REVIEW ARTICLE

Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions

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The Ras/Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) pathway is at the heart of signalling networks that govern proliferation, differentiation and cell survival. Although the basic regulatory steps have been elucidated, many features of this pathway are only beginning to emerge. This review focuses on the role of protein–protein interactions in the regulation of this pathway, and how they contribute to co-ordinate activation steps, subcellular redistribution, substrate phosphorylation and cross-talk with other signalling pathways.

Key words: MAPK pathways, multiprotein complexes, protein phosphorylation, scaffolding proteins, signalling complexes, signal transduction.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway is one of the primordial signalling systems that Nature has used in several permutations to accomplish an amazing variety of tasks. It exists in all eukaryotes, and controls such fundamental cellular processes as proliferation, differentiation, survival and apoptosis. The basic arrangement includes a G-protein working upstream of a core module consisting of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK (Figure 1). This set-up provides not only for signal amplification, but, maybe even more importantly, for additional regulatory interfaces that allow the kinetics, duration and amplitude of the activity to be precisely tuned. At present we can distinguish six MAPK modules, which share structurally related components, but seem to mediate specific biological responses. For recent reviews on MAPK pathways, the reader is referred to references [1–3]. Here we will focus on the regulation of the ERK (extracellular-signal-regulated kinase) pathway, which features Ras as G-protein, Raf as MAPKKK, MEK (MAPK/ERK kinase) as MAPKK and ERK as MAPK.

Despite enjoying a decade in the limelight of scientific interest and revealing a plethora of new insights into the circuitry of signalling pathways in general, this pathway still holds many secrets. These pertain mainly to the regulation of Raf, and to a deeper understanding of how specific biological responses are encoded by spatial and temporal changes in the activity and subcellular distribution of the pathway components, and how these fluctuations are orchestrated at the molecular level. Work from many laboratories has highlighted protein–protein interactions as powerful means of co-ordinating signalling processes, most strikingly exemplified by the assembly of multi-protein signalling complexes on activated receptors, or of transcription-factor complexes on gene promoters. However, it is becoming apparent that this regulatory motif is also widely used for the control of intracellular signalling networks. Here we will review the accumulating evidence for complex protein interactions in the Ras/Raf/MEK/ERK pathway, and discuss their significance for the regulation and function of this pathway.

STRUCTURE AND BASIC REGULATION OF THE Ras/Raf/MEK/ERK PATHWAY

This topic is summarized in Figures 2 and 3. Two components of this pathway, Ras and Raf, are proto-oncogenes. Thus it is not too surprising that major functions of this pathway pertain to growth control in all its facets, including cell proliferation, transformation, differentiation and apoptosis. A wide variety of hormones, growth factors and differentiation factors, as well as tumour-promoting substances, employ this pathway. Most of these stimuli activate Ras proteins by inducing the exchange of GDP with GTP, which converts Ras into its active conformation. This process relies on the recruitment of GDP/GTP exchange factors to the cell membrane where Ras resides. The archetypal Ras exchange factor, SOS (son of sevenless), is towed to the membrane by the growth-factor-receptor-bound protein 2 adapter protein, which recognizes tyrosine phosphate docking sites located on the receptors themselves or on receptor substrate proteins [4]. Feedback phosphorylation of SOS by the activated ERK pathway induces the disassembly of the SOS complex and termination of Ras activation (Figure 3). This motif of activation by subcellular redistribution is reiterated at the level of Ras. Activated Ras functions as an adapter that binds to Raf kinases with high affinity and causes their translocation to the cell membrane, where Raf activation takes place [5] (Figure 2).

raf genes encode serine/threonine-specific kinases that integrate the upstream input signals, and hence feature a complex regulation, as will be discussed below. Despite the conservation of Ras and other MAPKKKs, MAPKKs and MAPKs, there is

Abbreviations used: Bcr, Breakpoint cluster region; BXB, isolated Raf-1 kinase domain; CNK, connector–enhancer of KSR; CK2, casein kinase 2; CRD, cysteine-rich domain; DEF, docking site for ERK, FxFP; DLK, dual leucine-zipper-bearing kinase; ERK, extracellular-signal-regulated kinase; Hsp, heat-shock protein; KIM, kinase interaction motif; IκB, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; KSR, kinase suppressor of Ras; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MP1, MEK partner 1; MUK, MAPK upstream kinase; NF-κB, nuclear factor-κB; PAK, p21cdc42/rac1-activated serine/threonine kinase; PKA, cAMP-activated protein kinase; Rb, retinoblastoma protein; RBD, Ras-binding domain; RKIP, Raf kinase inhibitor protein; SEK, stress-activated protein kinase/ERK kinase; SOS, son of sevenless; SUR, suppressor of Ras; TNF, tumour necrosis factor; ZPK, leucine-zipper protein kinase.

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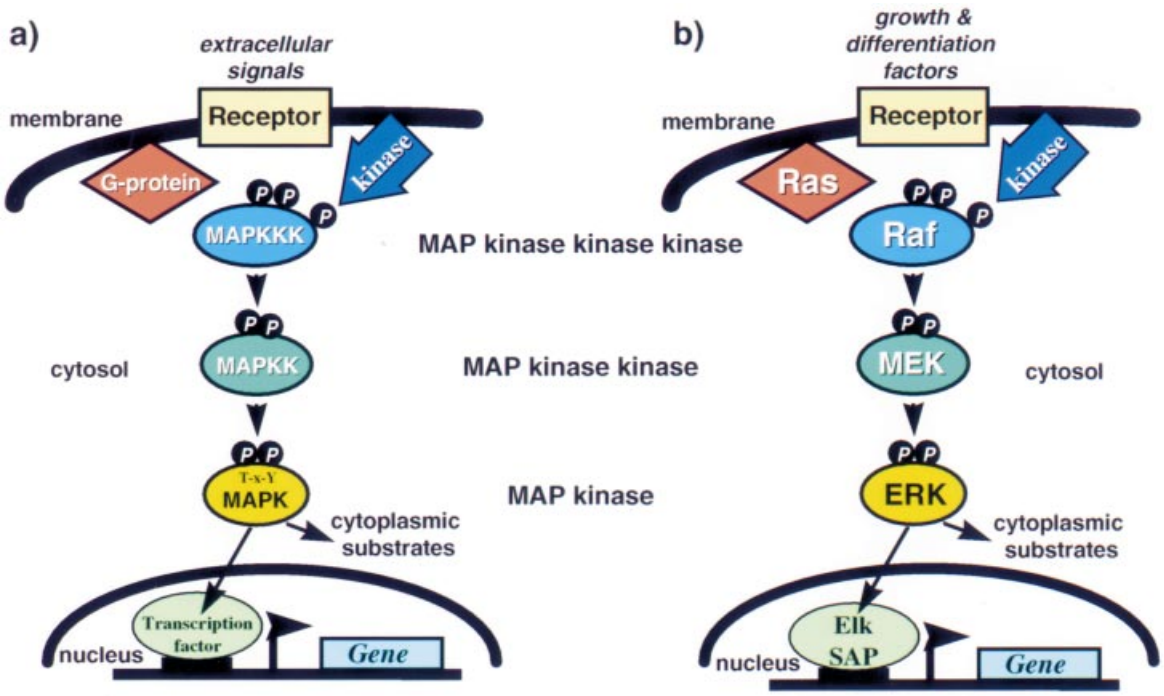

Figure 1 Schematic representation of the structure of MAPK pathways

(**a**) General set-up of MAPK pathways; (**b**) the ERK pathway in particular.

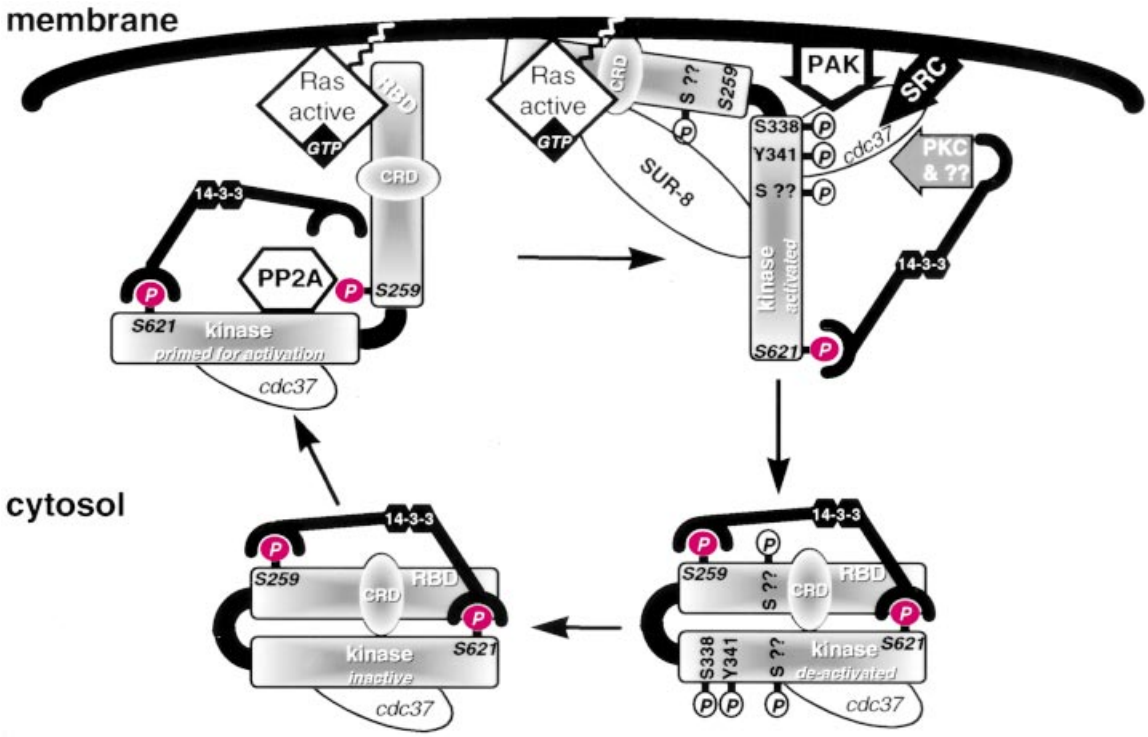

Figure 2 Model of Raf-1 activation

See the text for details. Abbreviations: PKC, protein kinase C; PP2A, protein phosphatase 2A.

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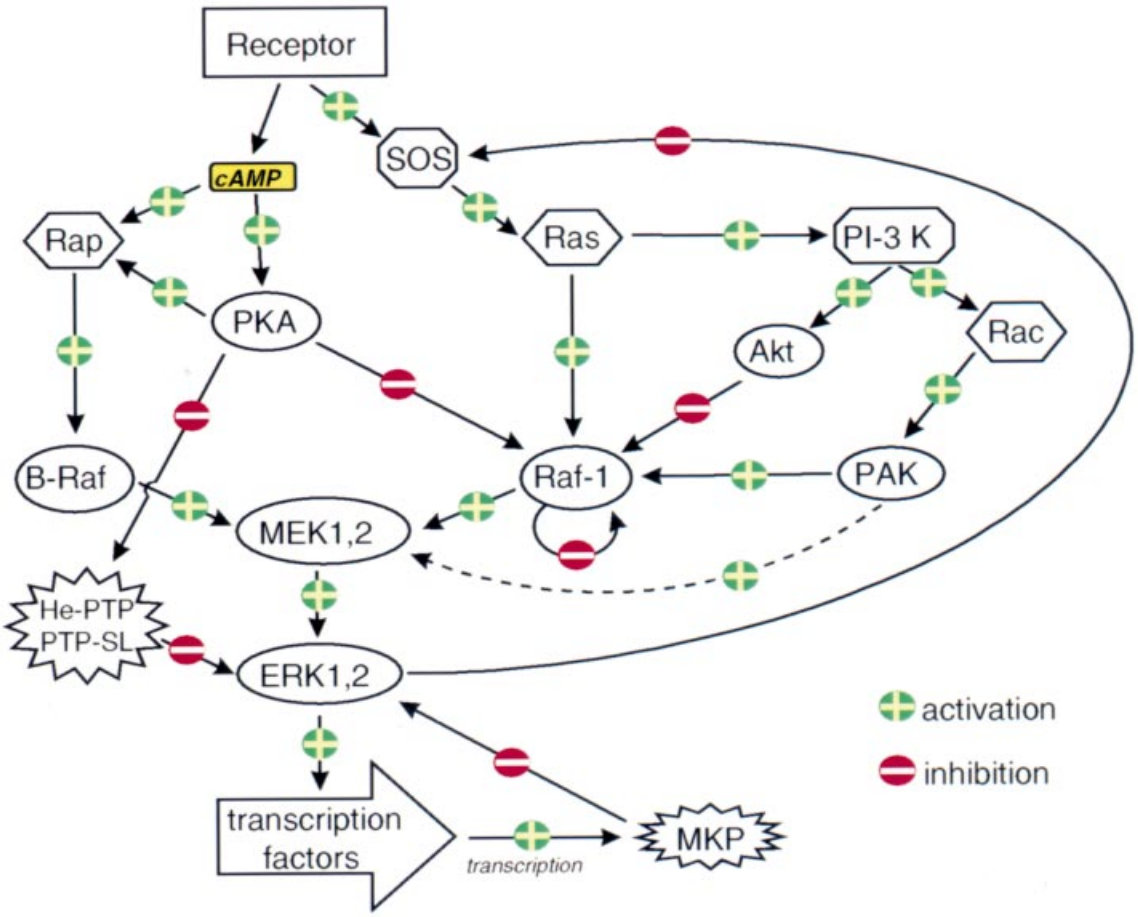

Figure 3 Regulation of the Ras/Raf/MEK/ERK signalling network

Solid lines represent direct effects on activity. The broken line indicates that PAK phosphorylation enhances the binding of MEK to Raf-1 and hence facilitates MEK activation indirectly. Abbreviations: PI-3 K, phosphoinositide 3-kinase; PTP, protein tyrosine phosphatase; MKP, MAPK phosphatase.

no Raf homologue in the yeast *Saccharomyces cerevisiae*. However, the yeast Ras homologues are not part of MAPK cascades, but function in nutrient-sensing pathways regulating adenylate cyclase. In contrast, the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* each contain at least one functional *raf* gene, the product of which functions downstream of Ras in an ERK pathway similar to that in mammalian cells. *Drosophila* Raf is essential for cell proliferation and for determination of cell fates during development, such as photoreceptors in the eye as well as head and posterior body structures [6]. The disruption of the Raf locus in the worm severely reduces the viability of the larvae, and the few survivors have defects in vulva differentiation [7]. Mammals possess three Raf proteins: Raf-1, A-Raf and B-Raf. The ubiquitously expressed Raf-1 is certainly the best studied, but probably least understood, isoform. A-Raf and B-Raf exhibit more restricted expression profiles. The very different phenotypes of Raf-1, A-Raf and B-Raf knock-out mice make a convincing case for these proteins being non-redundant and serving distinct functions [8]. Depending on the genetic background, the elimination of A-Raf produces intestinal and/or neurological defects, but the pups are born alive. In contrast, B-Raf knock-out mice have defects in neuroepithelial differentiation and in the maturation and maintenance of endothelial cells, and die *in utero* due to vascular haemorrhage. Knocking out the *raf-1* gene in an inbred background results in death during midgestation. In an outbred strain, Raf-1 knock-out mice die shortly after birth, showing general growth retardation and developmental defects that are most apparent in placenta, lung and skin. It should be noted that the Raf-1 knock-out mice still express a truncated Raf-1 protein, which is devoid of catalytic activity, but may nevertheless contribute to the observed abnormalities by acting as a dominant-negative mutant. Overall, the phenotypes of the knock-out mice are reasonably consistent with the expression data, and indicate that Raf-1 serves a general role in tissue formation, whereas A-Raf and B-Raf fulfil more specialized duties.

At present, the basis for this diversification is enigmatic, because all three Raf isoforms share Ras as a common upstream activator and MEK as the only commonly accepted downstream substrate [2,9]. MEK is activated by phosphorylation of two serine residues in the activation loop. Although other kinases such as MEKK-1 (MEK kinase-1), mos or Tpl-2 can phosphorylate the same serines, the predominant MEK activators in most cell types are Raf kinases [2]. Thus it came as a complete surprise that the chemical Raf inhibitors ZM336372 and SB203580 failed to block the activation of MEK and ERK by growth factors and phorbol esters. While these inhibitors abolish Raf activity *in vitro*, they paradoxically induce vigorous

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activation of Raf-1 when administered to cells [10,11]. A likely explanation for this phenomenon is that Raf-1 activity is normally bridled by a negative feedback initiated by Raf-1 itself. By cutting off this feedback, the Raf inhibitors allow activating modifications to accumulate, resulting in a massive activation of Raf-1 when measured *in vitro* in the absence of the drug. Thus Raf seems to be suspended in a balance between activation and auto-inhibition [10,11]. Unfortunately, the nature of the negative feedback is not known yet, but it has important ramifications. First, it casts doubt on the usefulness of Raf kinase inhibitors as anti-cancer drugs. Secondly, it shows that activation of Raf-1 can be efficiently achieved by removing an inhibitory constraint. It is currently unknown whether this principle is used physiologically. Thirdly, it also suggests that the coupling of Raf to MEK is a regulated process. Despite hyperactivating Raf-1, the drugs did not stimulate MEK or ERK, showing that efficient coupling to MEK needs more than Raf's catalytic activity. As will be discussed below, the Raf/MEK interface is indeed used for regulation.

Raf can activate both MEK-1 and MEK-2 (also called MKK-1 and MKK-2) with similar efficacy *in vitro*. However, some results from genetic model systems and transfection experiments suggest a preferential coupling between certain Raf and MEK isoforms [2]. The significance of this is still enigmatic, because both MEK isoforms can activate the downstream ERK kinases. MEK belongs to the rare breed of dual-specificity kinases which can phosphorylate both threonine and tyrosine residues [3]. They activate ERK-1 and ERK-2 (also called p44 and p42 MAPK) via phosphorylation of a -Thr-Glu-Tyr- motif in the activation loop. Again, most biochemical and transfection experiments suggest that ERK-1 and ERK-2 are functionally equivalent, and it is unclear why two ERK genes exist. However, the fact that both MEK and ERK isoforms are usually co-expressed, and the evolutionary conservation of two genes each for MEK-1/2 and ERK-1/2 down to the small, streamlined genome of worms [13,14], seems to indicate functional diversification. ERK is a serine/threonine kinase with an impressive portfolio of more than 50 substrates [15], which clearly puts it at the business end of this pathway. This, however, does not exclude the existence of branch-points at the level of Raf or MEK, which will be discussed below.

A COMPLICATED RELATIONSHIP: HOW Ras PROTEINS REGULATE Raf KINASES

Inactive Raf-1 is localized in a multi-protein complex of 300–500 kDa [16]. A seminal discovery was that activated Ras can bind to Raf-1 with high affinity [5], providing a simple and elegant explanation for earlier observations that had implicated Raf-1 as an essential effector of Ras-induced cell transformation [18] (Figure 2). But, surprisingly, this interaction does not augment Raf-1's catalytic activity, unless Ras is properly localized at the cell membrane [19]. Ras can interact with two domains in the Raf-1 N-terminus: the Ras-binding domain (RBD; amino acids 55–131) and the cysteine-rich domain (CRD; amino acids 139–184) [20,21]. The RBD alone is sufficient for the translocation of Raf-1 from the cytosol to the cell membrane, while the CRD is dispensable. However, the CRD is necessary for efficient activation [22–24]. The exact role of the CRD and the localization of its binding epitopes in Ras is controversial, but a consistent finding has been that point mutations in the CRD can influence the affinity of binding to Ras and can affect both basal and Ras-induced kinase activity [25,26]. This points to an important, yet complex, contribution of the CRD to Raf-1 activation by Ras. This interpretation is consistent with the observation that the artificial tethering of Raf-1 (Raf–CAAX) to the cell membrane results in only partial activation, and that Raf–CAAX can be further stimulated by growth factors or Ras–GTP [27,28]. This appears to be mediated by direct binding of Ras to Raf–CAAX [28], as well as by other Ras-initiated signalling processes [29,30]. Thus Ras supplies direct and indirect Raf activation signals.

The physical interaction may induce a conformational transition state in Raf-1 that is sensitized to activation. As Ras can spontaneously form dimers in a lipid bilayer [31], and since dimerization can activate Raf-1 [32,33], such a state could comprise Ras inducing Raf-1 dimerization. An alternative, but not mutually exclusive, possibility is that Ras dimerization is needed for simultaneous binding to the RBD and CRD, which have both been shown to be contacted by the Ras effector domain [21]. A Ras dimer would elegantly resolve the steric dilemma of how the tiny Ras effector domain can engage two different domains in Raf at the same time. In any case, Ras binding seems to relieve the inhibition which the N-terminal regulatory domain of Raf-1 exerts over the catalytic domain at the C-terminus [34]. Point mutations in the CRD, which can alleviate this inhibition, were isolated in a screen for Raf-1 mutations that increased the affinity for Ras [25]. An additional layer of complexity has been added by the discovery of a modulator protein, SUR-8 (suppressor of Ras-8), in a genetic screen in *C. elegans*. SUR-8 can form a ternary complex with Raf-1 and Ras–GTP, enhancing Raf-1 activation [35]. As SUR-8 interacts with the Raf-1 catalytic domain, SUR-8 could be a physical link that conveys Ras signals directly to the Raf-1 kinase domain.

In addition, Ras also supplies indirect regulatory signals (Figures 2 and 3). One such a signal is provided by phosphoinositide 3-kinase, whose phospholipid products can activate Rac, a small G-protein that binds and activates p21cdc42/rac1-activated serine/threonine kinase (PAK) [29]. PAK-3 has recently been shown to phosphorylate Raf-1 on serine-338, one of the sites whose phosphorylation is required for activation [36]. The other site is tyrosine-341, which is targeted by Src family kinases [37,38]. In addition, abl [39,40] and JAK (Janus kinase) [41,42] family tyrosine kinases also induce Raf-1 activation and tyrosine phosphorylation, but since the phosphorylation sites have not been mapped, it is not known whether they work through tyrosine-341. These tyrosine kinases can be co-immunoprecipitated with Raf-1, and, being found at the cell membrane, they may form part of the activation complex (Table 1 and Figure 4). Phosphorylated serine-338 and tyrosine-341 synergize to activate Raf-1 [38], but the complex pattern of phosphopeptides induced upon Raf-1 activation suggests that other, as yet unknown, sites contribute to full activation. In addition, phosphoinositide 3-kinase may also supply an inhibitory signal via Akt, which has been reported to suppress Raf-1 activity by phosphorylation of serine-259 [43]. A-Raf activation has only been scantly explored, but resembles Raf-1 activation in its dual need for both a Ras signal(s) and phosphorylation on the tyrosine residue corresponding to tyrosine-341 in Raf-1 [44].

In contrast, B-Raf seems to be mainly regulated by binding to Ras family proteins. Remarkably, the binding of Ras–GTP alone suffices to activate B-Raf *in vitro* and *in vivo* [45,46]. Ras must have first undergone post-translational isoprenylation, and the activation can be modulated by lipid cofactors [47]. In part, this difference may be attributed to B-Raf being able to bypass the necessity for essential kinase signals. B-Raf possesses a 'phosphomimetic' aspartate at the position equivalent to tyrosine-341 in Raf-1 and features constitutive phosphorylation of the serine-338 counterpart [38]. However, an elegant study using domain-swapping experiments between Raf-1 and B-Raf has also shown

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Table 1 Raf-associated proteins described in the literature

All observations refer to Raf-1, unless stated otherwise. *In vitro* interactions were demonstrated in the yeast two-hybrid system, in the baculovirus expression system or by *in vitro* pull-down assays. *In vivo* interactions were demonstrated by co-immunoprecipitation of endogenous or transfected proteins in mammalian cells. Raf association with receptors usually requires receptor overexpression, and its physiological significance has been debated. Abbreviations: n.r., not reported; Gβ/γ, G-protein β/γ subunits; PP2A, protein phosphatase 2A; PKC, protein kinase C; MKK, MAPK kinase; SEK, SAPK (stress-activated protein kinase)/ERK kinase; JAK, Janus kinase; STAT, signal transduction and activators of transcription; IL-2-R, interleukin-2 receptor; EGF-R, epidermal growth factor receptor; PDGF-R, platelet-derived growth factor receptor.

Interacting protein	Interaction seen		Proposed function	Selected references
	In vitro	*In vivo*		
G-proteins				
Ha-, Ki- and N-Ras	Yes	Yes	Activation of all three Raf isoforms	[162,163]
Rap1/Krev	Yes	Yes	Activation of B-raf; overexpression inhibits Raf-1	[22,50]
TC21/R-Ras-2	Yes	Yes	Activates Raf-1 and B-raf; no binding to A-raf	[164]
TC21/R-Ras-2	Yes/no	No	Interaction with isolated RBDs, but not full-length Raf proteins	[165]
Gβ/γ	Yes	Yes	n.r.	[115]
Adapters				
SUR-8	Yes	Yes	Enhances Ras–Raf interaction and Raf activation	[35]
CNK	Yes	Yes	Enhances Raf signalling in *Drosophila*	[122,123]
KSR	Yes	Yes	Scaffold protein for Raf/MEK/ERK	[78,111,112]
Grb10	Yes	Yes	Interacts with Raf-1 at mitochondria;	[166]
			also interacts with MEK-1	[167]
14-3-3	Yes	Yes	Facilitates Raf activation	[64–67,168]
Cytoskeleton				
Vimentin	Yes	Yes	Indirect target of Raf-1	[134]
Tubulin	Yes	n.r.	Unknown	[169]
Chaperones				
Hsp90	Yes	Yes	Required for Raf signalling	[95,103–105]
Hsp50/Cdc327	Yes	Yes	Enhances Raf activation	[95,96]
Hsp65	Yes	n.r.	n.r.; no interaction with B-Raf	[97]
Bag-1	Yes	Yes	Raf activation	[98]
Phosphatases				
PP2A	Yes	Yes	Facilitates Raf activation	[87]
Cdc25	Yes	Yes	Proposed Raf substrate; association mediated by 14-3-3	[170]
Kinases				
MEK-1,2	Yes	Yes	*Bona fide* Raf substrates	[171,172]
CK2α	Yes	Yes	Raf-1-associated IκB kinase	[144]
CK2β	Yes	n.r.	Selective association with A-raf	[147,148]
Tpl-2/Cot	n.r.	Yes*	Activates MEK and SEK-1/MKK-4	[137,138]
Akt	n.r.	Yes	Modulator of Raf signalling	[43,143]
PKCϵ	Yes	Yes	Unknown	[173]
PKCζ	Yes	Yes	No effect on Raf/MEK/ERK	[174]
	Yes	Yes	Binds via 14-3-3; activates Raf -1	[175]
Bcr	Yes	Yes	Unknown; association via 14-3-3	[73]
ERK-5	n.r.	Yes	Synergism in cell transformation	[149]
Lck	n.r.	Yes	Raf activation	[176]
Fyn and Src	Yes	n.r.	Raf activation	[177]
Jak	Yes	Yes	Raf activation	[41,42,178]
Miscellaneous proteins				
A20	Yes	Yes	Unknown; association via 14-3-3	[161]
Bcl-2	Yes	Yes	Translocates Raf to the mitochondria	[107a]
Rb	Yes	Yes	Proposed Raf substrate	[136]
p130	Yes	n.r	n.r.	[136]
RKIP	Yes	Yes	Disrupts Raf–MEK interaction	[127,128]
STAT-1	n.r.	Yes	Required for Raf activation by interferon γ and oncostatin M	[179]
Tvl-1	Yes	Yes	Substrate; enhances Raf-1 activation	[180]
PA28α	Yes	Yes	Proteasome component; selective B-raf interaction	[181]
Receptors				
IL-2-R β chain	n.r.	Yes	Unknown; IL-2 binding releases Raf from receptor	[182]
Ltk	n.r.	Yes	n.r.	[183]
EGF-R	n.r.	Yes	Transient association; EGF dependent	[184]
PDGF-R	Yes	Yes	Association is PDGF dependent	[185]

* Cited as data not shown.

that protein association via the CRD is crucial for this differential response [48]. These experiments were designed to find out why B-Raf is activated by Rap1, a Ras family member that blocks Raf-1 activation by Ras when overexpressed. Rap1 binds tightly to the Raf-1 CRD, preventing it from interacting with Ras. Thus when both Ras and Rap1 interact with the RBD and the CRD

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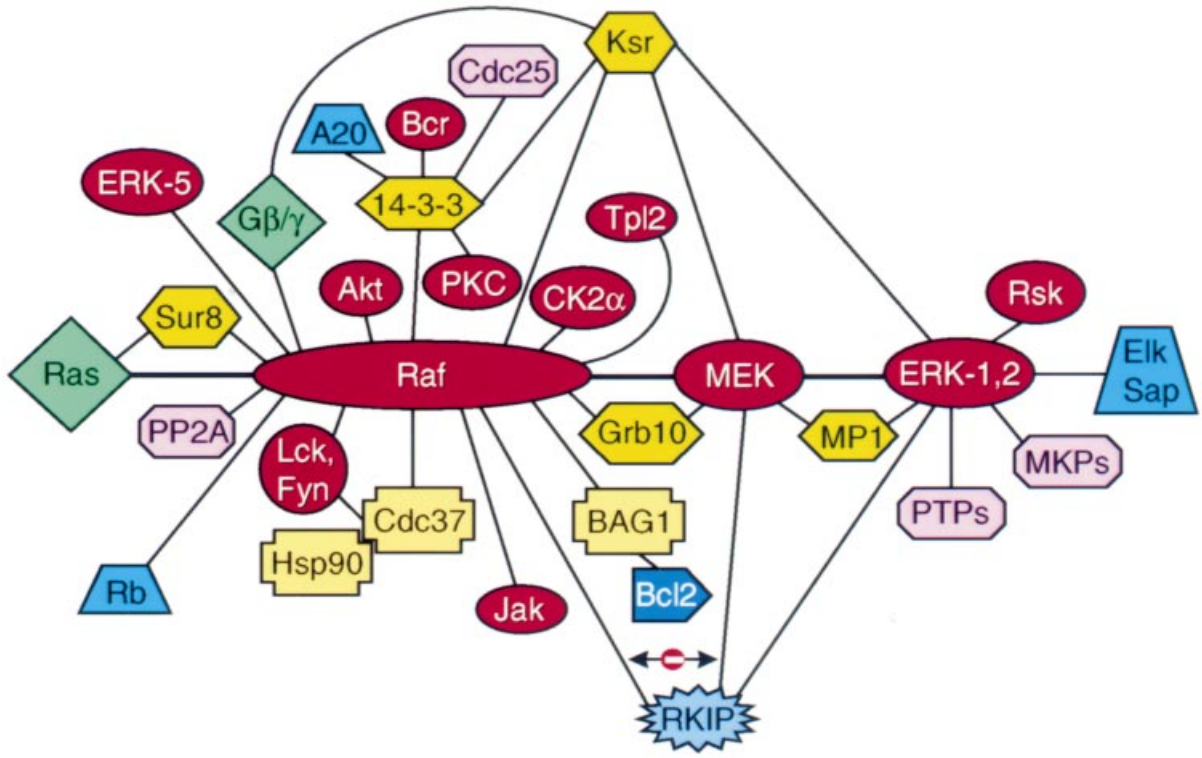

Figure 4 Multi-protein signalling complexes

Lines indicate physical interactions between proteins. Please note that all possible interactions are depicted, but that it is unlikely that all interactions are realized simultaneously within a cell. Kinases are shown in red, G-proteins in green, phosphatases in pink, adapters in dark yellow, chaperones in light yellow, and transcription factors in blue with black letters. A20 is a tumour-necrosis-factor (TNF)-induced gene that protects cells from TNF-mediated cytotoxicity. It associates with Raf-1 via 14-3-3 proteins [161]. Abbreviations: PKC, protein kinase C; JAK, Janus kinase; Rsk, ribosomal S6 kinase; MKP, MAPK phosphatase; PTP, protein tyrosine phosphatase; Gβ/γ, G-protein β/γ subunits; PP2A, protein phosphatase 2A. The minus symbol indicates that RKIP dissociates the interaction between Raf and MEK.

respectively, the Raf activation complex seems to be locked in a refractory state [22]. When the Raf-1 CRD is replaced by the lower-affinity B-Raf CRD, Rap1 is converted into an activator. Likewise, the Raf-1 CRD incorporated into B-Raf renders B-Raf insensitive to Rap1 activation [48]. These experiments imply that the conformational changes resulting in Raf activation must accommodate dynamic flexibility. If the CRD binds too strongly, activation does not occur. These findings could explain the paradoxical observation that ceramide enhances Raf-1 binding to Ras, but produces a complex unresponsive to activation [49]. In addition, these findings help to explain the paradoxical observation that in some cell types, such as PC12, cAMP can facilitate ERK activation despite inhibiting Raf-1 [50]. cAMP can activate Rap1 either via cAMP-dependent exchange factors [51] or via cAMP-activated protein kinase (PKA) [50]. The resulting activation of B-Raf can bypass the PKA-mediated Raf-1 suppression. Thus the expression of B-Raf can convert cAMP from an inhibitor into an activator of the ERK pathway (Figure 3).

This paradigm of the differential regulation of Raf isoenzymes by Ras family proteins is realized in PC12 cells. These cells are used widely as a model for neuronal differentiation. Differentiation is triggered by neurotrophic factors, such as nerve growth factor, which can support the long-lasting activation of the ERK pathway. In contrast, factors such as epidermal growth factor which elicit transient ERK activity are mitogenic [52]. Both epidermal growth factor and nerve growth factor induce transient ERK activation via Ras and Raf-1, but the latter can procure the sustained activation of the ERK pathway via the activation of B-Raf by Rap1 [53]. This neat example of achieving a specific biological response is based on the switching of allegiances from a Ras–Raf-1 to a Rap1–B-Raf signalling complex. Here the changes in protein interactions dictate the biological outcome.

This regulatory motif may also be utilized in another variation. The phosphorylation of Raf-1 on serine-43 by PKA reduces its affinity for Ras–GTP, thereby thwarting Raf-1 activation [54]. However, in specific cell types, such as PC12 cells, phosphorylation of serine-43 was reported to redirect Raf-1 to bind to Rheb, another member of the Ras family [55]. It is unclear how general this phenomenon is, and whether Rheb simply serves to sequester Raf-1 [56] or whether the Rheb–Raf-1 complex possesses a signalling capacity in its own right. In the latter case, as serine-43 is not conserved in the other Raf isoforms, Rheb could work as a PKA-controlled gate to a Raf-1 isoenzyme-specific pathway.

MAKING CONNECTIONS: 14-3-3 IS AT THE HUB

14-3-3's prosaic name does not foretell the fame it has received as the first phosphoserine-specific adapter protein to be discovered [57]. 14-3-3 is an abundant, ubiquitously expressed and evolutionarily highly conserved protein family that regulates cell-cycle checkpoints, proliferation, differentiation and apopto-

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sis [58,59]. All these task are probably achieved via the interaction with and modulation of the function of a wide range of signalling proteins (Figure 4). In many cases 14-3-3 inactivates the target protein by changing its subcellular localization or protein associations. For instance, the Bad protein promotes apoptosis by binding to Bcl-2 and Bclx at the mitochondrial membrane, annihilating their protective function [60]. Survival signals induce the phosphorylation of Bad, generating binding sites for 14-3-3. Phosphorylation and subsequent 14-3-3 binding not only causes Bad to disengage from Bcl-2 or Bclx, but also results in sequestration of Bad into the cytosol [61]. Likewise, 14-3-3 neutralizes the cell-cycle phosphatase Cdc25 [62] and forkhead-family transcription factors [63] by binding to and exporting the phosphorylated forms from the nucleus into the cytosol.

Raf-1 was among the first signalling proteins discovered to be associated with 14-3-3, but the functional consequences are still open to debate. The initial reports showed that 14-3-3 enhanced Raf-1 signalling in such diverse model systems as yeast [64,65], *Xenopus laevis* oocytes [66] and mammalian PC12 cells [67]. These were later corroborated by genetic screens in *Drosophila melanogaster* demonstrating that mutations in 14-3-3 disrupted photoreceptor development [68] and that 14-3-3 overexpression stimulated torso signalling [69], both of which are Raf/MEK/ERK-dependent processes. However, purified 14-3-3 was unable to activate Raf-1 *in vitro*, suggesting that, in the cell, 14-3-3 rather may increase the coupling of Raf-1 to an upstream activator or a downstream substrate [67]. This possibility appears plausible in view of the X-ray structure of 14-3-3, which shows a dimer forming a shallow groove that is wide enough to accommodate two large proteins simultaneously [70]. Indeed, subsequent experiments with dimerization-defective 14-3-3 mutants found active Raf-1 exclusively associated with the native 14-3-3 dimer, whereas the inactive fraction was bound to 14-3-3 monomers [71]. Although the possibility cannot be excluded that these mutations destroy another function in addition to dimerization, a prime role for dimerization is documented by the observation that 14-3-3 mutants deficient in phosphoserine binding act as dominant negatives by poisoning the function of endogenous 14-3-3 via heterodimerization [72].

Despite a host of candidates, the relevant binding partner to which 14-3-3 cross-links Raf-1 has remained elusive. It was demonstrated that 14-3-3 bridges Raf-1 with the serine/threonine kinase Breakpoint cluster region (Bcr), and that this occurs preferentially at the cell membrane where Raf-1 is activated. However, disappointingly, the association with Bcr did not impinge on Raf-1 activation [73]. Nevertheless, the Bcr–Raf-1 complex may gain relevance in pathological situations. Bcr can be joined with the abl tyrosine kinase due to a chromosomal translocation, and the resulting fusion protein is considered to be the transforming principle underlying chronic myelogenous leukaemia [74]. Raf-1 activation constitutes an essential step in Bcr–abl transformation [75]. Bcr–abl retains the 14-3-3 binding site [76], but it remains to be tested whether 14-3-3 plays a role in Raf-1 activation by Bcr–abl. Our own preliminary results show that, at least in overexpression systems, 14-3-3 can recruit upstream activating kinases into the Raf-1 activation complex, although this may be mediated indirectly via other Raf-1-associated proteins. A candidate for such a protein is KSR (kinase suppressor of Ras), which will be discussed in more detail below. KSR is a putative scaffolding protein for the Raf/MEK/ERK module that binds to MEK and ERK constitutively, but to Raf-1 only at the cell membrane. Since KSR also interacts with 14-3-3 [77], it is conceivable that 14-3-3 acts as a 'scaffold for the scaffold'. The inclusion of 14-3-3, whose adapter function is conditional on phosphorylation, would not only introduce an additional control element into the scaffolding complex, but also might increase its versatility, possibly by enabling switching between alternative MEK activators. In addition, the KSR–14-3-3 complex may enhance the coupling of Raf-1 to its substrate MEK [78]. *In vitro*, 14-3-3 failed to enhance MEK phosphorylation by Raf-1 under various conditions, but these experiments were done in the absence of KSR [67]. However, the reconstitution of working multi-protein complexes *in vitro* is technically difficult if functionality depends on the correct stoichiometry between components.

Finally, the Raf-1 binding partner could be Raf-1 itself. Using drug-dependent dimerizer systems it was shown that, in the cell, Raf-1 activation can be achieved by dimerization [32,33], and it was suggested that 14-3-3 may be the natural dimerizer [33]. A closer biochemical investigation led to a revised model whereby one 14-3-3 dimer interacts intramolecularly with the two binding sites in Raf-1, phosphoserine-259 and -621 [79]. The removal of 14-3-3 by competition with synthetic phosphopeptides disabled both basal and induced Raf-1 activity. Addition of recombinant 14-3-3 could revive Raf-1, but only if it had been activated previously. Based on these results, the authors proposed that the role of 14-3-3 is to stabilize both the inactive and the activated conformations of Raf-1. This mode of action very much resembles that of a chaperone, and as will be discussed below chaperones indeed seem to be critical for proper Raf-1 function.

Unfortunately, work with Raf-1 mutants in which serine-259 and -621 were changed added still more puzzles. Both sites are phosphorylated in resting cells [80], but can be hyperinduced by activation of PKA [81]. Replacing serine-621 with a number of other amino acids almost completely destroys the catalytic activity of Raf-1 and limits the usefulness of these mutants for activity studies [80,82–84]. Although this indicates an essential function of serine-621, *in vitro* biochemical experiments with the isolated Raf kinase domain, BXB, suggest an inhibitory influence of serine-621 phosphorylation. The constitutive activity of BXB can be suppressed by phosphorylation of serine-621 *in vitro*, and the selective removal of this phosphate re-activates catalytic activity [82]. This regulation also seems to occur *in vivo*, as the activity of BXB and v-Raf is down-regulated by PKA activation. Both mutants lack serines-43 and -259, and serine-621 is the only site that becomes phosphorylated in response to PKA activation [81,82]. However, other studies correlated serine-621 phosphorylation and 14-3-3 binding with an increase in activity [85]. The reason for this discrepancy is unclear at present.

Serine-259 is amenable to mutational analysis, and the results obtained clearly earmark it as a negative regulatory site, with its mutation inducing Raf-1 activation [43,86,87]. The extent of the increase in activity varies between cell types, and the phosphorylation is not mimicked by a negatively charged amino acid, but requires the physical presence of the phosphate group, suggesting that the effect of mutation of serine-259 is likely to be due to the destruction of a protein interaction site. As no other phosphoserine-259 binding partners are known, this hypothesis by default classifies 14-3-3 as negative regulator. This assumption is supported by an independent class of mutants in the Raf-1 CRD. Certain point mutations in the CRD induce Raf-1 activation, which – wherever tested – are correlated with a loss of 14-3-3 binding [86,88]. It is not entirely clear whether CRD contains a true phosphorylation-independent 14-3-3 binding site [89], or whether these mutations simply may affect the phosphorylation state of serine-259 or -621. In summary, these results depict an important, but highly contradictory, role for 14-3-3 in the regulation of Raf-1.

There are, however, some recent results which may be able to reconcile these controversies (Figure 2). In this respect, important

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findings were that Ras can displace 14-3-3 from the N-terminal binding site(s), serine-259 and the CRD [90]; that 14-3-3 facilitates the membrane translocation of Raf-1 [91]; and that dephosphorylation of serine-259 is one of the first changes in Raf-1 phosphorylation that is noticeable during the mitogen-induced activation process, and is required for activation [87]. Dephosphorylation of serine-259 appears to be executed by protein phosphatase 2A, which associates with Raf-1 at the cell membrane. The specific inhibition of protein phosphatase 2A by okadaic acid prevents both dephosphorylation of serine-259 and activation of Raf-1, while mutation of serine-259 renders Raf-1 resistant to inhibition by okadaic acid [87]. A role for protein phosphatase 2A in Raf activation was also confirmed by genetic epistasis experiments in *C. elegans* and *Drosophila* [92,93]. In combination, these findings suggest the following scenario, as depicted in Figure 2. In quiescent cells Raf-1 resides in the cytosol, tied into an inactive state by the binding of a 14-3-3 dimer to phosphoserines-259 and -621. When activation ensues, Ras–GTP binding not only brings Raf-1 to the membrane, where it can associate with protein phosphatase 2A, but also destabilizes the interaction of 14-3-3 with phosphoserine-259. This permits protein phosphatase 2A access to phosphoserine-259 to remove the phosphate, thereby freeing one arm of the 14-3-3 dimer, which is now available to recruit upstream activators and promote the activation process. According to this model, Raf serine-259 mutants are predicted to represent a transition state, with elevated basal activity, but still susceptible to further activation by growth factors and the indirect Raf activation signals described above. In this case the expected phenotype corresponds faithfully to the phenotype observed. The same holds true for the oncogenic deletion mutants that lack the whole N-terminal regulatory domain. These mutants are constitutively active, but are susceptible to further activation [82,84]. The phenotype of the serine-621 mutant is more difficult to rationalize. This mutation obviously must lead to the complete release of 14-3-3 when the second binding site, serine-259, is deleted, mutated or dephosphorylated, and hence the serine-621 mutant fails to conscript activators. This would satisfactorily explain why serine-621 mutants cannot be activated. However, this mutation also almost completely abolishes basal catalytic activity when introduced into the isolated Raf-1 kinase domain [9,82,84]. This phenotype is consistent with an essential structural role of serine-621, or a strict requirement for 14-3-3 to attract factors that allow Raf-1 activity. Among these factors could be the kinases that phosphorylate serine-338, because mutation of this residue also severely cripples both the basal and induced activities of Raf-1 [94]. The region encompassing serine-621 of Raf-1 is conserved in B-Raf, but the exchange of the equivalent serine-728 to alanine in B-Raf yields a kinase with substantial residual activity, further supporting a functional rather than a structural role of this serine residue [83]. This role seems to hinge on 14-3-3 binding rather than on phosphorylation itself. The displacement of 14-3-3 by competing phosphopeptides inactivates Raf-1, despite leaving phosphorylation intact [79]. This observation is compatible with the interpretation that phosphorylation of serine-621 on its own has a negative impact on catalytic activity, which is converted into a positive function by 14-3-3. While this model depicted in Figure 2 accommodates many salient aspects of the interaction between Raf and 14-3-3, it must be noted that 14-3-3 still holds many secrets.

STAYING IN SHAPE AND MORE: WHAT CHAPERONES DO FOR Raf

A number of chaperones have been found to associate with Raf-1, including Hsp90 (heat-shock protein of 90 kDa) and Hsp50/Cdc37 [16,95,96], FKBP65 (FK-506 binding protein) [97] and Bag-1 [98]. Bag-1 was isolated originally as an anti-apoptotic Bcl-2 binding partner, but subsequent experimentation has placed it among the chaperones by revealing that it regulates Hsp70 and Hsc70 [99,100]. Chaperones seem to be necessary for stabilizing Raf's feeble tertiary structure, as evidenced by the high propensity of purified Raf-1 to denature into insoluble aggregates, as well as by experiments with geldanamycin. This drug binds to Hsp90 and prevents it from interacting with and helping folding of its client proteins [101]. Treatment with geldanamycin almost completely rids cells of Raf-1 protein within a few hours by inducing its aggregation, ubiquitination and subsequent degradation [101,102]. However, besides these mundane maintenance roles, chaperones may serve more intricate functions in the regulation of signalling.

Mutations in Cdc37 and Hsp90 (called Hsp83 in *Drosophila*) were isolated in genetic screens as suppressors of Ras/Raf/MEK/ERK-dependent developmental pathways in *Drosophila* [103,104]. Mutated Hsp83 could still bind to Raf-1, but reduced its kinase activity [103]. This may not simply reflect a loss of Hsp90's chaperone properties, but could be related to a function in recruiting Raf-1 activators. A first hint was provided by the observation that, in PC12 cells, the preferential activation of B-Raf over Raf-1 was correlated with Hsp90 association [105]. Later on it was shown that the association of Raf-1 with Hsp90 was primarily mediated via Cdc37 [95,96]. The overexpression of a Cdc37 mutant deficient in Hsp90 binding impaired the growth-factor stimulation of the ERK pathway in mammalian cells, whereas the co-expression of Cdc37 in insect cells enhanced both the basal and the Ras- and Src-induced activity of Raf-1. Cdc37 could even partially restore the activity of a Raf-1 serine-621 mutant, suggesting functional resemblance to 14-3-3. In contrast, a Raf-1 mutant in which the tyrosine phosphorylation site had been replaced was resistant to Cdc37 activation [96]. As the Cdc37–Hsp90 complex is known to associate with v-Src and other tyrosine kinases [106,107], an intriguing possibility is that Cdc37 links Raf-1 to activation by tyrosine phosphorylation. Alternatively, but not necessarily exclusively, Cdc37 may facilitate coupling of Raf-1 to its substrate MEK, because MEK also is found in a high-molecular-mass complex with Cdc37 and Hsp90 [78].

A different sort of adapter function was also proposed for Bag-1. By mediating an interaction between Raf-1 and Bcl-2, Bag-1 may redirect Raf-1 to the mitochondrial membrane, where Bcl-2 resides [107a]. Here, Raf-1 can gain access to a new target, the pro-apoptotic Bad protein. Bad was portrayed originally as a direct substrate of Raf-1, being inactivated by Raf-1 phosphorylation [107a]; however, since Raf-1 fails to phosphorylate the two sites commonly identified as inactivating phosphorylation sites [108], Raf-1 may rather trigger Bad phosphorylation indirectly. In addition, Bag-1 was reported to activate Raf-1 directly *in vitro* [98], but this could be related to a chaperone function that maintains Raf-1 in an active state rather than an authentic activator function. These findings depict Bag-1 as a combined activator and adapter that can re-route Raf-1 signals into anti-apoptotic pathways. Although an attractive hypothesis, the activation of Raf-1 by Bag-1 *in vivo*, and the existence of a ternary Raf-1–Bad–Bcl-2 complex at the mitochondria, still need to be proven. But, given that the artificial targeting of the Raf-1 kinase domain provides efficient protection against apoptosis [75,107a], and that survival signals can induce the translocation of Raf-1 to mitochondria [75,109], the existence of Raf-1 adapter and activator molecules at the mitochondrial membrane is likely.

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HOLDING IT TOGETHER: SCAFFOLDS AND ADAPTERS

KSR and CNK (connector–enhancer of KSR)

All the components of the Ras/Raf/MEK/ERK pathway can interact with each other physically: Ras–GTP binds to Raf; Raf can bind to MEK; and MEK can bind to ERK. These interactions are of eminent importance for the proper transmission of signals down the pathway, and hence Nature does not rely solely on the intrinsic affinities of these proteins for each other. In yeast, MAPK modules are neatly organized by scaffolding proteins that ensure the efficiency and fidelity of signal transduction by joining the pathway components [2]. The quest for mammalian homologues or orthologues was unrewarding until the serendipitous discovery of KSR. KSR was isolated by three independent groups in genetic screens in flies and worms as the product of a gene that could suppress the phenotypes caused by activated Ras [110]. As its primary sequence exhibited identity with kinases, most closely with Raf-1, it was christened KSR, i.e. kinase suppressor of Ras. Although it shares no identity with the yeast scaffolds, the analysis of its mammalian homologues soon revealed several properties pointing to KSR being a scaffolding protein for the ERK pathway (Figure 5). KSR could co-operate with Ras to enhance oncogenic transformation of fibroblasts, as well as the maturation of *Xenopus* oocytes by accelerating MEK and ERK activation. This required the cysteine-rich CA3 domain in the N-terminal region, but not KSR kinase activity. In fact, the KSR kinase domain alone inhibited all these effects [111]. Further, KSR associated with Raf-1 at the cell membrane in a Ras-dependent manner and could enhance Raf-1 activation, a trait that was again traced to CA3, a cysteine-rich domain resembling the Raf CRD [112]. The CA3 region was also implicated in mediating the translocation of KSR to the cell membrane in an independent study, probably via an interaction with the β subunit of heterotrimeric G-proteins [113]. The β/γ dimer is located at the cell membrane and is responsible for stimulation of the ERK pathway by G-protein-coupled receptors [114]. Curiously, the Raf-1 CRD also was reported to bind to the same type of β subunit as KSR *in vitro* and in co-expression systems [115], but is not not clear whether this interaction occurs between endogenous proteins and what the functional significance is.

Later on, KSR was shown to bind MEK and ERK in the yeast two-hybrid system as well as in mammalian cells [116,117]. Overexpression of KSR inhibited ERK-dependent biological effects, whereas low-level expression facilitated ERK signalling [118]. Such a dose-dependent reversal of effects is typical for a scaffolding protein that only can assemble its client proteins when present in an appropriate stoichiometric ratio, but disperses signalling complexes when overexpressed. In all cases the kinase activity of KSR was dispensable, and the mutation of essential catalytic residues did not affect KSR function. To date no *bona fide* substrate for KSR has been found, and reports that KSR corresponds to ceramide-activated kinase [119] and phosphorylates Raf-1 [120] are disputed [49,112] and have not been widely accepted. It could well be that KSR is genuinely devoid of catalytic activity, and we are witnessing the transition from a kinase to a dedicated scaffolding protein. Given that in yeast PBS2 (where PBS = polymyxin B sensitivity) functions both as a MAPKK and as a scaffolding protein for the respective MAPK module [2], evolution may have taken this development a step further in KSR. Although none of these studies have yet shown a quaternary complex between KSR, Raf-1, MEK and ERK, all the properties of KSR suggest that it is a scaffolding protein.

KSR's main binding partner appears to be MEK. MEK binds to the kinase domain of KSR [121], which functions as a strong dominant-negative mutant [111,121] when severed from the N-terminus, presumably by sequestering MEK. The biochemical analysis of KSR protein interactions turned up a number of familiar faces, including Hsp90, Hsp70, Cdc37, MEK-1 and -2, and 14-3-3 [78]. Together with as yet unidentified proteins, they form a large signalling complex. Interestingly, three KSR mutations corresponding to *C. elegans* loss-of-function alleles selectively compromised MEK binding, highlighting the importance of this association [78]. As, like Raf-1, the binding of ERK to KSR is dependent on activated Ras [118], KSR appears primarily to nucleate MEK signalling complexes. This poses the provocative question of whether KSR could serve as interface for linking MEK to different upstream activators and downstream substrates. MEK kinases other than Raf have been described, for instance mos, some MEKK isoforms and Tpl-2, but MEK-1/2 are considered very specific kinases with ERK-1/2 as sole substrates [2,3]. Although the overexpression of KSR blocks ERK activation by growth factors, Ras, Raf and MEK [121], this does not necessarily exclude a function in another pathway. Alternatively, the conditional interaction with activated Raf and ERK could indicate a role for KSR in controlling the kinetics of ERK activation, maybe by converting an initial transient activation peak into a sustained stimulation. The duration of ERK activity has a decisive bearing on the biological outcome [52], and many of the biological systems in which KSR was tested, such as oocyte maturation and oncogenic transformation, rely on prolonged ERK activity. Such a role would also be consistent with the somewhat puzzling observation, made during the original suppressor screens, that KSR could revert the phenotype induced by oncogenic Ras, but did not affect normal Ras function [110], indicating that KSR is primarily important for the transduction of sustained Ras signals. In this context it may be relevant that Ras stimulates KSR phosphorylation, very probably executed through ERK. The mutation of these phosphorylation sites did not alter the ability of KSR to accelerate Ras-induced oocyte maturation, but this does not exclude a role in other settings [118].

A subsequent genetic screen in *Drosophila* for modifiers of the rough eye phenotype caused by expression of the dominant-negative KSR kinase domain led to the cloning of CNK (connector–enhancer of KSR) [122]. The primary structure of CNK contains no catalytic domain, but several protein–protein interaction domains, suggesting a function as a multivalent adapter protein. CNK enhanced the dominant-negative KSR phenotype and suppressed activated Ras, but not Raf, signalling, indicating that it works downstream of Ras and upstream of or parallel to Raf. The latter possibility is more likely, since CNK is associated with Raf in fly cells. A closer dissection of Ras pathways in *Drosophila* showed that CNK regulates the ERK pathway via its C-terminal Raf interaction domain, as well as an ERK-independent pathway via its N-terminus [123]. Thus CNK seems to modulate different Ras effector pathways, a function that could be crucial for the proper co-ordination of downstream signalling. Unfortunately, no interaction could be detected between the human CNK homologue and mammalian Raf-1. While this may not be too surprising, given that human CNK is only about half the size of the *Drosophila* protein, it leaves the function of CNK in mammals mysterious.

MP1 (MEK partner 1)

MP1 was isolated in a yeast two-hybrid screen using MEK-1 as bait [124]. It is a small protein featuring no exciting motifs and no revealing homologues. Nevertheless, it turned out to possess a very intriguing function as a specialized adapter protein (Figure

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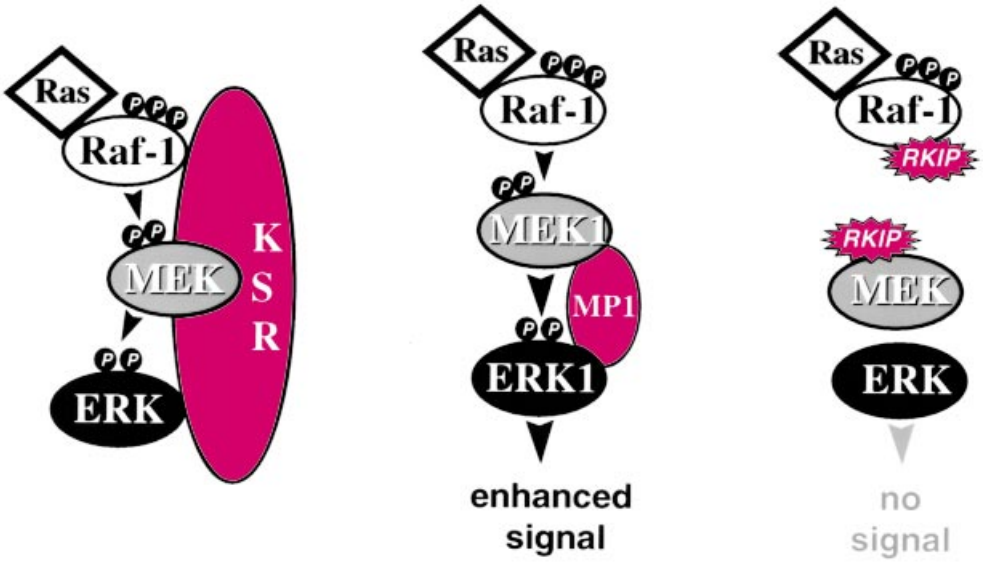

Figure 5 Function of KSR, MP1 and RKIP

5). MP1 also interacts with ERK, and by linking MEK-1 with ERK-1 it favours the activation of ERK-1 over ERK-2. The physiological significance of this is not understood, because in most scenarios MEK-1 and -2, as well as ERK-1 and -2 respectively, appear to be functionally equivalent. However, a few exceptions have been described. Raf-1 bound to Ras seems to interact preferentially with and activate MEK-1 rather than MEK-2 [125], and in rat fibroblasts v-*raf* selectively induced ERK-2 activity [126].

SPLITTING IT UP: THE CONTROL OF MEK ACTIVATION BY RKIP (Raf KINASE INHIBITOR PROTEIN)

As every coin has two sides, regulatory themes in biology often come in two antipodal variations. The counterpart of adapters would be proteins that interfere with specific connections. One such protein, RKIP, has recently been identified [127]. Isolated in a two-hybrid screen as a Raf-1-associated protein, RKIP could also bind to MEK and ERK *in vitro* and *in vivo*, initially suggesting that it may be a scaffold for the kinase module. However, both biochemical and biological properties clearly distinguish it from scaffolding proteins. When tested for its effects on the Raf/MEK/ERK cascade reconstituted *in vitro*, RKIP selectively impaired the phosphorylation of MEK by Raf, without affecting the phosphorylation of ERK by MEK or the phosphorylation of the transcription-factor substrate Elk by ERK. This inhibition was very specific indeed, because RKIP did not interfere with Raf autophosphorylation or the phosphorylation of an artificial substrate by Raf. It also did not prevent the phosphorylation of MEK by MEKK-1. The basis for this selectivity is that RKIP can disrupt the physical interaction between Raf-1 and MEK (Figure 5), behaving like a competitor for substrate [128]. The binding sites for Raf and MEK in RKIP overlap, making their binding mutually exclusive. In contrast, the minimally required binding sites for RKIP and MEK in Raf, as well as those for RKIP and Raf in MEK, are distinct. Both proteins interact with the catalytic domain of Raf-1, but the essential RKIP binding site lies at the beginning of the catalytic domain (subdomains I and II), while MEK association requires subdomains VI–VIII in the core of the kinase, suggesting that RKIP may reduce binding affinity by an allosteric mechanism. Alternatively, bound RKIP could pose a steric hindrance that is prohibitive to the interaction between Raf and MEK [128].

Importantly, RKIP seems to be a physiological regulator of ERK signalling. Its overexpression blocks ERK-dependent processes such as gene transcription and cellular transformation. In contrast, lowering RKIP protein levels by expression of antisense RNA or neutralizing RKIP function by antibody microinjection causes activation of the pathway. RKIP binding to Raf-1, but not to MEK, is controlled by growth factors, probably via modification of Raf-1. Stimulation induces the release of RKIP from Raf-1, allowing activation of MEK and ERK. Later, when ERK activity declines, RKIP re-associates with Raf-1 [127,128]. Since RKIP acts as a stoichiometric inhibitor, the RKIP expression level may set the threshold for activation of the ERK pathway. RKIP belongs to the family of phosphatidylethanolamine binding proteins, which are widely expressed and evolutionarily conserved [129]. These binding proteins have been cloned previously on several occasions, but were devoid of a clear function apart from their ability to bind phospholipids. This trait, however, seems to have no bearing on their inhibitory function within the ERK pathway [127].

Very recently, an inhibitor protein for MUK (MAPK upstream kinase)/DLK (dual leucine-zipper-bearing kinase)/ZPK (leucine-zipper protein kinase), a MAPKKK in the JNK (c-Jun N-terminal kinase) pathway, was cloned [130]. The inhibitor, called MBIP, binds to MUK/DLK/ZPK and interferes selectively with JNK activation by MUK/DLK/ZPK, but not by Tpl-2, another MAPKKK upstream of JNK. This resembles the mechanism whereby RKIP disables Raf activation of ERK, and highlights the potentially widespread utilization of this regulatory motif.

Interestingly, the Raf/MEK interface is also used for positive regulation. Several laboratories have observed a strong synergism in transformation assays between Ras and Rho-family GTPases [131]. It was shown subsequently that the Rho-family GTPase Rac could robustly enhance the activation of the ERK pathway [132]. This study traced the mechanism

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to MEK phosphorylation by the Rac-activated kinase Pak-1. The phosphorylation did not change the catalytic activity of MEK, but rather enhanced the interaction with Raf-1, thereby facilitating MEK activation. Thus Rac can impinge on the ERK pathway on two levels (Figure 3). First, it contributes to Raf activation by inducing phosphorylation of serine-338 [29] and, secondly, it increases the efficiency of MEK activation [132].

SIGNAL DIVERSIFICATION: MULTI-PROTEIN KINASE COMPLEXES

Being at the receiver end of the ERK module, Raf-1 collects and funnels a variety of upstream signals into the pathway. The ERK pathway is without doubt a major effector of Raf, but accumulating evidence suggests that it is not the only one, and that Raf-1 despatches signals into different downstream pathways. This evidence is based mainly on observations of Raf triggering biological effects in the absence of ERK activation. For instance, activated Raf-1, but not activated MEK mutants, can drive the differentiation of rat hippocampal neurons [133]. Another example is the observation that Raf-1 can induce the depolymerization of vimentin filaments. This effect is not prevented by MEK inhibitors, and is caused by as yet unknown Raf-1-associated vimentin kinases which are regulated by Raf-1 [134].

In addition, a number of novel Raf substrates have been inferred, although MEK remains the only widely accepted substrate at present. One of these alternative substrates proposed is Bad, which has been discussed above. Another interesting example is the retinoblastoma (Rb) tumour suppressor protein. To permit cell-cycle progression from G1 into S phase, Rb must be inactivated by phosphorylation. This is accomplished by the concerted action of cyclin D- and E-dependent cell-cycle kinases [135]. A recent report has also invoked Raf-1 as a Rb kinase contributing to Rb inactivation [136]. Mitogen stimulation induced the binding of Raf-1 to Rb, and Rb inactivation was dependent on Raf-1 binding. The interaction domain was mapped to the first 28 amino acids of Raf-1, which is unique to Raf-1. Thus, if the physiological relevance of this exciting observation can be confirmed, Rb would not only represent a direct link from Raf to the cell-cycle machinery, but also the first Raf-isoenzyme-specific substrate.

Another intriguing set of findings showed that Raf-1 signalling complexes comprise a number of other kinases, some of which appear to be regulated by Raf-1 or vice versa. The first kinase suspected in a complex with Raf-1 was Tpl-2/Cot. Tpl-2 can phosphorylate and activate MEK. Curiously, dominant-negative Ras and Raf mutants impaired ERK activation by Tpl-2, and dominant-negative Tpl-2 interfered with ERK activation by Raf. These results were interpreted to suggest that both kinases are part of a Ras nucleated multi-protein signalling complex and are mutually interdependent [137]. This hypothesis was challenged by a report showing that ERK activation by Tpl-2 was independent of Ras and Raf-1 [138], but this may have been due to the use of different cell types. This latter report added SEK-1 (where SEK = stress-activated protein kinase/ERK kinase) as a new Tpl-2 substrate. SEK-1 is a MAPKK for the stress-responsive JNK/MAPK, and hence suggested Tpl-2 as an entry point for two MAPK pathways. Tpl-2 has rather restricted expression, but notably is found in T-cells [137], where proliferation requires the concordant stimulation of both the ERK and JNK pathways [139]. While little is known about the physiological role of Tpl-2, Raf-1 is well established as an essential transducer of mitogenic signals in T-cells. Thus in these cells a Raf-1–Tpl-2 complex may expand the signalling capacity to provide activation of both MAPK pathways.

Tpl-2 has also been implicated in the activation of nuclear factor-κB (NF-κB) [140,141]. NF-κB is a ubiquitous transcription factor that is involved in proliferation, apoptosis and the inflammatory response. NF-κB activity is mainly controlled by IκB inhibitor proteins, which sequester NF-κB in the cytosol. Inflammatory signals initiate the degradation of IκB by stimulating phosphorylation of serines-32 and -36 [142]. Ras and Raf can activate NF-κB quite efficiently, but, despite vigorous efforts, the mechanism has remained elusive. Suggestions include Tpl-2 increasing the level of active NF-κB by enhancing the proteolysis of p105, an NF-κB precursor molecule [141], or inducing the phosphorylation of IκB, marking it for destruction [140]. Rsk, an ERK-activated protein kinase, can also phosphorylate IκB on serine-32 [142]. Two other IκB kinases have also been described as Raf-1-associated kinases. Akt/protein kinase B was reported to induce IκB phosphorylation [142], and also to associate with Raf-1 under conditions where the ERK pathway is repressed, for instance during myoblast differentiation [143]. Inhibition may be due to direct phosphorylation of Raf-1 at serine-259 by Akt [43]. Another IκB kinase was identified as casein kinase 2α (CK2α) during attempts to purify the protein kinase C- and Raf-1-dependent IκB kinases [144]. Although CK2 does not phosphorylate the signal-induced sites in IκB that acutely trigger its degradation, CK2-mediated phosphorylation is required for

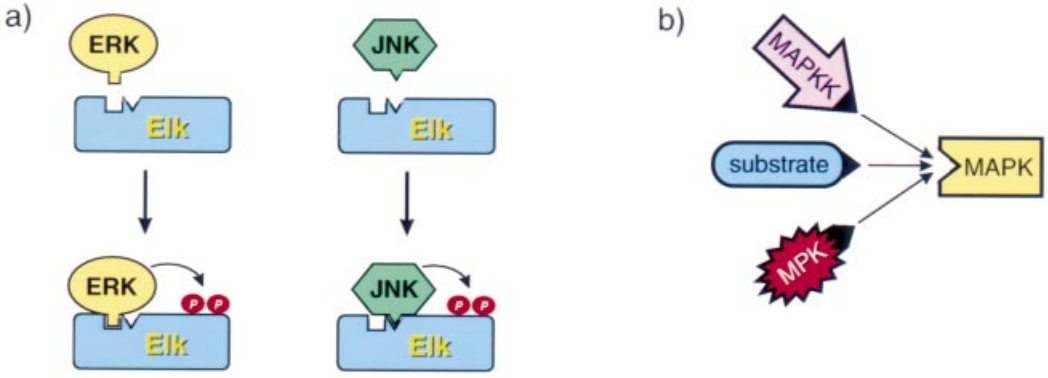

Figure 6 Docking sites

(**a**) MAPK substrates contain docking sites that interact with selected MAPKs. (**b**) MAPKs feature one common docking domain for MAPKKs, substrates and MAPK phosphatases (MPKs).

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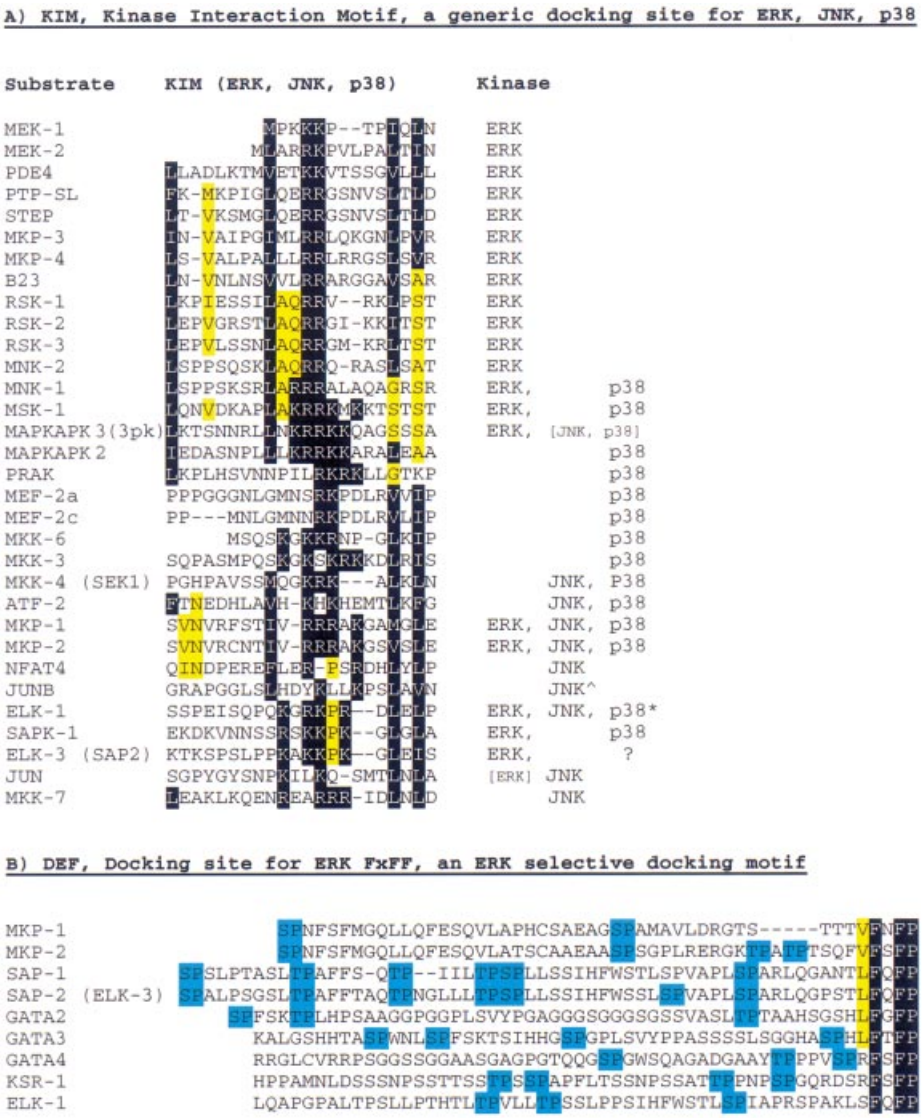

Figure 7 MAPK docking sites

The alignment shows the docking sites of various MAPK substrates with the corresponding kinases listed. Where assignments are uncertain, respective kinases are in brackets. Amino acids conserved in at least 50% of the sequences may be considered part of the core binding motif and are shaded black. Other similarities are shaded yellow. Ser-Pro and Thr-Pro consensus sites for MAPK phosphorylation are shaded blue. * p38 phosphorylation is KIM independent; ^ JNK binds to JUN-B, but does not phosphorylate it due to the lack of phosphorylation sites. Abbreviations: PDE, phosphodiesterase; PTP, protein tyrosine phosphatase; STEP, protein tyrosine phosphatase striatum-enriched; MKP, MAPK phosphatase; RSK, ribosomal S6 kinase; MNK, MAPK-interacting kinase; MSK, mitogen- and stress-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; PRAK, p38-regulated/activated protein kinase; MEF, myocyte enhancer factor; MKK, MAPK kinase; SEK, stress-activated protein kinase/ERK kinase; SAPK, stress-activated protein kinase; ATF, activating transcription factor; NFAT, nuclear factor of activated T-cells; SAP, SRF accessory protein; GATA, transcription factors binding to the nucleotide sequence GATA.

degradation [145]. CK2 phosphorylates a great number of regulatory molecules, but has become notorious for denying insight into its regulation by external cues [146]. Therefore it was remarkable that the activation status of Raf-1, as measured by MEK phosphorylation, was faithfully reflected in the activity of associated CK2α assayed by IκB phosphorylation [81], indicating that Raf-1 can regulate the activity of associated CK2α. In summary, IκB appears to be a node point at which several Ras/Raf-controlled signalling pathways intersect.

CK2 is a heterodimer consisting of a regulatory β and a catalytic α subunit. Only the latter was found in association with Raf-1 [144], suggesting that Raf-1 could physically and functionally replace the β subunit. As the only firmly established regulation of CK2 is exerted by the β subunit, Raf-1 conceivably could regulate CK2 by substituting for the β subunit. In addition, the CK2 β subunit was isolated in yeast two-hybrid screens as a protein that selectively bound to A-Raf, but not to the two other Raf isoforms [147,148]. Since the CK2 β subunit stimulated the catalytic activity of A-Raf in an insect cell overexpression system [147], CK2 β subunit may employ A-Raf as alternative catalytic subunit. It remains to be verified, however, whether these intimate *in vitro* liaisons between Raf kinases and CK2 exist in mammalian cells.

The motif of functionally interdigitating protein kinase complexes is further illustrated by the interaction of ERK-5 with Raf-1 [149]. ERK-5 is an unusually big MAPK which has a

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function in proliferation. Raf-1 forms complexes with ERK-5 and, although unable to activate ERK-5 on its own, contributes to Ras activation of ERK-5. However, this did not require the Raf kinase domain, but the regulatory domain. In turn, dominant-negative ERK-5 reduced Raf transformation, and activated MEK-5 (the respective ERK-5 MAPKK) synergized with Raf-1 in transformation. However, this did not involve enhancement of ERK-1/2 activation, but rather a new as yet undefined pathway. In many respects this facet of Raf-1 function very much resembles the way KSR contributes to the activation of the ERK-1/2 pathway, raising the possibility that a main role of Raf-1 is to participate in the assembly of multi-protein signalling complexes. Without presenting an exhaustive account of all cross-connections reported, these examples demonstrate that a high degree of communication exists between protein kinases, especially at the level of MAPKKKs.

PICKING ACTIVATORS AND SUBSTRATES: ERK DOCKING SITES

The roster of ERK-1/2 substrates ranges from cytoskeletal proteins to other kinases, phosphatases, enzymes and transcription factors [15]. While this diversified array of substrates can explain the pleiotropic functions of the ERK pathway, it poses the question of how a set of substrates required for a specific response is chosen. Several mechanisms seem to be in operation. Cell type- and situation-specific expression determines the subset of potential substrates available. Subcellular compartmentalization can regulate the accessibility of substrates. For instance, preventing ERK from translocating to the nucleus denies it access to its transcription factor substrates and abrogates the mitogenic response [150]. A simple mechanism for achieving specificity is provided by the recently discovered docking domains for MAPKs. Docking domains bind the appropriate MAPK and guide it to its phosphorylation target, thereby enhancing phosphorylation (Figure 6a). These domains have only been found recently and still await exact definition.

At present, a generic MAPK and an ERK-specific motif can be distinguished [151,152] (Figure 7). The ERK-specific motif features an Ser-Pro or Thr-Pro phosphorylation site in the vicinity of an ERK binding site (Phe-Xaa-Phe-Pro), and has been called DEF (docking site for ERK, FxFP) [151]. The generic MAPK binding site, KIM (kinase interaction motif), is usually rather remote from the phosphorylation site. KIMs come in different variations that can interact with any of the three MAPKs or only a subset [151,153,154]. A meticulous mutational dissection of the KIM in the transcription factor Elk has unveiled subtle differences between ERK and JNK binding to the KIM, and shown that p38 does not require the KIM at all to phosphorylate Elk [155]. However, sequence comparisons between different KIMs do not disclose designated binding motifs for specific MAPKs (Figure 7). KIMs consist of a basic amino acid centre flanked by hydrophobic residues on one or both sides. The basic amino acids interact electrostatically with a cluster of acidic amino acids in the C-terminus of the MAPKs [152]. This acidic site is evolutionarily conserved in the different MAPKs, and was termed CD (common docking) domain, because it serves as a common binding site for their activating MAPKKs, their substrates and their inactivating phosphatases [152] (Figure 6b). Some ERK substrates contain both a KIM and a DEF. The purpose of this is unclear, because they do not cooperate to bind ERKs, but rather seem to work independently [151]. It is possible that this arrangement serves to integrate signals that converge on a common substrate by providing docking sites for two different MAPKs.

WHERE TO GO: ERK ANCHORING PROTEINS

Another major role in choice of substrates is played by compartmentalization, and ERK-1/2 in particular have been shown to undergo activation-dependent intracellular redistribution to different sites, with that to the nucleus being most easily perceptible. It has been proposed that these directed redistributions are in great part specified by anchoring proteins. Such proteins are well known for PKA, but are only now being discovered for ERKs. It has been proposed that the upstream activator MEK-1/2 tethers inactive ERK-1/2 in the cytosol [156]. Phosphorylation triggers ERK release and eventually nuclear translocation. In the nucleus ERKs are retained by an anchoring protein that is induced by ERKs, and has been speculated to represent a ERK phosphatase [157]. This would couple ERK retention in the nucleus to de-activation, and also prevent this pool of ERK from being re-activated by MEK in the cytosol. Interestingly, the protein tyrosine phosphatases PTP-SL and HePTP have also recently been shown to serve as cytosolic anchors for ERK-1/2 [158,159]. Since they can dephosphorylate ERK-1/2, they ensure that ERKs bound to them are maintained in the inactive state. Interestingly, the KIM docking sites of these phosphatases contain a serine that can be phosphorylated by PKA, enabling ERK to disengage and become activated. This unexpected cross-talk may explain why, in some cell types, cAMP can activate ERK without the need for Raf kinase activation [160].

CONCLUSION

In this review I have tried to illustrate that protein interactions play a major role in the regulation of the Ras/Raf/MEK/ERK pathway. Making and breaking of connections is increasingly recognized as a regulatory motif for orchestrating signalling pathways in time and space. At the moment we are just able to see the tip of the iceberg. But, fortunately, we can be confident of progress. For one, Nature has helped us by having designed interaction motifs that are recognizable by sequence alignment or structural comparison. In addition, enlisting modern technology, such as proteomics and sophisticated large-scale yeast two-hybrid screening, should allow us to decipher the composition of multi-protein signalling complexes. This sets the stage for tracing the multiple interactions and understanding their functional consequences. Such an insight will be especially helpful in revealing the multiple layers of cross-talk between signalling pathways and how specific responses are achieved by the combinatorial utilization of a limited set of enzymic machinery.

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