Review

Partner exchange: protein–protein interactions in the Raf pathway

Reiner Wimmer and Manuela Baccarini

University of Vienna, Center for Molecular Biology, Max F. Perutz Laboratories, Doktor-Bohr-Gasse 9, A-1030 Vienna Austria

The three-tiered Raf–MEK–ERK kinase module is activated downstream of Ras and has been traditionally linked to cellular proliferation. Mammals have three *Raf*, two *Mek* and two *Erk* genes. Recently, the analysis of protein–protein interactions in the pathway has begun to provide a rationale for the redundancy within each tier. New results show that the MEK–ERK-activating unit consists of Raf hetero- and homodimers; downstream of Raf, MEK1–MEK2 heterodimers and ERK dimers are required for temporal and spatial pathway regulation. Finally, C-Raf mediates pathway crosstalk downstream of Ras by directly binding to and inhibiting kinases engaged in other signaling cascades. Given the roles of these interactions in tumorigenesis, their study will provide new opportunities for molecule-based therapies that target the pathway.

The Raf–MEK–ERK pathway

Mitogen-activated protein kinase (MAPK) cascades are signaling modules in which a signal, in the form of phosphorylation, is received by an entry point kinase and passed on to an intermediate kinase, which proceeds to activate the 'business end' of the cascade, the MAPK itself. This three-tiered array enables a significant increase in cumulative signal strength as well as diversification and temporal modulation of the signal as it progresses down the pathway. This basic pattern is repeated in cascades that implement very different biological outcomes, ranging from proliferation to differentiation, response to stress and cytokines and apoptosis [1].

Of the four MAPK cascades operating in vertebrates, the Raf–MEK–ERK pathway was the first to be discovered and remains the best studied. Typically, the pathway is induced downstream of growth factor receptors via the exchange of GTP for GDP on the membrane-associated small G protein Ras. GTP-bound Ras recruits the entry point kinase Raf to the membrane, where it is activated by complex, yet incompletely understood mechanisms. Raf in turn phosphorylates MEK, a dual specificity kinase whose only proven target is extracellular signal-regulated kinase (ERK). ERK, in stark contrast, regulates a vast array of targets distributed in different subcellular locations, including metabolic enzymes, structural proteins and transcription factors (Figure 1). Clearly, tight spatiotemporal regulation is crucial for steering the ERK signal in the right direction and implementing appropriate biological outcomes (see [2] for a recent, comprehensive review). Briefly, regulation can be achieved by the direct binding of pathway components to each other [3] or to scaffold proteins, which additionally provide localization signals [4,5], and also by the presence of inhibitors that disrupt these complexes [4]. *In vivo*, the differential expression of the pathway components themselves (for instance in the case of Raf [6]), of their scaffolds [4]) and of their regulators (as in the case of dual specificity phosphatases [7]) in various tissues contributes to the wiring of the pathway by generating different combinations of active signal transducers.

In spite of the more than 20,000 papers published in the past 20 years, the pathway still holds surprises that have important biomedical consequences. This review will focus on the recent advances made on the mechanistic aspects of pathway regulation, particularly on the role of heterodimerization of pathway components in the modulation of the signal, and on the biological functions of pathway components, some of which were entirely unexpected. Finally, we attempt to put these new discoveries in an evolutionary perspective.

Pros and cons of partnership: dimers in activation and negative feedback control

Dimerization is a recurring theme in the Raf–MEK–ERK pathway. Dimerization can activate the kinases (as in the case of Raf), but it can also be used to mediate negative feedback control (as in the case of MEK) or to enable concomitant binding to scaffold and substrates, allowing the localization of signaling complexes (as in the case of ERK). The following section will give an overview of these different scenarios.

Energizing partnerships: Raf dimers in activation

Raf activation requires a transition between a 'closed' conformation, in which the N-terminal regulatory domain of the molecule interacts with the C-terminal kinase domain, to an 'open' conformation, in which the kinase domain is now free to recruit and phosphorylate its substrates [6,8,9] (see below). This conformational change involves the dephosphorylation of negative regulatory phosphorylation sites, the best studied of which mediates the interaction between Raf and 14-3-3 proteins. These chaperones bind phosphorylated Ser residues in both the regulatory and catalytic domains of Raf [4]. Binding of 14-3-3 to the site in the catalytic domain has a positive function, at least in C-Raf; autophosphorylation of this site in *cis* and the ensuing 14-3-3 binding are required to prevent proteasome-mediated C-Raf degradation [10]. By contrast, binding of 14-3-3 to the sites in the regulatory

Corresponding author: Baccarini, M. (manuela.baccarini@univie.ac.at)

 0968-0004/$ – see front matter doi:10.1016/j.tibs.2010.06.001

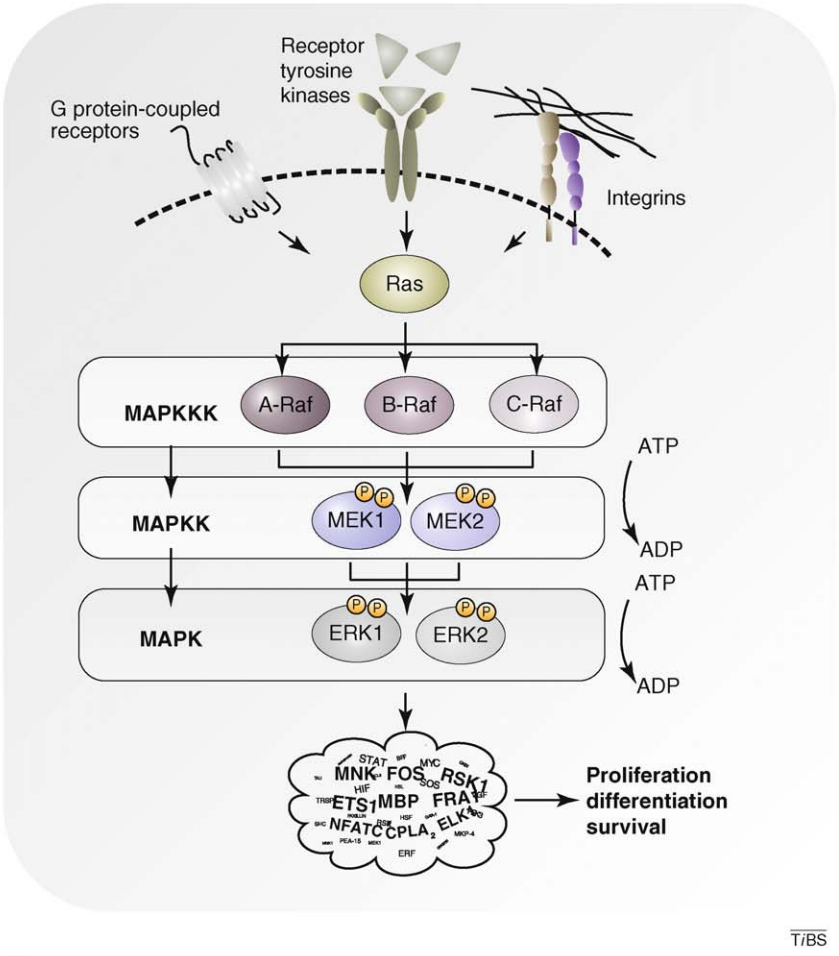

Figure 1. The Raf–MEK–ERK pathway. The Raf pathway is a typical three-tiered cytosolic kinase cascade, activated downstream of the small GTPase Ras by signal feeding into receptor tyrosine kinases, G protein-coupled receptors and integrins. The entry point kinase (MAPK kinase kinase; MAPKKK) Raf phosphorylates and activates MEK, a dual specificity kinase (MAPKK), which in turn transfers the signal to ERK (MAPK). ERK has a vast array of substrates whose activation leads to diverse biological outputs. Each tier consists of more than one kinase: there are three Rafs (A, B and C), two MEKs (1 and 2) and two ERKs (1 and 2).

domain might inhibit the translocation of Raf to the membrane by masking regions involved in its interaction with Ras; alternatively, simultaneous binding to the sites located in the regulatory and catalytic domains might stabilize the closed conformation and prevent activation. Dephosphorylation of the N-terminal 14-3-3 binding site loosens the closed conformation, and allows the interaction of Raf with Ras–GTP at the plasma membrane [4,6]. Here, phosphorylation occurs on the activation segment within the Raf kinase and on sites clustered at its upstream boundary, in the so-called N (negatively charged) region. The crystal structure of the B-Raf kinase domain revealed that phosphorylation of the activation segment breaks up its interaction with the P-loop (or glycine loop), the flexible region found at the N-terminal part of protein kinase domains, which positions the phosphates of ATP in the catalytic cleft, thus allowing the kinase to adopt its active conformation. The kinases phosphorylating the activation segment have not yet been identified. By contrast, several kinases, including Src, p21-activated kinase (PAK) [8], casein kinase (CK)-2 [11] and more recently, Raf itself [12], are known to phosphorylate the Ser and Tyr residues in the N-region. Phosphorylation of the N-region is thought to facilitate activation by weakening the interaction of the Raf regulatory domain with the catalytic domain. It is only necessary for the activation of C-Raf and, probably, A-Raf, because the corresponding B-Raf Ser is constitutively phosphorylated and the Tyr is replaced by an Asp. The presence of these constitutive negative charges and the resulting loose interaction between the regulatory and kinase domain are probably the reason why B-Raf has a higher MEK kinase activity than the other two family members [13]. Conversely, unique residues in the A-Raf regulatory domain are predicted to stabilize the 'closed' conformation and adversely affect activation, accounting for the low kinase activity of A-Raf [14].

In recent years, it has become clear that Raf proteins are able to heterodimerize. After an initial report that active Ras stimulates the formation of B-Raf–C-Raf heterodimers [15], interest in Raf dimers and in their role in ERK activation was renewed by the observation that oncogenic B-Raf mutants with reduced intrinsic MEK kinase activity stimulate the ERK pathway by activating endogenous C-Raf [16]. Follow-up studies showed that in normal cells, Raf heterodimerization is positively regulated by extracellular signals that activate the ERK pathway [15,17–19], and that the activity of Raf heterodimers towards MEK is higher than that of monomers or homodimers [18]. Dimers

in which only one subunit is kinase-competent are fully active. The kinase-dead subunit acts as an activator; however, it is currently unclear whether B- and C-Raf are equally proficient in this respect [18,19].

Consistent with these findings, work by the Therrien laboratory recently showed that the catalytically active Raf kinase consists of a side-to-side dimer in which helix αC is rotated in a productive conformation [20]. Interestingly, this conformational change is achieved both by Raf homodimerization and by heterodimerization with kinase suppressor of Ras (KSR), a scaffold protein that connects Raf and MEK, increasing the fidelity and strength of Raf–MEK–ERK signaling [4]. KSR contains a pseudokinase domain, similar to the kinase domain of C-Raf, which can allosterically activate Raf in the context of a heterodimer [20]. Collectively, these studies indicate that the ability of Raf to homodimerize or to heterodimerize (with other Raf proteins or with KSR) is crucial for Raf catalytic activity (Figure 2).

In wild-type cells, Raf dimerization is reversible and subject to negative regulatory feedback by ERK via the phosphorylation of B-Raf on multiple sites [18,21]. ERK is also capable of phosphorylating C-Raf on multiple residues, thereby preventing it from binding to Ras and entering a new activation cycle [22]. Whether this phosphorylation also contributes to the disruption of Raf dimers, although likely, is presently unknown.

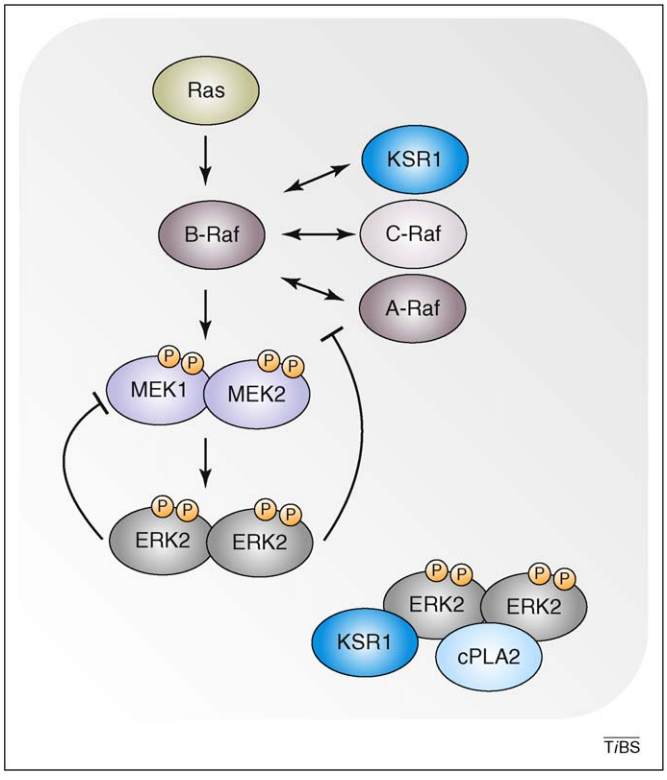

Figure 2. Dimer formation in the Raf pathway. Ras promotes the heterodimerization of B-Raf with the scaffold KSR1, C-Raf or A-Raf. These heterodimers have high MEK kinase activity and probably represent the MEK activating unit. ERK-mediated phosphorylation of B-Raf and probably C-Raf causes the dissociation of Raf heterodimers and prevents the re-association of Raf with Ras, limiting signal duration. In the next tier, phosphorylation of MEK1 by ERK represses the activation of stable MEK1–MEK2 heterodimers. Finally, ERK homodimers, which bridge scaffolds (KSR1) and substrates (phospholipase A2; cPLA2), are required to maintain signal specificity at different subcellular locations. Blunt arrows denote inhibitory effects, normal arrows indicate activation; double arrows indicate dimer formation.

Box 1. Partners in crime: inhibitor-induced Raf dimerization and cancer

PLX4032 [25–27] and other Raf inhibitors such as sorafenib [25,48] and GDC-0879 [27] efficiently inhibit MEK–ERK activation in cells harboring the B-Raf V600E variant, but they can activate the ERK pathway to different extents both in normal cells and in cells expressing *RAS* mutations. All groups involved in the discovery of this paradoxical effect concur that its molecular basis is the ability of the inhibitors to promote Raf dimerization. Their conclusions, however, differ in several respects. It is not clear, for instance, whether pan-Raf inhibitors [26,27], or only more selective B-Raf inhibitors will activate the ERK pathway [25]. In addition, three modes of action have been proposed (Figure 3): one that requires the presence of B-Raf and two that do not. In the first model, persistent B-Raf activation by oncogenic Ras confines B-Raf to an inhibitory cytosolic complex maintained by the kinase activity of B-Raf itself. By lowering this, Raf inhibitors mediate the release of B-Raf from the complex, allowing its relocalization to the membrane and the formation of a Ras–B-Raf–C-Raf complex, the ERK activating unit [25]. In the two other models, B-Raf is not required; by binding to the C-Raf gatekeeper residue, the inhibitors are envisioned to induce conformational changes that promote C-Raf homodimerization (Figure 3) [26,27]. These C-Raf homodimers are then responsible for MEK–ERK activation and increased proliferation. But how can the C-Raf homodimers phosphorylate MEK in the presence of the inhibitor? Two different explanations have been put forward: (i) the inhibitors capable of inducing MEK activation have fast off-rates and their transient binding to C-Raf is sufficient induce a conformational change that can promote dimerization but not a long-lasting inhibition of the kinase activity or (ii), the inhibitors might promote MEK activation only in low concentrations, at which one dimer subunit is bound to the inhibitor and transactivates the other, which binds ATP [25–27]. Regardless of the mechanism, the consequences of inhibitor-induced MEK–ERK activation and proliferation could be dire: rapidly developing drug-related skin tumors have been observed in melanoma patients treated with Raf inhibitors [23,24,28].

Les liaisons dangereuses: Raf inhibitors, dimerization and cancer

An unexpected new twist to the dimer story emerged from recent studies with chemical Raf inhibitors. *RAS* and *BRAF* are mutated at high frequency in a subset of human malignancies, particularly melanoma [9]. They are therefore considered prime targets for molecular anticancer therapy, and the search for inhibitors has been relentless for many years; indeed, several Raf inhibitors are currently being tested in preclinical and clinical trials. Recently, PLX4032, an inhibitor developed against active Raf, particularly against the B-RafV600E mutant found in melanoma, achieved an unprecedented 70% response rate in phase I trials [23,24]. Despite this success, three recent studies [25–27] have reported paradoxical activation of the MEK–ERK pathway and increased proliferation in wild-type cells and in tumor cells harboring *RAS* mutations (Box 1, Figure 3).

Before these reports, it seemed reasonable to predict that the clinical use of Raf inhibitors might have potentially deleterious effects resulting from ERK inhibition in normal cells. These studies reverse that scenario, and in fact predict that one danger of Raf inhibitors could be their ability to activate ERK in cells that do not harbor activating *BRAF* mutations but are otherwise prone to deregulated proliferation. Strikingly, keratoacanthomas and squamous cell carcinoma *in situ* have developed in patients treated with sorafenib and PLX4032 (10% and 23% of the

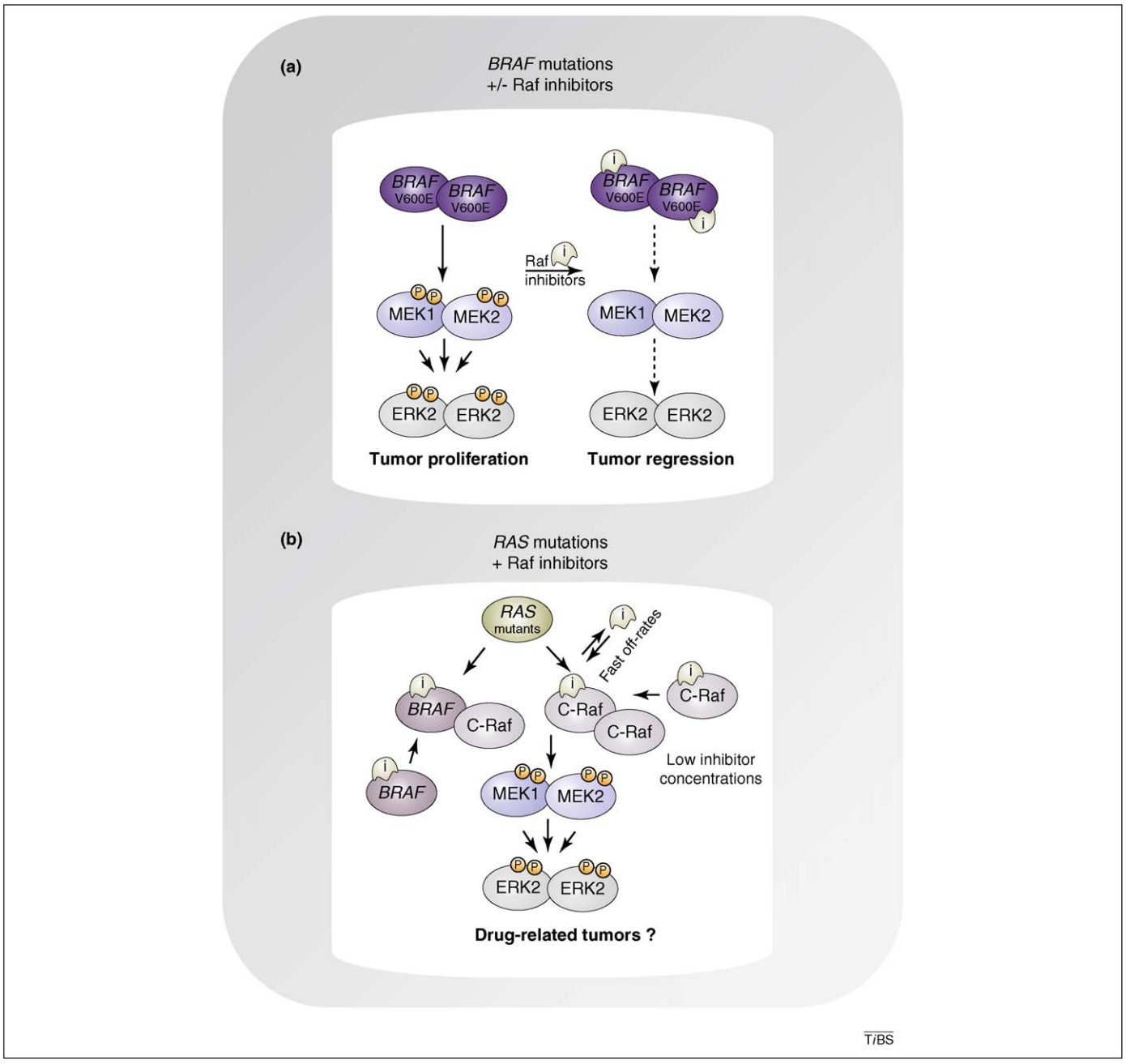

Figure 3. ERK activation by oncogenic B-Raf mutants and by Raf inhibitors. **(a)** The V600E B-Raf mutant found at high frequency in human melanoma has very high kinase activity and does not depend on Ras for activation. In cells harboring this mutant, B-Raf inhibitors blunt the V600E kinase activity, leading to a reduction in ERK activation and cell proliferation and subsequent tumor regression. **(b)** By contrast, in cells harboring *RAS* mutations, B-Raf hyperactivation confines this protein to an inactive complex in the cytosol; inhibitor treatment reduces B-Raf activity and enables it to relocalize to the membrane, where it forms a complex with C-Raf and MEK. Alternatively, in Ras-induced tumors, Raf inhibitors can bind to C-Raf, promoting its dimerization and the activation of MEK–ERK. Only one subunit of the C-Raf dimer is occupied by the inhibitor, whereas the other is free to phosphorylate MEK. This effect can be achieved either by transient binding (inhibitors with fast off-rates), or by using low concentrations of the inhibitor. Regardless of the specific mode of action, inhibition of Raf paradoxically leads to ERK activation, cell proliferation and possibly the development of drug-related tumors.

patients, respectively [23,24,28]), suggesting that the latter prediction might be correct. These skin tumors can be easily spotted and surgically removed by the clinicians closely monitoring patients with melanoma. Therefore, the benefits of inhibitor therapy remains enormous. However, long-term treatment with the drug might result in much more severe side effects if it instigates the same (pre)cancerous growth in organs less accessible to screening and surgery, such as the airways in smokers or the colon, sounding a note of caution regarding the clinical use of these inhibitors.

MEK dimers and the importance of negative feedback

MEK1 and MEK2 form homo- and heterodimers *in vitro* [29] and stable heterodimers *in vivo* [30]. The heterodimers are the target of ERK-mediated negative feedback phosphorylation of MEK1 Thr292, a residue missing in MEK2. *Mek1* ablation and mutations abolishing dimer formation or Thr292 phosphorylation all cause an increase in the intensity and duration of MEK2 and ERK phosphorylation. How the regulatory phosphorylation of the MEK1 subunit affects the phosphorylation of the activation loop of the MEK2 is unknown, but these data ascribe a clear regulat-

ory function to MEK heterodimerization in ERK signaling (Figure 2).

Kindred by choice: ERK dimers, scaffolds and the subcellular localization of signaling complexes

ERK1 and ERK2 can also form dimers, primarily homodimers relying on hydrophobic interactions [31]. Dimerization is not required for ERK nuclear translocation [32–34], as originally proposed [35], but is crucial for signaling. Specifically, dimerization is required to maintain the fidelity of the ERK signal in different cell compartments. To reach these subcellular locations, ERK must interact with specific scaffold proteins, including KSR1 (plasma membrane and cytosol), Sef (Golgi and cytosol), IQ motif-containing GTPase activating protein (IQGAP)1 and paxillin (cytoskeleton and focal adhesions), MEK partner (MP)1 (late endosomes) and MAPK organizer (MORG) (localization unknown) [36]. These scaffolds bind the same hydrophobic site on ERK that is necessary for substrate binding and phosphorylation [34]. Thus, simultaneous binding of ERK to scaffolds and substrates can only be achieved by using ERK dimers as binding platforms in which one ERK molecule interacts with the scaffold and the other with the substrate (Figure 2). This mechanism applies to the interaction of both ERK1 and ERK2 with a variety of scaffold proteins, and its abrogation inhibits cell proliferation and transformation *in vitro* and *in vivo* [34].

The acid test: is partnership essential?

The previous section summarized the biochemical evidence aspects of dimer formation in the Raf–MEK–ERK pathway, but what are the consequences of dimerization for the biology of the pathway? This section focuses on the role of dimer formation in modulating the output of the pathway and in mediating crosstalk with other pathways.

Raf heterodimerization: ERK activation and beyond

Is the formation of B-Raf–C-Raf heterodimers required for ERK activation in normal cells? Loss of function experiments in several cell types and organs are not consistent with this hypothesis. In general, ERK phosphorylation is resistant to the loss of one of the Raf kinases, with some exceptions: B-Raf is the crucial ERK activator in the placenta and in fibroblasts [37,38], in the β-cells of the Langerhans islets of the pancreas [39], and in oligodendrocytes [40]. In *Braf*-deficient oligodendrocytes, C-Raf is present in a complex with MEK and yet it cannot compensate for *Braf* ablation. Interestingly, the MEK complexes isolated from *Braf*-deficient oligodendrocytes are also devoid of KSR. Thus, MEK activation appears to depend on a minimal activator complex composed of B-Raf and KSR [41]. C-Raf might be part of this complex, but is neither sufficient nor required for MEK activation [40], perhaps because A-Raf can substitute for C-Raf in this context. In support of this idea, *Araf*; *Craf* compound knockout cells show a reduction in the early phases of ERK activation [42]. By contrast, most *Craf*-deficient cells and organs show an increase in ERK phosphorylation, indicating that C-Raf is dispensable for ERK activation and in fact mitigates it [10,40,43–47]. In the context of tumorigenesis, recent results show that Ras-induced complex formation between the oncogenic B-RafV600E mutant and C-Raf, but not wild-type B-Raf or A-Raf, reduces MEK–ERK activation and decreases proliferation in melanoma cells [48]. Thus, association with C-Raf appears to enhance the activity of dimers containing B-Raf with low intrinsic catalytic potential, and to reduce the activity of dimers containing active B-Raf.

In the past few years, it has become clear that C-Raf does more than simply activate MEK. *Craf* ablation causes defects in survival [43,44,49–53], maintenance of cell shape and motility [45], and differentiation of keratinocytes [46] and erythroblasts [54]. The defect in erythroblast differentiation correlates with lack of sustained ERK pathway activation, but whether *Craf* ablation impinges on the pathway directly or indirectly has not been ascertained [54]. All the other defects can be traced back to the ability of C-Raf to bind to and inhibit three fellow serine/threonine kinases: the proapoptotic mammalian sterile 20-like kinase (MST)2 [49,51,55]) and apoptosis signal-regulating kinase (ASK)1 [50]) kinases, and the cytoskeleton-based Rho effector Rok-α (or ROCK2) (Figure 4) [45,46,52,56]. Inhibition of Rok-α by gene silencing, expression of dominant-negative mutants, or treatment with chemical inhibitors reverts all the phenotypes of *Craf*-deficient cells in culture [45,52]. In addition, we recently showed that downstream of Ras, C-Raf operates as an endogenous Rok-α inhibitor to block keratinocyte differentiation, thereby allowing both the development and maintenance of Ras-driven epidermal tumors [46]. These data have established the *in vivo* relevance of the C-Raf–Rok-α interaction, and have prompted a more detailed investigation of the complex.

Both C-Raf and Rok-α are modular kinases featuring a catalytic domain kept inactive by the intramolecular interaction with a negative regulatory, autoinhibitory region

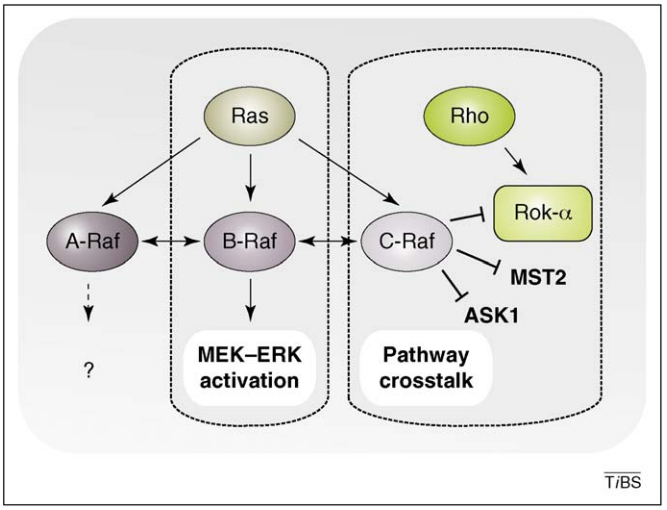

Figure 4. Essential functions of Raf downstream of Ras. Gene ablation experiments have revealed that B-Raf is essential for MEK–ERK activation downstream of Ras. Both A-Raf and C-Raf can participate in ERK activation by heterodimerizing with B-Raf (horizontal double-headed arrows). The function of A-Raf is unclear, but a role for C-Raf in mediating crosstalk between the Ras pathway and other signaling cascades has emerged. C-Raf implements pathway crosstalk by binding to and inhibiting three serine/threonine kinases: the two proapoptotic kinases ASK1 and MST2 and the cytoskeleton-based Rho effector Rok-α (blunt-headed arrows). Rok-α inhibition is required for maintenance of cell shape and motility and it is essential for preventing differentiation in Ras-driven epidermal tumors.

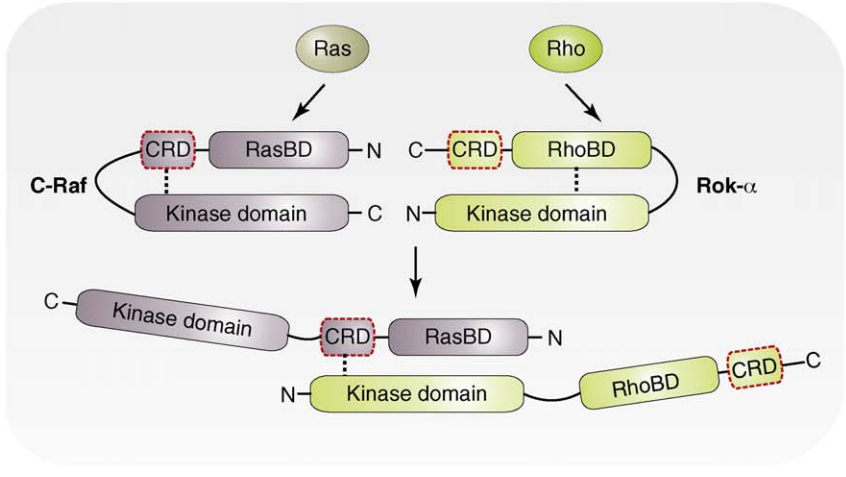

Figure 5. Kinase-independent inhibition of Rok-α by C-Raf. Inhibition of Rok-α by C-Raf is mediated by inhibition in *trans*, a kinase-independent mechanism relying exclusively on protein–protein interactions. Both kinases have a modular structure consisting of a kinase domain whose activity is restrained by the interaction with an autoinhibitory region. The autoinhibitory regions of C-Raf and Rok-α share a cysteine-rich domain (CRD; indicated by a broken red line). Upon binding to small GTPases [via the Ras binding domain (RasBD) of C-Raf and the Rho binding domain (RhoBD) of Rok-α], autoinhibition is relieved and both kinases assume an 'open' conformation, which enables substrate binding. In this situation, the autoihibitory domain of C-Raf binds to the kinase domain of Rok-α, attenuating its activity.

containing a cysteine-rich domain, which is very similar in C-Raf and Rok-α. Intramolecular inhibition is relieved upon kinase activation by the binding of small GTPases (Ras for C-Raf and Rho for Rok-α) to the negative regulatory domain of the proteins. In growth factor-stimulated cells or in tissues containing active Ras, in which C-Raf and Rok-α are activated concomitantly, the negative regulatory domain of C-Raf binds to the kinase domain of Rok-α and attenuates Rok-α downstream signaling [56]. This mechanism of inhibition in *trans* requires only the C-Raf regulatory domain, and is the first example of kinase regulation mediated by physical interaction rather than by the phosphorylation of negative regulatory residues (Figure 5).

Regulation of signaling in time and space: essential role(s) of MEK and ERK dimers

Temporal regulation of MEK2 activation is an essential function of MEK1 within a MEK1–MEK2 heterodimer. This function of MEK1 leads to the paradoxical activation of the ERK pathway in *Mek1*-deficient cells, embryos and tissues. At present, it is unknown whether MEK1 must be kinase-competent to restrict MEK2 activation (Figure 2). Depending on the context, however, MEK1 can also be essential for ERK activation. This is the case in adhesion signaling, which crucially depends on the selective phosphorylation of Ser298 of MEK1 by PAK [57], and in the developing mouse placenta [30,58,59]. It is therefore conceivable that a block in adhesion-dependent ERK signaling during early placentation might be the cause of the intrauterine death of *Mek1*-deficient embryos. Together with the observation that *Mek2* knockout animals are viable and show no major anomalies [60], these data define MEK1 as the crucial modulator of ERK signaling.

ERK1 and ERK2 have high homology, are equally efficiently phosphorylated by MEK1 and MEK2, target the same PxS/TP substrate sites [61] and share an interactome composed of 284 proteins [62]. Mice lacking *Erk1* are viable and fertile, with minor defects in T-cell development [63], decreased adiposity [64] and facilitated learning and memory [65]. *Erk2*–deficient embryos, by contrast, die *in utero* (embryonic day 6.5–11.5) due to trophoblast failure [66–68]; however, once these extraembryonic defects are circumvented by tetraploid aggregation, *Erk2*-deficient animals develop normally [66]. In addition, removal of *Erk2* leads to hyperactivation of ERK1, which can then substitute for ERK2 [69], and individual silencing of either ERK1 or ERK2 affects cell proliferation in a manner proportional to their expression level [70]. Together, these reports suggest that the function of ERK1 and ERK2 are redundant except in a restricted number of very specific contexts, and that the overall level of ERK signaling, rather than whether the signal comes from ERK1 or ERK2, is the key factor. By contrast, very recent work by the Blenis laboratory showed that ERK2, but not ERK1, is involved in Ras-induced epithelial to mesenchymal cell transition (EMT), a process that contributes to cancer cell spreading [71]. The molecular basis of this difference is the selective ERK2-mediated upregulation of the transcription factor Fos-related antigen (FRA)-1, which can activate a subset of EMT genes. The mechanism underlying ERK selectivity has not been elucidated in detail, but the data indicate that functions specific to ERK1 and ERK2, however subtle, might exist and play crucial roles in biomedically relevant processes.

Evolutionary considerations

One noticeable feature of the mammalian ERK pathway is redundancy. Mammals have three functional *Raf*, two *Mek and* two *Erk* genes (Figure 1). Each of the three Rafs can activate both MEKs and both MEKs can activate ERKs, albeit with different efficiency. Why then are there so many components in each of the three tiers? Analysis of the recent biochemical and biological data indicates that redundancy serves a purpose both within and outside of the Raf–MEK–ERK pathway At the level of Raf, *BRAF* bears the closest similarity to genes of lower organisms,

and its protein product has the highest MEK kinase activity. The fact that both C-Raf and A-Raf are more resistant to activation suggests that the expression of additional Rafs could only be tolerated if the overall MEK kinase activity did not change proportionally. A-Raf and C-Raf heterodimerize with B-Raf, leading to the formation of MEK-activating heterodimers. However, this is unlikely to be relevant, because B-Raf can be activated by heterodimerization with the scaffold KSR1 [20], which is present in lower organisms and is subject to a regulation as complicated as that of C-Raf [72]. The B-Raf–KSR1 dimer is an extremely efficient MEK activator, probably because KSR1 can concomitantly induce the activating conformational change in the B-Raf kinase domain and bind MEK, thereby bringing the activated Raf kinase and its substrate into close proximity [20]. Indeed, the existence of complexes containing B-Raf, KSR1 and MEK has been demonstrated *in vivo* [40,41]. Another possibility would be that heterodimerization between B-Raf and C-Raf might function in the temporal regulation of the signal, assuming, for instance, that in a heterodimer, C-Raf is the subunit targeted most efficiently by ERK-mediated negative feedback phosphorylation [21,22]. Although this is possible, what most clearly emerged from the *in vivo* study is that the 'younger' C-Raf is devoted to implementing the crosstalk of the Ras pathway with other signaling cascades such as the one regulated by Rho, thereby adding considerable flexibility to the system (Figure 4).

Downstream of Raf, MEKs are single-minded kinases whose only substrates are ERKs. The main differences between MEK1 and MEK2 are found in a proline-rich region containing phosphorylation sites unique to MEK1. This proline-rich region emerges first in the amniota and is conserved in this clade, thus correlating its appearance with the essential role of MEK1 in the development of extraembryonic tissue is an attractive idea. In our current working model, MEK1 is required in the context of a MEK heterodimer to control signal strength (Figure 2). This complex regulation emphasizes the importance of keeping the activation of the ERK pathway under tight control.

Heterodimerization and the possibility of regulating one isoform through the other is not an option in the case of ERK. In fact, it remains unclear whether ERK1 and ERK2 have a specific function in cells that co-express the two kinases, as suggested by a recent study [71], or whether the expression of both proteins simply serves the purpose of reaching a certain level of signal strength, as maintained by others [61].

Concluding remarks

Recent findings emphasize the importance of protein–protein interaction in Raf pathways: dimerization of Raf enzymes, or interaction of Raf with the structurally similar scaffold KSR1, bring about activation; only one partner needs to be catalytically competent, which is the basis of the paradoxical activation of the ERK pathway by low activity oncogenic B-Raf mutants as well as by Raf inhibitors. For MEK, heterodimerization allows the control of signal strength and duration by ERK-mediated negative feedback phosphorylation, and for ERK, homodimerization is necessary to form a platform essential for the simultaneous binding of substrates and adaptor molecules steering the signal to the appropriate subcellular localization. Finally, direct interaction with serine/threonine kinases operating in parallel signaling cascades is the basis of the ability of C-Raf to mediate pathway crosstalk downstream of Ras. In the case of Raf and ERK dimers and of the C-Raf–Rok-α complexes, it is clear that these protein–protein interactions play a role in tumorigenesis. If they can be targeted by drugs, these interactions would represent a unique window of opportunity for therapeutics. Many questions, which are not confined to the ERK pathway but concern the wiring of signaling networks in general, remain open (Box 2); as we learn more about the regulation of protein–protein interactions, the prospect of small molecule inhibitors that will disrupt them moves closer.

Box 2. Unanswered questions

The evidence reviewed in this article attests to the importance of protein–protein interactions in the Raf–MEK–ERK pathway. Several questions, however, remain open, which apply not only to this pathway but to signaling networks in general.

- If a signal transducer has more than one function, as in the case of C-Raf, how do extracellular signals couple to the specific intracellular biochemical functions necessary to achieve the intended biological outputs?
- How do dynamic changes in the assembly of signaling complexes specify biochemical and biological outcomes?
- Which of the interactions are essential in development and disease?
- Can we exploit these interactions for the purpose of molecule-targeted therapy?

Acknowledgements

We thank all the members of the Baccarini laboratory for helpful discussions and apologize to all the colleagues whose work, for reasons of space, could not be cited in this review. The Baccarini laboratory is supported by the Austrian Scientific Research Fund (grants P19530, SFB 021 and W1220) and by the European Community (grants INFLA-CARE and GROWTHSTOP).

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