The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity

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Summary

ERK MAP kinase signaling plays a pivotal role in diverse cellular functions, including cell proliferation, differentiation, migration and survival. One of the central questions concerning this signaling is how activation of the same protein kinase, ERK, elicits distinct cellular outcomes. Recent progress has demonstrated that differences in the duration, magnitude and subcellular compartmentalization of ERK activity generate variations in signaling output that regulate cell fate decisions. Furthermore, several molecules have been identified as spatial, temporal or strength-controlling regulators of ERK activity. Signaling by various extracellular stimuli thus could be modulated by these regulators to give qualitative and quantitative differences in ERK activity, which are then interpreted by the cells as determinants for appropriate responses.

Key words: Cell-fate decision, Cellular response, Growth factor, MAP kinase, Signal transduction

Introduction

Extracellular-signal-regulated kinase (ERK), also known as classical MAP kinase, is a highly conserved serine/threonine kinase that phosphorylates various substrates, including many enzymes, transcription factors and cytoskeletal proteins (Sturgill and Wu, 1991; Nishida and Gotoh, 1993; Treisman, 1996; Robinson and Cobb, 1997; Chang and Karin, 2001; Pouyssegur and Lenormand, 2003). In response to a wide array of extracellular stimuli, ERK is activated through sequential phosphorylation. It is involved in diverse cellular functions. Its role in growth-factor-stimulated cell-cycle progression is well known, but ERK also regulates cell differentiation, migration, survival and other biological processes (Marshall, 1995; Bonni et al., 1999; Tunquist and Maller, 2003; Huang et al., 2004). ERK activation can thus elicit opposite outcomes, depending on the situation: cell proliferation versus cell-cycle arrest, cell survival versus cell death, and so on. It remains unclear, however, how the activation of one signaling molecule ERK transduces multiple signals from extracellular stimuli to specific cellular responses. This cannot be fully explained by cell type specificity, because ERK activation has distinct outcomes even in the same cell type (Schaeffer and Weber, 1999; Tan and Kim, 1999). Accumulating evidence has demonstrated that differences in the duration, magnitude and subcellular compartmentalization of ERK activity determine signaling specificity (Fig. 1). In addition, recent studies have identified several proteins that could control the duration, magnitude or subcellular compartmentalization of ERK activity. Here, we discuss these findings and their implications.

The duration of ERK activity

The duration of ERK activity has been implicated as a critical factor in cell fate decisions. Treatment of PC12 cells with nerve growth factor (NGF) induces sustained activation of ERK and causes their differentiation into sympathetic-like neurons, which is characterized by neurite outgrowth. By contrast, epidermal growth factor (EGF) stimulates transient ERK activation and causes cell proliferation (Gotoh et al., 1990; Nguyen et al., 1993; Marshall, 1995). When the EGF receptor (EGF-R) is overexpressed in PC12 cells, ERK activity becomes sustained and the cells undergo differentiation in response to EGF (Traverse et al., 1994). The duration of ERK activity therefore appears to determine PC12 cell fate. Only sustained ERK activation can induce expression of p35, the neuron-specific activator of cyclin-dependent kinase (CDK) 5 required for neurite outgrowth (Harada et al., 2001), although the precise mechanism that links the sustained ERK activation to the induction of p35 remains unknown. Interestingly, repetitive depolarization of hippocampal neurons, which causes protrusion of dendrites, prolongs ERK activation (Wu et al., 2001). This raises the possibility that sustained ERK activation is involved in synaptic plasticity as well as in neuronal differentiation.

The ERK-signal-duration affects different cellular responses in other cell types. Sustained, but not transient, activation of ERK is required for quiescent fibroblasts to begin to proliferate (Dobrowolski et al., 1994; Balmanno and Cook, 1999). When quiescent fibroblasts are treated with thrombin or platelet-derived growth factor (PDGF), they display sustained ERK activation and enter S phase. By contrast, thrombin-mimicking peptide (TMP) or EGF stimulates transient ERK activation and cannot induce the onset of S phase (Vouret-Craviari et al., 1993; Murphy et al., 2002). Indeed, blocking ERK activity with a specific inhibitor of the upstream kinase MEK, even

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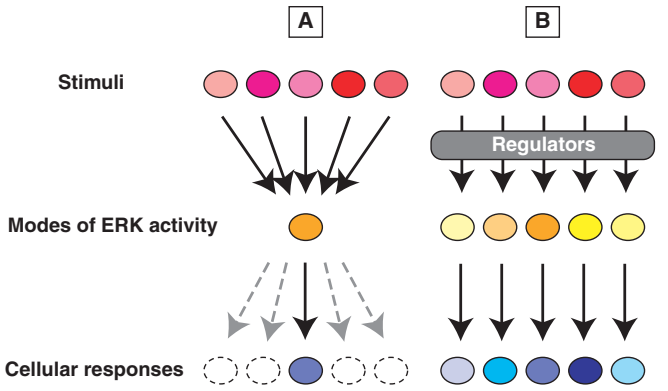

Fig. 1. A model for ERK-mediated signal transduction. (A) If various extracellular stimuli elicit a single mode of ERK activity, the ERK activation should lead to a single cellular response. (B) If several regulators control the duration, magnitude and/or compartmentalization of ERK activity upon stimulation, the resulting different modes of ERK activity can regulate multiple cellular responses.

several hours after growth factor stimulation, effectively blocks S phase entry (Weber et al., 1997).

Recently, Murphy et al. have provided clues to how sustained ERK activation causes fibroblast proliferation (Murphy et al., 2002; Murphy et al., 2004). Whereas both transient and sustained activation of ERK induce transcription of immediate early genes, such as *Fos*, *Jun*, *Myc* and *Egr1*, only sustained ERK activation causes phosphorylation and stabilization of the proteins they encode. Because most immediate early genes encode transcription factors, they should in turn change expression levels of other genes crucial for cell proliferation. In fact, the mRNA and protein levels of cyclin D1, which is a transcriptional target of the Fos-Jun complex and is important for S-phase entry, are elevated and maintained by sustained ERK activation (Weber et al., 1997; Balmanno and Cook, 1999).

Differences in the duration of ERK activity are also used to regulate cellular responses in yeast. The budding yeast proteins Fus3p and Kss1p show about 50% identity to human ERK. Sabbagh et al. have shown that Fus3p limits the duration of Kss1p activity, and that transient Kss1 activation is required for mating responses whereas sustained activation supports filamentous growth (Sabbagh et al., 2001).

Regulators of ERK activity duration

What causes the difference in the duration of the ERK signal? York et al. have reported that transient and sustained ERK activation in PC12 cells are mediated by the small GTPases Ras and Rap1, respectively (York et al., 1998). Both EGF and NGF induce transient Ras activation but NGF stimulation also leads to sustained Rap1 activation. An interfering mutant of Rap1 blocks the ability of NGF to stimulate sustained ERK activation without altering the initial phase of ERK activation. By contrast, a dominant-negative form of Ras blocks only the initial activation of ERK. Most recently, a combination of computational simulations and experimental validation has provided a more detailed analysis of Ras and Rap1 dynamics (Sasagawa et al., 2005). Ras-GAP, which is an inactivator of Ras, is recruited to the receptor complex in a stimulus-dependent manner. Both EGF and NGF lead to Ras activation and Ras-GAP recruitment, resulting in transient Ras activation. By contrast, Rap1-GAP activity is stimulus-independent and maintained at a constant level. Therefore, high levels of NGF can overcome the inhibition by Rap1-GAP and give sustained Rap1 activation.

Bhalla et al. have also conducted computational simulations and reported that sustained ERK activation in fibroblasts is brought by a positive feedback loop between ERK and protein kinase C (PKC) signalling (Bhalla et al., 1999; Bhalla et al., 2002). PKC stimulates ERK activation through activation of the MAP kinase kinase kinase (MAPKKK) Raf. ERK activates cytosolic phospholipase A2 (cPLA2), and the arachidonic acid produced by cPLA2 activates PKC. Inhibition of PKC signaling has no effect on initial ERK activation, but blocks sustained ERK activation.

In addition, differences in receptor dynamics among growth factor receptors might contribute to the different effects of the growth factors on the duration of ERK activity (Di Fiore and Gill, 1999). Upon EGF stimulation, the EGF-R undergoes rapid internalization followed by degradation, which terminates ERK activation and thus makes it transient. The internalization of other receptors is relatively slow.

Another temporal regulator of ERK activity is Sprouty – a negative feedback inhibitor of the ERK pathway (Hanafusa et al., 2002). Sprouty becomes tyrosine phosphorylated in response to growth factor stimulation, and tyrosine phosphorylated Sprouty is active as an inhibitor of ERK activation. Thus, Sprouty represses the later, but not initial, phase of ERK activation. Overexpression of Sprouty makes ERK activation transient and suppresses the differentiation of PC12 cells. By contrast, overexpression of a dominant-negative form of Sprouty results in sustained ERK activation and promotes their differentiation.

The magnitude of ERK activity

The magnitude of ERK activity is also a determinant of cell fate. Although activation of the ERK pathway usually promotes cell-cycle progression, it sometimes leads to cell-cycle arrest. By using cells overexpressing a fusion protein in which Raf is linked to a steroid hormone receptor, several studies have varied the magnitude of ERK activity and shown that strong activation of ERK causes cell-cycle arrest (Sewing et al., 1997; Woods et al., 1997; Roovers and Assoian, 2000). Moderate levels of ERK activity induce the expression of cyclin D1 and cyclin E, resulting in accumulation of active CDK complexes. By contrast, high levels of ERK activity stimulate the CDK inhibitor p21 (WAF) by both transcriptional and post-translational mechanisms (Coleman et al., 2003); this reduces CDK activity and in turn causes G1 arrest. Interestingly, p21-mediated G1 arrest can be bypassed by activation of the RhoA small GTPase or Akt kinase pathway (Olson et al., 1998; Mirza et al., 2004). Thus, the cell fate decision is clearly a complicated one, in which many signaling pathways can interact.

The ERK pathway is often related to oncogenesis, and the magnitude of ERK activity appears to influence the survival of carcinoma cells. High ERK activity reduces the apoptosis rate of colon carcinoma cells that have detached from the

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extracellular matrix and increases the expression of the Fos relative Fra-1 (Vial and Marshall, 2003). Moderate ERK activation is required to induce transcription of *Fra-1*, and higher levels of activation stabilize the Fra-1 protein. This is reminiscent of the stabilization of immediate early gene products by sustained ERK activation mentioned above. Depletion of Fra-1 abrogates the anti-apoptotic effect of ERK, which suggests that the prolonged expression of Fra-1 provides survival signals.

Regulators of ERK activity magnitude

Scaffold proteins could play an important role in regulation of the magnitude of ERK activity. KSR (kinase suppressor of Ras) is a scaffold protein that interacts with Raf, MEK and ERK, thereby potentiating ERK activation (Therrien et al., 1996; Morrison, 2001). KSR-null mice show reduced levels of ERK activity, which blocks T-cell activation and inhibits tumor development (Nguyen et al., 2002). The E3 ubiquitin ligase IMP (impedes mitogenic signal propagation) binds to KSR in quiescent cells (Matheny et al., 2004). The binding causes hyperphosphorylation and mislocalization of KSR, thus maintaining KSR in an inactivated state. Upon growth factor stimulation, IMP is recruited to Ras and undergoes autoubiquitinylation, which allows KSR to dissociate from IMP. Then, KSR translocates to the plasma membrane and forms the KSR-Raf-MEK-ERK complex. Reduction of IMP expression by RNAi leads to high levels of stimulus-dependent ERK activity without altering the timing or duration of the ERK activation and can enhance neurite outgrowth of PC12 cells in response to suboptimal doses of NGF. KSR and IMP thus combine to regulate ERK signal magnitude.

Other scaffold proteins for the ERK pathway exist (Morrison and Davis, 2003). MP1 (MEK partner 1) couples ERK to MEK (Schaeffer et al., 1998), and reduction of MP1 expression by siRNA results in decreased ERK activation in response to growth factor stimulation (Teis et al., 2002). MEKK1 (MEK kinase 1) and the clathrin adaptor protein β-arrestin are also scaffold proteins that bind to Raf, MEK and ERK, enhancing ERK activation (Karandikar et al., 2000; Luttrell et al., 2001). It is difficult, however, to discriminate between their effects on the magnitude and the duration of ERK activity, because strong activation generally leads to long-term activation. Further investigations will be needed to ascertain whether these scaffold proteins enhance both the magnitude and duration of ERK activity.

Subcellular compartmentalization of ERK activity

ERK is able to shuttle between the cytoplasm and the nucleus, although it is for the most part cytoplasmic in most quiescent cells. Neither a nuclear export signal (NES) nor a nuclear localization signal (NLS) has been identified in ERK1/2. However, ERK is reported to bind to the nuclear pore complex (Matsubayashi et al., 2001; Whitehurst et al., 2002). Cytoplasmic localization of ERK is maintained through its binding to MEK or MAP kinase phosphatase (MKP)-3, each of which contains an authentic NES (Fukuda et al., 1996; Karlsson et al., 2004). Following activation, ERK dissociates from cytoplasmic anchors such as MEK and translocates to the nucleus (Fukuda et al., 1997; Khokhlatchev et al., 1998; Adachi et al., 1999; Matsubayashi et al., 2001; Whitehurst et al., 2002), where it phosphorylates its nuclear substrates, including many transcription factors. The nuclear translocation of ERK is required for proliferation of fibroblasts and differentiation of PC12 cells (Robinson et al., 1998; Brunet et al., 1999).

Activated ERK does not always localize to the nucleus, however. Agonists of certain G-protein-coupled receptors activate ERK but do not induce its nuclear translocation or transcriptional responses (DeFea et al., 2000). In human foreskin fibroblasts (HFFs), EGF-stimulated ERK remains cytoplasmic for the most part whereas phorbol myristate acetate (PMA)-stimulated ERK translocates to the nucleus. Treatment with leptomycin B, a specific inhibitor of the NES receptor CRM1, enhances both the nuclear localization of ERK and the expression of *Fos*, which may promote cell proliferation, without altering the magnitude of ERK activity (Whitehurst et al., 2004). Thus, the subcellular compartmentalization of ERK activity is an important factor controlling cell fate decisions.

The spatial control of ERK activity also regulates several developmental processes. Treatment of embryonic carcinoma and stem cells with retinoic acid leads to differentiation into primitive endoderm-like cells. In the differentiated cells, serum induces significant ERK activation, but ERK is confined to the cytoplasm, which suppresses *Fos* expression and cell proliferation (Smith et al., 2004). Agents that disrupt the actin or microtuble cytoskeleton restore *Fos* expression, which suggests that an intact cytoskeleton is required for the cytoplasmic sequestration of ERK. In the morphogenetic furrow of the developing *Drosophila* eye, activated ERK is held in the cytoplasm for hours (Kumar et al., 2003). Interestingly, disruption of this cytoplasmic localization by addition of an NLS to ERK results in defective patterning of the furrow.

There are many cytoplasmic ERK substrates, such as p90RSK, cPLA2 and cytoskeletal proteins (Sturgill et al., 1988; Lin et al., 1993). For example, ERK phosphorylates and activates the Ca^{2+}/calmodulin-dependent enzyme MLCK (myosin light chain kinase) in FG carcinoma cells, which promotes cell migration (Klemke et al., 1997). Moreover, several transcription factors are activated by ERK in the cytoplasm and then translocate to the nucleus after phosphorylation. Therefore, cytoplasmic retention of ERK can actively determine cellular responses in addition to simply preventing the phosphorylation of nuclear substrates.

Spatial regulators of ERK activity

There are several known regulators of ERK compartmentalization. Recently, Torii et al. (Torii et al., 2004) have shown that Sef (similar expression to FGF genes), a known inhibitor of the ERK pathway, targets ERK activity to the cytoplasm. Sef binds to activated MEK in a stimulus-dependent manner and blocks the dissociation of activated ERK from MEK. This inhibits the translocation of ERK to the nucleus. Overexpression of Sef does not suppress ERK activity in the cytoplasm – cytoplasmic effectors, such as p90RSK, are phosphorylated and activated – but the nuclear effectors, such as Elk-1, cannot be activated in response to growth factor stimulation. Reduction of Sef expression by siRNA increases

ERK nuclear localization, Elk-1 activity, and expression of ERK target genes such as *Fos*, *Jun* and *Egr1*.

PEA-15 is a 15-kDa protein expressed in a broad range of tissues and enriched in astrocytes. PEA-15 contains an NES and binds to ERK without inhibiting its activation. Thus, PEA-15 is able to sequester ERK activity to the cytoplasm (Formstecher et al., 2001). Deletion of *PEA-15* increases ERK nuclear localization, *Fos* expression and cell proliferation in primary astrocytes. In addition, the cytoplasmic sequestration of ERK by PEA-15 correlates with senescence in fibroblasts (Gaumont-Leclerc et al., 2004). Reduction of PEA-15 expression by RNAi rescues the cells from senescence. The actin-binding protein calponin and β-arrestin can also restrict ERK activation to the cytoplasm (Menice et al., 1997; DeFea et al., 2000).

Subcellular compartmentalization includes not only nucleocytoplasmic trafficking but also other specific localization mechanisms. The multidomain scaffold protein paxillin facilitates formation of the Raf-MEK-ERK complex at the focal adhesions of hepatocyte growth factor (HGF)-stimulated epithelial cells (Ishibe et al., 2003). The activated ERK in turn phosphorylates paxillin. This leads to interaction

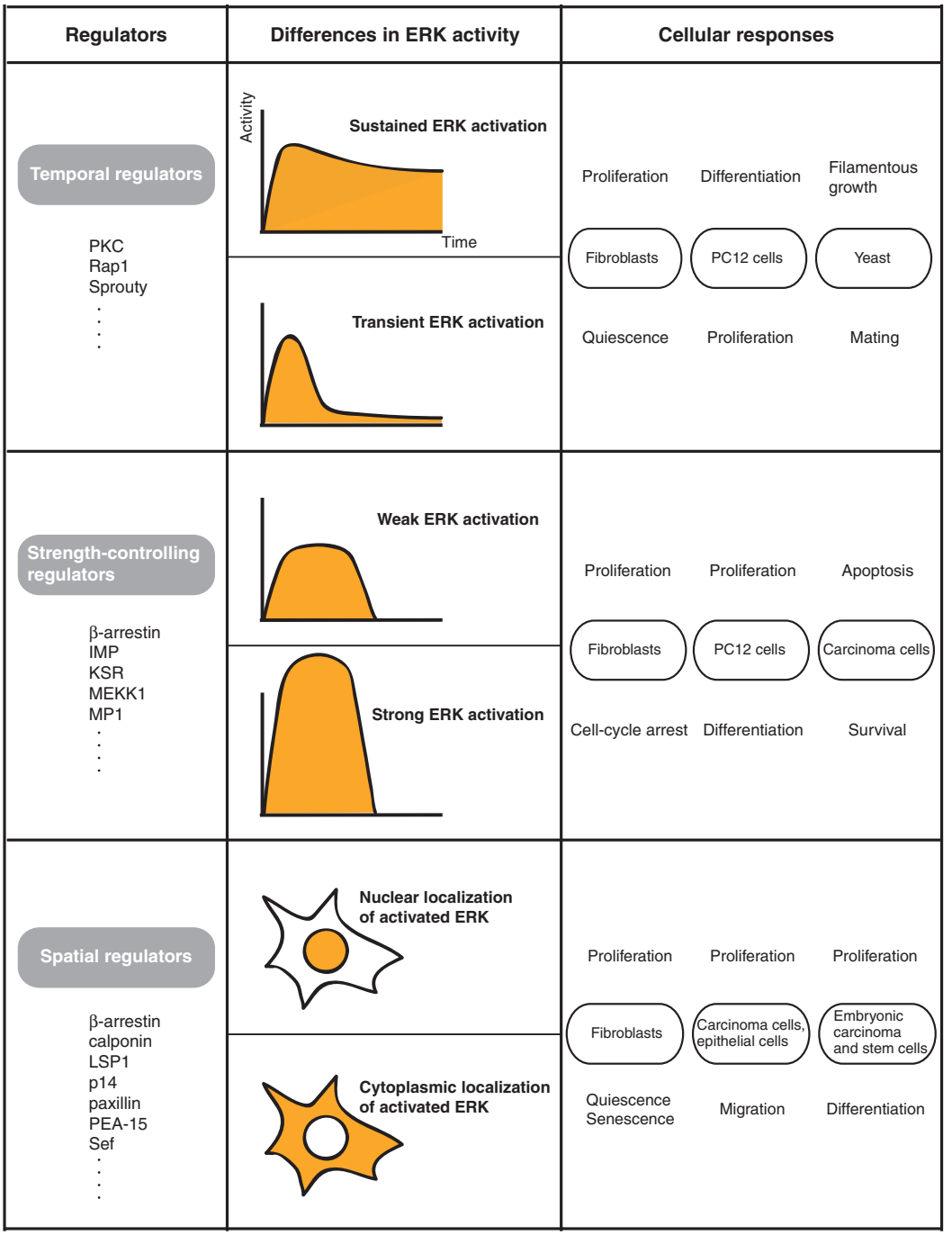

Fig. 2. Schematic representation of regulatory mechanisms for ERK activation and cellular responses.

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of paxillin with FAK (focal adhesion kinase) and subsequent activation of phosphoinositide 3-kinase (PI3K), which could contribute to cell migration or tubule formation (Ishibe et al., 2004). The actin-binding cytoskeletal protein LSP1 (leukocyte-specific protein 1) interacts with, and targets, the KSR-MEK-ERK complex to peripheral actin filaments (Harrison et al., 2004). Because overexpression of a dominant-negative form of LSP-1 increases apoptosis of lymphoma cells, ERK activity around actin filaments might have an anti-apoptotic effect. Furthermore, Sef anchors the MEK-ERK complex on the Golgi apparatus or the plasma membrane (Torii et al., 2004) and, p14, which interacts with MP1, targets ERK to endosomes (Teis et al., 2002; Bivona and Philips, 2003). Thus, the subcellular compartmentalization of ERK activity is regulated precisely, and should generate diversity in cellular responses.

Conclusion and perspectives

The studies discussed here reveal the significance of the duration, magnitude and subcellular compartmentalization of ERK activity as mechanisms for determining cell fate (Fig. 2). The crucial unsolved question is how the differences in ERK activity determine cellular responses. Is there a common mechanism? The key factor seems to be changes in gene expression. The cytoplasmic sequestration of ERK activity can limit ERK-mediated transcription. The duration and magnitude of ERK activity also affect the transcription of a number of crucial genes for differentiation or cell-cycle regulation. In other cases, the differences in ERK activity affect stabilization of proteins, including immediate early gene products. Therefore, the qualitative and quantitative differences in ERK activity might be interpreted as changes in gene expression that elicit specific responses.

Recent progress has also revealed the mechanisms that create the differences in ERK activity. Spatiotemporal and/or strength-controlling regulators could transduce signals from extracellular stimuli into different modes of ERK activity. What controls these regulators? Do their expression and functions vary according to the cellular context and stimulus? Because several studies have revealed the patterns of ERK activation during embryogenesis – sustained versus transient activation, strong versus weak activation, or nuclear versus cytoplasmic activation (Gabay et al., 1997; Christen and Slack, 1999; Corson et al., 2003) – it would be interesting to investigate the expression and activity of the regulators at the same time.

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