Intracellular signaling cascades

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In order to survive and perform their functions, cells need to respond to many extracellular signals such as mitogens, hormones, cytokines, physical changes of the environment and stress. These extracellular signals induce various distinct cellular processes, which are largely executed by induction of de-novo gene expression. Most of the extracellular agents bind to membranal receptors, and do not penetrate the cells in order to activate transcription. Rather, the extracellular signals are transferred from the membranes to the genes in the nucleus via several communication lines known as intracellular signaling pathways, which operate within a complex network. In many cases, the transmission of signals through these pathways involves sequential phosphorylation events by protein kinases, which are termed kinase cascades. (Fig. 1). Such a mechanism is used by four mitogen-activated protein kinase (MAPK) signaling cascades, which are important in the transmission of many extracellular signals. Each of these signaling cascades consists of up to five tiers (levels) of protein kinases that activate each other by phosphorylation. These MAPK cascades cooperate to transmit signals to their intracellular targets and thus to initiate cellular processes such as proliferation, differentiation, development, stress response and apoptosis. Another important intracellular signaling pathway operates via the lipid kinase phosphoinositide 3-kinase (PI3K), and uses a kinase cascade that includes PDK1, PKB (AKT) and GSK3, and is known as the PI3K-PKB pathway. This cascade is thought to be involved primarily in cell survival but can also function in proliferation, metabolism and stress response. Finally, a PKA dependent cascade is involved primarily in metabolic processes. Our laboratory is studying all of the above cascades, in an attempt to understand their mode of action, their regulation and particularly their role in various cellular systems and oncogenesis. The most studied signaling pathway in the lab is the extracellular signal-regulated kinase (ERK) cascade, which is activated in response to diverse stimuli, and can regulate a large number of distinct cellular processes. In the last two years we concentrated mainly on the study of the regulation of the ERK cascade and its downstream effects by subcellular localization, alternative splicing, and protein-protein interactions. We also studied the role of the MAPK cascades in apoptosis and angiogenesis in various cellular systems.

Regulation of the ERK cascade by subcellular localization

The subcellular localization of ERKs and the MAPK/ERK kinases (MEKs) plays an important role in the regulation of the signals transmitted via this cascade. We have previously shown that ERK1/2 and MEK1/2 are localized in the cytoplasm of resting cells due to interactions with anchoring proteins. Upon activation, ERK1/2 and MEK1/2 are detached from the anchoring proteins and translocate into the nucleus. Immediately after entering the nucleus, MEK1/2 are rapidly exported from this location by CRM1, whereas ERK1/2 are retained in the nucleus for 30-180 minutes. We also found that the ERK1/2 interact with their anchoring proteins mainly via a CRS/CD docking motif in their C terminus (Fig. 2,3). Recently we studied the protein-protein interactions that determine the subcellular signaling of ERKs both before and after stimulation. We found that inactive ERK2 interacts with a large number of proteins through its CRS/CD domain, whereas the phospho-ERK2 interacts with only few substrates. Varying calcium concentrations significantly modified the repertoire of ERK2-interacting proteins. The effect of calcium on ERKs' interactions influenced also the localization of ERKs, as calcium chelators enhanced nuclear translocation, while elevated calcium levels prevented it. This effect of calcium was apparent upon LPA stimulation, where ERKs translocation was delayed compared to that induced by EGF in a calcium-dependent manner. These results are consistent with a model in which in quiescent cells ERKs are bound to several cytoplasmic proteins. Upon stimulation, ERKs are phosphorylated and released from their cytoplasmic anchors to allow shuttling towards the nucleus. This translocation is delayed when calcium

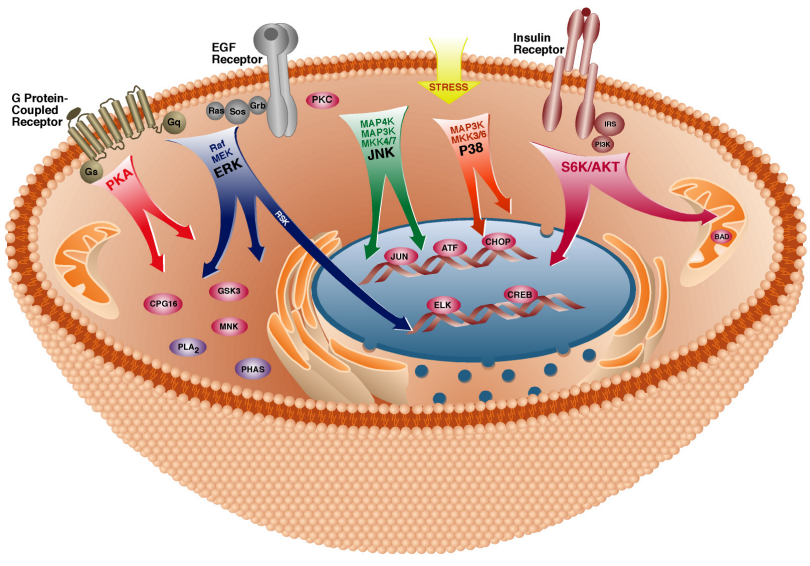

Fig. 1 *Schematic representation of intracellular signaling cascades.*

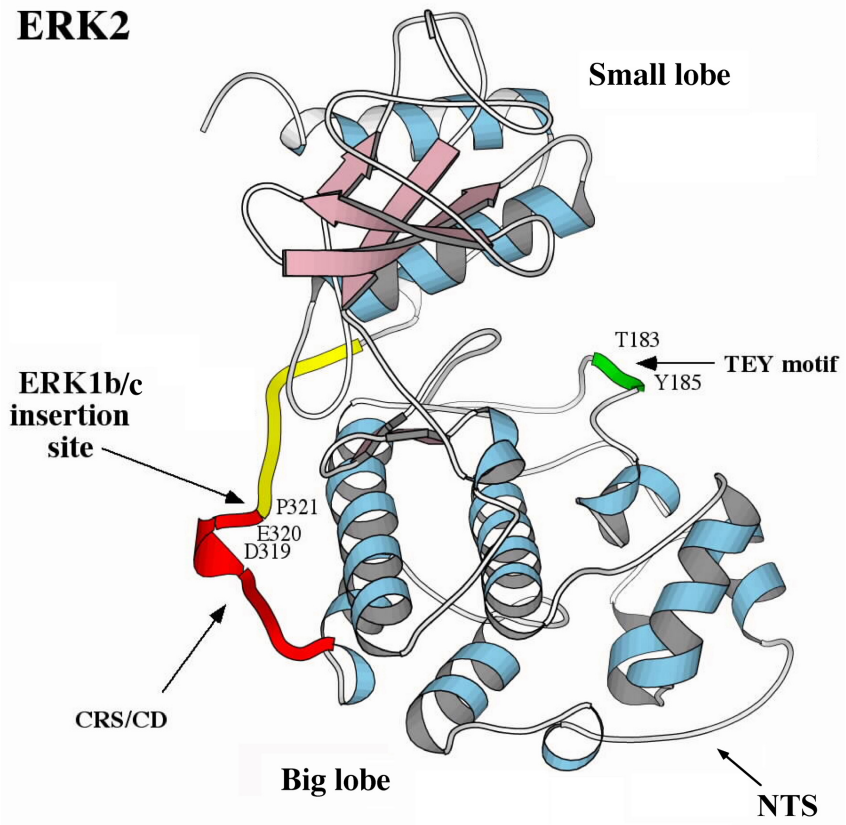

Fig. 2 *Three-dimensional structure of ERK2 with the sites of the CRS/CD, ERK1b/c insert, activation loop and the NTS*

levels are increased, and this modifies the localization of ERKs and therefore also their spatiotemporal regulation. The effects of calcium can be derived from its influence on the novel nuclear translocation signal that we have recently identified as the sequence that mediates the translocation of ERKs and MEKs into the nucleus. Thus, calcium regulates ERKs localization, which is important for the compartmentalization of ERKs with their proper substrates, and thereby their signaling specificity (Chuderland et al. 2008, Chuderland and Seger, 2008).

Another important subject that we studied in the past years is the mechanism by which ERKs are retained in the nucleus at later stages after stimulation. We found that the traslocated ERKs may interact with various nuclear proteins that do not allow their export, and often are activated by them or serve as nuclear scaffold proteins that facilitate ERKs activity. One of these anchors is PolyADP-ribose polymerase1 (PARP1), which is known to catalyze a post-translational modification of nuclear proteins by polyADP-ribosylation. We found that PARP1 interacts with ERKs via the CRS/CD domain of the latter. This interaction induces activation of PARP1 and thereby reveals an alternative mode of PARP1 activation, which does not involve the well-known interaction with damaged DNA. It also shows that ERKs can activate some of their substrates by a phosphorylation-independent mechanism. The activation of PARP-1 dramatically increased ERK2-catalyzed phosphorylation of the transcription-factor Elk1, and enhanced ERKs-induced histone acetylation and transcription of c-fos. Furthermore, the scaffolding activity of PARP1 causes retention of ERKs in the nucleus for a long while after mitogenic stimulation. Thus, PARP1 can serve as a nuclear scaffold of ERKs that determines their localizations and facilitate their nuclear activity (Cohen-Armon et al. 2007).

The subcellular localization is important not only for ERKs but also for the MEKs, which are mainly localized in the cytoplasm due to a nuclear export signal in their N-terminus. We previously found that upon stimulation this nuclear export signal induces a fast shuttle in and out of the nucleus. The role of the rapid shuttle is not clear, but we recently found that it may induce an export of nuclear proteins and thereby inhibits their nuclear activity. One of the examples that we recently published is PPARγ, which is a known target of the ERK cascade. It was previously demonstrated that PPARγ activity is attenuated upon mitogenic stimulation due to phosphorylation of its Ser84 by ERKs. During the study on the PPARγ as a target of the ERK cascade, we found that stimulation by TPA attenuates PPARγ's activity in a MEK-dependent manner, even when Ser84 is mutated to Ala. To elucidate the mechanism of attenuation, we found that PPARγ directly interacts with MEKs but not with ERKs, both in vivo and in vitro. Immunofluorescence microscopy and subcellular fractionation revealed that MEK1 exports PPARγ from the nucleus, and this finding was supported by small interfering RNA knockdown of MEK1 and use of cell permeable interaction blockers. Thus, we showed a novel mode of downregulation of PPARγ by its MEK-dependent redistribution from the nucleus to the cytoplasm. It is likely that MEKs also participate in the export of other nuclear proteins such as ER and ERKs (Burgermeisteir et al, 2007).

Regulation of the ERK cascade by phosphatases and alternative splicing

ERK activity is regulated mainly by the incorporation of phosphates to the regulatory Tyr and Thr residues in ERKs' activation loop. This incorporation of phosphates is mediated by the activating kinases MEKs, as well as by various phosphatases that remove the incorporated phosphates. The identity and mode of action of the phosphtases of ERKs is not fully understood yet, so we recently studied the role of the residues in the activation loop of ERKs

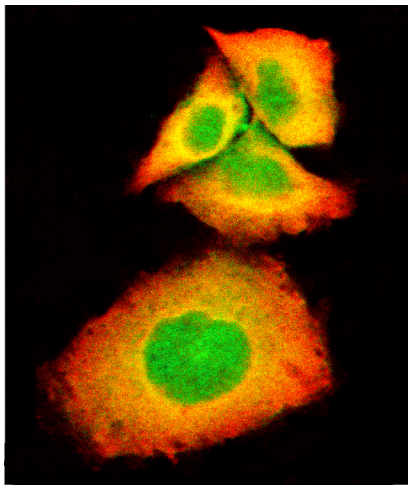

Fig. 3 *Localization of ERKs and MEKs in TPA-stimulated CHO cells. The accumulation of the green color in the nucleus indicates that most of the ERKs molecules were translocated while most of the MEKs molecules were detected in the cytoplasm.*

in determining ERK's dephosphorylation. We substituted residues within amino acids 173-177 of ERK2, and studied their role in ERK phosphorylation, substrate recognition, and subcellular localization. We found that substitution of residues 173-175 and particularly Pro174 to Ala reduces the EGF-induced ERK2 phosphorylation, without modifying its *in-vitro* phosphorylation by MEK1. Examining the ability of these mutants to be dephosphorylated revealed that 173-5A mutants are hypersensitive to phosphatases, indicating that these residues are important for setting the phosphorylation/dephosphorylation balance of ERKs. In addition, 173-5A mutants reduced ERK2 activity towards Elk-1, without affecting the activity of ERK2 towards MBP, while substitution of residues 176-8 decreased ERK2 activity towards both substrates. Substitution of Asp177 to Ala increased nuclear localization of the construct in MEK1-overexpressing cells, suggesting that this residue, together with His176, is involved in the dissociation of ERK2 from MEKs. Combining CRS/CD motif and the activation loop mutations revealed that these two regions cooperate in determining the net phosphorylation of ERK, but the role of the CRS/CD motif predominates that of the activation loop residues. Thus, we show here that residues 173-177 of ERK2 join other regulatory regions of ERKs in governing ERK activity mainly by phosphatases (Bendetz-Nezer and Seger, 2007).

The two most abundant isoforms of the ERKs are ERK1 and ERK2, which are regulated in a very similar manner. We have identified additional forms of ERK that seem to be activated by MEK1, but are subjected to a different mode of regulation and downstream targets. One such isoform is the rodent 46 kDa ERK1b, which has a unique mode of regulation, and its expression is increased upon oncogenic transformation. Another isoform that we have identified is ERK1c, which is produced in primates by a similar splicing process to that of the rodent ERK1b (inclusion of intron 7). ERK1c is a 42 kDa protein that is regulated differently from other ERK isoforms under various conditions. In a previous study we demonstrated that ERK1c can be localized in the Golgi of confluent cells and induce a cell density-dependent fragmentation. We further studied the Golgi function of ERK1c, and found that it plays an important role in the MEK-induced Golgi fragmentation during mitosis. Thus, in late G2 and mitosis of synchronized cells, the expression and activity of ERK1c was increased, and it colocalized with the Golgi marker p58. The activation of ERK1c was MEK-dependent, although its phosphorylation on Tyr204 alone was much stronger than the double Thr202 and Tyr204 phosphorylation. SiRNA of ERK1c significantly attenuated, while ERK1c overexpression facilitated, Golgi fragmentation during mitosis. These effects were also reflected in the progression of mitosis in the transfected cells, although the retardation in the pace of transitions between the different stages of mitosis did not fully correlate with the attenuation of fragmentation. Inhibition of all MEK isoforms had a much broader effect, indicating that mitotic progression is synchronized by Golgi fragmentation as well as by other MEK-dependent processes. However, the ERK1c seems to be activated primarily by the alternatively spliced form of MEK1, termed MEK1b and not by MEK1 or MEK2. In addition, the ERK1c effect could not be complemented by ERK1 or ERK2, making the Golgi effect a unique MEK1b-ERK1c effector in mitosis. This provides an alternative route for the ERK cascade and an additional mechanism for extending the specificity of the ERK signaling cascade (Shaul and Seger, 2006; Shaul and Seger,2007; Shaul et al 2008).

Regulation of apoptosis and angiogenesis by the ERK cascade

Apoptosis is crucial in regulating many aspects of the reproductive system. We found that several non-apoptotic G-protein coupled receptors (GPCRs) can induce apoptosis in some cell lines or when mislocalized. Since MAPKs and PI3K-dependent signaling play important roles in apoptosis regulation, we undertook to study their involvement in the induction of apoptosis in such systems. Two pro-apoptotic stimulants were investigated in two corresponding cell lines. One of them was prostaglandin F2α (PGF2α), which is known to be the principal physiologic luteolytic factor in mammals. It was found to exert direct apoptosis in human luteinized granulosa cells, and was thereby studied in SVOG-4O cell line. The second stimulant was gonadotropin-releasing-hormone-analog (GnRH-a), which is known to induce direct apoptotis in many malignancies, benign diseases and various cell lines. As a model system for this stimulant we used the mouse pituitary cell line, αT3-1, which was found here to undergo apoptosis upon GnRH-a treatment. We found that PGF2α and GnRH-a, operating via their cognate Gq-coupled receptors, directly induced apoptosis in SVOG-4O and αT3-1 cell lines, respectively. The apoptotic effect of PGF2α and GnRH-a is mediated by PKC upstream of the JNK cascade, and is inhibited by the PI3K-PKB pathway. PKC activation induced the assembly of the catalytic and regulatory subunits of PP2A, thus activating it. Activated PP2A was shown to bind to PKB, causing its dephosphorylation from both activatory pThr308 and pSer473. Furthermore, PKC activation inhibited of PI3K giving rise to a model in which

the reduction of PKB activity releases the PKB-induced inhibition of MLK3, which leads to elevated JNK activity and accelerated PGF2α and GnRH-a apoptotic effect. We concluded that both PGF2α and GnRH-a exert their pro-apoptotic effect via similar signal transduction pathways. Our results, revealing the signal transduction pathways governing apoptosis, may help both in the diagnosis and treatment of various abnormalities using specific signal transduction modulators.

In addition, we are also studying the signaling and physiological functions elicited by the plasma factor PEDF, which can act as an antiangiogenic factor. We found that PEDF purified from plasma is a phosphoprotein, which is extracellularly phosphorylated by protein kinase CK2 (CK2), and to a lesser degree, intracellularly, by protein kinase A (PKA). CK2 phosphorylates PEDF on two main residues, Ser24 and Ser114, and PKA phosphorylates PEDF on one residue only, Ser227. The physiological relevance of these phosphorylations was determined by using phosphorylation site mutants. We found that both CK2 and PKA phosphorylations of PEDF markedly affect its physiological function. The fully CK2 phosphorylation site mutant S24,114E abolished PEDF neurotrophic activity, but enhanced its antiangiogenic activity, while the PKA phosphorylation site mutant S227E reduced PEDF antiangiogenic activity. We recently studied the interplay between the PKA and CK2 phosphorylation of PEDF, and found that a PEDF mutant mimicking the CK2-phosphorylated PEDF can not be phosphorylated by PKA, while the mutant mimicking the PKA-phosphorylated PEDF is a good CK2 substrate. Using triple mutants that mimic the PKA and CK2 phosphorylated and non-phosphorylated PEDF, we found that PEDF can induce several distinct cellular activities dependent on its phosphorylation. The mutant mimicking the accumulative PKA plus CK2 phosphorylation, exhibited the strongest antiangiogenic and neurotrophic activities, while the mutants mimicking the individual phosphorylation site mutants had either a reduced activity or only one of these activities. Thus, differential phosphorylation induces variable effects of PEDF, and therefore contributes to the complexity of PEDF action. We are now studying a bacterial recombinant triple phospho-mimetic mutant, and our preliminary results indicate that it can be used to generate effective antiangiogenic drugs (Maik-Rachline and Seger, 2006).

The long-term objectives of our future studies are to obtain a comprehensive view on intracellular signaling in proliferation, apoptosis, angiogenesis and cancer. This will be achieved by studying (i) The protein-protein interaction, and the subcellular localization of signaling components. (ii) The regulation of nuclear processes by MAPK cascades. (iii) Mapping distinct intracellular signaling networks. (iv) Understanding the role of MAPK cascades in various systems such as apoptosis, angiogenesis and cancer. These approaches have, and will continue to allow the elucidation of the key regulators of most if not all cellular processes upon various stimulations.

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