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Biochemical Pharmacology 64 (2002) 755–763

Biochemical Pharmacology

Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling

Jacques Pouysségur, Véronique Volmat, Philippe Lenormand*

Institute of Signaling, Developmental Biology and Cancer Research, CNRS-UMR 6543, Centre Antoine Lacassagne, 33 Avenue de Valombrose, 06189 Nice, France

Received 13 March 2002; accepted 25 April 2002

We dedicate this manuscript to the memory of Véronique Volmat tragically deceased on April 22, 2002.

Abstract

Extracellular signals transduced *via* receptor tyrosine kinases, G-protein-coupled receptors or integrins activate Ras, a key switch in cellular signalling. Although Ras can activate multiple downstream effectors (PI3K, Ral ...) one of the major activated pathway is a conserved sequential protein kinase cascade referred to as the mitogen activated protein (MAP) kinase module: Raf > MEK > ERK. The fidelity of signalling among protein kinases and the spatio-temporal activation are certainly key determinants for generating precise biological responses. The fidelity is ensured by scaffold proteins, a sort of protein kinase ''insulators'' and/or specific docking sites among the members of the signalling cascade. These docking sites are found in upstream and downstream regulators and MAPK substrates [Nat Cell Biol 2 2000 110]. The duration and the intensity of the response are in part controlled by the compartmentalisation of the signalling molecules. Growth factors promote nuclear accumulation and persistent activation of ERK (p42/p44 MAP kinases) during the entire G1 period with an extinction during S-phase. These features are exquisitely well controlled by (i) the temporal induction of the MAP kinase phosphatases, MKP1-3, and (ii) the compartmentalisation of the signalling molecules. We have shown that MKP1-2 induction is strictly controlled by the activation of the MAP kinase module providing evidence for an autoregulatory mechanism. This negative regulatory loop was further enhanced by the capacity of ERK to phosphorylate MKP1 and 2. This action reduced the degradation rate of these MKPs through the ubiquitin–proteasomal system [Science 286 1999 2514].

Whereas the two upstream kinases of the module, Raf and MEK remained cytoplasmic, ERK anchored to MEK in the cytoplasm of resting cells, rapidly translocated to the nucleus upon mitogenic stimulation. This process was rapid, reversible, and controlled by the strict activation of the MAPK cascade. Prevention of this nuclear translocation, by overexpression of a cytoplasmic ERK-docking molecule (inactive MKP3) prevented growth factor-stimulated DNA replication [EMBO J 18 1999 664]. Following long term stimulation, ERK progressively accumulated in the nucleus in an inactive form. This nuclear retention relied on the neosynthesis of short-lived nuclear anchoring proteins. Nuclear inactivation and sequestration was likely to be controlled by MAP kinase phosphatases 1 and 2. Therefore we propose that the nucleus represents a site for ERK action, sequestration and signal termination [J Cell Sci 114 2001 3433].

In addition, with the generation of mice invalidated for each of the ERK isoforms, we will illustrate that besides controlling cell proliferation the ERK cascade also controls cell differentiation and cell behaviour [Science 286 1999 1374].

Keywords: MAP kinases; MAPK-phosphatases; Scaffolding proteins; Nucleus; Cell trafficking

1. Introduction

ERK (p42/p44 MAPK) constitutes a major signalling module conserved throughout evolution that is activated in mammalian cells *via* stimulation of receptor tyrosine kinases, G-protein coupled receptors and integrins [6]. These cell surface signals converge towards activation of the small G-protein Ras that recruits the serine/threonine kinase Raf to the membrane where it is fully activated by largely unknown mechanisms [7]. The signal is amplified *via* two downstream kinases, MEK and ERK that are

* Corresponding author. Tel.: +33-492-031-227; fax: +33-492-031-235.
E-mail address: philippe.lenorman@unice.fr (P. Lenormand).

Abbreviations: CDK, cyclin dependent kinase; Crm-1, chromosomal region maintenance 1; EGF, epithelial growth factor; ERK, extracellular regulated kinase; JNK, c-*jun* N-terminal kinase; KSR, kinase suppressor of Ras; MAPK, mitogen activated protein kinase; MEK, MAPK or ERK kinase; MKP, MAP kinase phosphatase; MNK1, mitogen and stress kinase1; MP1, MEK partner 1; NGF, nerve growth factor; PEA15, phosphoprotein enriched in astrocytes 15 kD; PI3K, phosphatidyl inositol 3 kinase; PP2A, protein phosphatase 2A; Ste5, sterile 5.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01135-8

uniquely activated since MEK is dually phosphorylated on two serine residues by Raf, and then ERK is dually phosphorylated on a tyrosine and threonine residue by MEK (sequence TEY). Amplification *via* this signalling cascade is such that it is estimated that activation of solely 5% of Ras molecules is sufficient to induce full activation of ERK [8].

Activated ERK phosphorylates numerous substrates on (S/T)P sites in all cellular compartments (review by [9]). Proper activation of the ERK pathway relative to the closely related JNK and p38 MAPK pathways and efficiency in transmitting activation occurs by two mechanisms. First, scaffolding proteins are expected to maintain in close vicinity the components of the ERK signalling cascade, and second, specific docking sites on substrates, activators and regulatory proteins maintain the specificity of activation. The first part of this presentation will unveil our current understanding of these scaffolding proteins and docking sites.

ERK activation is essential for cell growth [10] and provides an integrated response: it increases nucleotide synthesis, activates the transcription of many genes acting *via* transcription factors and chromatin phosphorylation, it stimulates protein synthesis *via* MNK1, and finally facilitates the formation of an active cyclinD–CDK4 complex, which is rate-limiting for cell growth (reviews by [11,12]).

The specific role of the two ERK isoforms is not yet fully understood. First, both isoforms are ubiquitously expressed, second, they are highly similar (overall 75% identity at the amino acid level, and up to 90% identity when the N-terminal stretch is not taken into account) and third, *in vitro* both isoforms present the same substrate specificity. However, isoform-specific invalidation in mice provides contrasting results, first ERK1−/− mice are viable, fertile and of normal size [5]. In these animals ERK2 can compensate for most of the functions of ERK1, solely thymocyte terminal differentiation is impaired. On the contrary, ERK2 invalidation is lethal at early embryonic stages, day 6.5 (Sylvain Meloche, personal communication). In these embryos, ERK1 cannot compensate for loss of ERK2, thus specific functions of ERK2 remain to be discovered. Alternatively, ERK1 is not expressed in some cells or at such low levels compared to ERK2 that it cannot provide the strength of activation required for embryonic survival.

Considering the pleiotropic substrates and the ubiquitous expression of ERK, cell specific regulation must occur to ensure conduction of the appropriate signal. For example, expression of the ERK regulator PEA15 is restricted to only a few cell types such as terminally differentiated astrocytes. In these cells, expression of PEA15 attenuates ERK-dependent transcription and proliferation by binding ERK and re-addressing ERK signalling in the cytoplasm [13].

In a single cell, activation of the ERK pathway can lead to antagonistic fates, for example in PC12 cells both differentiation and cell proliferation require ERK activation (following NGF or EGF stimulation, respectively). In these cells EGF causes a transient activation of ERK, whereas NGF causes a sustained activation of ERK, thus the duration of ERK activation specifies signal identity [14]. Similarly, we have observed a correlation between the strength of mitogenic signalling in CCL39 cells and the duration of ERK stimulation. We have shown that non-mitogenic factors induce transient activation of ERK (less than 15 min) that does not lead to cell cycle entry whereas mitogens induce cell proliferation and long term stimulation of ERK (up to 6 hr) [15]. Similarly, it has been shown that very potent ERK activation protects cells from apoptosis induced by anchorage and serum removal [16], whereas moderate ERK activation is required to permit apoptosis induced by anchorage and serum removal [17].

Clearly the ERK pathway must be tightly controlled in its duration of activation and sub-cellular localisation to ensure proper outcome of integrated biological responses such as cell proliferation, differentiation and survival. The Section 3 of this presentation will describe the phosphatases that control the duration of stimulation, and Section 4 will present the regulation of ERK trafficking.

2. Scaffolding and docking sites

As indicated previously, several MAPK cascades delivering specific biological responses are present in a particular cell. There is a high degree of homology between MAPK modules, in their general organisation but also at the protein level with a high percentage of similarity in the primary sequence of the different MAPKs (60% between ERK1/2 and either JNK or p38 MAPK). Furthermore, the substrates of the three main MAPKs: ERK, JNK, and p38 MAPK display similar phosphorylation consensus motifs: (T/S)P.

Two mechanisms prevent inappropriate cross-talk between the different MAPK modules. First, scaffold proteins create multi-enzyme complexes that bring together components of a single kinase cascade (review by [18]). These complexes insulate the module from activation by irrelevant stimuli and favour the rapid transmission of the signal through the cascade. Second, specific docking sites on MAPKs which serve for the binding of substrates, activators and regulators increase the fidelity and the efficiency of the enzymatic reactions.

The most studied MAPK scaffolding protein is Ste5 in the yeast *Saccharomyces cerevisiae* (review by [19]). Through distinct regions, Ste5 binds simultaneously to Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (one of the two MAPKs) but Ste5 binds weakly to the other MAPK Kss1. Fus3 is preferentially implicated in the mating pheromone response, whereas Kss1 is primarily involved in the filamentous response. Fus3 and Kss1 share the same activators, Ste11 and Ste7, however it seems that Kss1 may be better at transmitting low-level, long-duration, scaffold-independent signalling, whereas Fus3 preferentially

transmits scaffold-associated signalling (review by [20]). Contrary to that previously thought, scaffolded complexes are not stably assembled. During vegetative growth, recent work showed that the upstream activators Ste11 and Ste7 are predominantly cytoplasmic, while the scaffold Ste5 and the MAPK Fus3 are located both in the nucleus and in the cytoplasm and shuttle permanently between these two cellular compartments [21]. In pheromone-treated cells, Ste11, Ste7 and Fus3 are co-localised with Ste5 to tips of mating projections. However, subsequently activated-Fus3 dissociates rapidly from this multi-protein complex to translocate to the nucleus. The role of the scaffolding protein Ste5 in this signalling pathway is essential, since Ste5 that cannot transit *via* the nucleus is unable to localise to the cell periphery and is unable to activate the pathway [22]. This novel regulatory scheme may ensure that cytoplasmic Ste5 does not activate downstream kinases in the absence of pheromone.

In mammalian cells the role of scaffolding proteins is not as well understood. First, a two hybrid screen using MEK1 as a bait identified MEK partner 1 (MP1) as a scaffold protein that specifically binds MEK1 and ERK1 to the exclusion of MEK2 and ERK2, thereby enhancing the activation of ERK1 [23]. The functional role of this isoform-specific interaction is not yet understood. However, a partner of MP1, P14 was discovered recently and the MP1–P14 complex scaffolds MEK1 and ERK1 to the cytoplasmic surface of late endosomes/lysosomes where P14 is localised [24]. The presence of ERK1 on the surface of late endosomes/lysosomes may participate in signalling by internalised cell surface receptors, or could be involved in regulating lysosomal secretion.

Another putative scaffold protein for the ERK pathway is the kinase suppressor of Ras (KSR) protein. KSR was first identified by genetic screening in *Drosophila* and *Caenorhabditis elegans* as an activator of the Ras pathway since mutations in KSR resulted in attenuation of Ras-mediated signalling (review by [25,26]). A mammalian homologue has been isolated that interacts directly with MEK and ERK *via* distinct domains while interaction with Raf appears to be indirect. KSR1 translocates from the cytoplasm to the cell membrane in response to growth factor treatment. This process is controlled by the serine/threonine kinase C-TAK1 that phosphorylates KSR1 at a site that confers 14-3-3 binding, thus sequestering the KSR1 complex in the cytoplasm in absence of stimulation [27]. In response to growth factors, the KSR1 S392 site is dephosphorylated by an unknown phosphatase, and KSR1 is liberated from 14-3-3 binding and translocates to the plasma membrane where it brings in close vicinity MEK and ERK to the active Raf signalling complex. Therefore, KSR1 seems to act as a scaffold protein to maintain specificity and increase signalling through the ERK cascade.

From the work on Ste5 and KSR1 an emerging concept arises: scaffolding proteins are not only insulator between homologous signalling modules, but they play an important role as regulators of the sub-cellular localisation of kinase cascade components.

The three major MAPKs phosphorylate their substrates on the consensus (T/S)P sequence and many potential substrates contain this motif (review by [9]). Therefore MAPKs have acquired specific docking sites to specify interactions with relevant substrates. Docking sites also increase the local concentration of the kinase, hence favouring substrate phosphorylation. The main docking site of ERK is composed of a cluster of negatively charged amino acids conserved from *C. elegans* to human (common docking; CD) [1,28]. Data from the three-dimensional structure of ERK indicate that the CD site is localised on the opposite side of the kinase respective to the catalytic cleft, thus substrates must dissociate from the docking site to be phosphorylated, indicating that association *via* kinase docking sites is highly dynamic [29]. Docking sites on ERK interacting proteins have been identified on substrates, activators, scaffolding proteins and phosphatases. On interactor proteins, docking sites are constituted by a cluster of positively charged amino acids (D-domain), that interact on the same negatively charged ERK docking site. This implies that interaction of these proteins with ERK are mutually exclusive, thereby providing a molecular mechanism for the sequential activation and inactivation of ERK.

The specificity of ERK interaction with proteins may not be determined solely by the ERK negatively charged motif since interchanging by mutation the docking site present on ERK by the docking site present on p38 MAPK still allows the binding of MEK to ERK while no binding of MKK6 (the upstream activator of p38 MAPK) can be detected [1]. It is now thought that the docking region on ERK is contained in a docking groove where several interacting motifs co-operate to confer strong and specific binding for each MAPK-interacting molecules [29,30]. The spacing and organisation of these different motifs on the different MAPK interacting proteins is a feature that may account for the differential MAPK specificities observed [31]. Furthermore, in yeast, both the MKK-MAPK docking interaction and binding to the scaffolding protein Ste5 make mutually reinforcing contributions to efficiently conduct mating pheromone signalling [32].

Moreover, the phosphorylation state of partners can also modulate the affinity of the interaction. For example, the association of ERK with its substrate Elk-1 is enhanced upon ERK activation [33] whereas interaction of ERK with its activator MEK is reduced upon activation of the signalling cascade [34]. Interestingly, cross-talk with other signalling pathways can be mediated by regulating docking interactions. For example, in some cell types, the tyrosine phosphatase PTP-SL retains ERK in the cytoplasm by association *via* the docking site and maintains ERK in an inactive form by tyrosine dephosphorylation [35]. Upon phosphorylation of Ser231 of PTP-SL by PKA, binding

and dephosphorylation of ERK is impaired, which then permits activation and subsequent nuclear translocation of ERK.

On proteins substrates, there are two classes of docking sites, the D-domain (cluster of positively charged amino acids) and the FXFP motif whose binding pocket on ERK remains to be determined. A systematic study of docking sites on Elk-1 indicates that the D-domain, and the FXFP motif form a flexible modular system that has two functions [36]. First, the affinity of a substrate for ERK can be regulated by the number, type, position and arrangement of these docking sites. Second, docking sites can direct phosphorylation of specific (S/T)P residues. [29,36].

The discovery of these kinase docking sites has provided new tools to deregulate the ERK signalling cascade. For example, micro-injection into the nucleus of a peptide corresponding to the ERK binding site of MEK has been shown to disrupt the association of ERK and MEK in the nucleus hence significantly inhibiting the MEK driven export of ERK out of the nucleus [37]. Similarly, micro-injection into the nucleus of a peptide corresponding to the ERK binding site on $p90^{rsk}$, has been shown to disrupt interaction between ERK and nuclear phosphatases hence increasing active ERK in the nucleus [4]. This latter experiment confirms that several interacting proteins act *via* highly homologous docking sites since a peptide corresponding to the sequence of an ERK substrate can impede ERK association with phosphatases.

3. Regulation of ERK activation by phosphatases

Schematically, mitogenic stimulation elicits ERK activation in four phases. First, there is an initial burst of activation, second, there is a very rapid inactivation within minutes, third, there is a prolonged activation peaking from 2 to 4 hr poststimulation, and fourth, the activation gradually diminishes and ERK activity is reduced nearly to basal levels at the end of the G1 phase of the cell cycle. A burst of ERK activation has been described at the G2/M transition, but is beyond the scope of this presentation. Considering that dephosphorylation of either the threonine or the tyrosine residue within the ERK activation loop TEY motif is sufficient for total enzymatic inactivation [38], numerous phosphatases could be implicated in the two phases of inactivation: the rapid initial phase and the slower and delayed one.

The serine/threonine specific phosphatase PP2A has been implicated in the first inactivation of ERK observed within minutes of NIH-3T3 cells stimulation [39] and of *Xenopus* oocytes stimulation [40]. The remaining phospho-tyrosine residue must be removed by a constitutive phosphatase. Several related tyrosine specific phosphatases such as PTP-SL, STEP, He-PTP/LC-PTP show a good specificity towards ERKs [30,41,42]. However these cytosolic tyrosine phosphatases seem to present a restricted expression pattern and thus the ubiquitously expressed phosphatase(s) that may play the same role in most cells is (are) not yet identified. Interestingly, a cytosolic *Drosophila* tyrosine phosphatase PTP-ER, related to the tyrosine phosphatases mentioned above, plays an important role to down-regulate ERK activation during *Drosophila* eye development [43], however it is not known if PTP-ER plays a major role in the inactivation of the first peak of ERK activation.

The delayed phase of inactivation is dependent on protein synthesis, indicating that neosynthesised phosphatases are required [39,44]. Furthermore, these phosphatases have a tyrosine specificity since they are inhibited by vanadate treatment [4,39,40]. The phosphatases that fulfill these criteria are the MAPK phosphatases (MKPs). MKPs belong to the dual specificity phosphatases family (DUSP) since they are capable of dephosphorylating both the tyrosine and the threonine residues of MAPKs (reviews by [45,46]). Evidence indicates that MKPs are good candidates in controlling a negative feed-back regulation loop of ERK activation. First, it has been shown that MKP1 and MKP2 are induced upon activation of the ERK pathway [44], furthermore MKP1 and MKP2 proteins are stabilised by ERK phosphorylation [2]. Indeed MKP1 is phosphorylated on Ser359 and Ser364 by ERK, which does not modify phosphatase activity, but reduces the rate of proteasome-dependent degradation. Finally, MKP3 [47] and MKP1 [48] are catalytically activated upon ERK binding to their N-terminal non-catalytic moiety. Catalytic activation of MKP1 and of MKP3 occurs by binding of ERK *via* the classical docking site. Hence substrate-specificity is ensured by two means: protein–protein interaction, and catalytic activation of the phosphatase.

The precise role of each MKPs *in vivo* is not yet understood. Clearly expression of some MKPs is restricted to specific sub-cellular compartments, which must impinge on the range of available substrates. In addition some phosphatases are immediate early genes products induced by ERK activation, such as MKP1 and MKP2 whereas others are induced at a much slower pace, such as MKP3. Finally, one MKP may be effective in inactivating several MAPKs, thus providing the capacity to cross-regulate other signalling pathways when activated by a single-signalling pathway. It is likely that there is redundancy between MKPs since invalidation of the *MKP1* gene did not affect mice physiology [49]. New approaches will be required to assess the functional role of individual MKPs *in vivo*.

4. Regulation of ERK sub-cellular localisation

4.1. Trafficking

The sub-cellular localisation of ERK during serum stimulation in NIH-3T3 cells is presented in Fig. 1A. As

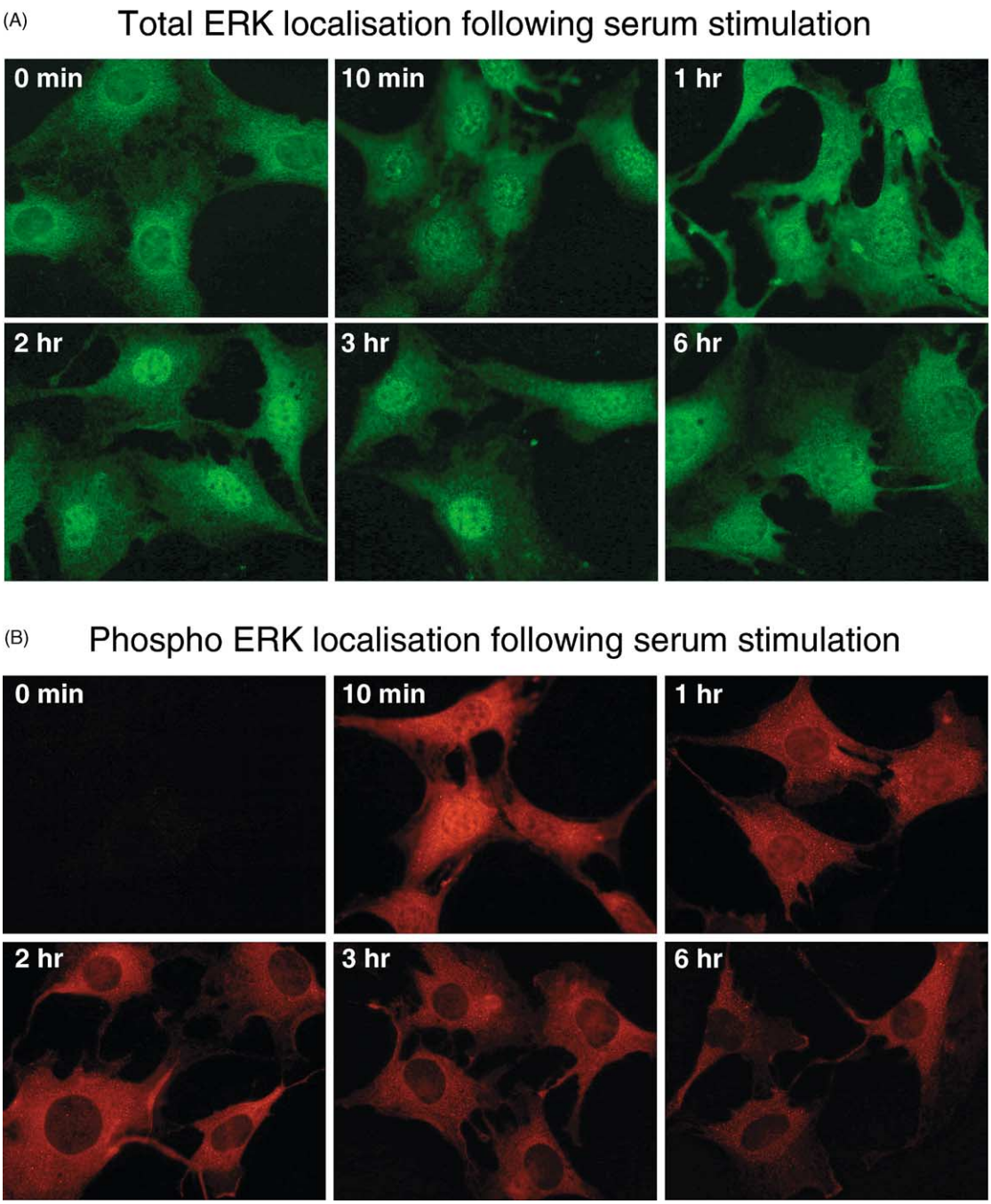

Fig. 1. NIH-3T3 cells were cultured in Dulbelcco's Modified Eagle's Medium (DMEM, Life Technologies Inc.) supplemented with 7.5% inactivated foetal calf serum. Cells were maintained in 5% CO_2 and serum was removed 12 hr prior to stimulation (0 min). Cells were then stimulated with 10% inactivated foetal calf serum for the indicated times, and processed for immunofluorescence as described for CCL39 cells in [4], (A) with total-ERK antibody, and (B) with anti-phospho-ERK antibody.

described for other cell lines, ERK is accumulated in the cytoplasm of arrested NIH-3T3 cells (first panel). Within 10 min of stimulation (second panel), a major part of the pool of ERK translocates into the nucleus. At 1 hr after stimulation, ERK is distributed throughout the cell, slightly more in the nucleus than in the cytoplasm (third panel). Interestingly, maximal nuclear accumulation of ERK is observed in NIH-3T3 cells following 2 hr of serum stimulation (fourth panel), which is faster than that observed with other cell lines such as CCL39 fibroblasts [50]. After 3 hr of stimulation, ERK is still accumulated in the nucleus (fifth panel), however at 6 hr, ERK is found mainly in the cytoplasm (sixth panel).

Hence, the intracellular redistribution of ERK following mitogenic stimulation occurs in two phases, first there is rapid entry into the nucleus without emptying the cytoplasmic pool and second ERK accumulates massively in the nucleus after several hours of stimulation. Non-mitogenic

stimuli induce the initial nuclear entry of ERK but fail to trigger the nuclear accumulation of ERK [4,50]. Similarly when the fate of cells is differentiation, only differentiating signals trigger the nuclear accumulation of ERK observed after several hours of stimulation [51].

Artificial retention of active ERK in the cytoplasm is sufficient to block growth factor-induced DNA replication [3] and cell transformation [52]. Alternatively, by restoring ERK nuclear translocation of senescent fibroblasts that are programmed for cell arrest, it is possible to re-establish normal c-*fos* activation [53]. Physiologically, the cytoplasmic retention of ERK may play a critical role in maintaining a differentiated phenotype in some cell types. For example, increased expression of the protein PEA15 traps ERK in the cytoplasm of astrocytes and blocks cell proliferation whereas genetic deletion of PEA15 increases astrocyte proliferation [13]. Regulation of ERK signalling by cytoplasmic trapping may be a frequent phenomenon since it was shown recently that beta-arrestin associates with ERK and enhances the ERK cytoplasmic activity while inhibiting ERK mediated transcription [54].Clearly, regulation of ERK nuclear translocation is a critical step to convey adapted biological responses for each cell type.

The mechanisms of ERK nuclear import and export are still largely unknown. It has been shown that ERK associates with MEK in the cytoplasm of resting cells *via* their docking sites, an interaction that is dramatically reduced upon activation of the MEK/ERK signalling pathway, thus allowing ERK to translocate to the nucleus [34]. As presented previously, recent work on the scaffolding protein KSR indicates that KSR participates in the regulation of the sub-cellular localisation of kinase cascade components. However the contribution of KSR in the release of ERK from the cytoplasmic complex has not yet been established. Clearly activation of the pathway is essential, since blocking MEK activity abrogates ERK nuclear translocation [55], however phosphorylation mutants of ERK still translocate to the nucleus in response to cell stimulation. [55–57]. Furthermore, in yeast, phosphorylation mutants of the Fus3-GFP fusion protein translocate to the nucleus at the same rate as wild-type Fus3-GFP [21].

It is now established that there is a continuous shuttling of ERK between the cytoplasm and the nucleus. Indeed, when quiescent cells are treated with Leptomycin B that blocks crm-1 dependent nuclear export, ERK appears within minutes in the nucleus [4]. This occurs in the total absence of ERK activation since it is not impeded by prior treatment with the MEK inhibitor U0126 [58]. Moreover the trafficking of Fus3-GFP fusion proteins has been studied in yeast by fluorescence recovery after photobleaching (FRAP). The half-life of Fus3-GFP fluorescence recovery in the nucleus is about 4 s, which is independent of stimulation although stimulated cells present a higher level of Fus3-GFP in the nucleus [21].

It seems that multiple mechanisms account for the rate at which ERK translocates to the nucleus. It has been proposed that ERK crosses the nuclear pore by passive diffusion since blocking active nucleocytoplasmic trafficking with wheat germ agglutinin does not impede ERK nuclear import [59]. However, in yeast cells Fus3-GFP trafficking is slower when active nucleocytoplasmic transport is impaired using temperature-sensitive mutants of gsp-1/gsp-2, two ran homologues [21]. The transport across the nuclear pore of Fus3-GFP is not halted, leaving the possibility that other mechanisms participate in translocating ERK through the nuclear pore. Anyhow the way by which ERKs cross the nuclear pore by active transport is not yet understood since ERKs do not possess any motif homologous to known nuclear localisation sequences (NLS), nevertheless recent work in *Drosophila* may provide clues to understand this phenomenon. Indeed deletion or mutations of the *Drosophila* importin alpha homologue DIM-7 or mutations of the importin beta homologue Ketel, reduce the nuclear localisation of D-ERK, the *Drosophila* homologue of ERK. Interestingly, DIM-7 associates with phosphorylated D-ERK which should allow to better understand how ERK can interact with the active import machinery while lacking a classical NLS sequence [60].

Phosphorylation-dependent dimerisation of ERK has also been proposed to explain ERK nuclear entry. Indeed, ERK-betagalactosidase fusion proteins are unable to enter the nucleus when ERK dimerisation motifs are mutated [59]. However, ERK dimerisation mutants appear to translocate normally ([59] and Philippe Lenormand, unpublished data).

Recently direct binding of ERK to nuclear pore complex has been revealed [57]. In that case, ERK transport across the nuclear pore would be propelled by Brownian motion. Indeed it has been shown in permeabilised mammalian cells that ERK associates directly with the nuclear pore complex and translocates to the nucleus independently of soluble factors and ATP. Furthermore ERK binds *in vitro* to an FG repeat region of nucleoporin CAN/Nup214.

Altogether, the relative contribution of these four different mechanisms in conducting ERK across the nuclear membrane remains to be determined.

The export of ERK out of the nucleus is not fully understood either. Clearly ERK1 and ERK2 protein sequences show no motif homologous to a nuclear export sequence (NES) and thus should not be exported actively *via* crm-1 dependent mechanism. However blocking active crm-1 nuclear export with Leptomycin B triggers the nuclear accumulation of ERK and MEK [4,61]. Therefore the active export of MEK *via* its NES could be one of the mechanisms that drives ERK out of the nucleus by piggyback mechanism.

5. Nuclear accumulation and inactivation

When activation of the ERK pathway is transient, ERK rapidly exits out of the nucleus [50], however during

sustained activation, ERK accumulates in the nucleus as shown in Fig. 1A. The nuclear accumulation of ERK in the nucleus requires the ERK-dependent transcriptional induction of short-lived nuclear anchoring proteins [55]. The identity of these nuclear anchors remains elusive, however the use of anti-phospho-ERK antibodies provided new clues in understanding this nuclear accumulation of ERK.

As shown in Fig. 1B, in serum-deprived NIH-3T3 cells there is no detectable ERK phosphorylation, hence no ERK activity (first panel). However, within 10 min of serum stimulation phosphorylated ERK is present both in the cytoplasm, site of activation by MEK and in the nucleus where it is expected to phosphorylate nuclear transcription factors (second panel). Within 1 hr of serum stimulation, ERK is still phosphorylated in the cytoplasm but remarkably it is less phosphorylated in the nucleus than 10 min poststimulation (third panel). Surprisingly at 2 and 3 hr poststimulation, most of the ERK present in the nucleus is dephosphorylated, whereas a major part of the cellular pool of ERK has massively accumulated in the nucleus at these time-points (compare fourth and fifth panels of Figs. 1A and B). At 6 hr poststimulation, ERK phosphorylation is still absent from the nucleus, but also much reduced in the cytoplasm, albeit still at higher levels than in arrested cells (sixth panel).

Complementary experiments indicate that nuclear phosphatases of tyrosine specificity are responsible for this inactivation of ERK when it has accumulated in the nucleus during persistent activation of the signalling pathway. Furthermore these phosphatases are neosynthesised in response to mitogenic stimulation, and require binding *via* ERK docking sites to dephosphorylate ERK [4].

MKP1 and MKP2 are good candidates for inactivation of ERK in the nucleus since they are: induced by activation of the ERK pathway, localised in the nucleus, possess ERK docking sites and inhibited by tyrosine phosphatase specific inhibitors [45,46]. Furthermore, MKP1 and MKP2 may participate in the nuclear anchoring of ERKs since these proteins present all the characteristics of ERK nuclear anchors, MKP1/2 are induced by ERK activation [44], and are short-lived nuclear proteins whose expression is virtually abolished within 1 hr upon traductional or translational block [2]. Unfortunately we have not been able so far to identify the nuclear anchors of ERK nor apprehend the role of MKP1/2 in the nuclear inactivation of ERKs. The use of RNA interference to abrogate expression of each MKP isoforms, may provide answers to these questions.

6. Conclusions

ERK activation plays a major role in the integration of multiple biological responses. Hence, exquisite regulation of ERK activation is essential in conveying appropriate signals. The intensity, duration and sub-cellular localisation of ERK activation are well regulated. Scaffolding proteins and docking sites provide the means to avoid cross-activation between MAPK signalling pathways, and permit precise and even cell-specific sub-cellular localisation of ERKs.

We propose that the nucleus plays an important role, first to convey ERK transcriptional action, then as a site of signal termination by sequestering ERK away from its activator MEK and dephosphorylating ERK by feed-back regulated phosphatases.

Unanswered questions remain despite intense research efforts world-wide, are there other ERK scaffolding proteins in mammalian cells? What is the role of MP1 and P14 and more generally, what are the specific roles of ERK1 vs. ERK2? Which tyrosine phosphatases complement the threonine dephosphorylation by PP2A during the first stages of inactivation? What is the identity of the ERK nuclear anchors and of the phosphatases that inactivate ERK in the nucleus? Answering these question will provide an even better understanding of the ERK activation pathway, a key transduction signalling cascade controlling growth, differentiation and cell survival.

Acknowledgments

We thank all the members of the laboratory for helpful discussions, and are particularly grateful to Dr. C. Brahimi-Horn for carefully reading the manuscript.

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