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Biochimica et Biophysica Acta 1773 (2007) 1213–1226
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamcr

Review

The MEK/ERK cascade: From signaling specificity to diverse functions

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Received 9 August 2006; received in revised form 10 October 2006; accepted 13 October 2006
Available online 19 October 2006

Abstract

The ERK signaling cascade is a central MAPK pathway that plays a role in the regulation of various cellular processes such as proliferation, differentiation, development, learning, survival and, under some conditions, also apoptosis. The ability of this cascade to regulate so many distinct, and even opposing, cellular processes, raises the question of signaling specificity determination by this cascade. Here we describe mechanisms that cooperate to direct MEK-ERK signals to their appropriate downstream destinations. These include duration and strength of the signals, interaction with specific scaffolds, changes in subcellular localization, crosstalk with other signaling pathways, and presence of multiple components with distinct functions in each tier of the cascade. Since many of the mechanisms do not function properly in cancer cells, understanding them may shed light not only on the regulation of normal cell proliferation, but also on mechanisms of oncogenic transformation.

Keywords: MAPK; ERK; MEK; Signaling specificity

1. Introduction

Even though cells are surrounded by a membrane that provides a separation between the outer and inner environments, they are able to respond to extracellular stimuli such as mitogens and hormones, and convert their signals into cellular processes. This conversion is usually mediated by the binding of extracellular ligands to specific transmembranal receptors, which are consequently activated to further transmit the signals of the different ligands through intracellular signaling pathways. These pathways, operating within complex networks of interacting proteins, transmit the signals to various intracellular targets, thereby regulating inducible cellular processes such as transcription, translation, proliferation, differentiation and apoptosis. Central building blocks in the intracellular signaling network are the mitogen-activated protein kinase (MAPK) cascades [1–4]. Transmission of signals via these cascades is usually initiated by activation of a small G protein (e.g., Ras), which is followed by a sequential activation of several sets of cytoplasmic protein kinases. Each of the cascades is composed of three to five tiers (MAP4K, MAP3K, MAPKK, MAPK and MAPKAPK), and three tiers out of them, MAP3K, MAPKK, and MAPK are considered to be the core cascade. One or more kinase component in each of these tiers phosphorylate and activate components in the next tier, until a downstream component phosphorylates target regulatory molecules that initiate the required physiological process. Four distinct MAPK cascades (ERK, JNK, p38 and BMK) have been elucidated, although their number is likely to increase, due to the identification of novel MAPKs that are not specified to any known pathway [5]. Each of these cascades is named after the subgroup of its MAPK component, but the MAPKK and, to some extent, the other tiers, also contain components that are selective to each cascade. All these cascades cooperate in transmitting signals from many ligands, and thereby determine the cell's fate upon distinct extracellular stimulations. In this review we focus on the extracellular signal-regulated kinase (ERK) cascade, which was the first MAPK to be elucidated [6], and describe mainly the components in the MAPKK and MAPK tiers of the cascade (MEKs and ERKs). As other MAPK cascades, the ERK cascade is activated by various stimuli, and participates in the regulation of proliferation, differentiation, survival, learning, apoptosis and more. We devote a big part of the review to describe the mechanisms that dictate the signaling specificity of the cascade, regulate its downstream activity, and

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doi:10.1016/j.bbamcr.2006.10.005

allow it to initiate so many distinct and even opposing cellular processes.

2. Components and regulation of the ERK cascade

2.1. MAP3Ks and MEKs

The ERK cascade is activated by a large number of extracellular stimuli and various internal processes. Upon its activation, the cascade plays a central role in the induction of processes such as proliferation, differentiation, development, and under certain conditions also in cell survival, learning, migration, apoptosis, morphology determination and oncogenic transformation [6–9]. The signaling via this cascade (Fig. 1), is usually initiated by activation of small G proteins (e.g. Ras), which transmit the signal further by recruiting the MAP3K tier Raf kinases to the plasma membrane, where they are activated [10]. Other MAP3K components that participate in the activation of ERKs under specific conditions are (i) c-Mos that acts specifically in the reproductive system [11]; (ii) the protooncogene TPL2 [12]; and (iii) MEKK1 [13] that acts mainly under stress conditions. An additional protein kinase in this tier might be the kinase suppressor of Ras (KSR), but the role of its catalytic activity is still controversial [14], as it seems to act mainly as a scaffold protein for the ERK cascade [15]. All these MAP3Ks transmit the signals further by phosphorylating and activating the MAPKK tier proteins, MAPK/ERK kinases (MEKs; [16,17]), which funnel upstream signals into the characteristic linear cascade, as observed in lower organisms [18].

The MEKs constitute an evolutionarily conserved group of three highly homologous (~85%) mammalian isoforms [19–21]. These isoforms are the 45 kDa MEK1, its alternatively spliced form, MEK1b (43 kDa) that is thought to be inactive, and the 46 kDa MEK2. These proteins are composed of a catalytic kinase domain, which is surrounded by a regulatory N-terminal domain (~80 amino acids) and a shorter C-terminal region (~30 amino acids). X-ray analysis of truncated human MEK1 and MEK2 revealed that they are indeed similar in structure to other protein kinases [22]. MEKs are activated by phosphorylation of two Ser residues in their activation loop (Ser218 and Ser222 in MEK1) located within a Ser–Xaa–Ala–Xaa–Ser/Thr motif [23], typical to all MAPKKs. This phosphorylation may be regulated in part by a direct interaction of the domain for versatile docking (DVD) region in MEKs with their upstream activators [24]. The activity of MEKs is regulated by additional phosphorylation/dephosphorylation processes as well. Amongst these are the phosphorylation of Ser386 of MEK1 by ERKs [25], which can either inhibit ERKs activity [26], or under other conditions, facilitate the activation by enhancing the binding of MEK1 to the scaffold Grb10 [27]. Phosphorylation of Ser298 of MEK1 by p21-activating protein (PAK1) plays an accessory role in MEKs' activation [28,29], a process that can be inhibited by a feedback phosphorylation on Thr292 of MEK1 by ERKs [30]. Several other phosphorylation sites on MEK1, which might regulate its activity are detailed elsewhere [31–33]. Finally, the downregulation of MEKs involves a rapid dephosphorylation of pSer218 and pSer222, mainly by the protein Ser/Thr phosphatase PP2A [34], but possibly also other phosphatases.

Upon activation, MEKs act as dual specificity kinases and phosphorylate the regulatory Tyr and Thr residues of ERKs at the next tier of the cascade, thereby causing their activation [35]. Importantly, ERKs seem to be the only physiological substrates of MEKs, suggesting that these kinases serve as the specificity determining components of the ERKs within the

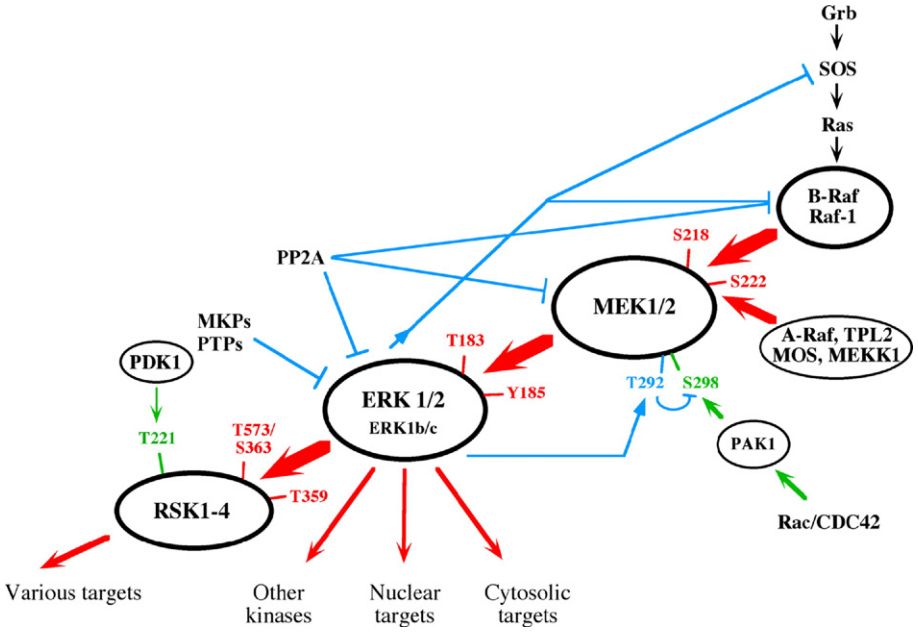

Fig. 1. Schematic representation of the ERK signaling cascade. Activation and inactivation processes are indicated. Dashed lines indicate indirect activations, and bold arrows stand for the main pathway upon growth factor activation. For more details see text. Red—activatory phosphorylation. Green—accessory phosphorylation. Blue—inhibitory phosphorylation and dephosphorylation.

cascade. Aside from their role as ERK activators, MEKs seem to function as cytoplasmic anchor proteins for ERKs [36,37]. In addition, it was shown that MEKs translocate into the nucleus of resting cells [38], and this process is facilitated upon stimulation [39,40]. However, the nuclear MEKs are rapidly exported out of this location by a NES region in their N-terminal domain. This shuttling allows MEKs to serve as a nuclear exporting platform that operates on ERKs [41], PPARγ, and possibly other nuclear proteins. In addition, this translocation is probably required for the phosphorylation of the nuclear ERK isoforms such as ERK1b and ERK1c [42,43].

2.2. ERKs and MAPKAPKs

The next tier in the cascade, which contains the MAPK components, is composed of the ERK proteins that are the evolutionary conserved products of the two genes, erk1 (Mapk3) and erk2 (Mapk1) [44–46]. Aside from the two main protein products, the 44 kDa ERK1 and the 42 kDa ERK2, several other alternatively spliced forms have been described, including the rodent 46 kDa ERK1b [42], the primate ERK1c [43] and the ERK2b [47]. All these kinases are composed of a catalytic kinase domain wrapped by regulatory stretches, and also contain an unique insert sequence within their kinase domain [46]. As mentioned above, the ERKs are all activated by dual phosphorylation on their regulatory Tyr and Thr residues located within the Thr–Xaa–Tyr motif [48]. This unique dual phosphorylation seems to be mediated solely by MEKs, which recognize only the native conformation of ERKs [35]. The molecular mechanism that allows full activation of ERKs by MEKs was extensively studied over the past decade. Mutational analysis has uncovered a region located at the C-terminus of ERKs (amino acids 312–320 in ERK2), important for its interaction with MEKs [37,49]. This region was termed initially cytoplasmic retention sequence (CRS), but was later shown important for the interaction of ERKs with many other proteins, and therefore, also termed common docking (CD) domain [50]. This region is one of the docking sites in the ERK molecule that allows binding of the kinase to a variety of proteins as described below. The most important interacting residues in this region are three acidic amino acids (Asp316, Asp819 and Glu320 in ERK2), as well as a few hydrophobic ones. This region was shown to interact with three basic, and two hydrophobic residues in the N terminus of MEKs, termed the D domain. Other regions in ERKs that can contribute to the binding energy between ERKs and MEKs are (i) the β7–β8 and αD-αE groove that interacts mainly with the hydrophobic region of the D-domain [51]; (ii) several residues within the kinase insert domain in the C-terminal lobe that may either associate with MEK or secure the proper conformation required for the binding [52]; and (iii) possibly also the N terminal domain [53]. Crystallization studies have revealed that binding of the D domain of MEKs to the CRS/CD domain of ERKs induces a conformational change that exposes the regulatory Thr and Tyr residues to the environment [54], and thereby allows the attached MEKs to phosphorylate them. The phosphorylation leaves out critical ion pair interactions between the phosphorylated sites and the HRD motif and C-helix. This induces a rotation and depression in the surface of the catalytic pocket of ERKs to form a pocket that allows the binding of the ERKs' substrates, and fits the phosphorylated Ser/Thr residue in the substrate to its right position towards the γ phosphate of the ATP [55,56]. These changes allow a full catalytic activity of the ERK2, which is 5–6 orders of magnitude higher than its basal activity [57].

The inactivation of ERKs is mainly mediated by removal of the phosphates from either one, or both of the regulatory Thr or Tyr residues of ERKs [58]. This process can be mediated by either protein Ser/Thr phosphatases (PPs) such as PP2A [59], by protein Tyr phosphatases, such as PTP-SL [60], or by dual specificity phosphatases, generally termed MAPK phosphatases (MKPs, [61]). Interestingly, the PTPs and MKPs interact with the ERKs via a D domain, similar to that of MEKs [50]. Upon binding, PTPs remove only the phosphate from the Tyr residues, which is sufficient to induce complete inactivation of the ERKs [35]. However, this initial dephosphorylation is usually followed by removal of the second phosphate from the Thr residue by the PPs, which is the reason for the lack of significant amounts of pTyr-ERK in the cells [62]. MKPs inactivate MAPKs by simultaneously removing phosphates from both pTyr and pThr residues, although the dephosphorylation of pTyr may slightly precede that of pThr [63]. Apart from the downregulation by phosphatases, ERKs seem to participate in multiple feedback loops that are important for the reduction of their activity at later stages after stimulation (Fig. 1). These include inhibitory phosphorylation of the upstream exchange factor SOS [64], Rafs [65,66], and MEKs [29].

Upon phosphorylation of the regulatory Tyr and Thr residues, ERKs become a potent protein Ser/Thr kinases, which phosphorylate substrates on their consensus sites, which is in most cases, Pro–Xaa–Ser/Thr–Pro [67]. However, the shorter sequence Ser/Thr–Pro alone is sometime sufficient to direct ERKs' phosphorylation, and the phosphorylation of SOS seems to occur on a site that does not contain any adjacent Pro residues [64]. In addition, many of the substrates can interact with ERK via specialized docking domain including the D domain described above for MEKs. Another important domain on ERK interacting proteins is the docking site for ERK–FXF (DEF domain), which consists of Phe–X–Phe–Pro consensus sequence [68]. This site binds to a hydrophobic cluster in the bigger lobe of ERKs known as the DEF binding domain [69]. Additional docking motifs in specific substrates are likely to emerge in the future. Thus, the combination of docking motifs together with catalytic site interactions, often function in a tripartite manner to direct interactions between ERKs and their substrates to participate in the determination of their substrate specificity.

Upon stimulation, ERKs have been demonstrated to phosphorylate a large number of substrates [9]. Some of these substrates are localized in the cells' cytoplasm, while others are phosphorylated in the nucleus by ERK molecules that are translocated into this organelle upon stimulation [9]. Notably, ERKs phosphorylate and activate a series of transcription factors such as Elk1 [70], c-Fos [71], p53 [72], Ets1/2 [73], and

even c-Jun [74], which are important for the initiation and regulation of proliferation and oncogenic transformation. Alternatively, the ERKs can transmit the signal further by phosphorylating and activating protein kinases at the MAPKAPK tier. The main MAPKAPK of the ERK cascade are the 90 kDa ribosomal S6 kinases RSKs [75], which can independently translocate into the nucleus and phosphorylate a distinct set of substrates there. Additional MAPKAPKs are the mitogen- and stress-activated kinase (MSK; [76]), and the MAPK-interacting kinases (MNKs; [77,78]), that are equally activated by the related p38 cascade or by other kinases [79]. Finally, MAPKAPK3 [80], and MK5 [81] are only slightly activated by ERKs, as they are more responsive to p38s [82]. MAPKAPKs can regulate the activity of additional kinases (e.g. Myt1 [83]), but these are usually not considered to be genuine members of the ERK cascade. All the above substrates change their activity upon ERKs phosphorylation, and thus regulate ERKs-dependent processes upon most stimulations.

3. Specificity determination

The ability of ERKs to transmit different, and even opposing, signals in the same cells raises the question as to how the specificity of the different signals transmitted by the ERK cascade is regulated. Several mechanisms that participate in this specificity determination have been proposed in the past years (Fig. 2), including: (i) duration and strength of the signals [84–86]; (ii) interaction with various scaffold proteins [15,49,87]; (iii) subcellular localization [88,89]; (iv) extensive cross-talk and interplay between the ERK cascade and other intracellular signaling pathways [1]; and (v) presence of several similar isoforms, at each tier of the cascade [90], including alternatively spliced forms [43], which are all described below. Although these mechanisms could independently determine signaling specificity, they are often cooperating with each other to secure proper downstream effects. In addition, the effect of similar ligands on different cells is influenced by other mechanisms, such as the expression of specific substrates [9], but these aspects are not discussed here.

3.1. Duration and strength of the signals

The first model that was proposed to explain signaling specificity by the ERK cascade involved changes in the duration and strength of the signals transmitted by the cascade [84]. The model was mainly based on the kinetics of ERKs activation in PC12 cells upon stimulation with EGF that causes proliferation, as opposed to stimulation with NGF that leads to neurite formation (differentiation). Thus, it was shown that EGF stimulation causes a strong but transient activation of the ERK cascade, while NGF causes an equally strong but sustained activation [91]; both effects were shown to be dependent on the activity of ERKs. It was also shown that artificial prolongation of ERKs activity upon EGF stimulation changes the effect of this factor to differentiation rather than proliferation [92]. This correlation between the duration of ERK activity and its effects on cellular processes has also been observed in other systems such as oncogenic transformation of fibroblasts [93], and even in yeast, where sustained activation of the MAPK homolog KSS1 is required for filamentous growth, while a transient activity of this kinase results in a mating response [94]. It should be noted that much of the

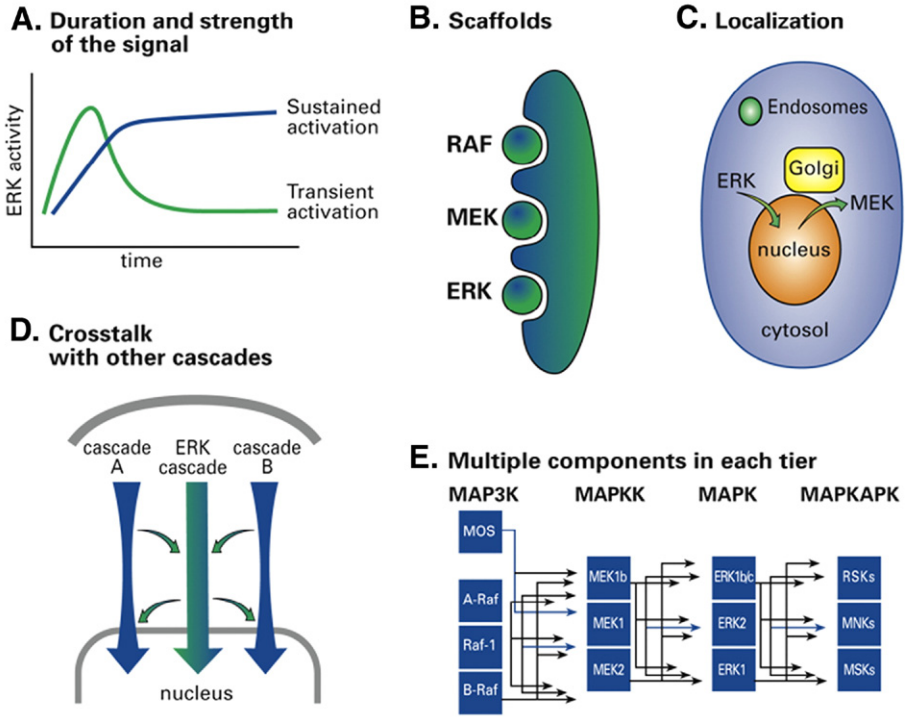

Fig. 2. Schematic representation of mechanisms that determine the signaling specificity of the ERK cascade. For more details, see text.

duration of ERKs activity is regulated by phosphatases (see above), or through downregulation of upstream tiers of the cascade [95].

The regulation of the kinetics of ERKs activation by extracellular signal has been extensively studied in the past decade, and was shown to involve several unrelated mechanisms that operate separately in each tier of the receptor-mediated pathways [86,95]. However, less is known about the way by which the duration of the signals influences the downstream processes. Interestingly, such a mechanism has been recently proposed by Murphy et al. [71,86,96], who demonstrated that signal duration is likely to influence the transcription and activity of immediate early genes, such as c-Fos, c-Jun, c-Myc and Egr1. The most prominent example for this mechanism is the transcription factor c-Fos, a known target of the ERK cascade [86] that induces both its expression and stabilization upon stimulation. Thus, when ERKs activation is transient, they are able to induce the expression of c-Fos, although ERKs activity declines before the c-Fos accumulates, which prevents the stabilization of the latter. On the other hand, when ERKs activity is sustained, c-Fos can be phosphorylated and activated by ERKs and RSKs. As a consequence, the phosphorylated c-Fos can induce expression of a larger repertoire of genes, and thus participate in relatively late processes such as cell cycle progression. Although this is an attractive mechanism, it cannot explain all of the effects of a longer duration of ERKs activity. Additional mechanisms, such as protection of ERK phosphorylation by specialized interacting proteins [97], stabilization of additional proteins [98], effects on chromatin remodeling [99], and others, are likely to emerge as key steps in regulating ERKs specificity and functions.

3.2. Scaffolds

A second mechanism that contributes to the specificity of the ERK cascade is the formation of multi-protein complexes, primarily due to interaction with scaffold proteins [49,87]. The importance of scaffolds for MAPK signaling was first demonstrated in yeast [100], where different scaffolds could direct signaling components to regulate distinct processes. An example is the Ste11 protein, which acts as a MAP3K of two distinct pathways. In the mating response, Ste11p is recruited by the scaffold protein Ste5p to interact with, and activate, the MAPKK Ste7p and the MAPKs Fus3/Kss1 [101]. Conversely, in response to osmotic pressure, Ste11p is recruited by the MAPKK Pbs2, which serves as a scaffold that directs the signals to the MAPK Hog1 [102]. Importantly, Ste5p not only affects Ste11p signaling, but can also act as a switch between the MAPKs, Fus3 and Kss1, in the mating response [103].

In the past few years, scaffold proteins were also implicated in the regulation of signaling cascades in mammals [104]. For example, the JNK cascade is highly regulated by several distinct scaffold proteins, and other scaffolds have been identified for the ERK and p38 signaling cascades [105]. Notably, most of these scaffolds have a very little, or no sequence similarity to the yeast scaffolds, and therefore, their effects need to be studied mainly in mammalian systems. The importance of scaffold proteins in mammals lies mainly in their ability to recruit proteins belonging to a specific signal transduction event, and bring them to a close proximity to each other [106]. In addition, scaffold proteins can provide better stability to some of the cascade components, to determine the threshold of signaling and lead to different functions of a given cascade by recruiting of different substrates to individual cascade components [87,107,108]. Finally, scaffolds can determine the localization of the cascade components [109], or protect signaling components from phosphatases, thereby regulating their activity [97]. By doing so, scaffold proteins induce faster kinetics of activation of their signaling cascade, modify signaling duration and intensity, secure better interaction between distinct signaling components, regulate the localization of the cascade's proteins, and modify crosstalk with other signaling pathways [87]. Thus, scaffolding supports several other mechanisms that can independently determine signaling specificity, and therefore, are key components in the signaling of most cascades. It should be noted that the term scaffold is usually reserved to proteins that interact with at least two, or more often, three or four components of a given cascade. Mono-interactions with certain component also exist, but those are referred to as interacting or anchoring proteins, rather than scaffolds. As of today, more than 50 scaffolding and anchoring proteins have been described for the ERK cascade [49], and description of many of them is found in another review in this issue. Nonetheless, several scaffolds that play a key role in the determination of ERK cascade specificity are described below.

One of the best studied scaffolds of the ERK cascade is the KSR1, initially identified as a key component in the ERK signaling in *D. melanogaster* and *C. elegans* [110–112]. Although KSR1 has high homology to Raf1, it is still controversial whether it can physiologically phosphorylate any substrate involved in ERK signaling [14]. In fact, many studies support a kinase-independent function of KSR1 [113], which appears to serve as a scaffold protein [15,114]. Thus, it was demonstrated that in quiescent cells, KSR1 is associated with MEKs [106], but not with ERKs or Raf kinases. In addition, it interacts with c-Tak1, which constitutively phosphorylate its Ser392 [115], with the adaptor protein 14-3-3 [116], with inactive PP2A [117], and with the inhibitory E3 ubiquitin ligase, IMP1 [118], which assists in maintaining the cytosolic localization of the KSR1 complex, [15]. Upon stimulation, IMP1 is recruited by Ras-GTP, and this is followed by a polyubiquitination of the IMP1 and its subsequent degradation. In parallel, the associated PP2A is activated to dephosphorylate Ser392 in KSR1, which is essential for dissociation of 14-3-3 protein [115]. These two processes together allow the rest of the KSR1 complex to translocate to and interact with the plasma membrane, where it recruits an activated Raf1 [118], which causes a facilitated activation of MEKs. Simultaneously, ERKs are recruited to the activated complex, and this facilitates their phosphorylation and activation [106], which are followed by the release of ERKs from the complex. The released active ERKs are then able to translocate to their sites of action in the cytoplasm or the nucleus and induce most ERKs-dependent cellular functions [15].

Besides KSR1 that seems to serve as a general scaffold upon various stimulations, other scaffolds seem to attract portions of the MEK and ERK molecules to specialized signaling events. One such protein is β-arrestin [119], that participates in ERK signaling upon G-protein-coupled-receptor (GPCRs) stimulation [120]. The interaction of ERKs, as well as MEKs and Raf1 with β-arrestin seems to be irreversible [119], thereby, the β-arrestin-facilitated activation of the ERK cascade in the membrane prevents ERKs from translocating into the nucleus, resulting in a preferential phosphorylation of cytoplasmic substrates [121]. Another scaffold that directs the ERK cascade to specific signaling is paxillin, which is a member of the focal adhesion protein family that plays a central role in focal adhesion assembly and is essential for cell spreading and migration [122]. Interestingly, it was shown that upon stimulation with hepatocyte growth factor, paxillin interacts with components of the ERK cascade, to regulate epithelial morphogenesis [123,124]. Similar to KSR1, paxillin seems to be constitutively associated with MEKs, whereas ERKs and Raf1 are recruited to paxillin only upon activation [123]. However, unlike KSR1, paxillin interaction partially prevents the nuclear translocation of ERKs and directs ERKs' signal to cytoplasmic targets. Other prominent scaffolds are Sef1 [125] and MEK partner 1 (MP1 [126]), which seem to be responsible mainly for directing the ERK cascade into certain compartments within the cytoplasm, and therefore, will be described under Subcellular localization below. Overall, it would appear that many of the functions of the ERK cascade are funneled through designated scaffolds, which dictate the kinetic, localization, and components of the cascade, in determining much of its specificity.

3.3. Subcellular localization

A third mechanism that contributes to the specificity of the ERK cascade is the restriction of components of the ERK cascade to specific cellular compartments, and the dynamic changes in their localization after stimulation [9,88,89]. These processes direct signaling components such as Ras and constituents of the ERK cascade into specific targets or organelles (e.g. Golgi [7,89]), where they may induce distinct functions [127]. Therefore, the signaling in one cellular compartment may have a different outcome from a similar signaling event in another localization. For examples, forcing nuclear localization of an ERK2–MEK1 chimera resulted in an increased transcriptional activity [128], and forcing membranal association of ERKs resulted in an attenuated transcriptional activity [129]. In addition it was shown that restricted cytoplasmic ERK2 activity protected, while ERK2 activation restricted to the nucleus antagonized proapoptotic stimuli in myeloid leukemia cells [130]. More on the molecular mechanisms that explain these differences in signal propagation in distinct subcellular localization are followed.

In resting cells, all components of the ERK cascade seem to be localized primarily in the cytoplasm. The mechanisms that govern this localization in resting cells are distinct for the different components. The exact mechanisms that anchor Raf1 to the cytoplasm in close proximity to the plasma membrane is not yet clear, but might involve interaction with liposomes, lipid rafts, or other lipid moieties [131]. MEKs seem to be localized in the cytoplasm of resting cells mainly due to its nuclear export signal in its N-terminal domain [38,39]; it is possible however, that residues in the C-terminal domain are also involved in this localization [132]. Finally, the interaction of MEKs with a large number of scaffold and anchoring proteins [32] may contribute to their precise localization as well.

ERKs and RSKs seem to be attracted to the cytoplasm, mainly by a relatively strong interaction with a large number of anchoring proteins. These anchors are probably responsible for the diffuse cytoplasmic appearance of the kinases, as well as their localization in specific microenvironments within the cells [49]. Among these interacting proteins, one can find microtubules [133] MEKs, [36,37], phosphatases [134], scaffolds, and other anchoring proteins [49]. The interaction of ERKs with many of these proteins is mediated by the cytoplasmic retention sequence (CRS/CD) of ERKs [37,50]. Other anchoring interactions might occur through different part of the ERK molecule such as residues 91–95 of ERK2, responsible for the interaction with microtubules [135], or even the DEF binding domain in the bigger lobe of the kinase domain [69]. Interestingly, the interactions mediated by the CRS/CD seem to be reversible due to conformational changes that occur upon phosphorylation of ERKs [136], whereas interactions through other regions in ERKs are more stable, as they are usually not reversed upon cellular stimulation [133].

The cytoplasmic distribution of the components of the ERK cascade is dramatically altered upon extracellular stimulation. Thus, Raf1 is recruited to the plasma membrane due to its interaction with activated Ras [137], while MEKs, ERKs and RSKs are released from their cytoplasmic anchors, causing much of their molecules to translocate into the nucleus [39,138]. Since these components of the ERK cascade do not contain nuclear localization signal (NLS), the mechanism of their nuclear translocation is not fully understood. It was shown that the ERKs [41] as well as MEKs [139] translocate to the nucleus using either a non-regulated or a facilitated mechanisms. The facilitated mechanism was proposed to require homodimerization of ERKs that may occur upon extracellular stimulation [41,140]. However, other studies found that mutating the dimerization sites does not significantly influence the stimulated translocation [136], and therefore, the importance of dimerization is still controversial. Recently, it was shown that this cyto-nuclear shift may occur by a direct interaction with several nuclear pore proteins (NUPs, [141,142]) but more studies are required in order to fully elucidate the mechanisms of nuclear translocation of ERKs and MEKs. Shortly after stimulation, MEKs are exported from the nucleus by the exportin system that utilizes their NES sequence [39]. ERKs and RSKs stay in that location longer, possibly due to the interaction with newly synthesized nuclear anchors [143]. Interestingly, the ability of MEKs to undergo nucleocytoplasmic shuttling may also result in a MEKs-induced export of

ERKs out of the nucleus [144], bringing the situation back to the initial basal conditions.

As mentioned above, the localization of ERKs, both before and after stimulation, is largely dependent on their interaction with various regulatory proteins. One such protein is phosphoprotein enriched in astrocytes (PEA-15), a widely expressed 15-kDa protein with a death-effector-domain [145,146]. In the cytoplasm, PEA-15 binds ERKs, but not any other component of the ERK cascade. This binding prevents ERKs' nuclear translocation, without inhibiting their kinase activity, therefore, causing a reduction in the nuclear activity of ERKs without much effect on the cytoplasmic activity [145]. The mechanism by which PEA-15 prevents nuclear translocation of ERKs is still unclear, but may involve PEA-15-dependent interference with ERKs' binding to NUPs [146]. Alternatively, it is possible that the NES of PEA-15 attracts ERKs to the cytoplasm, as previously reported for MEKs. Thus, PEA-15 acts as a cytoplasmic regulator that redirects ERKs' signaling to different targets.

Another protein that influences ERKs and MEKs localization is the MP1 [126]. This is a 13.5 kDa protein, which interacts with MEK1 and ERK1, but not with MEK2 and ERK2. In vivo studies demonstrated that MP1 does not significantly enhance ERK1 activation, indicating that MP1 may play another role in the regulation of the ERK cascade. Indeed, it was later found that the MP1–ERK1 complex can interact with the endosomal protein P14, which directs the localization of ERK1 and MEK1 to the endosomes/lysosomes, where they execute a distinct regulatory function [147]. Interestingly, MP1 can also interact with the scaffold protein MORG1, which directs it to different compartments, allowing it to regulate another set of intracellular signals [148]. Finally ERKs and MEKs can interact with an additional anchoring protein, termed Sef1, which direct MEKs and ERKs to the Golgi apparatus, prevents nuclear translocation of ERKs and may regulate cell cycle [7,125]. In summary, the localization of components of the ERK cascade, determined by specific sequences in ERKs MEKs and their interacting proteins, is important in directing signals to their appropriate substrates, and thereby, is a key component in dictating signaling specificity of the ERK cascade.

3.4. Cross-talk and interplay with other cascades

A fourth mechanism that contributes to the specificity of the ERK cascade is the interplay of the cascade with other signaling cascades. Although the 3–5 tiers of the ERK cascade usually form a linear pathway, several other signaling cascades can regulate the activity of components in several tiers of the cascade, and thus, modify their activation upon distinct physiological conditions. These modifications, which usually affect the kinetics strength and localization of the signals, are mediated mostly due to phosphorylation/dephosphorylation by kinases and phosphatases that are not always part of the ERK cascade. In addition, many of the signals by other cascades are likely to converge with that of the ERK cascade at downstream components, such as transcription factors and downregulating molecules [1], but this will not be discussed much in the current review. On the other hand, we bring here two examples of direct effects by unrelated cascades that can either activate or inhibit the ERKs signaling, and thereby significantly influence the specificity of the signals.

One of the signaling pathways that contributes to the activation of the ERK cascade is the Rac1/CDC42-PAK1 cascade. Rac1 and cdc42 are members of the Rho subfamily of small GTP binding proteins, which are activated by cell adhesion, to mediate lamellipodia extension and focal adhesion formation through the downstream effector, PAK1 [149]. The localization and activation of PAK1 in focal adhesion complexes lead to dynamic changes in cytoskeleton organization, which is often mediated by phosphorylation and activation of LIM kinase [150], or by inhibition of myosin light chain kinase [151]. Interestingly, aside from these kinases, PAK1 can also phosphorylate MEK1 in the focal adhesion complexes, which is an important step in the regulation of cell adhesion and spreading. Therefore, this phosphorylation is a site of convergence for integrin and growth factor signaling [28, 29].

In the past few years, the role of MEKs phosphorylation by PAK1 has been elucidated. Thus, adhesion of fibroblasts to fibronectin induces PAK1-dependent phosphorylation of MEK1 on Ser298. This phosphorylation is insufficient to induce activation of MEK1, a process that is mediated under these conditions by Raf1-induced phosphorylation of residues Ser218 and Ser222. However, this phosphorylation seems to support association of MEK1 with Raf1 [28], and to further enhance the association of MEK1 with ERKs that generates a more efficient activation of the ERK cascade in newly adhering cells [152]. Interestingly, the phosphorylation of MEK1 by PAK1 is subjected to a negative feedback regulation, as it was demonstrated that upon activation, ERKs can phosphorylate Thr292 of MEK1 [153], which is located in close proximity to the PAK1 phosphorylation site. This additional phosphorylation was recently shown to block the ability of PAK1 to phosphorylate Ser298, and thereby, reduce the Rac1-PAK1-dependent MEK1-ERKs complex formation [30]. Therefore, this phosphorylation is likely to participate in the down-regulation of the ERK cascade in later stages after stimulation, which seems to be a common negative feedback loop of the ERK cascade, also operating on Sos and Raf kinases (Fig. 1). It was recently reported that inhibition of PAK1 did not affect MEK1 activity induced by EGF, although it did affect the activity induced by PDGF [154]. Moreover, the scaffold complex MP1-p14 seems to participate in this differential regulation as it allows MEK1, not MEK2 activation upon adhesion, but not PDGF stimulations [155]. Therefore, the phosphorylation by PAK1 provides a mechanism to confer specificity upon certain converging stimulations.

Another group of kinases that may participate in the regulation of the ERK cascade, and therefore, exhibit a crosstalk between two regulatory systems is the cyclin dependent kinases (CDKs). This group is composed of several Ser/Thr kinases that are key components in the regulation of cell cycle progression [156]. Several studies demonstrated that members of this family, such as CDK1 [153] and CDK5 [157], can phosphorylate

MEK1, and consequently modify its activity. The regulation by these kinases is likely to play a role in different stages of the cell cycle, as CDK1 is a key regulator of the G_2/M phase of the cell cycle [156], while CDK5 may participate in cell cycle-independent processes [158]. Both CDKs were demonstrated to phosphorylate MEK1 on Thr286 and Thr292 [153,157,159]. However, unlike the PAK1-dependent phosphorylation, which acts as a positive regulator, the CDKs phosphorylation leads to the inhibition of MEK1 activity. This is probably not due to reduction of PAK1 activity, as proposed for ERKs phosphorylation on Thr292 described above, since the inhibition was observed under conditions with no PAK1 activation. A mechanism that was proposed for this inhibition was a phosphorylation-dependent photolytic cleavage of MEK1 at its N-terminal ERK-binding domain [159]. It should be noted however, that the role of CDKs in MEK1 regulation is still controversial, as a recent study demonstrated that the phosphorylation of Thr286 of MEK1 is unable to inhibit constitutively active MEK1 [160], and it is, thus far, not clear how Thr292 phosphorylation induces cleavage of the N-terminus of MEK1. Nonetheless, it is clear that CDKs can regulate ERKs activation during cell cycle progression [160], and this, as well as the phosphorylation by PAK1, provides a tool to determine the specificity of the ERK cascade under unique conditions.

3.5. Multiple isoforms in each tier of the cascade

A fifth specificity-determining mechanism might be the existence of various components with distinct regulations or functions in each tier of the ERK cascade. Indeed, the MAP3K tier of the cascade contains quite a few components, including Raf kinases, c-Mos, TPL2, MEKK1 and others, which operate under distinct conditions as described in other reviews in this issue [4]. On the other hand, the extensive sequence similarity between the components in the next tiers (MEKs and ERKs), and their identical substrate recognition, led to the initial conclusion that the isoforms in these tiers (MEK1 and MEK2 as well as ERK1 and ERK2) are functionally redundant. However, based on some biochemical, and especially genetic studies, it is now clear that some differences in regulation between the different isoforms do exist. In addition, the existence of specific alternative spliced isoforms of the ERKs and MEKs clearly extend the number of targets, and thereby, expand the number of processes regulated by the ERK cascade. Some of these components and their specific targets are described below.

In the MEK tier, MEK1 and MEK2 are ~80% identical to each other [21], and are essentially identical in most of their kinase domain. In accordance to this similarity, it was initially shown that MEK1 and MEK2 phosphorylate ERKs equally well, both in vivo and in vitro. Moreover, the constitutively active MEK1 and MEK2 mediate the activation of similar transcriptional and morphological responses [161], and both MEKs are required for proliferation in HeLa cells [162]. However, unlike the kinase domains, the N-termini and the characteristic Pro-rich inserts (residues 285–296 that are phosphorylated by PAK1, ERKs and CDKs, as described above), are quite divergent between the two MEKs (40% identity). Indeed, differential functions of MEK1 and MEK2 were demonstrated using MEK1 and MEK2 knockout mice. Thus, knockout of MEK1 causes an embryonic lethality of the *MEK1*$^{-/-}$ mice, which die at 10.5 days of gestation, due to a lack of angiogenesis in the labyrinthine region of the placenta. [163]. On the other hand, *MEK2*$^{-/-}$ mice are viable and fertile, with no observed morphological alternation [164]. Consistently, molecular differences in the activity of MEK1 and MEK2 have been reported by several studies over the years. It was initially shown that Ras and Raf1 form a signaling complex with MEK-1, but not MEK-2 [165], and that upon EGF stimulation in HeLa cells, Raf1 can activate both MEK1 and MEK2, whereas A-Raf only activates MEK2 [166]. This may affect MEKs activity during cell cycle progression, as reduction in MEK1, but not MEK2, levels results in a G_2/M arrest [162], and they also demonstrate a differential effect on the G1/S phase of the cell cycle [167].

Aside from MEK1 and MEK2, an alternative spliced isoform of MEK1, termed MEK1b, which lacks 26-amino acid residues within its kinase domain, was identified in human [20,21]. Initial studies on this isoform indicated that it might be catalytically inactive, and therefore, can be considered as a dominant negative isoform of MEK1 [21]. However, recent studies in our laboratory indicate that this isoform may be active upon certain cellular stimulations (data not shown), and therefore can function separately from MEK1 and MEK2 under restricted conditions. From the description above, it seems that MEK1 and MEK2 might be functionally redundant in the regulation of certain processes, but can exhibit unique activities under other conditions to extend the specificity of the ERK cascade.

In the MAPK tier, ERK1 and ERK2 are also very similar proteins, with about 70% similarity between them, which is higher in the kinase domain and lower in the flanking N- and C-terminal regions [46]. Numerous studies using anti-ERK and anti-phosphoERK antibodies revealed that the two isoforms are expressed in essentially all cells and tissues in variable relative amounts, whereby ERK2 is the predominant isoform in most cells [168]. Usually, both proteins share similar activation kinetics, cellular localization, and a set of substrates [168], indicating that under most circumstances the two isoforms function in a similar fashion. However, as described for MEKs, some differences between the two isoforms do exist under certain restricted conditions, and those differences are best exemplified by the use of ERK1 and ERK2 knockout mice. Thus, ERK1-deficient mice are viable, fertile, with normal size, but manifest a deficit in thymocyte maturation [169]. Moreover, these mice exhibit an elevated synaptic plasticity in the striatum, [170] which could be a result of a stimulus-dependent elevation in ERK2 phosphorylation, which was observed in neurons [170] as well as fibroblasts [169] of these mice. Conversely, ERK2-deficient mice die early in development, showing that ERK1 can't compensate for ERK2 in the embryo [171–173].

More detailed studies with T-cell-specific knockout of ERK1 and ERK2 revealed that both kinases are essential for the positive selection of T-cells. However, some of the downstream

activities, such as activation of the transcription factor Egr1 in this tissue were dependent on the total ERKs activity, and therefore, could be activated only by the more abundant ERK2 [174]. These results may therefore, indicate that some of the differences in ERK1 and ERK2 functions are related to their expression levels, and not so much to their actual substrates. Nonetheless, recent results indicate that the changes in expression are not always the cause of differences between ERK1 and ERK2. Accordingly, it was shown that ERK1 participates in MEKs signaling during G_2/M, while ERK2 seems to be essential during the G1 phase of the cell cycle [162]. Finally, unexpected differences between the two isoforms were found during Ras-dependent signaling in fibroblasts [90,175], where ablation of ERK1 promotes cell proliferation, while ERK2 knockdown blocks this effect. These data suggest that unlike the proliferation promoting effects of ERK2, ERK1 may actually inhibit proliferation of Ras-transformed cells. Thus, in similarity to MEKs, ERKs may have different effects under certain conditions, and thereby, expend the specificity of the cascade upon various stimulations.

Aside from the 44 kDa and 42 kDa ERK1 and ERK2, several alternatively spliced isoforms seems to operate downstream of MEKs in many cell lines and organisms. In rodents, a 46-kDa ERK was identified, which is a product of a 26 amino acid insertion into the CRS/CD of ERK1 [42]. Under many circumstances, ERK1b behaves similarly to ERK1 and ERK2. However, in Ras-transformed Rat1 cells, the expression levels of ERK1b are elevated, the protein is activated in a different kinetic than ERK1 and ERK2, and due to a phosphatase-dependent lower activation of ERK1 and ERK2, ERK1b becomes the major isoform that transmit extracellular signals upon oncogenic transformation [42,176]. The unique function of ERK1b is mainly caused by the interference with the function of the CRS/CD domain, which changes its subcellular localization, its regulatory phosphorylation/dephosphorylation processes, and its interactions with its various substrates [176].

In primates, similar splicing events to those in rodents results in the insertion of the same intron in close proximity to the region encodes for CRS/CD. However, in this case the insert contains a stop codon, and therefore, results in a 42-kDa protein, termed ERK1c [43]. As for ERK1b, this protein has reduced interactions with MKPs, MEKs and ERKs' substrates as compared to ERK1 and ERK2, and therefore, is regulated differently from the other ERKs. Interestingly, ERK1c was demonstrated to possess unique functions that are not observed in any of the other ERKs [43,177]. It was recently demonstrated that ERK1c expression, phosphorylation and activity are elevated during mitosis and in confluent cells as well. These events are probably important for the transmission of MEKs signals [178] that are required for the completion of mitotic and elevated density Golgi fragmentation [177]. ERK1 and ERK2 do not participate in the regulation of these processes, indicating that the alternatively spliced forms of ERKs exhibit unique functions that extend the signaling specificity of the ERK cascade. More functionally relevant, alternatively spliced forms of ERKs are likely to be identified in the future, which will even further extent the repertoire of ERKs targets.

4. Summary

The ERK cascade is a central signaling component, composed of protein kinases that sequentially phosphorylate and activate each other. This cascade is stimulated by a large variety of extracellular signals, and, as a consequence, regulates many distinct and even opposing cellular processes, including proliferation, differentiation, survival and even apoptosis. The ability of the ERK cascade to initiate and regulate all these effects raises the question as to how its specificity is determined. In order to execute all its functions, the ERK cascade is heavily regulated at several levels, mainly by different phosphatases, but also by other interacting proteins. These regulatory processes can be categorized into five distinct mechanisms that are important for the specificity determination of the ERK cascade. These include: (i) changes in the duration and strength of the signals that are regulated mainly via inhibitory signaling components, such as phosphatases that govern the induction of various immediate early genes. (ii) Scaffold proteins that bring components of the cascade to a close proximity to each other, and direct them to the proper upstream and downstream effector to facilitate their kinetic of activation. (iii) Subcellular localization that is often influenced by certain anchoring proteins, and direct the signals into their proper compartments and targets. (iv) Crosstalk with other signaling pathway that may influence the strength of the signals, and often modulates the activity of downstream targets of the ERK cascade. (v) The existence of multiple components with distinct functions in each tier of the cascade, which can track the signal into different specific targets for each of these components. As shown in this review, many of these mechanisms can cooperate in order to determine the final specificity of the cascade. Interestingly, many of the mechanisms do not function properly in cancer cells, and thereby, are probably involved in the induction and maintenance of oncogenic transformation of many cell types. Studying all these regulatory mechanisms can, therefore, lead to a better understanding of oncogenic transformation and may induce to a better design of drugs that can be used in the combat of cancer.

Acknowledgments

We thank Prof. Abraham Amsterdam, Mrs. Tamar Hanoch and Mrs. Marti Spiegel for their help in the preparation of the manuscript. Supported by a grants form La Foundation Raphael et Regina Levi and a grant from the European Community's Sixth Framework Program project IST-2004-027265-SIMAP. Rony Seger is an incumbent of the Yale S. Lewine and Ella Miller Lewine professorial chair for cancer research.

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