificity phosphatases that modulate the distal steps in the MAPK pathway, calcineurin also has a critical role in control of ERK1/2 in pancreatic β cells. Glucose metabolism and the increases in second messengers, noted above, increase cytosolic calcium by inducing calcium entry and/or its release from intracellular stores [39, 50]. We found that increased cytosolic calcium in β cells directs the calcineurin-dependent activation of ERK1/2 that can be pharmacologically blocked with calcineurin inhibitors, e.g., cyclosporin A, FK506, and its analog FK520 [39] (see Figure 2 and below). Calcineurin knockout mice have been generated. Mice with β-cell-specific deletion of the calcineurin regulatory subunit, calcineurin b1, were found to develop diabetes that is distinguished by decreased β-cell proliferation and mass as well as reduced pancreatic insulin content [51]. Interestingly, these defects could be rescued by expression of active NFAT. Attempts to understand the mechanism by which calcineurin activates MAPK kinase cascades have yielded interesting and perhaps unexpected outcomes.

In both a pancreatic β cell line and a neuroblastoma cell line KSR2 but not KSR1, is a calcineurin substrate [32]. Activity of the phosphatase contributes to the localization of KSR2 to the membrane, where it can activate the ERK1/2 pathway [32, 39]. Inhibition of calcineurin activity under these conditions reduces activation of ERK1/2. This regulation is calcium-dependent, providing mechanistic insight into activation of ERK1/2 by calcium. We had previously shown that dominant negative mutants of both Ras and Raf inhibited glucose-dependent ERK1/2 activation in pancreatic β cells, which is consistent with the idea that both are required for glucose-dependent ERK1/2 activation [39]. Importantly, we found that in pancreatic β cells both B-Raf activity and B-Raf/C-Raf dimerization are enhanced in a calcineurin- and glucose-dependent manner [52].

Negative feedback

Positive and negative feedback loops have critical roles in modulation of signal transduction cascades. Growth factors that activate the MAPK pathway have been suggested to regulate the MAPK phosphatases that turn off the kinases in the same pathways [53, 54]. Expression of B-Raf, but not C-Raf, in 293 cells led to increased ERK1/2 activation; however, B-Raf is subject to ERK1/2-mediated feedback inhibition as we found that treatment with the MEK inhibitor, U0126, in the absence of calcineurin led to increased B-Raf activity [52] (Figure 2). This suggests that calcineurin promotes B-Raf activation by interrupting the negative feedback effects of ERK1/2. ERK1/2 have been shown to phosphorylate B-Raf on residues S151, S750, T401, and T753, to disrupt not only active Ras interaction but also homo- and heterodimer formation [55]. Phosphorylation of T401 by ERK1/2 is reduced by U0126 and can specifically be

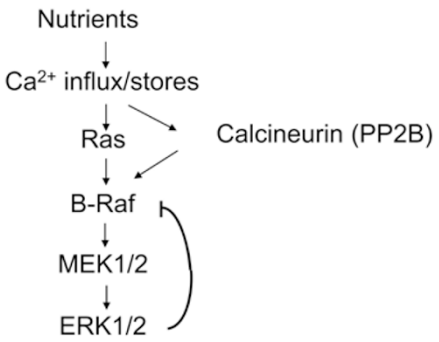

Figure 2 Calcineurin reverses negative feedback on B-Raf to permit ERK1/2 activation by nutrients in pancreatic β cells. Once activated, ERK1/2 can phosphorylate multiple upstream components in the pathway. In β cells, in response to nutrients ERK1/2 phosphorylate B-Raf preventing its continued stimulation of MEK1/2. B-Raf is required for the activation of ERK1/2 by nutrients. The calcium-dependent phosphatase calcineurin can dephosphorylate a key inhibitory site on B-Raf, allowing continued ERK1/2 activation.

removed by calcineurin to promote B-Raf activation and interaction with C-Raf [52].

ERK2 is fully activated by dual phosphorylation of both threonine-183 and tyrosine-185 residues [56, 57]. Canonical MAPK phosphatases or dual-specificity (DUSP) enzymes dephosphorylate both phosphothreonine and phosphotyrosine residues within ERK1 and ERK2 [58, 59]. In addition to the various extracellular stimuli that activate MAPK phosphatase-1, the second messenger cAMP has also been shown to stimulate activation of MAPK phosphatase-1 [60]. The ability of the cAMP pathway to inhibit growth of tumors transformed by activation of MAPK was a topic of much interest for many years [61]. Later it was discovered that there is a cross talk between cAMP and MAPK pathways, and the effects were differentially depending on cell type and context.

Cross talk of cAMP and MAPK pathways

In cells such as melanocytes, thyroid cells, cells of the anterior pituitary, and β cells, cAMP can enhance proliferation or differentiation; however, in the eye, lung, certain fibroblasts, astrocytes, and hepatocytes to name a few cAMP decreases proliferation [39, 61-66]. Activation of MAPK pathway by cAMP occurs in cells that are neural crest-derived, such as melanocytes and the neuroendocrine pheochromocytoma PC12 cell line [67, 68].

Effectors of cAMP include the multifunctional cAMP-dependent protein kinase (PKA) as well as the guanine nucleotide exchange factors, Epac1 and 2. Epacs are exchange factors for the small G proteins Ras-related protein 1 and 2 (Rap1, Rap 2) [69]. Activation of ERK1/2 by cAMP that induces proliferative signals is Ras- and B-Raf-mediated in melanocytes, whereas the differentiation program may solely rely on Epac activation [70, 71]. Proper signaling in the cAMP pathway is also achieved by compartmentalization of PKA and Epacs by the scaffolding proteins known as A-kinase anchoring proteins. Our laboratory identified the only AKAP shown to bind directly to ERK1/2, radial spoke protein 3 (RSPH3) (Figure 3). RSPH3 was identified in a yeast two-hybrid screen and it binds to ERK1/2 [72]. ERK1/2 phosphorylates the anchoring protein; this phosphorylation regulates the ability of RSPH3 to bind to the regulatory subunits of PKA. Inhibition of ERK1/2 activity in this setting negatively regulates the AKAP function of RSPH3. Several A-kinase anchoring proteins have been shown to mediate cross talk between PKA and the MAPK pathway by interacting with other components of the cascade. Recently AKAP-Lbc has been shown to amplify the positive effect that cAMP has on the MAPK pathway via its interaction with KSR1. In addition to its known function as a guanine nucleotide exchange factors, AKAP-Lbc is also a scaffold protein [73]. AKAP-Lbc binds to KSR1, which facilitates cAMP-dependent phosphorylation of KSR1 by PKA, ensuring sustained activation of ERK1/2 [74]. While these findings support the idea that PKA has a positive function in ERK1/2 activation, this is not always the case.

As mentioned above, cAMP activation of ERK1/2 that leads to proliferation in melanocytes is mediated by B-Raf. At the same time, C-Raf is inhibited by PKA via phosphorylation on several sites. Mechanistically, these phosphorylations both inhibit its interaction with Ras and alter the binding with the adaptor protein 14-3-3 [75, 76]. Ras-dependent activation of C-Raf requires not only activation loop phosphorylation but also proper binding and dimerization of 14-3-3 [77, 78]. In melanomas, Ras mutations and B-Raf mutations are usually mutually exclusive. If Ras is mutated, the pathway switches from B-Raf to C-Raf to activate ERK1/2 [67]. Overexpression of activated N-Ras in melanocytes hyperactivates ERK1/2, causing phosphorylation of S151 on B-Raf. This promotes the switch to C-Raf and melanocyte transformation [79]. In this context, cAMP is degraded by phosphodiesterases (PDE) [79]. Two isoforms of PDE4 are upregulated in N-Ras-transformed melanocytes, and are activated by ERK1/2. Loss of PDE4B2 reduces the ability of N-Ras to transform melanocytes, suggesting the requirement for PDE4 in the process [79].

Even though cAMP has been shown to inhibit proliferation, the inhibition is not ERK1/2-dependent. The ΔRaf1:ER fusion protein lacks amino terminal residues and is thus resistant to cAMP. ERK1/2, activated in this context, are not sensitive to elevated cAMP, although DNA synthesis, CDK2 activity, and cyclin A expression are [80]. In addition to the inhibitory effects of cAMP on ERK1/2, we find that ERK5 is also uncoupled from acti-

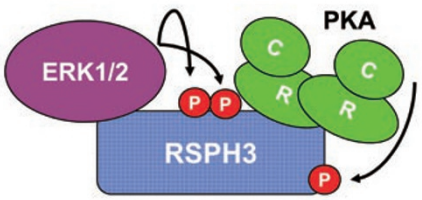

Figure 3 RSPH3 3 is a mammalian AKAP that binds ERK1/2. RSPH3 is the human homolog of a protein found in motile cilia that forms part of radial spokes that connect the inner to the outer pairs of microtubules. RSPH3 is also an AKAP. ERK1/2 bind directly to RSPH3 and phosphorylate it, altering its interactions with the regulatory subunit of PKA.

vating signals in a PKA-dependent manner [81].

MAPK pathway in other cancers

Historically aberrant activation of the ERK1/2 pathway either by mutations found in receptor tyrosine kinases or Ras or Raf, have been shown to lead to increased survival and/or proliferation of malignant cells. This general view is challenged by the behavior of small cell lung cancer (SCLC). Lung cancer is usually classified as SCLC or non-small cell lung cancer (NSCLC). Patients with SCLC often have metastases at the time of diagnosis, leading to a very poor long-term prognosis [82]. These two types of lung cancer are characterized by the differences in mutation profile. Activating mutations in the ERK1/2 pathway are found frequently in NSCLC and almost never in SCLC [83]. In contrast to most of the systems discussed above, in SCLC activated Raf-1 causes growth arrest [84]. ERK1/2 can also be activated by receptor tyrosine kinases, such as c-Met and its ligand hepatocyte growth factor, and cKit and its ligand stem cell factor, which establishes an autocrine loop in SCLC [85-87]. cKit and stem cell factor are expressed frequently in SCLC, but not in NSCLC. Inhibition of MEK1/2 does not impair growth of SCLC. This may, in part, be due to the presence of mutant retinoblastoma protein 1, as expression of wild-type retinoblastoma protein 1 can sensitize these cells to the MEK1/2 inhibitor [87]. Thus, it appears that MEK inhibitor insensitivity in SCLC arises from the loss of retinoblastoma protein 1 function. Loss or mutation of retinoblastoma protein 1 and p53 in pulmonary neuroendocrine cells is sufficient to cause a mouse model of SCLC [88, 89]. When various isoforms of PI 3-kinase are overexpressed in SCLC they increase stem cell factor-stimulated activation of AKT, but not ERK1/2 [86]. Additionally, an analysis of 42 patients with SCLC revealed that while about half were found to have active ERK1/2, AKT and c-Kit, the active ERK was cytoplasmic, not nuclear, and correlated positively with patient survival [90].

Conclusion

The ERK1/2 signaling cascade is central to regulation of many, sometimes opposing, cellular functions. Here, we point to new findings that determine signaling specificity in tissue-dependent contexts in both normal and pathological states. Indeed, dysregulation of the ERK1/2 signaling cascade has been noted in neurodegenerative disease [91] and diabetes, [92, 93] as well as in cancer. The effective targeting of this pathway in disease relies upon the understanding of intricacies in modulating all of the signaling components. Additionally, elucidating cross talk of MAPK components with other signaling pathways that induce and promote pathologies could also provide new ground for combination therapies.

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