MECHANISM AND REGULATION OF ERK2 SUBCELLULAR LOCALIZATION

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by

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MECHANISM AND REGULATION OF ERK2 SUBCELLULAR LOCALIZATION

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Dynamic changes in the localization of activated proteins can be obligatory events in signaling networks that control cell behavior. ERK1/2 activation contributes to regulated processes such as proliferation, differentiation and survival through the phosphorylation of multiple nuclear and cytoplasmic substrates. The pleiotropic effects of ERK1/2 activation suggest that regulated compartmentalization of the kinases and substrates may contribute to the fidelity of phenotypic changes in response to specific cell stimuli. Therefore, elucidating the mechanism of translocation as well as how this process is controlled is important for understanding how MAP kinases transmit signals. In vitro studies using a permeabilized cell system indicate that nuclear import of ERK2 is not regulated by soluble transport factors, but requires access to nucleoporins. While this process is not influenced by classical import machinery, it can be modulated by

anchoring proteins that bind to ERK2 and sequester the kinase in the cytoplasm. One of these proteins, PEA-15, prevents ERK2 import in an in vitro system by inhibiting the kinases' ability to interact with nucleoporins. In vivo assays of phosphorylated ERK1/2 show discrete subcellular localization patterns in response to different stimuli that are independent of the level of ERK1/2 activation. Under conditions in which ERK1/2 is concentrated in the cytoplasm, the nuclear substrate of the kinase, c-Fos, is not expressed, while the cytoplasmic substrate of ERK1/2, p90RSK, is phosphorylated.

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List of Tables and Figures

Abbreviations

ATP	Adenosine 5'-triphosphate
BRL	Buffalor rat liver
BSA	Bovine serum albumin
CD	Common docking
CDK	Cyclin-dependent kinase
CPM	Counts per minute
CRM1	Chromosomal region maintenance 1
DED	Death effector domain
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetracetic acid
ERK	extracellular signal-regulated protein kinase
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
HFF	Human foreskin fibroblast
IF	Immunofluorescence
JNK	c-Jun-N-terminal kinase

KOH	Potassium hydroxide
KSR	kinase suppressor of Ras
Kd	dissociation constant
LB	Leptomycin B
LPA	Lysophasphatidic acid
MAPK	Mitogen-activated protein kinase
MAP3K	MAP kinase kinase kinase
MAP2K	MAP kinase kinase
MEK	MAP kinase/ERK kinase
MEKK	MEK kinase
MgCl	Magnesium chloride
MKP	map kinase phosphatase
MLCK	myosin light chain kinase
MP1	MEK binding partner 1
NaCl	Sodium chloride
NaVO3	Sodium orthovanadate
NES	Nuclear export sequence
Ni^{+}NTA	nickel-nitriloaetic acid
NGF	Nerve growth factor
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
NTF2	Nuclear transport factor 2

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC12	Pheochromocytoma 12
PDGF	Platelet derived growth factor
PEA-15	Phosphoprotein dnriched in astrocytes – 15 kDa
PKC	Protein kinase C
PKD	Protein Kinase D
PLA_2	Phospholipase A_2
PMA	phorbol myristate acetate
PTP	Protein tyrosine phosphatase
RANGAP	Ran GTPase-activating protein
RanBP1	Ran binding protein 1
RCC1	Regulator of chromose condensation 1
REF	Rat embryo fibroblast
SDS	Sodium dodecyl sulfate
SOS	son of sevenless
TPA	12-O-tetradecanoylphorbol-13-acetate
TRITC	Tetramethylrhodamine B isothiocyanate
WGA	Wheat germ agglutinin

Chapter 1: General Introduction

Statement of Objectives

The MAP kinases, ERK1/2, are the terminal proteins in a three-tiered signaling cascade that impacts a majority of cellular functions. Numerous ligands activate ERK1/2, including growth factors, hormones, cytokines, serum and transforming agents. ERK1/2 substrates are involved in many cellular activities including growth, proliferation, survival, migration, glucose homeostasis and regulation of calcium entry [1, 2]. Thus, understanding how ERK1/2 are regulated to induce specific responses may reveal how cells interpret discrete environmental cues.

Patterns in the duration, amplitude and localization of active ERK1/2 are correlated with specific phenotypic changes [3-8]; however, how these characteristics are regulated at the molecular levels is unclear. The goal of my project was to understand how the subcellular localization of ERK1/2 is regulated, and how localization impacts phenotypic change. My project had three specific aims: 1) I wanted to determine the mechanism that ERK2 uses to move through the nuclear pore complex; 2) I planned to understand how a putative anchoring protein antagonizes ERK2 nuclear accumulation. 3) determine if activation of ERK1/2 can be uncoupled from nuclear localization. In the process of completing the last aim, I was able to examine some aspects of the amplitude of ERK1/2 activation not previously described in mammalian cells.

MAP Kinases

Protein phosphorylation serves as a powerful signal transducer by altering the activity, stability and subcellular localization of proteins. Protein kinases are the enzymes that catalyze the transfer of γ phosphoryl groups from ATP to serine, threonine or tyrosine residues on substrates. Recent analysis of the human genome has identified 518 putative protein kinases, which can be classified broadly into 9 major groups. MAP kinases are members of the CMGC group of kinases and are specific for serine and threonine residues on substrates. Twenty MAP kinases are known and although they respond to a surprisingly similar group of stimuli, many phosphorylate a discrete spectrum of substrates [9].

The activation of well-characterized MAP kinases involves a three-tiered protein signaling module (Figure 1-1). Extracellular signals induce the phosphorylation of a MAP kinase kinase kinase (MAP3K), which phosphorylates a dual specificity MAP kinase kinase (MAP2K), or MEK, which phosphorylates one or more MAP kinases on threonine and tyrosine residues in their activation loops (Figure 1-2). Once activated, MAP kinases phosphorylate serine and threonine residues within a motif best represented as PXS/TP. The proline next to the phosphorylation site, the P+1 residue, is a major requirement for substrate recognition by all MAP kinases; the only other residue found at this site, albeit rarely, is glycine[1, 10].

Well-studied MAP kinases include extracellular signal regulated kinase 1 and 2 (ERK1/2), p38 (four genes), c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (three genes), and ERK5 (Figure 1-1). In each of the signaling modules, only specific MAP2Ks are capable of phosphorylating the downstream MAPK, demonstrating their remarkably strict substrate specificity. Similarly, MAP2Ks are the

only enzymes identified to date taht are capable of phosphorylating MAPKs. In contrast, some MAP3Ks are not module specific and can activate more than one MAP2K, providing a mechanism to integrate multiple extracellular signals [1, 10].

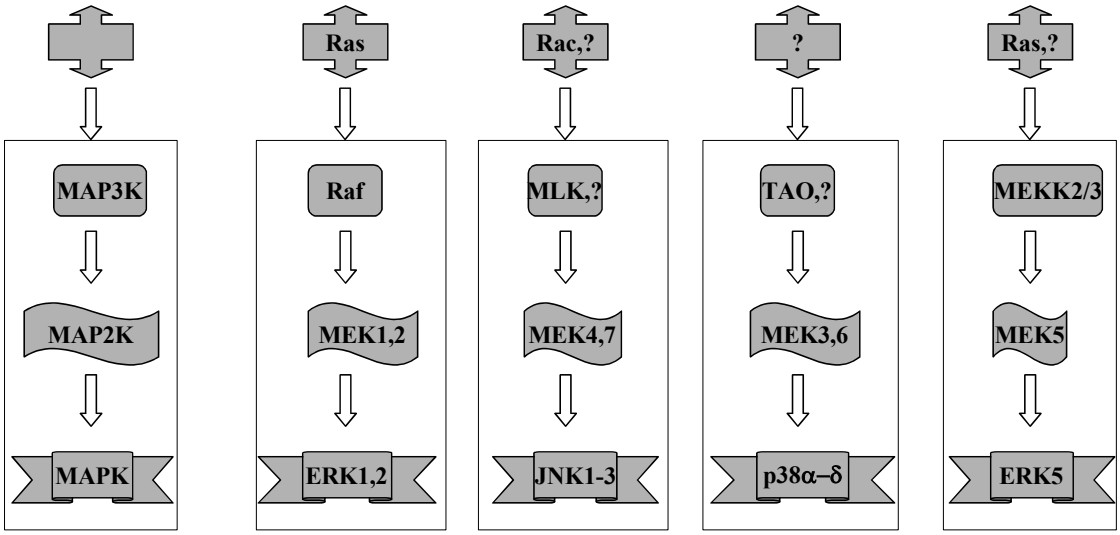


Figure 1-1: MAP Kinase Modules: MAP kinase cascades are composed of three kinases activated in sequence following stimulation. The left hand box indicates the general module. The four right hand boxes illustrate the best studied MAP kinase cascades.

ERK1/2 Module

ERK1 and 2 are 43 and 41 kDa kinases, respectively, that share 85 % overall sequence identity [11, 12]. Most in vitro studies have been conducted on ERK2, frequently the rat form. In this document, all residues listed will be those in the rat form of ERK2 unless otherwise noted. The term "ERK2" will be used in describing studies specifically on this form, while "ERK1/2" will be used in describing studies in which observation of the behaviors of the two isoforms are indistinguishable (e.g,

immunofluorescence using an antibody specific for both forms). Both forms of ERK1/2 have been found in all tissues and cells examined. Either form may be present in several fold excess of the other[1]. The functions of the two isoforms appear to be similar, but differences were revealed in mouse knockout studies. ERK1 knockout mice are viable but their thymocytes are unable to mature past the CD8+CD4+ stage[13]. This phenotype contrasts with that of the ERK2 knockout, which dies by embryonic day 12.5 due to a lack of vascularization of the placenta[14]. Thus, ERK2 may be redundant for many of ERK1 functions, but the opposite does not appear to be the case.

The X-ray crystal structures of both the unphosphorylated and phosphorylated form of ERK2 have been solved [15-17]. Like most protein kinases, ERK2 contains a catalytic core of two folding domains that harbor the active site in the cleft at their interface. This core is generally 270-300 amino acids and is composed of twelve subdomains that determine the kinase structure and contain catalytic residues [18]. ERK2, typical of the family, has an N-terminal domain composed primarily of β strands and a C-terminal domain formed by α-helices. Both of these domains contribute to the formation of the active site [15]. The N-terminal domain contains the phosphate anchor ribbon required for ATP binding. K52 in the β3 strand is crucial for properly orienting ATP; mutation of this residue creates a kinase-dead form of ERK2, which is the most commonly used mutation to inactivate protein kinases [19]. A second residue required for ATP interaction is E69 in the αC helix, an essential element of the N-terminal domain. The C-terminal domain contributes several other catalytic residues. In addition, protein substrate binding occurs on the surface of the C-terminal domain [15, 16].

In contrast to many kinases that contain a single activating phosphorylation site and/or autoinhibitory domain, activation of ERK2 requires dual phosphorylation. The activating phosphorylation sites, T183 and Y185, are located on the surface of the C-terminal domain in a region called the activation loop or phosphorylation lip[20]. When these residues are not phosphorylated, the two core domains are rotated apart and the catalytic residues are misaligned. Dual phosphorylation induces conformational changes that reposition residues in the activation loop, the αC helix and the P+1 site. These structural changes increase both the] activity and substrate recognition of ERK2 [16].

Inserts of variable length between protein kinase subdomains provide unique surface determinants that confer substrate and interaction specificity. ERK2 contains a 50- residue insert, called the MAP kinase insert, common to the MAP kinase family. This region, comprised of two helices (α1L14 α2L14), is found in the C-terminal domain adjacent to the substrate docking surface. The insert forms an interaction with the phosphorylation lip in the unphosphorylated form, but it becomes more mobile following dual phosphorylation. The role of this insert in ERK2 function is discussed below [15, 16].

Dually phosphorylated ERK2 formed dimers during crystalization and follow-up gel filtration analysis and equilibrium sedimentation experiments supported this observation [16, 21]. The dissociation constants for the phosphorylated and unphosphorylated dimers are ~20 nM and 20 μM, respectively. Analysis of the dimers formed in crystals revealed that the interface involved a leucine zipper and two ion pairs. The mutations L333A, L336A, L341A and L344A in combination with the charge reversal mutation, H176E, produce a dimer-deficient form of ERK2 [16, 21].

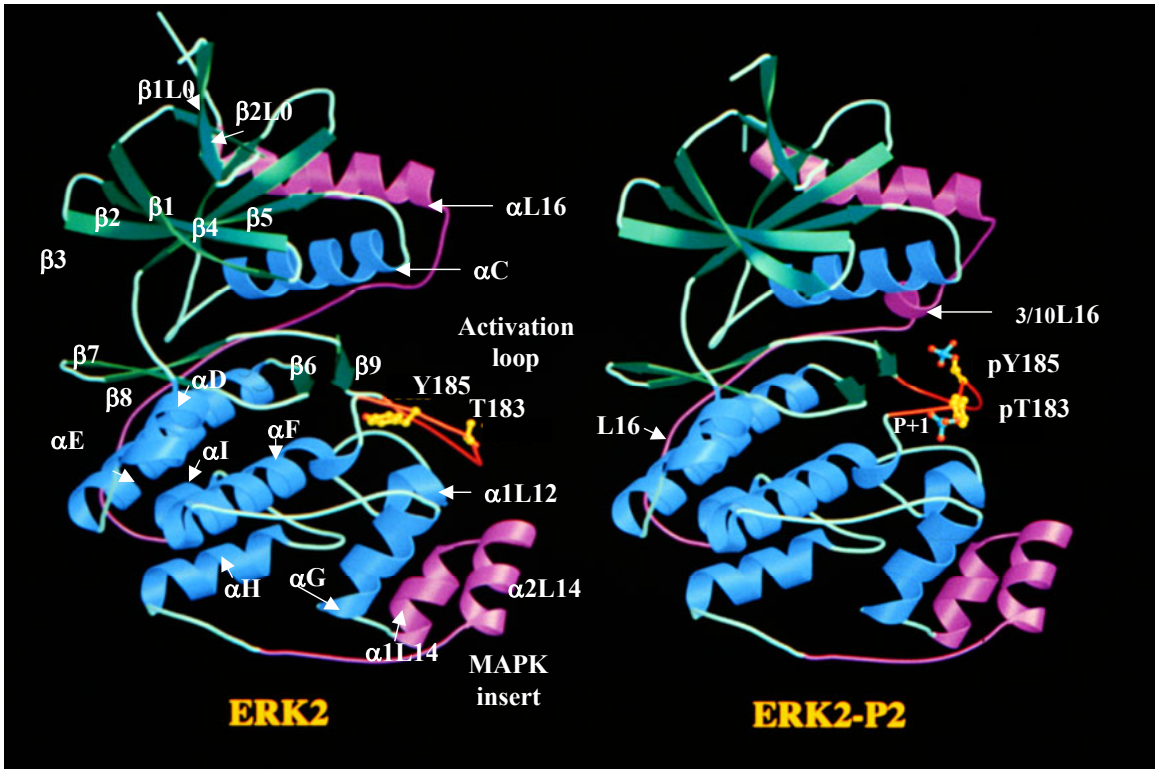


Figure 1-2: Crystal structure of unphosphorylated and dually phosphorylated ERK2. The structure on the left is unphosphorylated ERK2. The structure on the right is dually phosphorylated ERK2.

Dual phosphorylation of ERK1/2 is catalyzed by the MAP2Ks, MEK1/2 [22-25]. The MEK1 form has been studied extensively and will be the primary form referred to here unless otherwise noted. Similar to ERK2, MEK1 knockout mice lack proper placental vsacularization and die at embryonic day 10.5 [26]. MEK2 knockout mice are normal and do not have the thymocyte maturation defect observed in ERK1 knockout mice [27].

Activation of MEK1/2 is catalyzed by the MAP3K, Raf, which has three isoforms: Raf-1 (or c-Raf), B-Raf and A-Raf [28, 29]. The majority of studies have centered on Raf-1. Activation of MEK1 by Raf-1 occurs through the phosphorylation of serines 217 and 221 [30]. Once dually phosphorylated, MEK1 activity increases 7000-fold with changes in both k_{cat} and K_m [30, 31]. Substitution of the serines on MEK1 with acidic residues in combination with a small N-terminal deletion yields a highly active form of MEK1, termed MEK1R4F [31]. The overexpression of this mutant kinase in cells induces constitutive activation of ERK1/2 [32].

Pharmacological inhibition of MEK1/2 is achieved through the inhibitors PD98059, U0126, and PD184352, which are used extensively in animal and cell culture systems. The mechanism of action of these compounds is not clear but they do not appear to compete for ATP binding [33-36]. A crystal structure of MEK1 bound to the PD98059 compound shows a displacement of the αC helix on the kinase (noted on ERK2 in Figure 1-2), suggesting PD98059 induces a conformation change on MEK1 that interferes with catalysis (M. Cobb personal communication).

MEK1 phosphorylates ERK2 on T183 and Y185 and thereby dramatically increases ERK2 activity. Following dual phosphorylation of ERK2, the change in k_{cat} is nearly 50,000 fold [37]. Studies of MEK1 phosphorylation of ERK2 indicate that ERK2

is phosphorylated first on tyrosine and then on threonine in vitro and in vivo. Although kinetic parameters are affected, phosphorylation on tyrosine does not increase the activity of ERK2 in any manner detectable in cells. Therefore, the non-processive mechanism suggests that a threshold of mono-phosphorylated ERK1/2 must be reached before the kinases can be dually phosphorylated and activated [38, 39]. The K_d for the MEK1-ERK2 interaction has been placed in the μM range [40]. This interaction is weakened upon phosphorylation of ERK2 with the initial tyrosine phosphorylation apparently having the greatest effect.

Various methods have been used to examine the association of MEK1 and ERK2, including co-immunoprecipitation, in vitro binding and two hybrid tests. These methods, in combination with chimeras and mutagenesis, have identified a number of important regions for the interactions between the two proteins. Using chimeras, Brunet et al. reported that the ERK1/2 αC helix (Figure 1-2) is an important specificity determinant [41]. Subsequently, elements in the C-terminus also were implicated in the interaction between ERK2 and MEK1. A conserved region on MEK1 containing three lysines and a number of hydrophobic residues, called the docking (D) domain, has been identified as required for ERK2 binding. Interestingly, this region is also found in a number of ERK2 substrates (see below) [42].

Additional studies show that MEK1 binds to ERK2 in or around the MAP kinase insert, a 50 residue region that is unique to MAP kinases, cyclin-dependent kinases (CDKs) and glycogen synthase kinase (GSK) (Figure 1-2). MEK1 is unable to bind and activate ERK2 lacking the MAP kinase insert. Point mutations identified the insert that weaken the ability for MEK1 to activate ERK2[40]. A proline-rich insert found

exclusively in MEK1/2 is apparently an additional determinant for MEK1 and ERK2 binding. Deletion analysis suggests that this region is required for ERK1/2 activation in cells but not in vitro [43].

Activation of ERK1/2 typically occurs in response to stimulation of cell surface receptors. Growth factors, cytokines, transforming agents and G-protein coupled receptor (GPCR)-activating ligands all induce the ERK1/2 cascade [1, 2, 44]. Stimulation of tyrosine kinase receptors leads to activation of the small G protein, Ras, by its guanine nucleotide exchange factor (GEF), son of sevenless (SOS). In the GTP-bound form, Ras recruits Raf-1 to the membrane, where Raf-1 is phosphorylated and activated [1, 45]. In addition to growth factors, phorbol esters such as phorbol myristic acid (PMA, also referred to as TPA) potently activate the ERK1/2 cascade (Figure 1-3) [46].

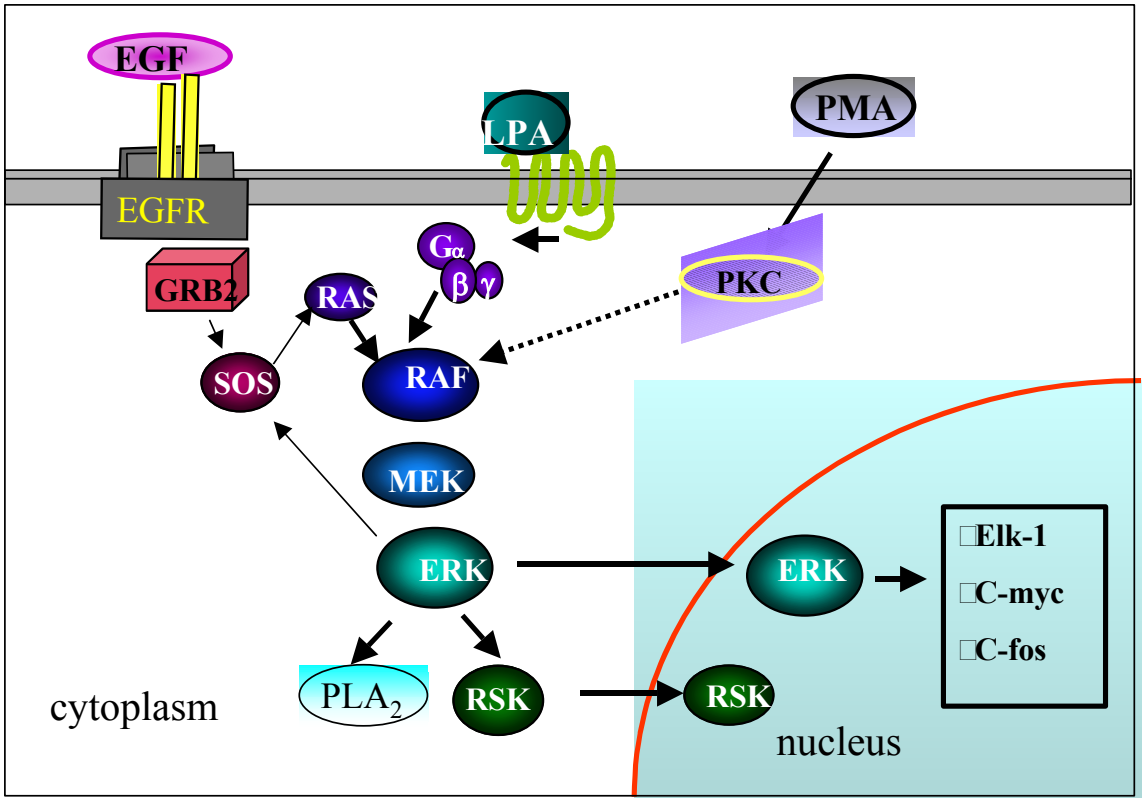


Figure 1-3: Stimuli and effectors for ERK1/2. Activation of Raf can occur through recruitment to the membrane after RAS activation. Following activation, ERK1/2 phosphorylates cytoplasmic substrates and can also move into the nucleus and phosphorylate substrates.

Following activation, ERK1/2 phosphorylate a number of downstream target proteins containing the minimal consensus sequence PXS/TP [47]. ERK1/2 effectors are localized in the nucleus as well as in the cytoplasm including on the membrane and cytoskeleton. Substrates include cytoskeletal proteins such as the microtubule associated protein, MAP-2 [11], protein kinases such as p90 ribosomal protein S6 kinase (RSK) [48, 49], MAP kinase-interacting kinases (MNK1&2) [50](Cooper 1997, EMBO) and myosin light chain kinase (MLCK) [51]. ERK2 also phosphorylates the apoptotic enzyme, caspase-9 [52], as well as many transcription factors such as the ternary complex factor, Elk-1, c-Myc and c-Fos [53-56]. In addition, ERK1/2 has been shown to phosphorylate and activate cytoplasmic phospholipase A_2 (PLA_2) (Figure 1-3) [57].

The plethora of ERK1/2 activating agents and effectors suggests a role for the kinase in a number of cellular programs. Indeed, ERK1/2 are crucial for growth, proliferation, differentiation and survival as well as differentiated functions including glucose homeostasis in pancreatic β cells and the reduction of calcium entry into cells[1, 2, 10]. Because ERK1/2 are downstream of Ras, which is mutated to an active form in many cancers, ERK1/2 have been implicated in abnormal proliferation. A 5-10 fold increase in activity of the kinases has been documented in primary breast carcinoma. Overexpression (5-20 fold) of the kinases also has been demonstrated in breast cancer tissue [58]. The relationship of the increase in ERK1/2 activation and abnormal proliferation has been confirmed by the use of the MEK inhibitors. In a study using PD184352, colon cancer cells were unable to undergo a G1 to S transition, or to form tumors in nude mice. PD184532 also reduced the invasiveness of these colon cancer

cells [36]. Although it seems clear that ERK1/2 themselves are not the primary defects in neoplasia, the sufficiency of ERK1/2 in the contribution to transformation phenotypes in model systems has been confirmed. For example, overexpression of the constitutively active MEK1, MEK1R4F, is sufficient to induce transformation of 3T3 cells[6].

The proliferative response to ERK1/2 activation occurs through the phosphorylation of ternary complex factors (TCF) such as Elk-1, and the related stress activated transcription factors, SAP-1a and SAP-2 [59]. These proteins bind to serum response elements often found in the promoters of immediate early genes such as *c-fos, egr-1, fra-1* and *fra-2*. Furthermore, ERK1/2 can phosphorylate and stabilize the expression of these immediate early genes, which in turn are required for the expression of cyclin D1 and cell cycle entry [5, 60, 61].

Inactivation of ERK1/2 occurs through the dephosphorylation of either tyrosine or threonine residues. Serine/threonine, tyrosine and dual specificity phosphatases dephosphorylate ERK1/2 in vitro, but the contribution of these phosphatases to ERK1/2 function in vivo is unknown [62]. Treatment of cells with the simian virus 40 small t-antigen, which inhibits the serine/threonine phosphatase, PP2A, is sufficient to increase ERK2 and MEK1 activity but not that of Raf-1 [63]. Numerous tyrosine phosphatases, including tyrosine phosphatase-SL (PTP-SL), hematopoietic protein tyrosine phosphatase (HePTP) and striatal-enriched phosphatase (STEP) dephosphorylate the Y183 residues of ERK 1/2 in vitro [64, 65].

A class of dual specificity phosphatases, MAP kinase phosphatases (MKPs), has are suggested to remove phosphate from both the threonine and tyrosine residues specifically on MAP kinases in a temporally and spatially regulated manner. MKP-1,

which is nuclear, dephosphorylates ERK1/2, JNK and p38. MKP-1 is transcribed during the immediate early gene response and is rapidly degraded by the proteasome, but phosphorylation of MKP-1 by MAP kinases stabilizes expression [66]. MKP-3, found primarily in the cytoplasm, appears to be specific for ERK1/2 dephosphorylation. MKP-3 activity is regulated by ERK2, which when bound, increases catalytic activity [7, 67]. While MKPs have the capacity to dephosphorylate both tyrosine and threonine on ERK1/2, it is not clear whether MKPs dephosphorylate both T183 and Y185 in cells.

Because all MAP kinases recognize phosphorylation sites in the context of a proline at the P+1 position, additional mechanisms exist to ensure proper interactions between individual MAP kinases and specific substrates. The identification of a number of motifs on ERK1/2-interacting proteins has suggested that ERK1/2 associates with multiple regions on substrates. Two motifs have been identified and well studied: the docking (D) and the FXF motif. The D motif is typified by $(R/K)_{1-3}X_{2-4}\Phi X\Phi$ (where X represents any residue and Φ indicates a hydrophobic residue) and is found in MEK1/2, substrates Elk-1 and SAP, and also the phosphatase, MKP-3 [68]. A reverse motif, LAQRR, is found on the ERK1/2 substrates p90RSK and MNK1 [69]. A region on ERK2 is required for binding to the D motif. Two sets of charged residues were identified by Nishida and colleagues: Asp316 and Asp319 (named the common docking (CD) site) and Glu160 and Asp161 (named the ED site) (Figure 3-1)[42, 70]. Additional studies found that mutation of Tyr314 and 315 reduced binding of ERK2 to MEK1 [71]. MEK3 and MEF2A, kinase and substrate for p38α respectively, also have D motifs. Co-crystallization of p38α with peptides from MEK3b and MEF2A revealed a docking groove on p38α that makes significant hydrophobic interactions with the D motif.

Binding experiments demonstrated that while mutations of charged residues in the CD domain reduced binding of MEF2A to p38α, mutations of the hydrophobic residues in the docking groove dramatically reduced the interaction between the two proteins. The MEK1 D domain binds to ERK2 through a hydrophobic groove across the αD helix that includes D316, D319 and E320. The hydrophobic interactions are likely the primary mechanism for the association between the D domain and MAP kinase [71, 72].

While the D domain is found on interacting proteins for ERK2, JNK2 and p38α, the FXF motif has thus far been found solely on ERK2 effectors. Elk-1, Lin-1, c-Fos, Egr-1, Fra-1, Fra-2, MKP-1 and the scaffold protein kinase suppressor of Ras (KSR), all contain one (or two in the case of KSR) FXF motif [60, 61, 73, 74]. Mutation of both phenylalanines within the motif increases the Km for phosphorylation of a peptide substrate 9-fold in vitro. The addition of two copies of the FXF motif to a peptide substrate increases the affinity for ERK2 10-30 fold. Many ERK1/2 interacting proteins contain both a D and FXF motif. Mutation studies measuring phosphorylation of D and FXF motif-containing substrates have found that these domains bind independently to ERK2, but function cooperatively to increase binding affinity [74, 75]. The region in ERK2 required for interaction with FXF has not been identified.

Scaffold proteins have been identified for MAP kinases in yeast, flies, worms and mammals. These proteins are hypothesized to serve multiple functions including increasing the efficiency of activation, inducing spatial and temporal regulation and promoting stimulus-specific activation of the cascade [1, 76]. Initial studies of scaffold proteins in yeast concluded that these proteins, often non-catalytic, are required for activation of the MAPK cascade of the pheromone-mediated mating pathway. For

example, in *S. cerevisiae*, Ste5 was recognized to bind to all three components of the MAP kinase cascade [77]. Yeast strains lacking Ste5 are unable to mate despite the presence of mating pheromones [78].

A well-studied mammalian scaffold is kinase suppressor of Ras (KSR), which was identified by genetic analysis in *C. elegans* and *D. melanogaster.* Deletion of both KSR isoforms in *C. elegans* impairs vulval development, while deletion of the only isoform in *D. melanogaster* is lethal [79, 80]. Loss of KSR from S2 cells by RNAi interference prevents activation of ERK1/2 in response to insulin [81]. Mammals have at least two KSR genes, with KSR1 being the predominantly studied form. KSR1 interacts with Raf-1, MEK1/2 and ERK1/2; it is hypothesized that KSR1 interacts with Raf-1 and/or MEK1/2 constitutively [76]. Morrison and colleagues hypothesize that in unstimulated cells, KSR1 is primarily localized to the cytoplasm and is quickly recruited to the plasma membrane following cell stimulation [82]. Mice lacking KSR1 are grossly normal, but stimulation of MEFs with EGF induces activation of half as much ERK1/2 as wild type mouse embryonic fibroblasts (MEFs). This reduced activation of ERK1/2 is associated with impaired T-cell activation and reduced tumor growth of epithelial cells [8]. In KSR1 -/- MEFs, Raf-1 activation is attenuated in response to EGF, but not to the phorbol ester, PMA as compared to wild-type cells [83]. These studies suggest that KSR is required for the activation of a subset of ERK1/2 by interacting with multiple components of the cascade and in response to discrete stimuli.

MP1 is another ERK1/2 pathway scaffold that was identified through a yeast two-hybrid screen for MEK1 interactors. MP1 has been shown to preferentially bind MEK1 and enhance ERK1 activation. When MP1 expression is reduced by RNAi in mammalian

cells, MEK1/2 and ERK1/2 activation are reduced, with the largest effect on ERK1 [84]. MP1 is found on late endosomes where it localizes with dually phosphorylated ERK1/2 10-30 minutes following EGF stimulation [85]. A number of other scaffold proteins bind to members of the ERK1/2 kinase module, but it is not clear how these proteins contribute to cascade regulation.

ERK1/2 are found in both the cytoplasmic and nuclear compartments [86]. Numerous studies indicated that ERK1/2 moves from the cytoplasm to the nucleus following activation. Localization of the active form to the nucleus is thought to be critical for the activation of certain transcription factors and proliferative and differentiation responses [7, 87-89]. Overexpression of a plasmid containing ERK2 fused to a constitutively nuclear and active MEK1, MEK1R4F-LA induces proliferation in PC12 cells and transformation of 3T3 cells [6]. Unphosphorylated ERK1/2 are also capable of localizing to the nucleus, although the function of this localization is unknown[90]. A more detailed introduction to ERK1/2 subcellular localization will be presented following an explanation of nuclear transport mechanisms.

Nuclear Transport

Many signaling proteins are activated in the cytoplasm and target nuclear substrates, making movement into the nucleus a requirement for signal transmission. The nuclear envelope is composed of an inner and outer membrane separated by the perinuclear space. Movement across the nuclear envelope is only possible through 90-120 MDa channels called nuclear pore complexes (NPCs). Each NPC is composed of a central framework, cytoplasmic filaments and a nuclear basket. The central framework is a 90 nm high channel with an hourglass shape. On the cytoplasmic and nuclear faces,

the pore is ~70 nm wide, while in the center the width is 30-40 nm. It is through this structure that proteins and ions move into and out of the nucleus [91-93].

The functional transport constituents of the NPC are nucleoporins, which are attached to the central framework, cytoplasmic filaments and nuclear basket. Typically, nucleoporins contain a number of motifs with phenylalanine and glycine: FG, FXFG and GLFG. Biochemical analysis of the yeast NPC has identified 29 nucleoporins, which are present in multiple copies in a single NPC. A comprehensive analysis found that most yeast nucleoporins are distributed symmetrically on both sides of the NPC [94]. Based on stokes radius, sedimentation coefficient and proteinase K experiments, FXFG containing nucleoporins are thought to have many disordered regions. [95]. It is hypothesized that the disordered nucleoporins form a filamentous barrier in the NPC, which can only be permeated by proteins capable of interacting with nucleoporins [94, 95]

Ions and small proteins (< 40 kDa) can diffuse through the network of nucleoporins, but analysis of passive transport using colloidal gold particles has revealed that this process is very slow for particles with a diameter greater than 5 nM[96]. Therefore, the movement by passive diffusion of most cellular proteins is highly inefficient, and typically requires soluble transport receptors that interact with nucleoporins to facilitate translocation through the pore [91, 92].

Receptor-mediated import and export processes are illustrated in Figure 1-4. The soluble transport receptors that facilitate the movement of cargo are called karyopherins and are classified as either α or β; numerous isoforms of both of these proteins exist. Karyopherin-α binds cargo through an arginine- and lysine-rich region called a nuclear localization sequence (NLS). The NLS was first identified in the SV40 large tumor

antigen as PKKKRKV [97, 98]. Additional sequences that can act as an NLS have also been described, including a bipartite sequence that has two basic amino acid stretches separated by 10 to 12 residues. Cargo-bound karyopherin-α forms a complex with karyopherin-β, which specifically interacts with the FG motifs on nucleoporins to move the tripartite complex through the NPC in a process called facilitated translocation. Because nucleoporins are also post-translationally modified by N-acetyl glucosamine residues, microinjection of wheat germ agglutinin, which binds with high affinity to these sugar and carbohydrate moieties, inhibits the interaction of nucleoporins with karyopherins as well as nuclear import [91, 92, 99].

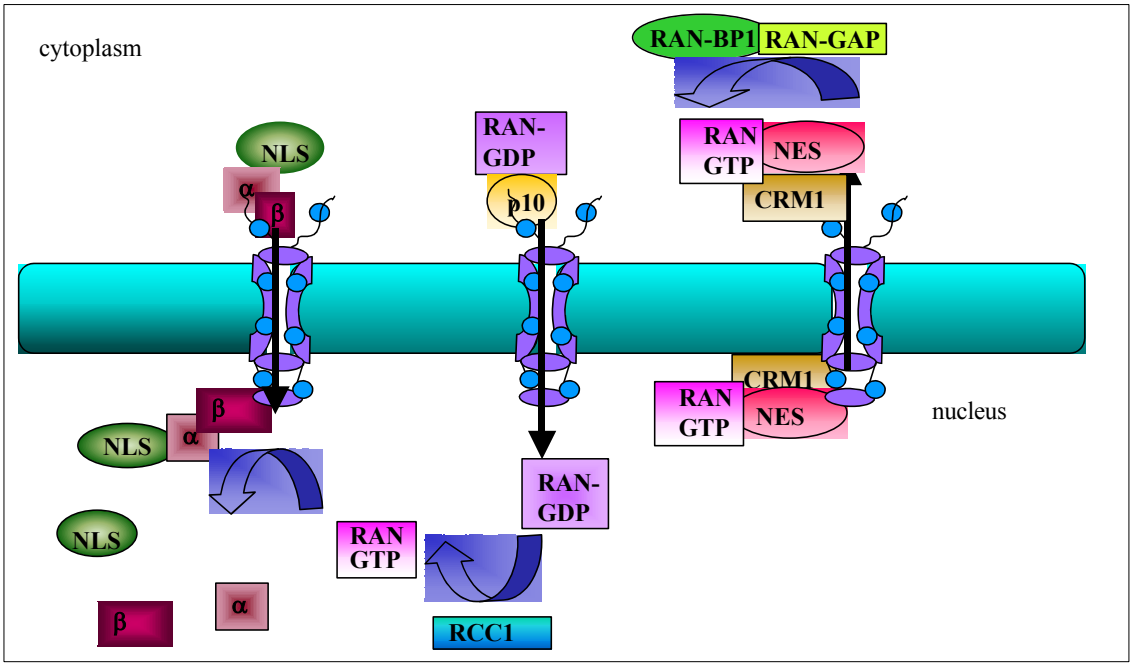


Figure 1-4: Receptor Mediated Transport. α = Karyopherin- α, β = Karyopherin- β. NLS and NES represent proteins with a nuclear localization or nuclear export signal, respectively.

Ran, a member of the Ras superfamily of GTPases, regulates the binding of karyopherins to cargo. Ran-GTP is localized to the nucleus while Ran-GDP is found predominantly in the cytoplasm [100-102]. This gradient of Ran is thought to provide a mechanism for the ability of cytoplasmic cargo proteins to move against a concentration gradient into the nucleus [103]. In the GTP-bound form, Ran interacts strongly with karyopherin-β (nM range) [99]. This interaction induces a conformational change that weakens the interaction of karyopherin-β with both nucleoporins and karyopherin-α inducing the release of cargo. Ran-GDP is unable to interact with karyopherin-β and therefore, the presence of Ran-GDP in the cytoplasm allows the formation of the cargo-karyopherin complex and nuclear entry [104, 105].

A cytoplasmic GTPase activating protein (GAP), and a nuclear guanine nucleotide exchange factor (GEF) maintain the Ran gradient. RanGAP hydrolyzes GTP in the cytoplasm. The GEF, regulator of chromosomal condensation (RCC1), exchanges GDP on Ran for GTP in the nucleus. Ran is continually cycling between the nucleus and cytoplasm [99, 106, 107]. The movement of Ran-GDP into the nucleus requires p10/NTF2, which binds specifically to the GDP form of Ran. In a manner similar to karyopherin-β, p10/NTF2 binds to nucleoporins and moves through the NPC [108, 109].

Export of proteins from the nucleus involves both Ran and karyopherins. Chromosomal region maintenance 1 protein (CRM1) is a well-characterized nuclear export receptor that binds to both substrates containing leucine-rich nuclear export sequences (NES) as well as Ran-GTP [110]. CRM1 binds directly to nucleoporins and moves the tripartite complex through the NPC. On the cytoplasmic side, Ran-GTP is

hydrolyzed to Ran-GDP and the complex dissolves, releasing both Ran-GDP and cargo into the cytoplasm [111, 112]. CRM1 is the target for the antibiotic, leptomycin B, which alkylates a single cysteine residue on CRM1 that is required for the interaction with nucleoporins [113]. Treatment of cells with leptomycin B inhibits export of CRM1 as well as its cargo [114].

Karyopherin-β binds to nucleoporins via hydrophobic a interaction with FG motifs. Structurally, karyopherin-β is composed of 19 HEAT (for Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2a and TOR1) repeats, which consist of two α-helices. The structure of karyopherin-β in a complex with an FG containing peptide has been solved and demonstrates HEAT repeats 5 and 6 are the primary FG binding regions [115]. NTF2/p10 also requires an interaction with FG repeats to move through the NPC, but NTF2/p10 must dimerize for proper translocation. The NMR structure of the NTF2/p10 dimer bound to a FXFG peptide revealed a "hydrophobic stripe" across the dimerized protein that is required for nucleoporin interaction [109, 116]. These analyses indicate that the interaction of proteins with nucleoporins is primarily hydrophobic without a strict structural requirement. The interactions between import receptors and nucleoporins is weak (μM range); however, the binding is required for nuclear entry [117, 118]. The mechanism by which these interactions promote the directional movement of the receptor through the NPC remains unclear.

Entry into the nucleus of at least three signaling proteins requires binding to nucleoporins.. The first identified protein was β-catenin, which is a component of the Wnt/wingless pathway and contains a number of armadillo (ARM) repeats. ARM repeats are structurally similar to HEAT motifs. Once activated, β-catenin translocates to the

nucleus and interacts with transcription factors. Several studies have demonstrated that β-catenin is capable of moving into the nucleus independently of karyopherin-α and β and Ran, but the movement does require an interaction with nucleoporins [119, 120]. We have proposed a similar model for ERK2, which will be described in chapter 2, and subsequently, the transcription factor, Smad2, was shown to traverse the NPC through an interaction with nucleoporins [121, 122]. Smad2 does not contain HEAT repeats, but does have a hydrophobic patch that is required for direct interaction with FXFG containing nucleoporins [122].

ERK1/2 Subcellular Localization

Translocation of signaling proteins from one cellular compartment to another is required for the activation of specific substrates and proper signal transmission. Most MAP kinases translocates to the nucleus following stimulation of cells [7, 86, 123, 124]. Blenis and colleagues, using both subcellular fractionation and immunofluorescence techniques, originally demonstrated that ERK1/2 localize to the cytoplasm in resting cells and are present in the nucleus following mitogen stimulation [86].

A physiological role for nuclear, active ERK1/2 is inferred from its ability to phosphorylate and activate nuclear transcription factors involved in proliferation. These substrates include Elk-1, c-Myc, and c-Fos [54, 55, 125]. Davis and colleagues have demonstrated that nuclear localization of overexpressed ERK2 correlates with an increase in c-Myc phosphorylation as well as transactivation. Overexpressed kinase dead ERK2 does not increase activity of the c-Myc transactivation domain [55].

Subsequent studies demonstrated that roughly half of the cellular activated ERK1/2 are associated with microtubules following stimulation with serum, indicating that only a fraction of the activated kinase is localized to the nucleus [126]. Because

ERK1/2 have cytoplasmic, cytoskeletal, membrane and nuclear substrates, regulatory mechanisms must exist to ensure proper subcellular distribution [1].

A number of studies of endogenous ERK1/2 have suggested that activation is sufficient for ERK1/2 nuclear entry. Pouyssegur and colleagues have used the hamster fibroblast cell line, CCL39, to examine ERK1/2 nuclear localization. In these cells, ERK1/2 are activated transiently (< 10 minutes) in response to non-mitogenic ligands, such as a thrombin receptor peptide agonist (TRP). In response to mitogenic stimuli, such as serum, ERK1/2 are activated in a prolonged manner (up to 6 hours). Following 2 minutes of stimulation with TRP, low levels of activated ERK1/2 were observed in the nucleus. At 5 minutes, significant nuclear accumulation occurred, and at 10 minutes active ERK1/2 were localized throughout the cell. In response to serum, low levels of ERK1/2 were again observed in the nucleus at 2 minutes. At 5, 10 and 15 minutes, active ERK1/2 were localized in the nucleus, with the highest amount of accumulation observed at 10 minutes. This observation led the authors to hypothesize that activation is sufficient for the nuclear entry of ERK1/2 [127]. In support of this hypothesis, the addition of the MEK1/2 inhibitor, PD98059, in combination with serum to CCL39 fibroblasts inhibited the majority of both ERK1/2 activation and nuclear localization [7].

Activation has also been associated with nuclear localization in PC12 cells stimulated with NGF. In response to NGF, which induces neurite outgrowth, ERK1/2 are activated in a prolonged manner (~24 hours) [87, 128]. Total ERK1/2 have been observed in the nucleus following NGF treatment at 5 and 45 minutes [87]. These experiments were performed prior to the development of ERK1/2 phospho-specific antibodies; therefore it is not clear if this nuclear ERK1/2 is active. In response to EGF,

which induces a transient (less than 10 minutes) activation of ERK1/2, no accumulation was observed in the nucleus at 5 or 45 minutes [129]. The investigators in this study did not examine earlier time points, and because ERK1/2 are active at 2 minutes following EGF stimulation, the possibility that nuclear entry occurs at an earlier time point cannot be ruled out [130].

The studies in CCL39 and PC12 cells suggest a strong correlation, in most cases, between ERK1/2 activation and nuclear entry. However, overexpression studies have suggested that activation is not a requirement for ERK1/2 nuclear localization. When ERK1/2 are grossly overexpressed (100X physiological concentration), immunofluorescence staining reveals the presence of the kinases in both the cytoplasm and nucleus of resting cells [131]. A similar pattern is observed when a kinase dead mutant of ERK2, K52R, is overexpressed [88]. Further analysis with mutants of ERK2 revealed that phosphorylation of the kinase is also not required for nuclear localization. The activation loop mutants, T183A and Y185F are localized to the nucleus when overexpressed [132].

Increasing the cellular concentration of ERK2 by overexpression may prevent anchoring in specific compartments due to a change in the stoichiometric ratios of ERK2 and the proteins that control its localization. Therefore, when endogenous and overexpression data are considered, one could conclude that cytoplasmic anchors may exist for the unphosphorylated form of ERK1/2. The activation of ERK1/2 may not be required for movement into the nucleus, but could be important for release from cytoplasmic anchoring proteins.

Among suggested cytoplasmic anchors for ERK1/2 are MEK1/2. In contrast to ERK1/2, MEK1/2 are found constitutively in the cytoplasm of resting and stimulated cells; overexpressed MEK1 is also cytoplasmic. This localization pattern is probably due to a leucine rich NES in the N-terminal region (residues 33-44) of MEK1 [133]. The NES is near an ERK2 binding region on MEK1 that will be discussed later. Mutation of the NES induces nuclear accumulation of MEK1 [134]. Treatment of cells with the nuclear export inhibitor, leptomycin B, allows the observation of MEK1/2 nuclear accumulation, indicating a CRM1-dependent export mechanism that maintains the bulk of MEK1/2 outside of the nucleus [132].

Nishida and Seger have demonstrated that the nuclear accumulation of overexpressed ERK2 is reversed in resting fibroblasts by co-expression with MEK1[135, 136]. The ability of microinjected or overexpressed MEK1 to sequester overexpressed ERK2 in the cytoplasm suggests that endogenous MEK1/2 may anchor endogenous, inactive ERK1/2 in the cytoplasm [132]. Stimulation of cells overexpressing both kinases induces nuclear localization of ERK2, an observation in agreement with the decreased affinity of the active ERK2 for MEK1. Conversely, when D domain mutants of MEK1 are co-expressed with wild-type MEK1 or ERK2, nuclear localization of overexpressed ERK2 is observed[71].

To demonstrate that a direct interaction between MEK1 and ERK2 is required for cytoplasmic sequestration of ERK2, Nishida and coworkers made a series of GST-MEK1 truncation mutants and tested their ability to bind ERK2. In a binding assay, the first 32 residues of MEK1 were required for ERK2 interaction with GST-MEK1. Next, the authors co-microinjected GST-MEK1 (1-60), which contains the putative ERK2 binding

region and the NES, with ERK2. Immunofluorescent staining revealed that in the presence of a 3-fold excess of GST-MEK1 (1-60), microinjected ERK2 was cytoplasmically localized. Lower amounts of GST-MEK1 (1-60), were not sufficient to cytoplasmically localize microinjected ERK2. The authors concluded from this study that cytoplasmic localization of ERK2 is a result of direct binding to MEK1 [134, 135]. However, the region of MEK1 used in this assay contains the previously described D domain, which is present in a number of ERK2 interacting proteins. Therefore, these experiments do not address the possibility that any D domain containing protein could sequester ERK2 in the cytoplasm. For example, MKP-3 contains a D domain, and when overexpressed also induces a cytoplasmic localization of ERK1/2 [90].

One hypothesis for MEK1's ability to induce the cytoplasmic localization of ERK2 is through the constitutive piggyback export of ERK2, which lacks a nuclear export sequence. Nishida and colleagues have proposed a model in which MEK1 is continually moving into the nucleus and being rapidly exported [137]. Their model suggests that following activation, ERK2 accumulates in the nucleus and phosphorylates target proteins. ERK2 is not exported when active, because the dually phosphorylated form has a low affinity for MEK1. Once dephosphorylated, nuclear ERK2 binds to MEK1 and the bipartite complex is exported from the nucleus. This model also suggests that MEK1 and ERK2 are continually shuttling between the nucleus and cytoplasm. Any inactive ERK2 that is present in the nucleus is therefore rapidly exported. Evidence for this hypothesis comes from experiments in which ERK1/2 and MEK1/2 accumulate in the nucleus of leptomycin B treated, serum starved cells [132, 137] .

Pouyssegur and colleagues have suggested that nuclear accumulation of ERK1/2 is the result of its ability to form interactions with nuclear anchoring proteins that are synthesized following ERK1/2 activation. This hypothesis was supported by experiments in which reduced retention of endogenous ERK1/2 was observed when cells were treated with the protein synthesis inhibitor, cycloheximide [7]. While this experiment is an indirect demonstration that nuclear anchors may be unstable, there is evidence that a prolonged activation of ERK1/2 correlates with expression of transcription factors that bind to ERK1/2 such as c-Fos [60]. Nuclear binding partners including topoisomerase II and kinetochores have also been identified to bind active ERK1/2 [138, 139]. It is currently unclear if accumulation of activated ERK1/2 in the nucleus is the result of the loss of interaction with cytoplasmic anchors, an increase in nuclear anchors for ERK1/2 binding an increase in the rate of ERK1/2 activation and nuclear import or some combination of all of these possibilities.

Studies of phosphorylated ERK2 demonstrated that it is capable of forming homo or hetero dimers with either phosphorylated or unphosphorylated ERK2. Microinjection of wild-type and mutant ERK2 into REF52 was performed to determine if dimerization was important for nuclear entry [21]. This approach allows for precise modulation of phosphorylation state and protein concentration. In these studies, microinjected, wild type and kinase dead thio-phosphorylated ERK2 both accumulated in the nucleus in serum-starved and growth factor-stimulated cells. The phosphorylation site mutant of ERK2, T183A and Y185F, did not accumulate in the nucleus in serum-starved cells unless co-injected with a phosphorylated form of ERK2. These experiments prompted the authors to suggest that nuclear localization of inactive ERK2 occurs through the

formation of a dimer with phosphorylated ERK2, which is competent for nuclear entry. Correspondingly, in serum-starved cells wild-type ERK2 accumulates in the nucleus following microinjection, while the dimer-deficient form does so less well. These experiments suggested that the ability to form dimers contributes to ERK2 nuclear entry and that unphosphorylated ERK2 can enter the nucleus by forming a dimer with the dually phosphorylated form [21].

Numerous studies have examined the requirement for protein-protein interactions in regulating the localization of ERK2. However, the mechanism that ERK2 uses to move across the nuclear pore complex has remained obscure. Many proteins have targeting motifs such as an NLS or an NES that associate with karyopherins[99]. ERK2 contains no identifiable NLS or NES. Because of its relatively small size, 42 kDa, it has been suggested that ERK2 can passively diffuse through the nuclear pore. Nishida and coworkers demonstrated that microinjection of WGA, which inhibits binding of transport factors to nucleoporins, does not inhibit the stimulus-dependent nuclear localization of overexpressed ERK2 causing them to propose that monomeric ERK1/2 enter the nucleus passively. These experiments are not conclusive because they used only one concentration of WGA (1 mg/ml) and ERK2 (2 mg/ml). It is possible that this concentration of WGA is not sufficient to block nuclear entry of such a large amount of ERK2[135].

Passive diffusion can also not account for translocation of the ERK2 dimer, which is 84 kDa. In agreement with the existence of a non-passive mechanism for nuclear entry, microinjection of WGA does inhibit the nuclear localization of an overexpressed form of ERK2 bound to β-galactosidase. In this assay, much lower amounts of β-

galactosidase-ERK2 were microinjected (100 μg/ml) and higher amounts of WGA (2 mg/ml) were used [132]. Active transport of ERK2 is supported by studies in *D. melanogaster* where the ERK1/2 homolog, D-ERK, interacts with a karyopherin family member, DIM-7. Biochemical interaction studies found that the association of these two proteins was positively regulated by dual phosphorylation of D-ERK. Furthermore, embryos lacking DIM-7 have reduced nuclear accumulation of D-ERK [140].

In summary, inactivated ERK1/2 are primarily localized to the cytoplasm in resting cells and activation of ERK1/2 is correlated with nuclear accumulation. Movement through the nuclear pore has the characteristics of both a receptor-dependent and passive mechanism. The preceding studies presented the basis for two questions that have shaped my thesis research. First, what is the mechanism for the translocation of ERK2 across the nuclear pore complex, and how might this process be regulated? Second, does nuclear translocation of ERK1/2 always follow activation, or can active ERK1/2 be targeted to other cellular locations?

Chapter 2: ERK2 Enters the Nucleus by a Carrier-Independent Mechanism

Abstract

In stimulated cells, the MAP kinase ERK2 concentrates in the nucleus. Evidence exists for CRM1-dependent, MEK-mediated nuclear export of ERK2, but its mechanism of nuclear entry is not understood. To determine requirements for nuclear transport, we tagged ERK2 with green fluorescent protein (GFP) and examined its nuclear uptake using an in vitro import assay. GFP-ERK2 entered the nucleus in a saturable, time- and temperature-dependent manner. Entry of GFP-ERK2, like that of ERK2, required neither energy nor transport factors and was visible within minutes. The nuclear uptake of GFP-ERK2 was inhibited by wheat germ agglutinin, which blocks nuclear entry by binding to carbohydrate moieties on nuclear pore complex proteins. The nuclear uptake of GFP-ERK2 was also reduced by excess amounts of recombinant transport factors. These findings suggest that ERK2 competes with transport factors for binding to nucleoporins, which mediate the entry and exit of transport factors. In support of this hypothesis, we showed that ERK2 binds directly to a purified nucleoporin. Our data suggest that GFP-ERK2 enters the nucleus by a saturable, facilitated mechanism, distinct from a carrier- and energy-dependent import mechanism and involves a direct interaction with nuclear pore complex proteins.

Introduction

MAP kinases are ubiquitous protein kinases that integrate cell surface signals by phosphorylating proteins throughout the cell. The MAP kinase, ERK2, targets proteins in multiple cell compartments following an activating stimulus. Stimuli induce the nuclear accumulation of ERK2 from its resting location in the cytoplasm of many types of cells, including fibroblasts, epithelial cells, and neuroendocrine cells [1, 2].

The location of ERK2 is a significant factor in determining its ability to phosphorylate key substrates and thereby influence cell behavior. Controlling the localization of ERK2 is a mechanism by which cell function may be influenced. For example, PEA-15, which promotes the cytoplasmic retention of ERK2, is overexpressed in disease states, such as Type II diabetes and breast cancer[141, 142]. ERK2 is active and constitutively nuclear in certain breast cancer cells, but excluded from the nucleus in certain non-small cell lung cancers [58], [Muneer and Minna, personal communication]. In addition to extensive data suggesting a correlation between phenotypes and ERK2 localization, some cell behaviors have been demonstrated to occur only if ERK2 accumulates in the nucleus. For example, the nuclear localization of ERK2 is essential for morphological transformation of 3T3 fibroblasts and neurite extension in PC12 cells [3, 6]. An understanding of how ERK2 localization is regulated will provide important insights into its normal function and disease mechanisms.

ERK2 subcellular localization is mediated by interactions with various proteins [21, 132, 135, 137]. Unphosphorylated ERK2 is anchored in the cytoplasm by its upstream activator, MEK1, which, like ERK2, is present at near micromolar concentrations. Following stimulation, active ERK2 translocates to the nucleus, possibly in a dimeric form [21, 132]. Phosphorylation is sufficient to cause the nuclear accumulation of ERK2, but its kinase activity is not required [21].

Nuclear binding sites for ERK2 may retain it in the nucleus, and binding partners include topoisomerase II and kinetochores [7, 138, 139, 143]. Nuclear export of ERK2 is also mediated by MEK1, which has a nuclear export sequence (NES) that binds to the nuclear export receptor, CRM1 [114]. Dephosphorylated, nuclear ERK2 can bind MEK1 and be transported out of the nucleus, a process inhibited by Leptomycin B, which disrupts CRM1-mediated export [137]. Furthermore, fusion of ERK2 to MEK1 yields a protein that is excluded from the nucleus, unless the NES is inactivated by mutagenesis, demonstrating the dominance of the MEK1 NES[6]. Interactions with several other proteins in addition to MEK1, including the phosphatases, PTP-SL and MKP-3, and the death effector domain-containing protein, PEA-15, promote localization of ERK2 in the cytoplasm [144-146].

A complex series of events controls ERK2 cytoplasmic and nuclear localization, but the mechanism for nuclear entry of ERK2 is not known. To examine this mechanism, we investigated the entry of ERK2 into nuclei of permeabilized cells under conditions not supporting CRM1-mediated export. We conclude that ERK2 enters the nucleus through nuclear pore complexes (NPCs) by an energy- and carrier-independent, facilitated mechanism.

Experimental Procedures

Constructs and recombinant proteins. The cDNA encoding rat ERK2 was subcloned into pRSETB-His_6-GFP using KpnI and HindIII restriction sites. GFP-ERK2, ERK2 and thiophosphorylated ERK2 (ERK2-P2) were prepared as described using the *E.coli* strain BL21 [21]. His_6-GFP, p38 and c-Jun N-terminal kinase 2 (JNK2) were purified as described in [21] for ERK2. Karyopherin- 1, karyopherin- 2, Ran, p10, and GST-NUP153c were purified as described [147-149]. To reconsititute classical import, rhodamine labelled BSA was coupled to a peptide containing the nuclear localization sequence (NLS) of the SV40 T antigen [148].

Cell Culture. BRL and REF52 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% glutamine.

Import Assays. Import assays were performed as described [147]. Briefly, BRL (Fig. 1C only) or REF52 cells were plated on coverslips 24 h before permeabilization. Cells were washed once with import buffer (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol) and permeabilized with 70 _g/ml digitonin in transport buffer for 5 min on ice. Cells were washed, and inverted over 40 l of import mix, which contained 0.8 M GFP-ERK2 (50 g/ml), 10 g/ml NLS-BSA-TRITC (~0.14 M) or indicated amounts of ERK2 or thiophosphorylated ERK2 (ERK-P2) [147]. To determine requirements for import, the import mix also included cytosol from *Xenopus* oocytes ([148], Fig. 1C only) or 0.5 M karyopherin- 2, 0.25 M karyopherin- 1, 2 M Ran, 0.4 M p10/NTF2, plus 15 M BSA (1 mg/ml), and as indicated energy in the form of an ATP/GTP-regenerating system (1 mM ATP, 1 mM GTP, 5 mM creatine phosphate, and 20 U/ml creatine kinase). Permeabilized cells were incubated for 15 min unless otherwise noted and were then washed and fixed in 3.7% formaldehyde for 15 min. Coverslips were mounted in 1 mg/ml phenylenediamine

in 90% phosphate buffered saline plus 10% glycerol. GFP and TRITC were visualized by fluorescence microscopy using a Zeiss Axiocam microscope equipped with a Hamamatsu Orca II camera. Uptake was quantified using Open Lab Software. His_6-ERK2 was detected by immunofluorescence using an anti-His_6 antibody (Clontech) at 1:200 dilution and an Alexa anti-mouse secondary antibody (Molecular Probes) at 1:3000 dilution [150].

Binding experiments: GST-Nup153c was incubated for 30 min with glutathione-agarose equilibrated in import buffer. His_6-ERK2 (200 nM) was added and mixed end over end at 23^{o} C for 45 min. Beads were washed with 1 M NaCl, 1 % Triton X-100 and 0.1% deoxcholate in 20 mM Hepes (pH 7.4) and analyzed on a 10% polyacrylamide gel in sodium dodecyl sulfate. Proteins were transferred to nitrocellulose and immunoblotted with Y691 for one h at 1:2500 dilution [6].

Results

ERK2 and GFP-ERK2 accumulate in nuclei by an energy- and carrier-independent mechanism. To examine the mechanism of nuclear entry of ERK2, we used an import reconstitution assay in permeabilized cells. Permeabilized cells lose transport factors and thus a complementation assay with cytosol or recombinant transport factors and an ATP/GTP (energy) regenerating system can be used to investigate nuclear uptake of ERK2. ERK2, at 0.8 μM (50 μg/ml), close to its intracellular concentration, was added with or without energy or cytosol to permeabilized REF52 cells and its localization was detected by immunofluorescence (Fig. 1A and not shown). ERK2 accumulated in the nuclei of the permeabilized cells in an energy-independent manner, even in the absence of cytosol. Because ERK2 is only ~41 kDa, it may have entered nuclei by diffusion. We increased the size of ERK2 to 68 kDa by incorporating a GFP tag; GFP-ERK2 was expressed and purified (Fig. 1B). Like ERK2, but unlike the control NLS-BSA-TRITC, GFP-ERK2 (0.8 μM) was taken up into nuclei of BRL and REF52 cells in the presence of apyrase, added to scavenge nucleotide (Fig. 1C and not shown). Over time, the concentration of GFP-ERK2 in the nucleus became greater than that in the import mixture (data not shown) and neither recombinant transport factors nor cytosol were required for its uptake (Fig. 1C). Quantification of the fluorescence intensity in the nuclei revealed no significant differences in GFP-ERK2 uptake in the presence or absence of these factors or cytosol in either cell type (data not shown). The control import substrate TRITC-NLS-BSA behaved in the expected manner, requiring energy and a source of transport factors for nuclear entry. We confirmed that the nuclear import of ERK2 was Ran independent, as the inclusion of Q69L Ran (defective in GTP hydrolysis) with cytosol inhibited import of NLS-BSA but not that of GFP-ERK2 (not shown) [151].

GFP-ERK2 enters nuclei by a saturable, facilitated mechanism. To determine if the nuclear entry of GFP-ERK2 occurred by a facilitated mechanism, we examined the concentration dependence, time course, and temperature-dependence of its nuclear uptake. The nuclear entry of GFP-ERK2 was time-dependent (Fig. 2). To confirm that there was no energy requirement for GFP-ERK2 entry, a time course of its uptake in the presence and absence of an ATP/GTP regenerating system was examined at room temperature. No energy requirement was revealed at any time point (Fig. 2A). Furthermore, non-hydrolyzable analogs of ATP and GTP had no effect on ERK2 uptake (data not shown). Time courses of nuclear uptake were performed at both room temperature and at 4°C. At the higher temperature, GFP-ERK2 uptake was readily detected after 10 min of addition to the permeabilized cells. The import of ERK2 was slowed but not abolished at 4°C (Fig 2B), unlike that of NLS-BSA, which is completely inhibited at this temperature. This behavior of ERK2 is reminiscent of the movement of karyopherin-β in the absence of cargo and Ran [152]. Using unfixed cells under these conditions, we could detect more ERK2 in the cytosol than in the bathing solution, possibly due to microtubule binding[126]; but even so, some nuclear entry was detected within 1.5 min (not shown) [153, 154].

Nuclear uptake of GFP-ERK2 was concentration-dependent (not shown). At 80 nM, rim staining of nuclei but little entry of GFP-ERK2 was observed. At 0.8 μM, GFP-ERK2 entry was readily observed. Pretreatment of ERK2 with an activated mutant of MEK1 under phosphorylating conditions to generate the phosphorylated form had no effect on its uptake, suggesting that phosphorylation of ERK2 does not influence its entry under these conditions (not shown).

To examine whether the uptake system was saturable, increasing amounts of untagged ERK2 and ERK2-P2 were added along with a fixed amount of GFP-ERK2 (Fig. 3A). Both

ERK2 and ERK2-P2 reduced the uptake of GFP-ERK2 in a concentration-dependent manner, indicating that the uptake system was saturable. These findings also support the above conclusion that ERK2 is equally effective in being taken up into the nucleus in the unphosphorylated and phosphorylated states. Because a 10-fold excess of the untagged proteins decreased GFP-ERK2 uptake by roughly half (Fig. 3B), the system is saturated at a concentration of ERK2 above that used for these assays. However, neither JNK2 nor p38, two other MAP kinases, competed for ERK2 entry (Fig. 3 C and D).

ERK2 enters the nucleus through the NPC and interacts directly with nucleoporins. Some nucleoporins contain the covalently attached sugar moiety, N-acetyl glucosamine, which binds wheat germ agglutinin (WGA) with high affinity. Blockade of nuclear uptake by WGA is diagnostic for nuclear entry via the NPC [155]. Therefore, we examined the effect of WGA on the nuclear entry of GFP-ERK2. The uptake of GFP-ERK2 was blocked by WGA in a concentration-dependent manner (Fig. 4), consistent with the conclusion that ERK2 enters the nucleus through the NPC. The nuclear accumulation of 27 kDa GFP, which enters the nucleus by diffusion, was unaffected by WGA (Fig 4B).

Our results indicate that ERK2 enters the nucleus through the NPC without a carrier and without the need for energy. Many nucleoporins contain FG repeats which are often found as part of a larger motif such as FXFG or GLFG. These repeats are recognized by nuclear carriers that transport their cargo across the NPC [92, 115, 156-160]. MAP kinases have been shown to interact with many proteins through relatively well-defined targeting motifs. Interestingly, one of these is the FXF motif, which was identified by Kornfeld as an ERK1/2-specific targeting domain [74]. We considered the hypothesis that ERK2 can interact directly with nucleoporins.

We tested this hypothesis using the import assay. Carriers can compete with each for shared docking sites [161]. If ERK2 binds to FG repeat docking sites, it should compete with the import of NLS-BSA. Thus, we tested its capacity to interfere with import of NLS-BSA. Both ERK2 and ERK2-P2 reduced the uptake of NLS-BSA in a concentration-dependent manner (Fig. 5). As a further test, if ERK2 is binding directly to nucleoporins, the import receptors should compete for these binding sites and reduce ERK2 entry. We examined the effects of two carriers, karyopherin-β1 and p10/NTF2, on uptake of GFP-ERK2 [148]. In support of the hypothesis, micromolar concentrations of both karyopherin-β1 and p10/NTF2 compete with GFP-ERK2 for nuclear entry (Fig. 6).

To determine if ERK2 can interact directly with a repeat-containing nucleoporin, we expressed the 153c fragment of the nucleoporin 153 as a GST fusion in bacteria and tested its binding to ERK2 in vitro. ERK2 associated with GST-Nup153c but not with GST or empty beads (Fig. 7). Their binding can be detected with as little as 20 nM Nup153c and 200 nM ERK2. ERK2 phosphorylates Nup153c only weakly (data not shown), suggesting that the binding is not due solely to an enyzme-substrate relationship. We hypothesize that the rapid entry of ERK2 into the nucleus is due to facilitated entry via a direct interaction with nucleoporins.

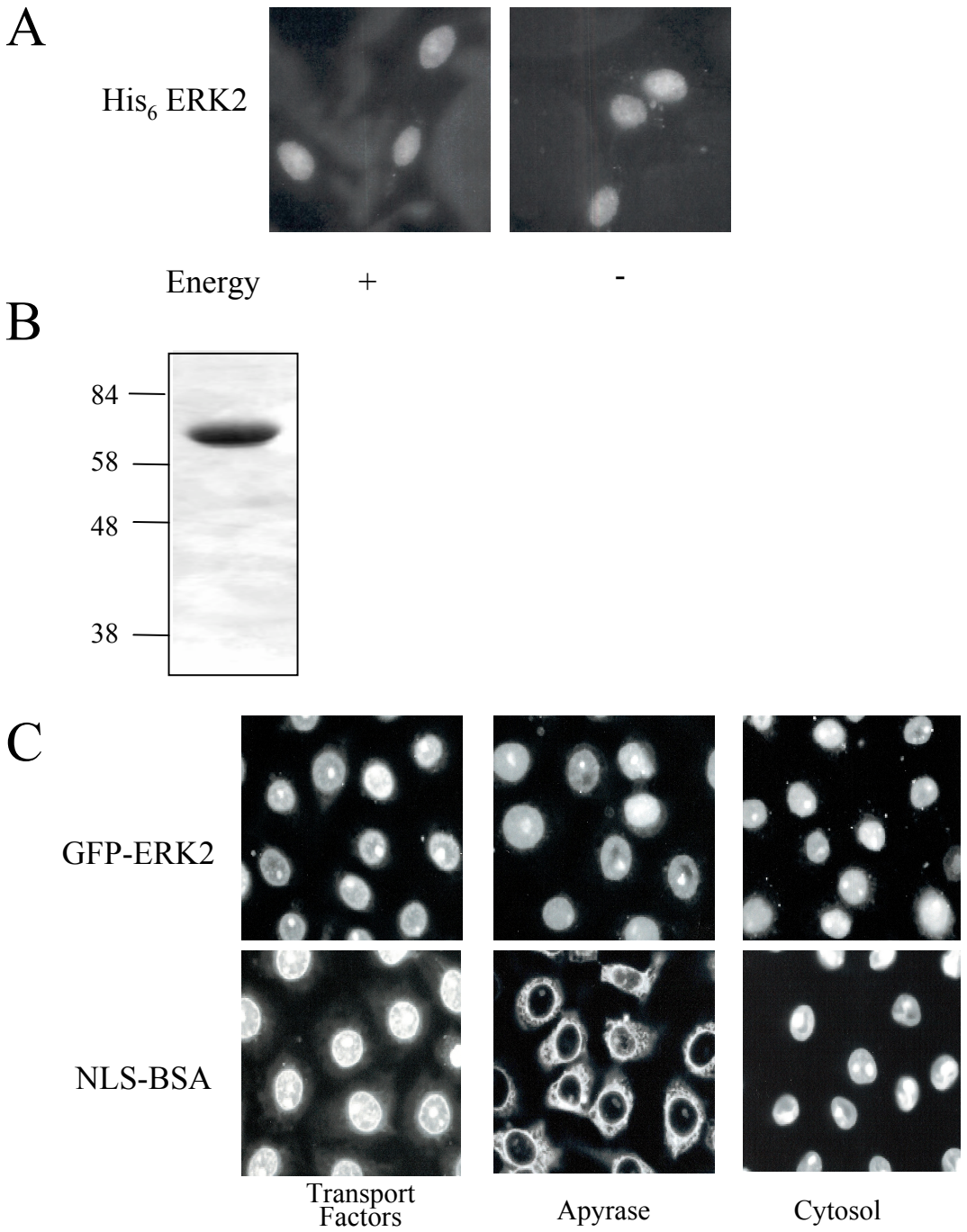


Figure 2-1. Characterization of ERK2 import in permeabilized REF52 and BRL cells. A. Nuclear import of His_6-ERK2 (0.8 □M, 50 □g/ml) in REF52 cells with or without an energy regenerating system (see Methods) in the absence of transport factors was visualized by immunofluorescence. B.Coomassie-stained polyacrylamide gel of purified GFP-ERK2. C. Nuclear import of GFP-ERK2 (0.8 □M 50 □g/ml) and TRITC-NLS-BSA (1.4 □M) in BRL cells in the presence of an energy regenerating system plus the indicated factors.

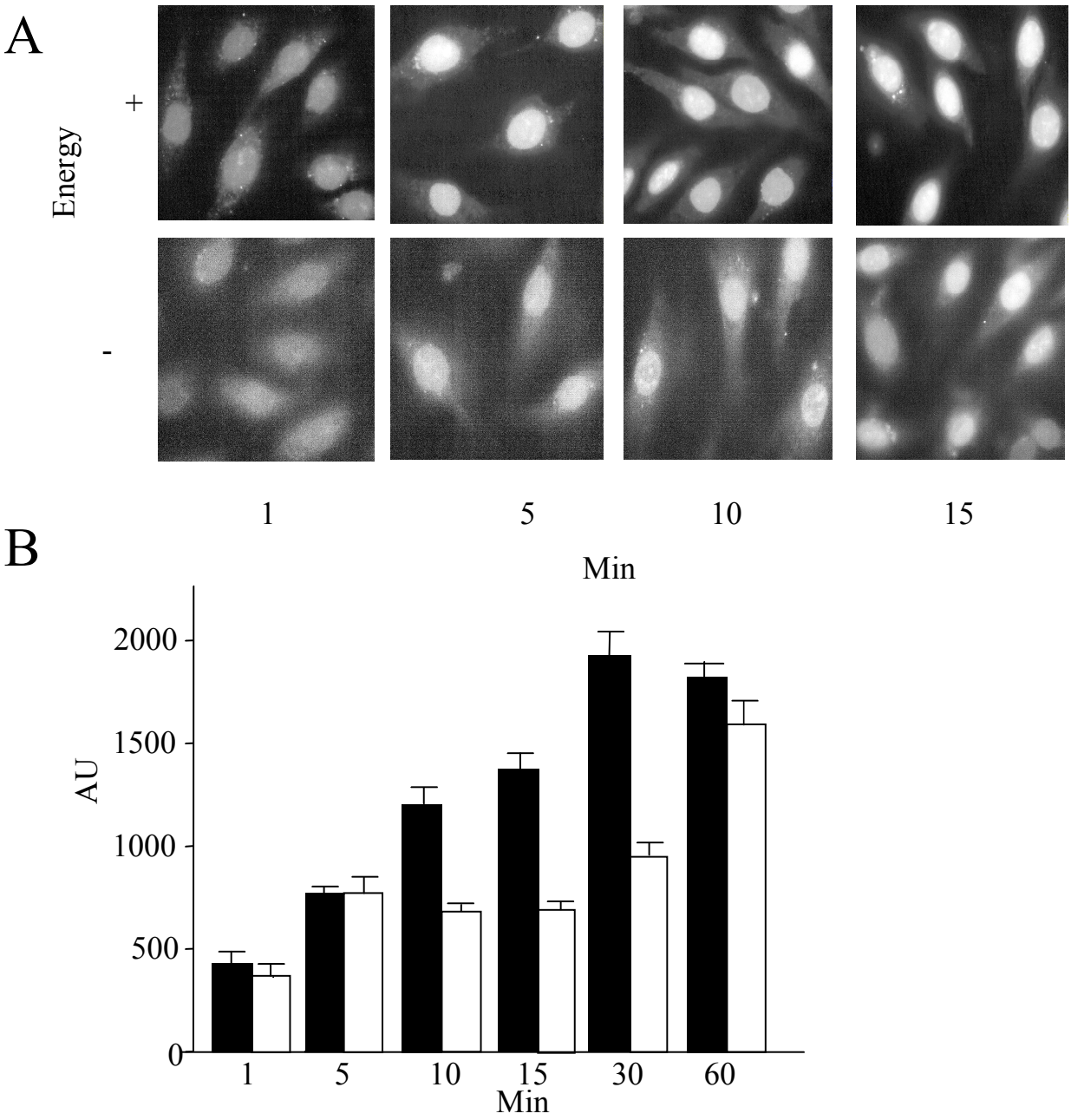


Figure 2-2. GFP-ERK2 import is energy independent. A. Import assay using GFP-ERK2 (0.8 □M, 50 □g/ml) was performed in REF52 cells with or without energy mix as indicated. Cells were fixed at indicated time points. B. Quantitation of REF52 nuclear fluorescence following import assay at room temperature (filled bars) or 4° C (open bars) in arbitrary units (AU) at indicated time points. Over a number of experiments, the relataive rate of import as room temperature was approximately that at 4°C

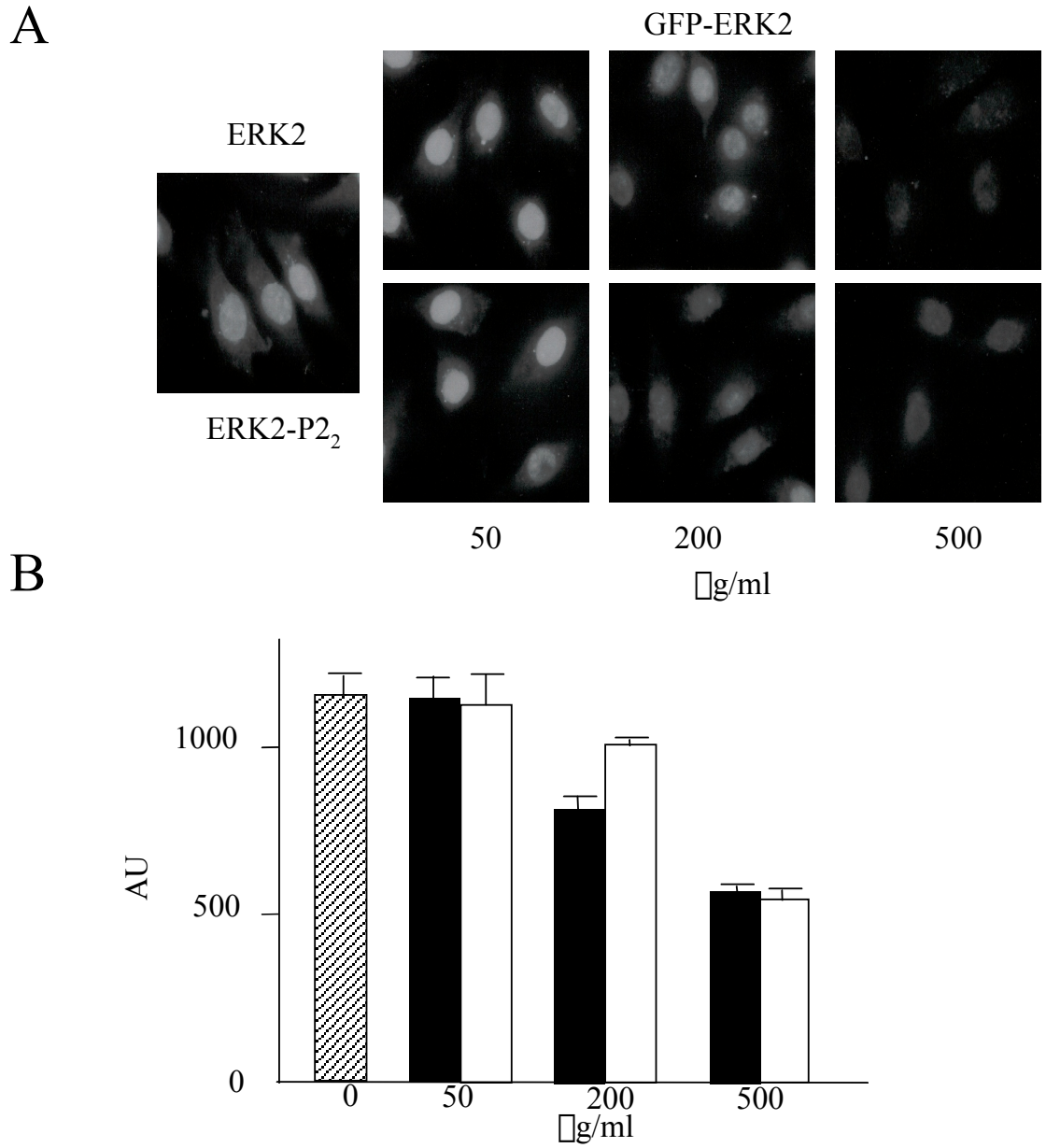
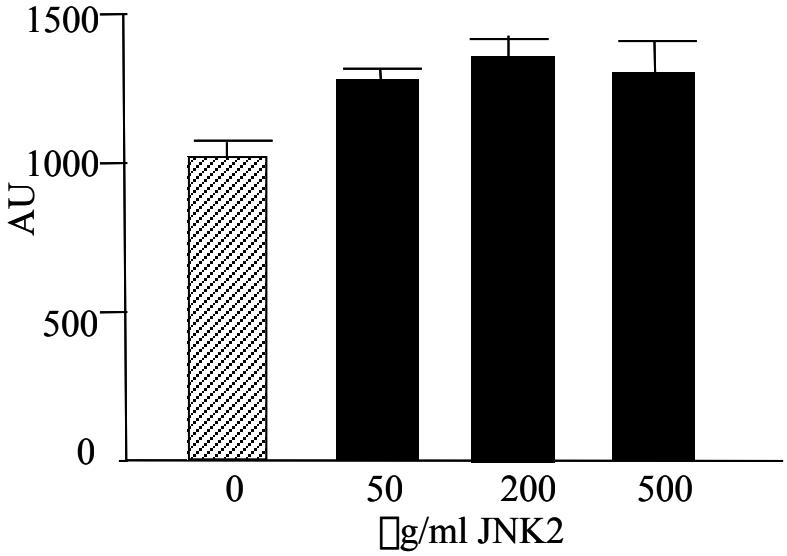
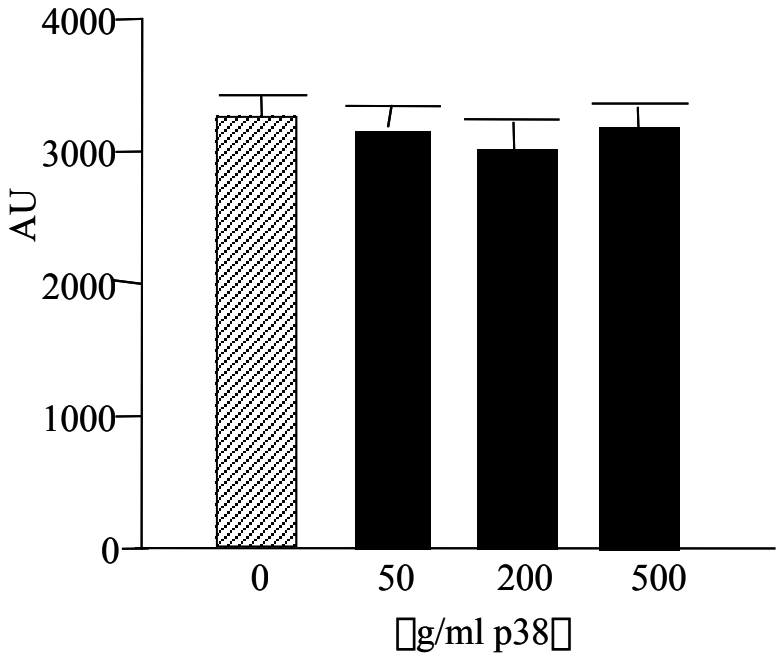


Figure 2-3. Effect of MAP kinases on nuclear uptake of GFP-ERK2. Import assay (A) and quantitation (B) using GFP-ERK2 (0.8 μM, 50 μg/ml) was performed in REF52 cells in the presence of increasing amounts of ERK2 (filled bars) and ERK2-P2 (open bars). Hatched bar is accumulation without excess ERK2. C&D. Quantitation of nuclear fluorescence from import assay using GFP-ERK2 (0.8 μM, 50 μg/ml) in REF52 cells in the presence of increasing amounts of JNK2 (C) and p38_ (D). Hatched bar is accumulation without excess MAP kinases.

C


1500
1000
500
0
AU
0
50
200
500
g/ml JNK2

D


4000
3000
2000
1000
0
AU
0
50
200
500
g/ml p38

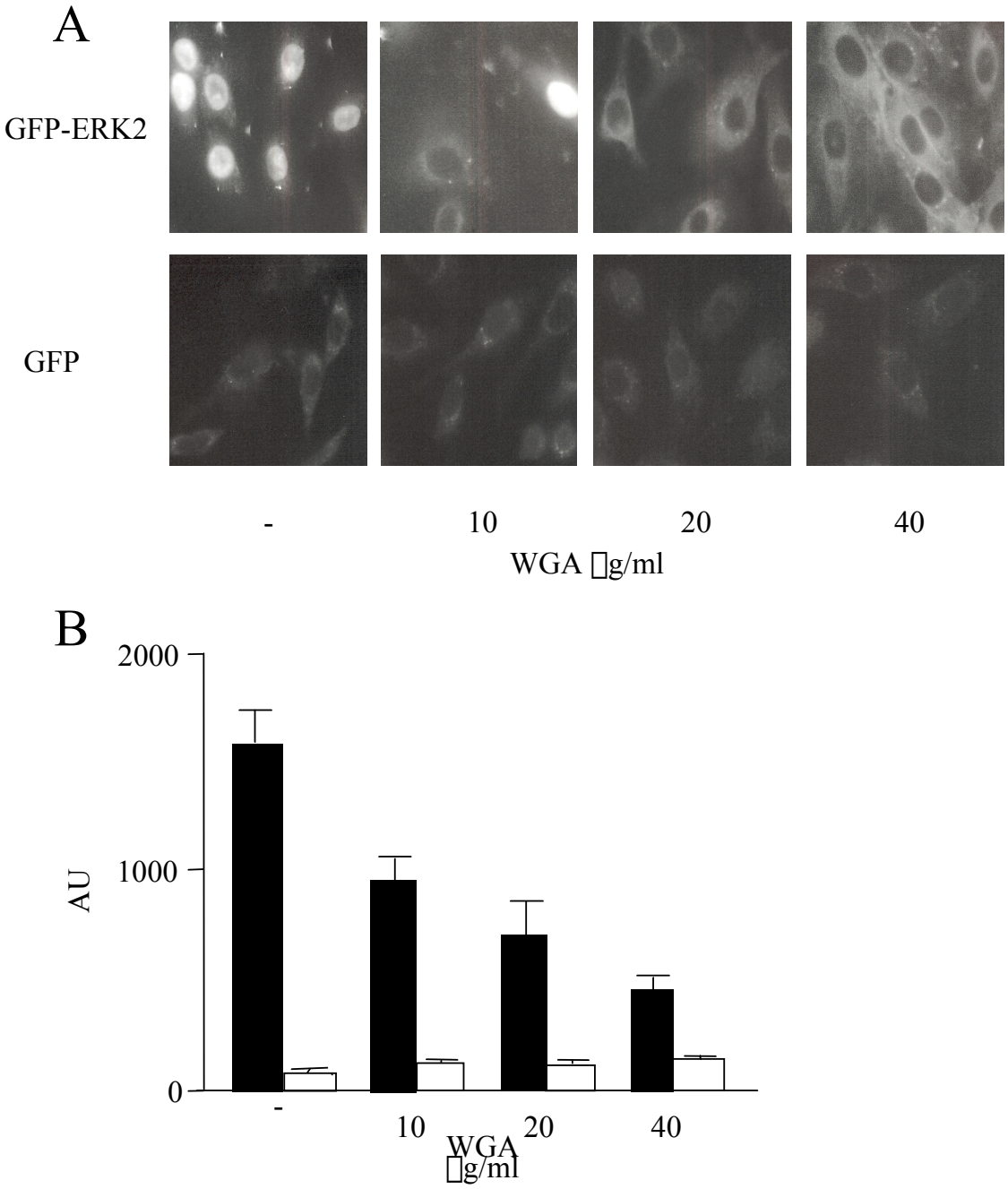


Figure 2-4. Effect of WGA on nuclear import of GFP-ERK2. Import assay (A) and quantitation (B) using GFP-ERK2 (0.8 μM, 50 μg/ml) (filled bars) and GFP (0.8 μM, 20 μg/ml) (open bars) was performed in REF52 cells in the presence of increasing amounts of WGA and energy mix.

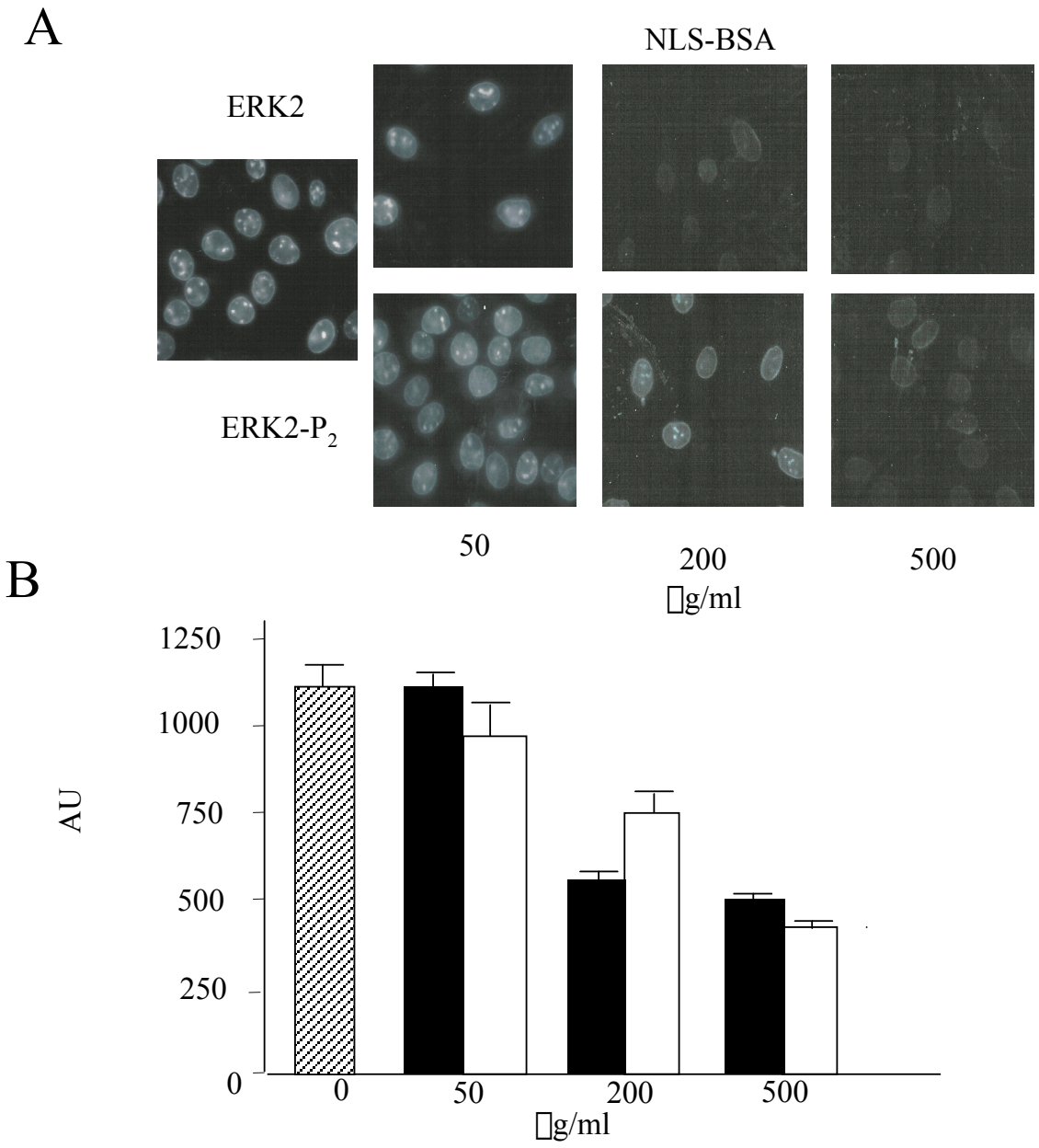
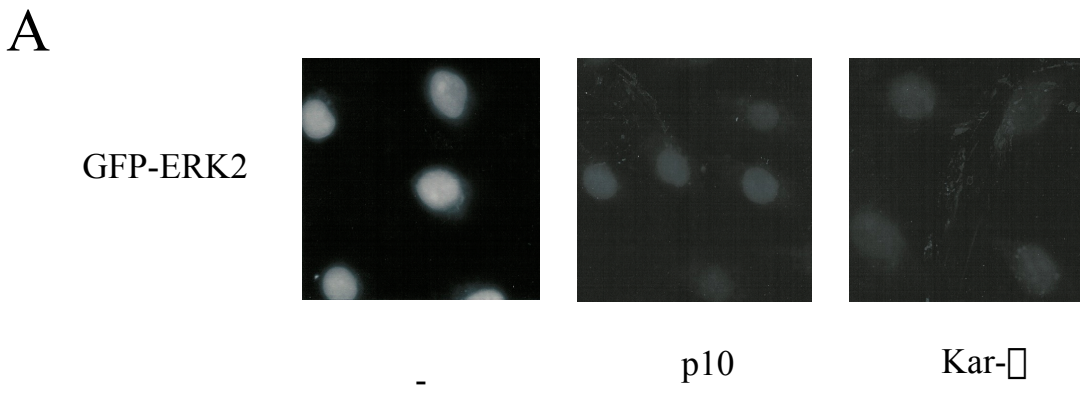


Figure 2-5. Effect of ERK2 on import of NLS-BSA. Import assay (A) and quantitation (B) using TRITC-NLS-BSA (1.4 μM) was performed in REF52 cells in the presence of transport factors, energy mix and increasing amounts of ERK2 (filled bars) and ERK2-P2 (open bars). Hatched bar is TRITC-NLS-BSA fluorescence in the absence of ERK2 or ERK-P2.



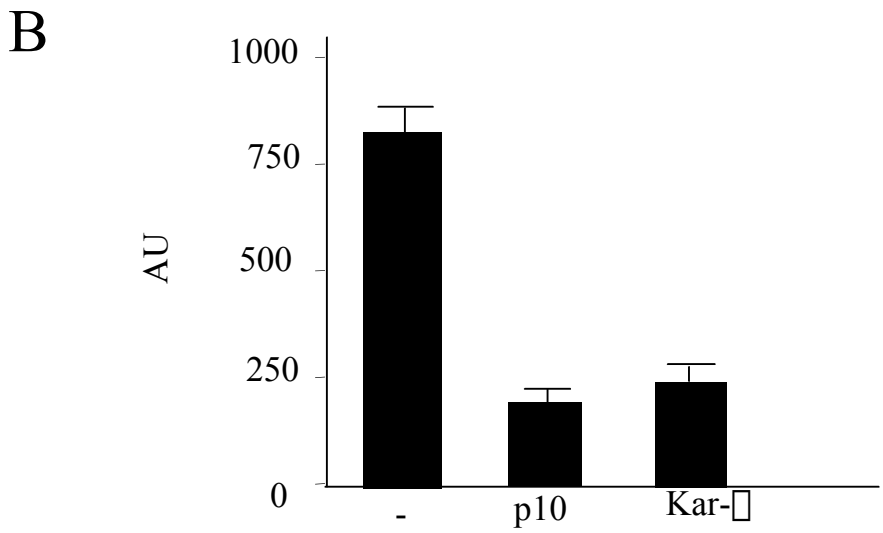


Figure 2-6. Effect of NPC binding proteins on import of GFP-ERK2. Import assay (A) and quantitation (B) using GFP-ERK2 (0.8 M, 50 g/ml) was performed in REF52 cells in the presence of energy mix and the indicated amounts of karyopherin-1 or NTF2/p10.

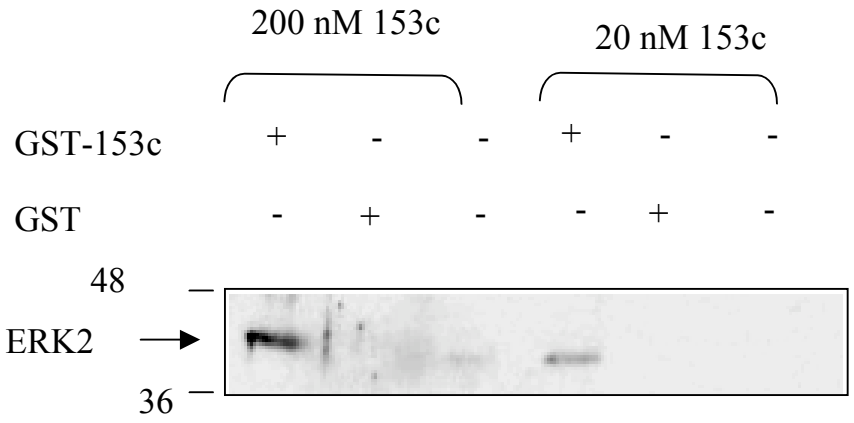


Figure 2-7. ERK2 binds to GST-Nup153c. GST-pulldown assay using GST-Nup153c at the indicated concentrations and 200 nM of ERK2 or buffer as indicated. Immunoblots were performed using Y691 for ERK2.

Discussion

We find that ERK2 enters the nucleus by a saturable, facilitated process, distinct from the conventional import mechanism. Our data indicate that the rapid uptake of ERK2 into the nucleus, like that of nuclear carriers, is dependent on its direct interaction with FG repeat-containing nucleoporins, as demonstrated through competition studies and the finding that ERK2 binds directly to a repeat nucleoporin. This mechanism is attractive not only because ERK2 binds FXF motifs in other proteins, but also because ERK2 has been reported to affect the nuclear import of proteins in a manner consistent with interaction with the import machinery [74, 162]. While our manuscript was in preparation, another group published a report concluding that ERK2 enters nuclei via an interaction with nuclear pore complex proteins; ERK2 was found to bind to a different FG repeat-containing protein, Nup214, in addition to the binding to Nup153 that was shown here [163]. These findings, together with ours, suggest that ERK2 binds to multiple nuclear pore complex proteins, supporting the proposed mechanism. These data in permeabilized cells are supported by previous findings in intact cells. We examined time courses of ERK2 localization after microinjection and found that the unphosphorylated protein rapidly entered the nucleus within 2 min, and then re-localized to the cytoplasm by 5-10 min [21]. These previous experiments suggested that the localization of ERK2 is controlled by active export as well as import.

The facilitated import mechanism found here has not been previously reported for other protein kinases, but has been suggested for the transcriptional regulator β-catenin (40,41). The major control of the nuclear function of β-catenin is its regulated association with the cytoskeleton. Once released, β-catenin binds nucleoporins without a requirement for carriers, competes with carriers for nuclear uptake, and enters the nucleus in an energy-independent

manner, similar to the results of our studies of ERK2 import. In the case of ERK2, its nuclear accumulation appears to be regulated primarily by its export; it does not need an import carrier because it interacts directly with nuclear pore proteins. Thus, direct binding to nucleoporins may be a generally employed mechanism for nuclear entry of proteins whose accumulation in the nucleus is controlled at other levels. This facilitated uptake mechanism is apparently not used by two other MAP kinases, JNK2 and p38, based on their inability to compete for ERK2 entry. This result is consistent with the apparently selective interaction of FXF motifs with ERK2 among MAP kinases [74].

Phosphorylation of ERK2 promotes its nuclear localization in vivo. However, we were unable to find effects of phosphorylation on its uptake in permeabilized cells. This result is consistent with the observation that overexpressed ERK2 accumulates in the nucleus without being phosphorylated [136]. Blockade of export, by favoring the dissociation of ERK2-MEK1 complexes, may be the primary event controlled by ERK2 phosphorylation. ERK2 forms dimers upon phosphorylation that promote its nuclear accumulation in vivo [21]. An ERK2 dimer, as a consequence of having twice the sites to interact with FX motifs, might bind more tightly to NPCs and be imported more rapidly than a monomer. Alternatively, other import mechanisms for dimers may exist that could not be detected in this system. Finally, dimeric ERK2 may be further impaired in its interaction with proteins such as MEK1 that may promote its piggyback export.

In summary, multiple regulated events lead to the stimulus-dependent nuclear accumulation of ERK2. ERK2, phosphorylated or not, appears to enter the nucleus by a rapid, facilitated, energy- and carrier-independent mechanism that relies on its direct interaction with nucleoporins. A second entry mechanism may also exist for ERK2-P2, perhaps as a dimer,

and/or by association with one or more other proteins that contain an NLS. ERK2 in the nucleus binds to proteins that retain it there even after it has been dephosphorylated. In stimulated cells, ERK2 that does not enter the nucleus is bound to cytosolic anchor proteins. Binding of ERK2 to proteins in the cytoplasm and nucleus may be as important as the transport mechanisms in determining its subcellular distribution. Unphosphorylated ERK2 is believed to be exported from the nucleus in a CRM-1-dependent manner primarily as a complex with MEK1 via its NLS. Other proteins may also mediate ERK2 export normally and under pathophysiological conditions. This model suggests strategies for therapeutic intervention in disease states in which the inappropriate localization of ERK2 is believed to contribute to symptoms.

Chapter 3: PEA-15 and ERK2 Nuclear Entry

Abstract

ERK2 nuclear-cytoplasmic distribution is regulated in response to hormones and cellular state without the requirement for karyopherin-mediated nuclear import. One proposed mechanism for the movement of ERK2 into the nucleus is through a direct interaction between ERK2 and nucleoporins present in the nuclear pore complex. Previous reports have attributed regulation of ERK2 localization to proteins that activate or deactivate ERK2, such as the MAP kinase kinase MEK1 and MAP kinase phosphatases. Recently, a small non-catalytic protein, PEA-15, has also been demonstrated to promote a cytoplasmic ERK2 localization. We found that the MAP kinase insert in ERK2 is required for its interaction with PEA-15. Consistent with its recognition of the MAP kinase insert, PEA-15 blocked activation of ERK2 by MEK1, which also requires the MAP kinase insert to interact productively with ERK2. To determine how PEA-15 influences the localization of ERK2, we used a permeabilized cell system to examine the effect of PEA-15 on the localization of ERK2 and mutants that have lost the ability to bind PEA-15. Wild type ERK2 was unable to enter the nucleus in the presence of an excess of PEA-15; however, ERK2 lacking the MAP kinase insert largely retained the ability to enter the nucleus. Binding assays demonstrated that PEA-15 interfered with the ability of ERK2 to bind to nucleoporins. These results suggest that PEA-15 sequesters ERK2 in the cytoplasm at least in part by interfering with its ability to interact with nucleoporins, presenting a potential paradigm for regulation of ERK2 localization.

Introduction

The mitogen-activated protein (MAP) kinase, ERK2, plays critical role in promoting cellular changes in response to both mitogenic and non-mitogenic stimuli. ERK2 is the multi-functional kinase in a kinase cascade that is activated by various stimuli acting through tyrosine kinase receptors, G protein-coupled receptors, and others [2, 164]. Activation of this cascade results in the dual phosphorylation of ERK2 and the consequent phosphorylation of target kinases, transcription factors, and other proteins throughout the cell. The effectors of ERK2 are localized in both the cytoplasm and the nucleus, making its subcellular localization important for its ability to induce cellular changes [6, 87]. Regulation of ERK2 localization has been characterized mostly by overexpression and/or using antibodies to N- and C-terminal epitopes [7, 88]; these epitopes are not readily accessible in ERK2 that is associated with microtubules, which constitutes a large portion of the cytoplasmic protein [126]. ERK2 and its upstream activator the MAP/ERK kinase MEK1(also known as MKK1) interact stably through multiple sites with an affinity in the micromolar range [40, 41, 133, 165, 166]. Overexpressed ERK2 accumulates in the nucleus in a stimulus-independent manner. This suggests that cytosolic binding is important in determining the distribution of ERK2 in cells. Coexpression with MEK1 shifts the distribution in favor of the cytoplasm [136]. From these studies, it has been suggested that inactive ERK2 is anchored in the cytoplasm by MEK1, and that once active, ERK2 loses affinity for MEK1, moves through the nuclear pore complex (NPC), and accumulates in the nucleus [135].

Overexpression studies have shown that ERK2 export can be facilitated by MEK1, which has a nuclear export sequence (NES) and requires the NES-binding protein

CRM1 for movement out of the nucleus [114, 134](16,17). Nuclear entry of ERK2 has recently been described as a facilitated mechanism in which ERK2 moves through the NPC by binding directly to nucleoporins [121, 163]This process, which has been described for at least two other signaling proteins [119]{Xu, 2002 #185, does not rely on the classical, karyopherin-mediated import mechanism. Thus, regulatory mechanisms governing the localization of ERK2 appear to differ significantly from those for many other proteins displaying regulated nuclear import.

Because ERK2 responds to a wide range of stimuli and controls numerous effectors throughout the cell, a number of mechanisms most likely exist to regulate its subcellular localization and thereby maintain fidelity between stimulus and stimulus-specific phenotypic change. Recently, the small anti-apoptotic protein, PEA-15, has been demonstrated to bind to ERK2 and localize it to the cytoplasm [146]. PEA-15 was originally characterized in astrocytes, where it became highly phosphorylated in response to phorbol ester treatment [167]. Neuronal cells express relatively high amounts of PEA-15; nevertheless, it is detected in most cultured cell lines. Astroctyes from PEA-15-knockout mice display a heightened sensitivity to tumor necrosis factor- □ induced apoptosis, indicating a protective effect of PEA-15 in the central nervous system [168]. It has also been shown to bind to the TRAIL receptor to inhibit apoptotic signaling [169]. Various cancer cell lines also express relatively high amounts of PEA-15, and an increase in its mRNA is correlated with neoplastic changes in mouse mammary gland [142]. Consistent with its anti-apoptotic phenotypes, PEA-15 contains a death effector domain (DED), which is required for PEA-15 to reverse Ras-mediated suppression of integrin activation [170].

PEA-15, when overexpressed, localizes ERK2 to the cytoplasm, even in the presence of stimuli that activate ERK2 [146]. PEA-15 has an NES and accumulates in the nucleus in cells treated with leptomycin B, an inhibitor of CRM1-dependent nuclear export. These data suggested that PEA-15 may influence the localization of ERK2 in a similar manner to that proposed for MEK1, by binding to ERK2 and mediating its nuclear export. Thus, these studies describe one potential mechanism for the regulation of ERK2 subcellular localization by non-catalytic proteins, which may contribute to targeting ERK2 to specific cellular compartments and substrates. To understand better the mechanism by which PEA-15 regulates ERK2 subcellular localization, we examined the behavior of ERK2 and PEA-15 using a permeabilized cell reconstitution assay, and found that PEA-15 prevents ERK2 nuclear entry. Further, PEA-15 prevents ERK2 from binding to nucleoporins in vitro, indicating it may assist in localizing ERK2 to cytoplasmic sites of action by inhibiting its movement into the nucleus.

Experimental Procedures

Constructs and Recombinant Proteins-- The cDNA encoding rat ERK2 was subcloned into pRSETB-His_6-green fluorescent protein (GFP) by using KpnI and HindIII restriction sites. Mutants were generated using the QuickChange kit (Stratagene). The cDNA encoding hamster PEA-15 was a gift from Mark Ginsberg and Joe Ramos (Scripps Research Institute and Rutgers). pGAD-ERK2 was cloned and two-hybrid vectors encoding LexA-PEA-15 or kinase dead MEK1 were generated by PCR as previously described [40]. The constructs encoded the proteins fused to the C-terminus of the LexA DNA-binding domain.

Yeast Two-hybrid Experiments-- Pairwise interaction tests were carried out in the yeast strain L40 co-transformed with pGAD-ERK2, or the indicated ERK2 mutants, and either the empty pVJL11 (LexA) vector or pVJL11-based constructs

encoding LexA fusions with sequences from PEA-15, MEK1, or MNK1 as described (11). The ERK2 mutants were originally identified and described in a study of MEK1-ERK2 interactions or in an earlier study of ERK2 nuclear localization [21, 40]. Co-transformants were selected and protein-protein interactions were tested by examining growth of co-transformed isolates on medium lacking His, Leu, and Trp. The expression of all LexA and GAD fusion proteins in yeast cells was confirmed by immunoblotting with antibodies specific for either LexA or ERK1/2 .

Protein Purification-- GFP-ERK2, and GFP-ERK2 mutants, were prepared as described [21]. GST-ERK2 was purified as described [171]. PEA-15 and PEA-15 D74A each containing a His6 tag were purified on Ni^{2+}-NTA-agarose (Qiagen) and MonoQ. Elution from the latter was performed with a step salt gradient. Karyopherin-1, karyopherin-2,

Ran, p10, and glutathione S-transferase (GST)-NUP153c were purified as described [147, 172, 173]. Rhodamine-labeled bovine serum albumin (BSA) was coupled to a peptide containing the nuclear localization sequence (NLS) of the simian virus 40 T antigen to make the control import substrate NLS-BSA [172].

Import Assays-- Import assays were performed as described using REF52 cells grown in medium supplemented with 10% FBS and 1% glutamine [147]. Cells plated on coverslips 24 h before permeabilization were washed once with import buffer (20 mM Hepes-KOH, pH 7.3/110 mM potassium acetate/2 mM magnesium acetate/1 mM EGTA/2 mM dithiothreitol) and permeabilized with 70 μg/ml digitonin in transport buffer for 5 min on ice. Cells were washed and inverted over 40 μl of import mix, which contained 0.6-0.8 μM GFP-ERK2 (38-50 μg/ml) or 5 μg/ml NLS-BSA-tetramethylrhodamine B isothiocyanate (TRITC) (0.07 μM); for NLS-BSA import, cells were incubated with 0.5 μM karyopherin-1, 0.25 μM karyopherin-2, 2 μM Ran, and 0.4 μM p10/NTF2 [147]. Permeabilized cells were incubated for 15 min or the indicated times and were washed and fixed in 3.7% formaldehyde for 15 min. Coverslips were mounted in 1 mg/ml phenylenediamine in 90% PBS plus 10% glycerol. GFP and TRITC were visualized by fluorescence microscopy with a Zeiss Axiocam microscope equipped with a Hamamatsu Orca II camera. Pictures for individual experiments were taken using identical exposures. Uptake was quantified by using Improvision OPEN LAB software.

Binding Experiments-- The method described by Werner and colleagues was used to measure binding of purified GST-ERK2 wild type and mutants to His_6-PEA-15, except that bovine serum albumin (3 mg/ml) was included in the incubations and interactions were detected by immunoblotting with an anti-His6 monoclonal antibody

(Clontech)[174]. To measure ERK2 binding to Nup153c, GST-Nup153c (2 M) was incubated for 30 min with glutathione-agarose equilibrated in import buffer at 4° C. His_6-ERK2 (200 nM) was added with or without the indicated ratios of PEA-15 and mixed end over end at 4° C for 2 h. Bovine serum albumin (6 mg/ml) was present in the binding reactions. Beads were washed 4 times with 1 M NaCl, 0.1% Triton X-100 in import buffer and analyzed on a 10% polyacrylamide gel in SDS. Proteins were transferred to nitrocellulose and immunoblotted with antiserum Y691 for 1 h at 1:1000 dilution [175].

Kinase Assays-- Kinase assays were performed as previously described. ERK2(0.8 M) was incubated with 120 nM MEK1R4F [32], 10 M ATP, and increasing amounts of PEA-15. Reactions were incubated for 60 min at 30° C. Proteins were resolved on gels as above and subjected to autoradiography. Cpm incorporated into ERK2 were measured by liquid scintillation counting.

Results

To analyze the possible site of interaction of PEA-15 with ERK2, we first demonstrated that we could measure their binding using a directed two-hybrid test (Fig. 1A, Table 1). We then assessed the binding of PEA-15 to the following ERK2 mutants that are defective in interactions with other proteins, some of which were identified from an ERK2 mutant library [40]: 1) ERK2 D316A, D319A (DDAA), which lacks two aspartic acid residues required for binding to proteins with basic/hydrophobic docking (D) motifs including MEK1, the substrates p90 Rsk, MNK1, Elk1, and c-Fos, and the MAP kinase phosphatase MKP-3[40, 42, 60, 73, 74]; 2) ERK2 lacking the MAP kinase insert (residues 241-272; ERK2 Δ241-272), which is not activated by MEK1 or MEK2; and 3) four other ERK2 point mutants, Y261N/C, L235P, N236I, G243R, that are variably defective in binding to MEK1 (Fig. 1B). PEA-15 bound to ERK2 (DDAA) as well as it bound to wild type ERK2, indicating that PEA-15 does not bind to ERK2 through a basic/hydrophobic D domain. However, PEA-15 did not interact with ERK2Δ241-272, although this mutant retained binding to MNK1 and MKP-3 [40]. Loss of a two-hybrid interaction suggests a substantial decrease in the affinity of their association. The point mutants that displayed reduced binding to PEA-15 were all located in or near the MAP kinase insert and included Y261N/C, which is the most defective in binding to MEK1 based on its inability to be activated in cells (Table 1; [40]). No functions of the MAP kinase insert are known other than for productive binding of ERK2 to MEK1. The binding data here indicate that a second function of the MAP kinase insert is to interact with PEA-15.

To validate the results of the two-hybrid tests, the binding of the two key GST-ERK2 mutants to PEA-15 was assessed using glutathione-agarose to harvest bound proteins (Fig. 1C). As expected, ERK2 (DDAA) associated with PEA-15 in the pull down assays, but ERK2 Δ241-272 bound poorly to GST-PEA-15, consistent with the two-hybrid findings.

As indicated above, the region of ERK2 that was necessary for the interaction with PEA-15 overlaps that previously identified as required for the capacity of MEK1 to activate ERK2 in cells [40]. This observation suggested that PEA-15 should also interfere with the interaction of ERK2 with MEK1. To determine if this was the case, we included PEA-15 in kinase assays with ERK2 and a constitutively active form of MEK1, MEK1 R4F. Under these conditions, we could assess competition as a loss of ERK2 phosphorylation and activation (Fig. 2). A 10-fold molar excess of PEA-15 in the kinase assays was sufficient to reduce ERK2 activation by MEK1 to less than 20% of that in its absence, consistent with the idea that PEA-15 interferes with a productive interaction of MEK1 with ERK2.

Overexpression studies demonstrated that PEA-15 can induce accumulation of ERK2 in the cytoplasm. This accumulation was attributed to regulation of ERK2 export by PEA-15, which has an NES . To determine effects of PEA-15 on ERK2 localization, we included PEA-15 in an import reconstitution assay with GFP-ERK2. In vitro import assays are performed by permeabilizing the plasma membrane with digitonin. Digitonin permeabilization results in the loss of soluble proteins including proteins required for conventional import and export processes, notably the small G protein Ran, but leaves the nucleus and NPC intact. We have shown previously that the control import substrate,

NLS-BSA, is not imported in the absence of added transport factors and energy in this system [121]. In contrast, GFP-ERK2 enters the nucleus in the absence of added soluble proteins and energy in a manner facilitated by interactions with the FXF-rich nucleoporins, which are major component of the nuclear pore [121, 163]. Protein outside the nucleus that is not tightly bound will wash away prior to visualization. Entry of GFP-ERK2 into the nucleus is inhibited by the lectin wheat germ agglutinin, which binds to N-acetylglucosamine residues on nucleoporins and occludes the NPC . If we included PEA-15 in these assays, we found a significant inhibition of GFP-ERK2 entry at 15 min. The inhibition of entry was dependent on the PEA-15 concentration. Reduced entry of GFP-ERK2 was clearly detectable if a 7.5-10-fold molar excess of PEA-15 was included in the assay (Fig. 3A). We also performed additional assays including recombinant transport factors, □-karyopherin, □-karyopherin, p10, Ran, and an energy reconstitution system (19), to determine if nuclear accumulation of GFP-ERK2 was influenced by PEA-15 in the presence of transport factors. Under these conditions, nuclear accumulation of GFP-ERK2 was still inhibited by PEA-15. On the other hand, PEA-15 had no effect on the entry of the control import substrate NLS-BSA (Fig. 3C), indicating that PEA-15 does not generally disrupt conventional nuclear import. Because an energy regenerating system, Ran, and CRM1 were not required in the permeabilized cell system, inhibition of the nuclear accumulation of GFP-ERK2 is most likely not due to CRM1-mediated export of a PEA-15-GFP-ERK2 complex. Our findings with PEA-15 are consistent with work by Nishida and coworkers who reported previously that MEK1 can prevent the nuclear entry of GFP-ERK2 in similar reconstitution assays [163]. Thus, we conclude that the

ability of PEA-15 to prevent nuclear accumulation of ERK2 in this assay must be due to its sequestration outside the nucleus.

To determine if cytoplasmic retention of GFP-ERK2 caused by PEA-15 required a direct interaction with ERK2, we tested the effect of PEA-15 on nuclear import of ERK2 with mutations noted above. GFP-ERK2 DDAA entered the nucleus like wild type ERK2, and its entry was reduced by PEA-15 (Fig. 4A and 4B). This result was consistent with the binding data that showed that mutation of the two aspartic acid residues in ERK2 DDAA did not reduce PEA-15 binding (Fig. 1A and 1C). GFP-ERK2Δ241-272 was also capable of nuclear entry, but, in contrast, its entry was much less sensitive to the presence of PEA-15; reduced entry was only observed at the highest PEA-15 concentration (Fig. 4A and 4B). In the presence of PEA-15 no entry of wild type ERK2 was observed even at 1 min, the shortest time of observation; nor was it observed at any time over the time course up to 30 min (Fig. 4C). ERK2 Δ241-272 displayed a similar accumulation over time even in the presence of PEA-15 (Fig. 4D). No inhibition of nuclear entry was observed with ERK2 Y261N (Fig. 5A, Table 2), which was the point mutant most impaired in binding to MEK1 and PEA-15 by two-hybrid test [40]. In addition, a mutant in the DED domain of PEA-15, D74A, that reportedly fails to bind to ERK2 was also tested in the reconstitution assay. In the presence of PEA-15 D74A, nuclear entry of GFP-ERK2 was detected; the extent was intermediate between that observed in the absence and presence of wild type PEA-15 (Fig. 5B). This result is consistent with the decreased ERK2 binding of this mutant. Thus, we conclude that binding of PEA-15 to ERK2 induces a cytoplasmic localization of ERK2 in this assay by directly preventing its nuclear entry. We showed previously using microinjection of

recombinant proteins into the cytoplasm of REF52 cells that ERK2 K52R, a kinase-dead mutant, and ERK2 T183A, lacking one of two essential phosphorylation sites in the activation loop, could also accumulate in the nuclei of stimulated cells [21]. Thus, we wished to test the ability of these ERK2 mutants to be imported into the nucleus in the import reconstitution system and the effect of PEA-15 on their entry. We found that GFP-ERK2 K52R and T183A both accumulated in the nucleus in a manner similar to wild type ERK2 (Fig. 6). In both cases, PEA-15 blocked their nuclear accumulation. We had also shown previously that the mutant ERK2 L4A H176E, which was unable to form dimers due to mutations in the dimer interface, displayed a reduced ability to accumulate in the nucleus of stimulated cells [21]. Interestingly, this dimer mutant localized to the nucleus in the import reconstitution assay in a manner similar to wild type ERK2 (Fig. 6). This suggests that a process distinct from facilitated entry causes the abnormal distribution of this mutant in cells. As with wild type ERK2, PEA-15 was able to prevent the nuclear entry of the dimer mutant. The dimer interface of ERK2 consists of residues in the N-terminal domain and the activation loop, all distant from the MAP kinase insert. These results are consistent with the earlier assessment that the MAP kinase insert is the major site of interaction of PEA-15 with ERK2.

Current evidence suggests that ERK2 binding to nucleoporins is required for its rapid nuclear entry in the import assay; therefore, we wished to determine the effect of PEA-15 on the ERK2-nucleoporin interaction. We and others have shown previously that ERK2 binds to nucleoporins (NUP 153c and NUP214) in GST-pull down assays [121, 163]. Both of these porins contain numerous FXF repeats, which are found in a number of ERK2 substrates [74]. Therefore, we tested the ability of ERK2 to bind to the

nucleoporin NUP153c in the presence of increasing concentrations of PEA-15 (Fig. 7). We found that an excess of PEA-15, similar to the amounts used in the import assay, significantly decreased ERK2 binding to NUP153c. These results suggest that PEA-15 interferes with the association of ERK2 with nucleoporins. In view of the two-hybrid data and the assays here, we estimate that the Kd for ERK2-PEA-15 binding is near 1 M, consistent with the concentration required for inhibitory effects in the import assays. PEA-15 itself, does not appear to bind to nucleoporins, consistent with import assay findings that PEA-15 does not inhibit import of NLS-BSA, which uses the classical nuclear import system (Fig. 3C); the karyopherins themselves must bind to nucleoporins to escort their import cargo into the nucleus.

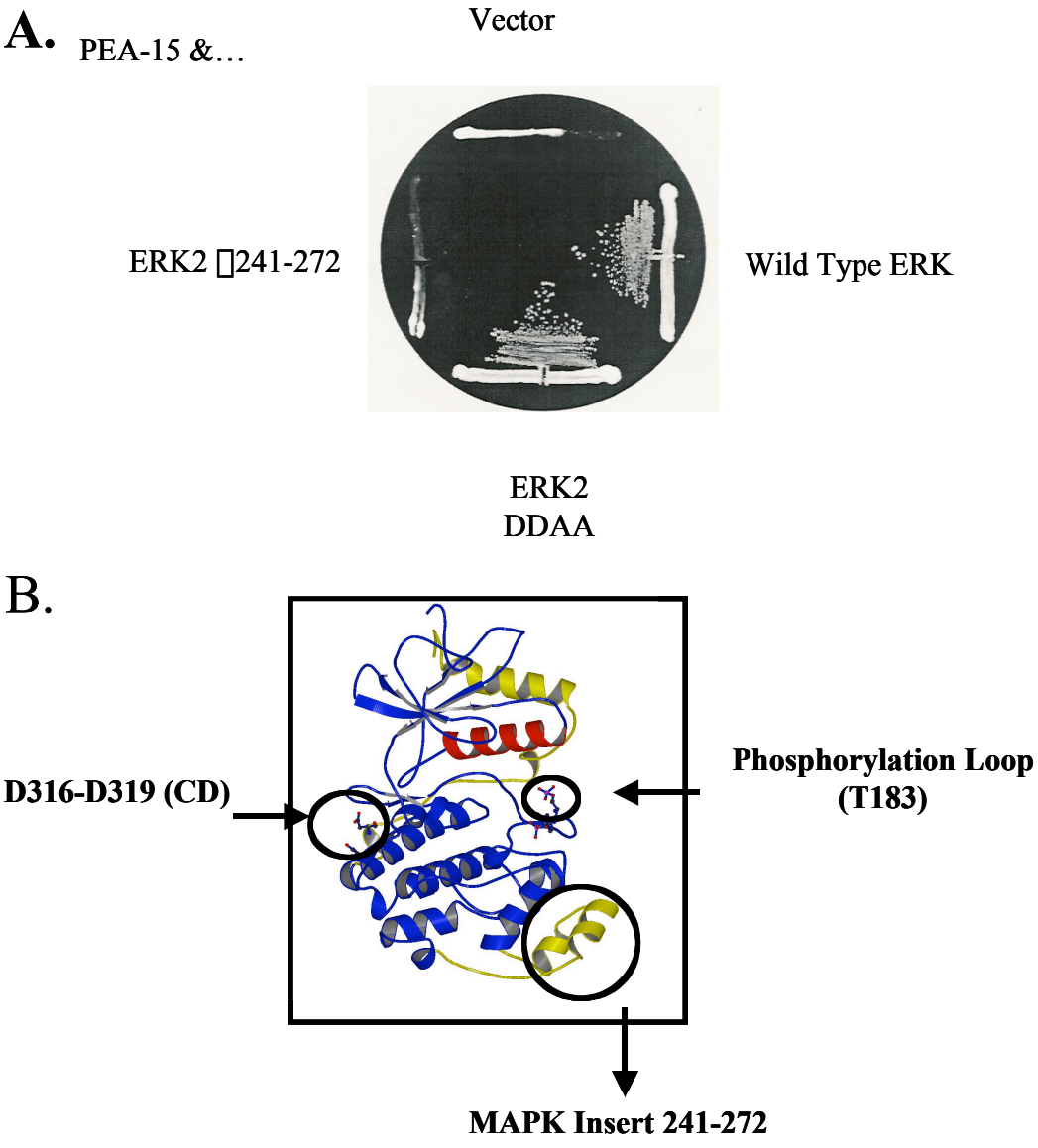


Figure 3-1. The interactions of wild -type and mutants of ERK2 with PEA -15. (A) Yeast were co-transformed with empty pGAD vector, pGAD-ERK2, pGAD-ERK2 DDAA or pGAD-ERK2*241-272 and pLexA-PEA-15. Binding is shown by growth on medium without His, Leu, and Trp. (Fred Robinson) (B) Crystal structure of phosphorylated ERK2 w ith the CD domain, MAPK insert and T183 phosphorylation site in the activat ion loop indicated (51). (C) GST-binding experiments were performed with GST -ERK2 wild type, D316A,D319A, and *241-272 incubated with His6-PEA-15. One of two experiments is shown.

C.

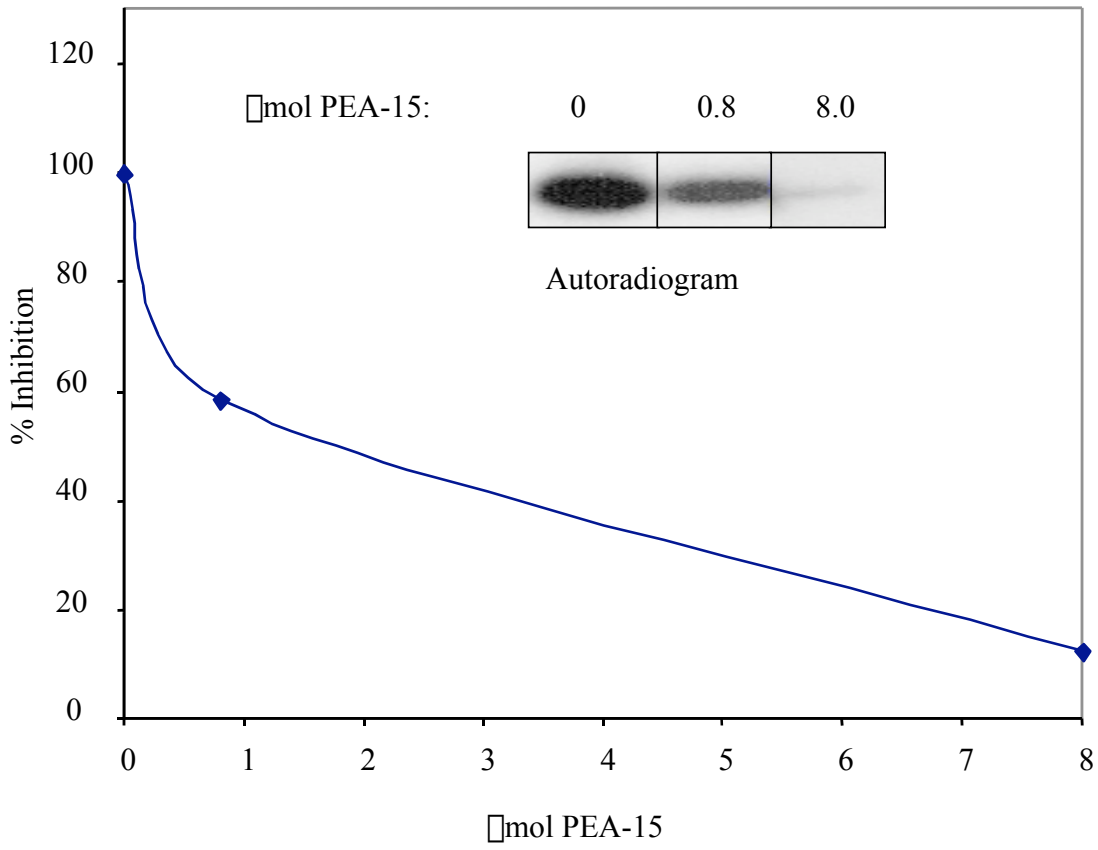


Figure 3- 2. PEA-15 inhibits activation of ERK2 by MEK1R4F. Kinase assays were performed using 120 nM MEK1R4F and 0.8 M ERK2 alone or with 0.75 or 7.5M PEA-15. The graph is an average of three experiments.

Mutation	MEK1-K97M	PEA-15	MNK1
WT	+	+	+
L235P	-	-	+
L235P,N236I	+	-	+
G243R	-	-	+
Y261C,N	-	-	+

Table 3-1: The ability of ERK2 wild type and mutants to bind to PEA-15 by yeast two-hybrid analysis. Plus signs (+) indicate an interaction; minus signs (-) indicate no detectable interaction.

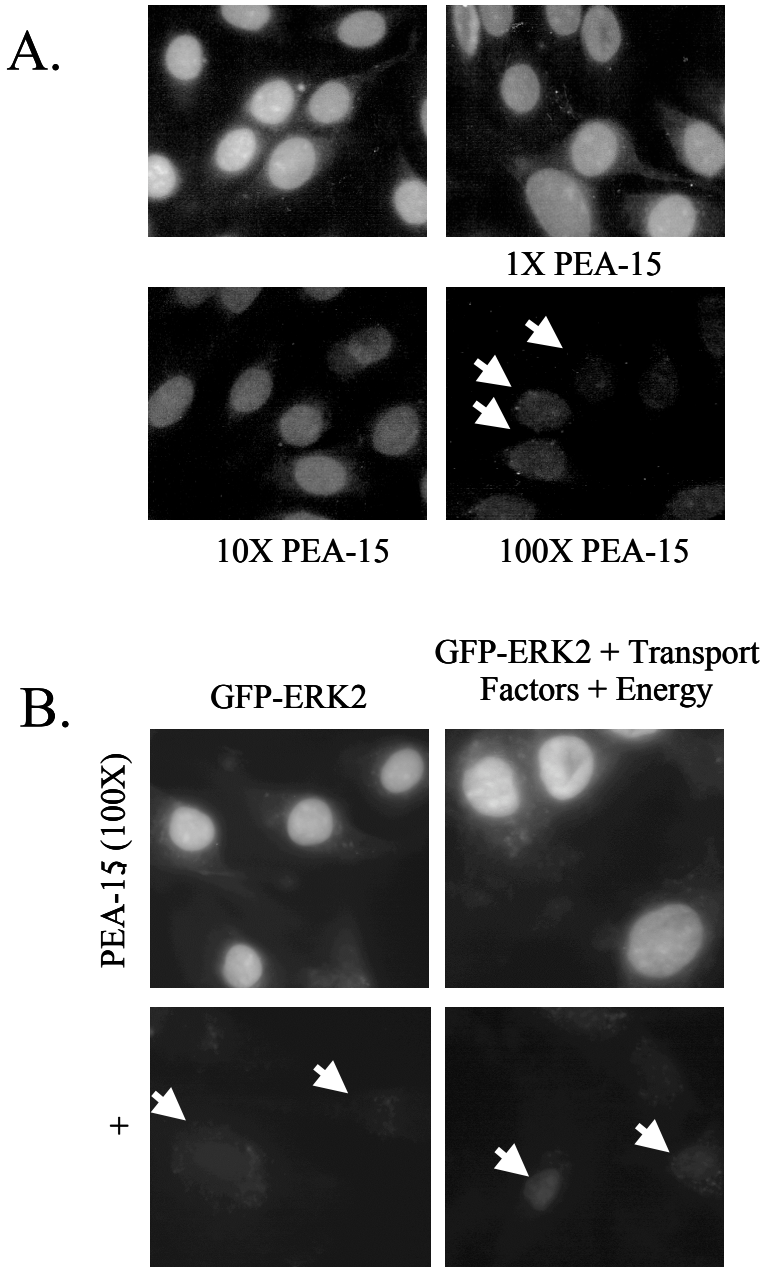
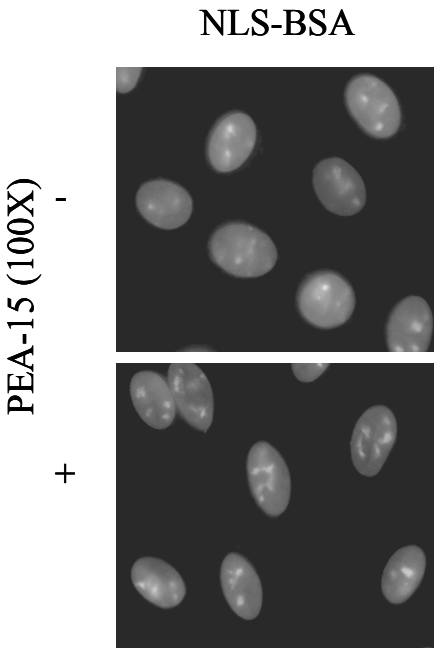


Figure 3-3. PEA-15 inhibits nuclear entry of GFP-ERK2. (A) GFP-ERK2 (0.8 □M, 50 □ g/ml) was added alone or with PEA -15 (0.6, 6 or 60 □ M) for 15 min. Arrows indicate nuclei. One of six experiments performed in duplicate.(B) Impor t assays with 0.8 □ M GFP-ERK2 alone and with 80 □ M PEA-15 with or without transport factors and an energy regenerating system for 15 min. Arrows indicate nuclei. One of two experiments.(C) As in A but NLS-BSA (5.0 □ g/ml) was added alone or with 80 □ M PEA-15 for 15 min. One of two experiments.

C.



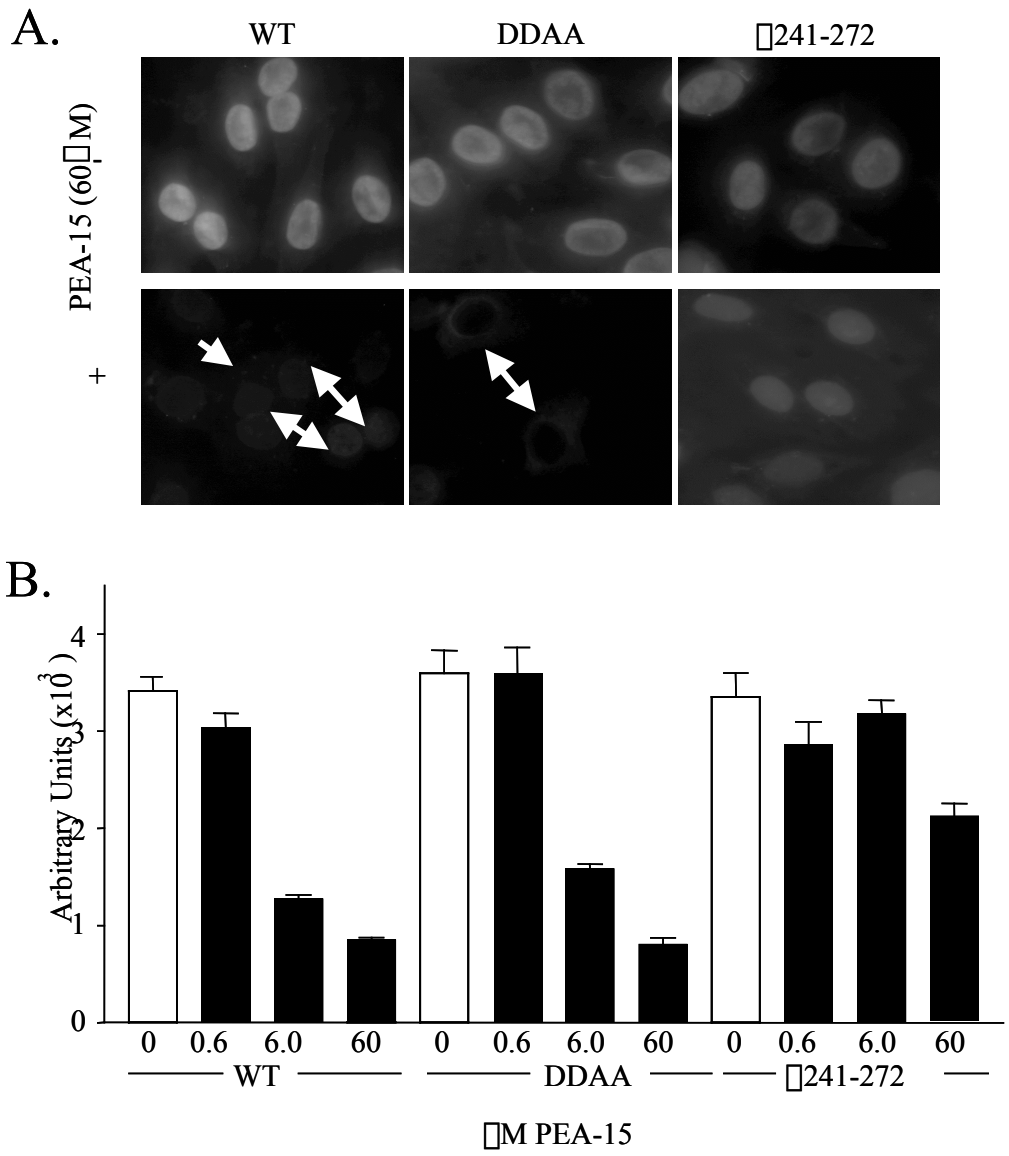
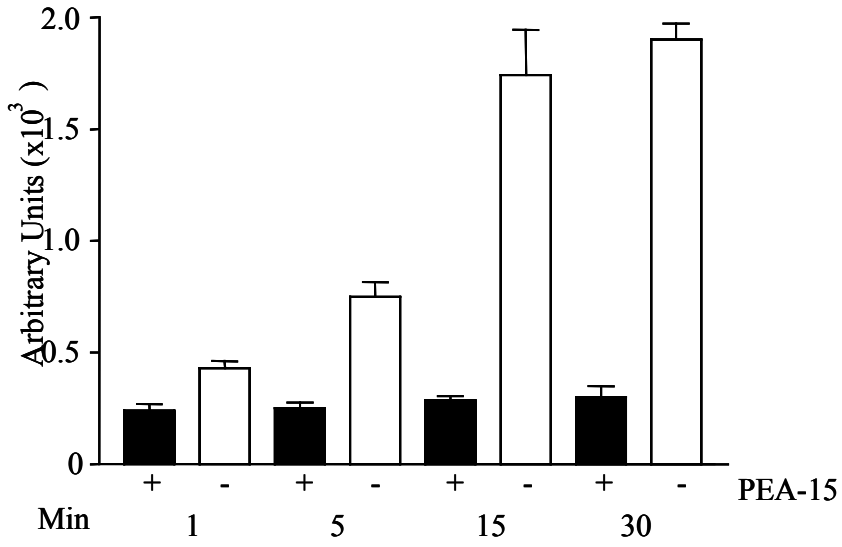
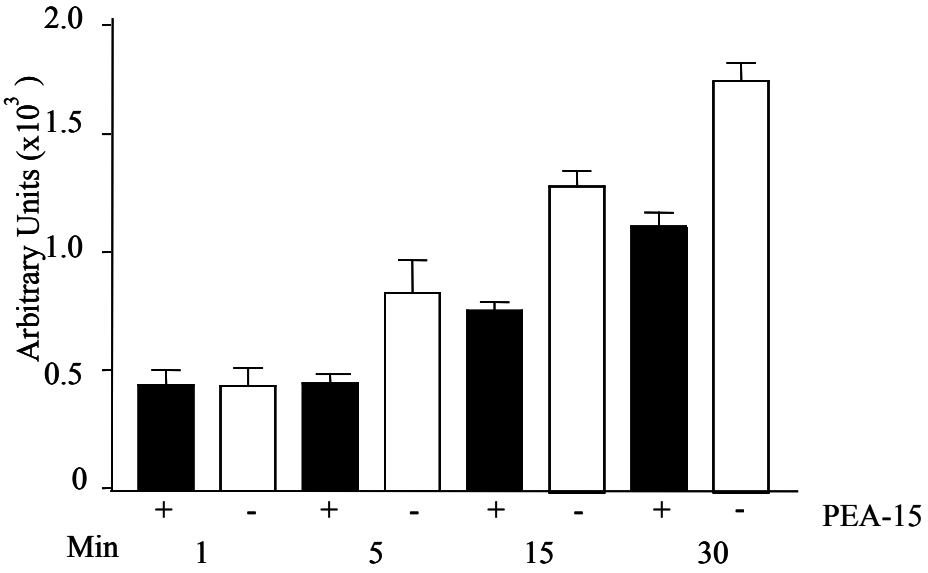


Figure 3-4. Nuclear accumulation of GFP-ERK2 and mutants in the presence of PEA-15. (A) Import assays with GFP-ERK2 (one of five experiments), GFP-ERK2 D316A, D319A (one of two experiments), and GFP-ERK2Δ241-272 (on of five experiments) with 60 μM PEA-15 for 15 min. (B) Quantitation of nuclear staining following import assays with GFP-ERK2 (one of three experiments), GFP-ERK2 DDAA (one of two experiments) or GFP-ERK2 Δ 241-272 (one of three experiments) alone (white bars) or with 0.6, 6 or 60 μM PEA-15 (black bars) for 15 min.

C.



D.



(C) Quantitation of nuclear staining following a time course import assay with GFP-ERK2 alone (white bars) and 60 □ M PEA -15 (black bars). One of eight experiments. (D) Quantitation of nucleus fluorescence in time course import assays with GFP ERK2 □241-272 alone (white bars) or with 60 □ M PEA-15 (black bars). One of eight experiments.

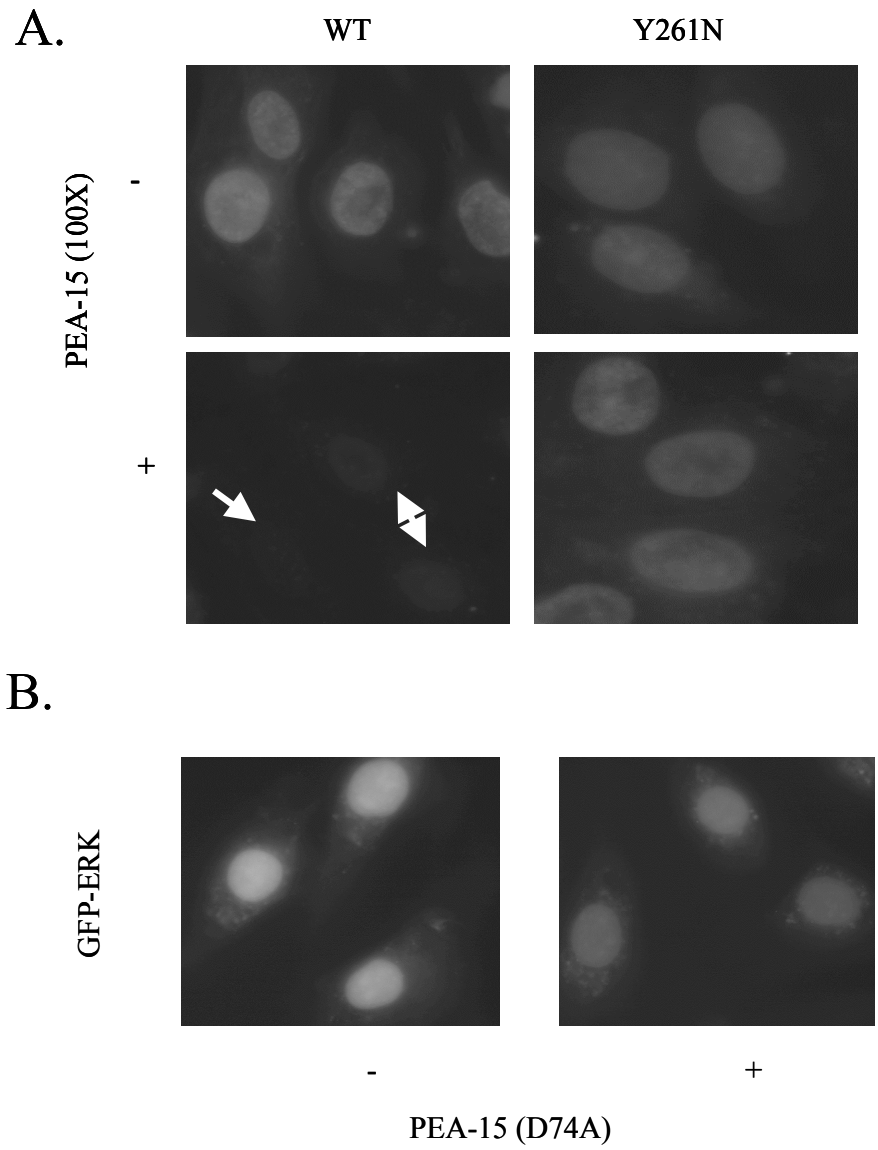


Figure 3-5. ERK2 and PEA-15 mutants in the import assay. (A) Import assay as in Fig. 4 with GFP-ERK2 and GFP-ERK2 Y261N with and without 60 μM PEA-15 for 15 min. Arrows indicate nuclei. One of four experiments. (B) Import assay as above with GFP-ERK2 and 60 μM PEA-15 D74A for 15 min. One of four experiments.

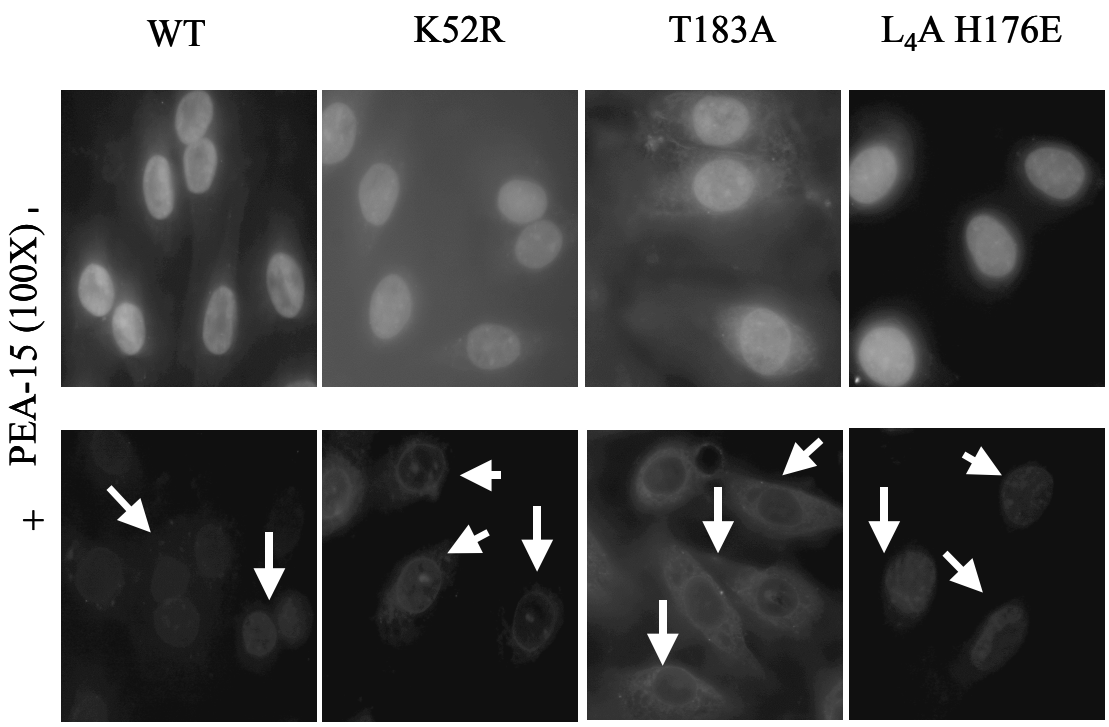


Figure 3-6. Nuclear accumulation of GFP-ERK2 mutants in the presence of PEA-15. Import assays as in Fig. 4 with GFP -ERK2 and the mutants K52R, T183A, H176E/L4A, and 241-272 with and without 60 M PEA-15 for 15 min. Arrows indicate nuclei. Note binding of ERK2 T183A to cytoskeletal elements. Import experiments were performed three times in the absence and once in the presence of PEA-15.

Mutation	PEA-15 sensitive
WT	+
D316A,D319A	+
H176E,L333A, L336A,L341A, L344A	+
K52R	+
T183A	+
Δ241-272	-
Y261N	-

Table 3-2: Effect of PEA -15 on import of ERK2 and mutants in the import assay. Plus signs (+) indicate accumulation into nucleus in 15 min; min us signs (-) indicate lack of nuclear entry.

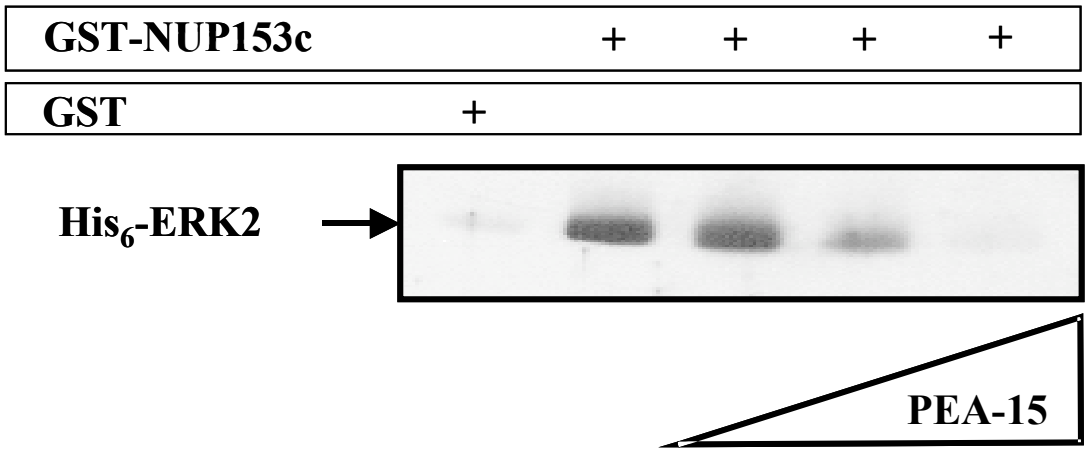


Figure 3-7. PEA -15 inhibits ERK2 binding to NUP 153c. GST-pull down assay using 2 μM GST or GST-NUP153c and 0.2 μM ERK2 alone or with 0.2, 2 or 20 μM PEA-15. One of five experiments.

Discussion

ERK2 phosphorylates proteins in multiple subcellular localizations. Movement of ERK2 within the cell is essential for targeting ERK2 to substrates in different compartments to produce discrete, ligand-specific phenotypes. The growth factor-dependent movement of ERK2 from the cytoplasm to the nucleus has been amply documented in PC12, 3T3, REF52, HeLa, CCL39, CHO and other cell types[86, 87, 90, 123, 126, 176]. Unlike most other proteins displaying this behavior, ERK2 apparently does not enter the nucleus by the classical import mechanism; instead ERK2 is capable of moving bidirectionally across the NPC independently of soluble carrier proteins by directly binding to FXF motifs in nucleoporins [121, 163]. ERK2 also activates substrates in the cytoplasm. In some cases a fraction of it may be tethered in the cytoplasm until a ligand-dependent release; perhaps its behavior is like that of β-catenin, which is retained in the cytoplasm through interaction with cytoskeletal elements until signaled for release to carry out its nuclear transcriptional regulatory function [177]. β-catenin, like ERK2, enters the nucleus by binding to nucleoporins [119]. The identification of proteins that mediate regulation of ERK2 movement and anchoring at the membrane, in the cytoplasm, and in the nucleus, as well as their mechanisms of action will be important in understanding how localization of ERK2 is controlled in a stimulus-dependent manner.

Several proteins have been implicated in preventing the nuclear accumulation of ERK2. In addition to MEK1, these include calponin, a protein highly expressed in smooth muscle, and PEA-15 [135, 146, 178]. Overexpression of PEA-15 is sufficient to maintain a cytoplasmic localization of ERK2, even under conditions that activate it and would normally result in its nuclear accumulation. PEA-15 reportedly binds to ERK2 in

both inactive and active forms [146]. Because PEA-15 itself has an NES, it has been proposed that export of PEA-15 from the nucleus via CRM1 also results in export of ERK2 complexed to it. However, no direct evidence for this mechanism has been reported. Here, we have examined the mechanism by which PEA-15 alters the localization of ERK2. Our results do not rule out a contribution to the PEA-15-dependent localization of ERK2 through accelerated export. Nevertheless, we demonstrate the unexpected result that PEA-15 has the capacity to sequester ERK2 outside of the nucleus, in a manner independent of nuclear export. We found no significant nuclear uptake of PEA-15 in the import assay in the absence or presence of energy and cytosolic transport factors (data not shown), suggesting that it may enter the nucleus at a relatively slow rate. If so, the effect of PEA-15 to block ERK2 import may be more significant than the action on ERK2 export. Under the conditions of the import reconstitution assay, NES-mediated export is not observed without the addition of CRM1 and Ran. Together with the kinetics of entry in the presence and absence of PEA-15, these findings indicate that the lack of nuclear ERK2 signal in our experiments can only be attributed to failed nuclear entry. Using a random mutagenesis strategy, we found that MEK1, the upstream activator of ERK2, binds to the ERK2 MAP kinase insert [40]; the two-hybrid and in vitro binding results here indicate that this region of ERK2 is also important for binding to PEA-15, supporting a report from Werner and colleagues [174]. MEK1 contacts a significant portion of the ERK2 surface [165].

Among well defined binding contacts, the MAP kinase insert is the site which is required for productive interaction between ERK2 and MEK1 that mediates ERK2 phosphorylation by MEK1 [40]. Because PEA-15 requires the MAP kinase insert to bind

tightly to ERK2, it is not surprising that PEA-15 reduces the in vitro activation of ERK2 by MEK1. This finding seems to conflict with data indicating that overexpression of PEA-15 increases ERK2 activation, both duration and amplitude, in CHO cells [146]. Perhaps in cells, inhibition of ERK2 activation by overexpressed PEA-15 is not observed either because the relocalization of ERK2 caused by PEA-15 prevents ERK2 inactivation by phosphatases or the reduced interaction of ERK2 with MEK1 is compensated for by other proteins such as scaffolds that promote their association. PEA-15 apparently anchors ERK2 in the cytoplasm. Previous reports demonstrate that this event is independent of ERK2 activation state [146]. In agreement with this finding, we find that a kinase-dead mutant and a phosphorylation site mutant of ERK2 are sensitive to PEA-15-mediated inhibition of nuclear import, although these proteins enter the nucleus like wild type ERK2 in the absence of PEA-15. This latter result is consistent with the lack of a requirement for ERK2 activity in the facilitated entry process.

The ability of the active form to be retained at sites of action may be substantially due to its affinity for substrates at those sites. For example, there is evidence that ERK2 is anchored in the nucleus in stimulated cells, perhaps through interactions with the mitotic apparatus or transcriptional regulators [7, 138, 139]. Based on results of an earlier study, we suggested that dimerization of phosphorylated ERK2 might cause retention of ERK2 in the nucleus, perhaps due to its inability to be exported when dimeric [21]. The current findings show that the dimer-defective mutant of ERK2 enters the nucleus like wild type ERK2. This movement suggests that the ability to dimerize is not required for nuclear entry, and is consistent with the hypothesis that the entry of ERK2

into the nucleus is not the predominant regulated step in its stimulus-dependent nuclear accumulation.

The effect of PEA-15 on ERK2 nuclear entry depends on its direct interaction with ERK2, rather than general interference with import mechanisms, because mutations in ERK2 or PEA-15 that decrease their affinity reduce the ability of PEA-15 to inhibit ERK2 import. Our work suggests that at least one mechanism of action of PEA-15 is through the inhibition of the interaction of ERK2 with nucleoporins in the NPC, thereby preventing ERK2 nuclear entry. Interfering with nucleoporin binding of ERK2 by other means has been shown to prevent entry of ERK2 into the nucleus [121]. The regions on ERK2 that bind FXF motifs of nucleoporins or other FXF-bearing proteins, such as Fos or Elk, have not been unequivocally identified. The fact that ERK2 which lacks the MAP kinase insert enters the nucleus in the import assay indicates that the insert itself is not an essential element of the FXF binding site. However, the interference of PEA-15 with ERK2 binding to NUP153c and the ability of PEA-15 to prevent the nuclear entry of ERK2 both support the hypothesis that PEA-15 directly interferes with the association of ERK2 with nucleoporins. Alternatively, through its contact with the MAP kinase insert, PEA-15 may induce a conformational change elsewhere on ERK2 that disorders the FXF binding site. A further hint that binding of a protein to the MAP kinase insert might block nuclear entry comes from the observation that ERK2Δ241-272 displays a subtle defect in nuclear uptake, most obvious as slower kinetics of entry. Finally, in support of this argument, MEK1, which binds to the MAP kinase insert of ERK2, is well documented to localize ERK2 to the cytoplasm. This equilibrium may be due in part to nuclear export of an ERK2-MEK1 complex, but Nishida's group has also shown that

MEK1 retains ERK2 in the cytoplasm in import reconstitution assays comparable to those used here [163]. Thus, the MAP kinase insert, although not essential for FXF binding, may be located near to or allosterically impact on the FXF binding motif. The weak interaction of ERK2 with FXF motifs has, thus far, prevented us from using the two-hybrid approach to identify the binding site. The identification of FXF binding regions on ERK2 is under intense study in a number of laboratories.

Expression of PEA-15 is low in most cell lines, except those of neuronal origin, indicating that only a fraction of ERK2, which is relatively abundant, is interacting and being sequestered by PEA-15. This small fraction of ERK2 may be sufficient to prevent apoptosis in certain cellular contexts. PEA-15, or other non-catalytic proteins, may be important for retaining ERK2 in the cytoplasm to perform specific functions. One of these may be its role in survival; PEA-15 is an anti-apoptotic protein, and a recent study has shown that phosphorylation of caspase-9 by ERK2 prevents the activation of the apoptotic cascade [52]. These cytoplasmic functions have often been neglected in comparison to the role of ERK2 in phosphorylating nuclear substrates such as transcription factors, but nevertheless are essential to ERK2 function. Furthermore, the ability of small non-catalytic proteins to integrate multiple signals, may also be crucial for ensuring proper cellular responses to multiple stimuli.

Chapter 4: Regulation of P-ERK1/2 Concentration and Location

Abstract

Stimulation of ERK1/2 contributes to diverse cellular behaviors including proliferation, differentiation, migration and survival. While ERK1/2 have the capacity to influence many phenotypes, their activation by discrete stimuli typically results in the generation of specific biological outcomes. Therefore, mechanisms must exist to ensure that ERK1/2 interact with appropriate substrates to induce stimulus-specific phenotypes. A number of different mechanisms including regulation of the duration and amplitude as well as subcellular localization have been proposed to impact the ability of ERK1/2 to initiate cellular programs. Work in PC12 cells has demonstrated a strong correlation between the duration of ERK1/2 activation and specific phenotypic consequences in response to discrete stimuli [4, 87]. Here we establish that amplitude and subcellular localization of ERK1/2 are also regulated in a stimulus-specific manner. In single cells, we find that different amounts of ERK1/2 are activated in response to different concentrations of ligands. The capability of the cascade to be activated in this graded manner suggests that different extents of ERK1/2 activation may have specific phenotypic consequences. We also demonstrate that the subcellular localization pattern of active ERK1/2 is dependent upon discrete stimuli. Furthermore, we find that restricted compartmentalization of ERK1/2 prevents the activation of certain effector proteins. These results suggest that, in addition to duration, both amplitude and subcellular localization of ERK1/2 may be regulated in a manner dependent on the nature of the activating ligand.

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Introduction

Intracellular signaling pathways integrate environmental cues into specific cellular behaviors. Because the enzymatic components of these signaling modules have the capability to activate numerous substrates in vitro, their ability to access effectors in vivo must be regulated to ensure appropriate interactions with substrates. The MAP kinases, ERK1/2, are typical of such signaling proteins as their stimuli and substrates are numerous and diverse. Activation of ERK1/2 has been demonstrated to promote cellular proliferation, differentiation, migration and survival [1, 2]. ERK1/2 effectors are located throughout the cell and include the nuclear transcription factors c-Fos and Elk-1, cytoplasmic protein kinases such as p90RSK and myosin light chain kinase (MLCK), and other enzymes such as phospholipase A_2 (PLA_2) [48, 49, 54, 56, 57]. The pleiotropic effects of ERK1/2 signaling indicate that the interaction between activated ERK1/2 and its substrates must be regulated to ensure the appropriate cellular response to distinct stimuli. Two potential methods for regulation of ERK1/2 are the modulation of the amount and subcellular localization of the active kinase; however, it is unclear if these properties are regulated differently by distinct stimuli in mammalian cells.

Previous reports have indicated that the amount of ERK1/2 activated can impact a specific biological outcome. Measurements of the amplitude of ERK1/2 activation in these studies has been examined in a population of cells; no evidence exists that different amplitudes in individual mammalian cells (Figure 4-1). For example, in KSR-deficient mice, the amount of ERK1/2 activated in response to stimuli is reduced by half compared to wild type. This observation is correlated with reduced T-cell proliferation and tumorigenic capacity of epithelial cells [179]. In colon carcinoma cells, a high amplitude of ERK1/2 activation induces expression of Fra-1, but lower levels have no effect on

expression of this immediate early gene. Fra-1 expression appears to be required for the prevention of anoikis in this cell type [5]. The correlation of reduced amplitude of ERK1/2 activation with a specific phenotype suggests that the amount of activated ERK1/2 may be important for the generation of specific phenotypes. However, it is unclear if the partial response of ERK1/2 is generated by a decrease in ERK1/2 activation in all cells or the reduction of the fraction of cells maximally activating ERK1/2 (Figure 4-1). *Xenopus* oocytes are particularly amenable to studying ERK1/2 behavior at the single cell level due to the large size of a single cell. Ferrell and colleagues demonstrated that above a specific concentration of progesterone, all the ERK in a single oocyte is activated. Below this threshold concentration, no ERK is active [180]. This bistable response of ERK to different concentrations of stimulus has been suggested to also occur in mammalian cells [181] The response of ERK1/2 in single cells to different ligand concentrations has not been examined in mammalian cells.

Compartmentalization is a well-established mechanism for regulating the interaction between activated signaling proteins and their substrates. Regulated compartmentalization could promote activation of relevant substrates and prevent interaction with inappropriate effectors. The nuclear accumulation of active ERK1/2 and the consequent phenotypic changes are well established. ERK1/2 are localized to cytoplasm or evenly distributed throughout resting cells [86] (A.W.W. and M.H.C. unpublished observations). Following activation, ERK1/2 have been shown to accumulate in the nucleus, a localization pattern required for proliferation of 3T3 cells and differentiation of PC12 cells [4, 6, 87]. ERK1/2 also has a number of cytoplasmic substrates that are involved in cellular behaviors, such as motility and inflammation [51,

57]. The location of these substrates implies that ERK1/2 must, under some circumstances be localized to the cytoplasm. Furthermore, the association of ERK1/2 with nuclear substrates may be undesirable in response to certain ligands. Despite the existence of ERK1/2 substrates in numerous cellular compartments, ligand-specific localization patterns of the active kinase have not previously been identified.

To understand the mechanisms that regulated active ERK1/2 better, we examined both the amplitude and localization of ERK1/2 in primary foreskin fibroblasts and some continuous cell lines. Our findings indicate that ERK1/2 activation in mammalian cells is graded in proportion to the concentration of the activating ligand. Furthermolre, discrete cytoplasmic and nuclear localization patterns of ERK1/2 occur following the stimulation of cells with specific ligands. Nuclear entry of ERK1/2 does not appear to be an obligatory step following activation, but may be regulated to target ERK1/2 to specific substrates.

Experimental Procedures

Cell Culture. HeLa cells were obtained from ATCC and fibroblasts were obtained from human foreskin specimens (<20 population doublings). Cell lines were maintained in DMEM (Gibco #12100-061) supplemented with 10% fetal bovine serum (Gibco #26140-095) and 1.0 % glutamine (Invitrogen #25030-081). HeLa cells were incubated in DMEM + 0.5 % serum for 6 hours prior to analysis. HFF's were a kind gift from F. Grinnell. HFF's were plated on 50 □g/ml collagen (Vitrogen #FXP-019) 24 hours before removal of serum. HFFs were incubated in 0.5 % serum overnight before analysis. EGF was obtained from BD Biosciences (#354001) and PMA was obtained from Sigma (#P8139). Leptomycin B (Sigma #L2913) was used for 90 minutes at a final concentration of 200 ng/ml prior to stimulation of cells.

Immunoblotting. HeLa cells were lysed in buffer containing 50 mM Hepes, 150 NaCl, 80 mM B-glycerolphosphate, 1 mM NaVO3, 5 % Triton X-100 and protease inhibitibors. HFF cells were lysed in 2X sample buffer. Lysates were separated n 10% poly-acrylamide gels. Total ERK (#SC-93) and c-fos (#SC-7202) antibodies were obtained from Santa Cruz. Phospho-ERK (#M-8159) and cyclin D1 (#C-5588) antibodies were obtained from Sigma and phospho-RSKT57 (#9346) obtained from Cell Signaling Technologies. Anti-serum Y691 was used for Total ERK analysis in HeLa cells as previously described [175].

Immunofluorescence and Fluorescence Measurements HeLa cells were fixed and permeabilized for 10 minutes in methanol at –20°C. HFF's were fixed in 3.7 % formaldehyde for 10 minutes at room temperature followed by methanol permeabilization at –20°C for 10 minutes. All cells were then blocked for a minimum of 30 minutes in

PBTA (1X PBS, 5 % BSA, 0.1 % Tween-20). Primary antibodies were used at a 1:100 dilution in PBTA for 1 hour at room temperature followed by washing and secondary antibody staining using Alexa 488 or 546 (Molecular Probes) for 30 minutes at 37°C. Cells were washed and mounted using Polymount. Cells were visualized using a Zeiss Axiocam equipped with an Orca II Hamamatsu black and white camera. Fluorescence intensities were acquired , pictures were taken of a minimum of 50 cells. Individual cells were circled and quantitated using Open Lab 3.1 software.

Results

To examine ERK1/2 activation at the single cell level, we immunostained HeLa cells stimulated with EGF with an active ERK1/2-specific antibody. We find, qualitatively and semi-quantitatively, that the fraction of dually phosphorylated ERK1/2 appears to be proportional to the concentration of stimulus (Figure 4-2A and B). Above concentrations of 0.2 ng/ml of EGF, we did not observe a further increase in the amount of ERK1/2 activated, a result in agreement with immunoblot analysis.

We observed a similar trend in individual human foreskin fibroblasts (HFFs) stimulated with EGF (Figure 4-3A). Further studies focused on HFFs because HeLa cells contain genetic aberrations that may impact both the activation and localization of MAP kinase. The same graded response of ERK1/2 activation in individual cells was observed using phorbol myristate acetate (PMA) to stimulate the HFFs (Figure 4-3B). These graded responses were also detectable by immunoblot analysis (Figure 4-6, middle panel). The range of responses observed in the population indicates that these cells have the capability to activate different amounts of ERK1/2 in proportion to ligand concentration.

Single cell analysis of HFFs demonstrated that EGF and PMA appeared to activate the same relative amounts of ERK1/2, but the localization patterns of ERK1/2 in response to these ligands were very different. At 5 minutes, nuclear, dually phosphorylated ERK1/2 staining was evident in response to PMA but not EGF (Figure 4-4). This result was surprising because previous reports indicated that once active, ERK1/2 accumulates in the nucleus [7, 86, 90]. At different concentrations, as well as at very short (~2 min) and longer (45 minute and 2 hour) times, no significant nuclear

accumulation of ERK1/2 was observed in EGF-treated cells (Figure 4-4 and 4-5). The total ERK1/2 (unphosphorylated and phosphorylated) staining pattern was similar to the phosphorylated pattern (Figure 4-9A). The difference in these localization patterns was independent of the amount of phosphorylated ERK1/2, because our previous single cell analysis indicated PMA and EGF activate similar quantities of the total pool of ERK1/2 (Figure 4-3A and 4-3B and Figure 4-6 middle panel). We also examined two other ligands, platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA). In cells stimulated with PDGF and LPA, phosphorylated-ERK1/2 localized to the nucleus, but the accumulation was not as dramatic as in cells stimulated with PMA (data not shown).

To determine how different amplitudes and localization of ERK1/2 impact the activation of effectors, we examined p90RSK phosphorylation and c-Fos expression in stimulated cells. p90RSK is a cytoplasmic kinase that is phosphorylated by PDK1 and ERK1/2 in the cytoplasm and consequently moves into the nucleus [48, 182]. At 5 minutes, the amount of p90RSK phosphorylated on the ERK1/2 specific site, T573, was dependent on the concentration of EGF or PMA used to treat cells (Figure 4-6) [183]. This activation was inhibited when cells were pretreated with 10 uM of the MEK1/2 selective inhibitor, U0126 (data not shown) [35]. Thus, the quantity of ERK1/2 activated correlates with the amount of phosphorylated substrate as detected by immunoblot analysis. Furthermore, ERK1/2 activated by either EGF or PMA is capable of phosphorylating cytoplasmic substrates.

We next wanted to examine if stimulation with EGF or PMA impacts the activation of ERK1/2 effectors localized to the nucleus. For this analysis, we examined c-Fos expression. C-Fos is a nuclear protein expressed following ERK1/2 activation of the

serum response element through serum response factor and ternary complex factors such as Elk-1 [56]. Additionally, ERK1/2 has been shown to phosphorylate and stabilize c-Fos [60, 125]. Following PMA stimulation, c-Fos was expressed in approximately 60 % of cells. In contrast, approximately 15 % of EGF-treated cells expressed c-Fos (Figure 4-7A &B). By immunoblot analysis c-Fos protein expression was only detectable in response to PMA (Figure 7C).

C-Fos is a component of the AP-1 transcription factor that activates cyclin D1 transcription [184]. To determine if PMA-induced c-Fos expression was sufficient to increase target gene expression, we immunoblotted cyclin D1 in cells stimulated with PMA and EGF. Cyclin D1 was not significantly induced over background in response to EGF; however, it was increased in response to PMA (Figure 4-8). Thus, the capacity of a ligand to induce cyclin D1 correlates with its ability to promote the nuclear accumulation of phosphorylated ERK1/2.

Because EGF activates many components of the intracellular signaling network, we wondered if the lack of c-Fos expression was due primarily to the absence of active, nuclear ERK1/2. To explore this question, we used the CRM1-dependent nuclear export inhibitor, leptomycin B. Previous studies have shown that leptomycin B induces nuclear accumulation of ERK1/2 [132, 137]. Figure 4-9A shows that nuclear accumulation of ERK1/2 is increased in EGF- and PMA- stimulated cells treated with leptomycin B. PMA induced more nuclear, active ERK1/2, consistent with our previous findings that phosphorylated ERK1/2 localizes to the nucleus more readily in response to PMA. C-Fos expression was elevated in the leptomycin B- and EGF- treated cells compared to EGF alone. C-Fos accumulation in response to PMA was unchanged in the presence of

leptomycin B (Figure 4-9B). The change in c-Fos expression observed by EGF in the presence of leptomycin B was not due to increased ERK1/2 or p90RSK activity because they were the same in all groups (Figure 4-9C). While we cannot rule out the possibility that additional proteins trapped in the nucleus by leptomycin B contribute to c-Fos expression, these data indicate a correlation between nuclear localization of active ERK1/2 and c-Fos expression.

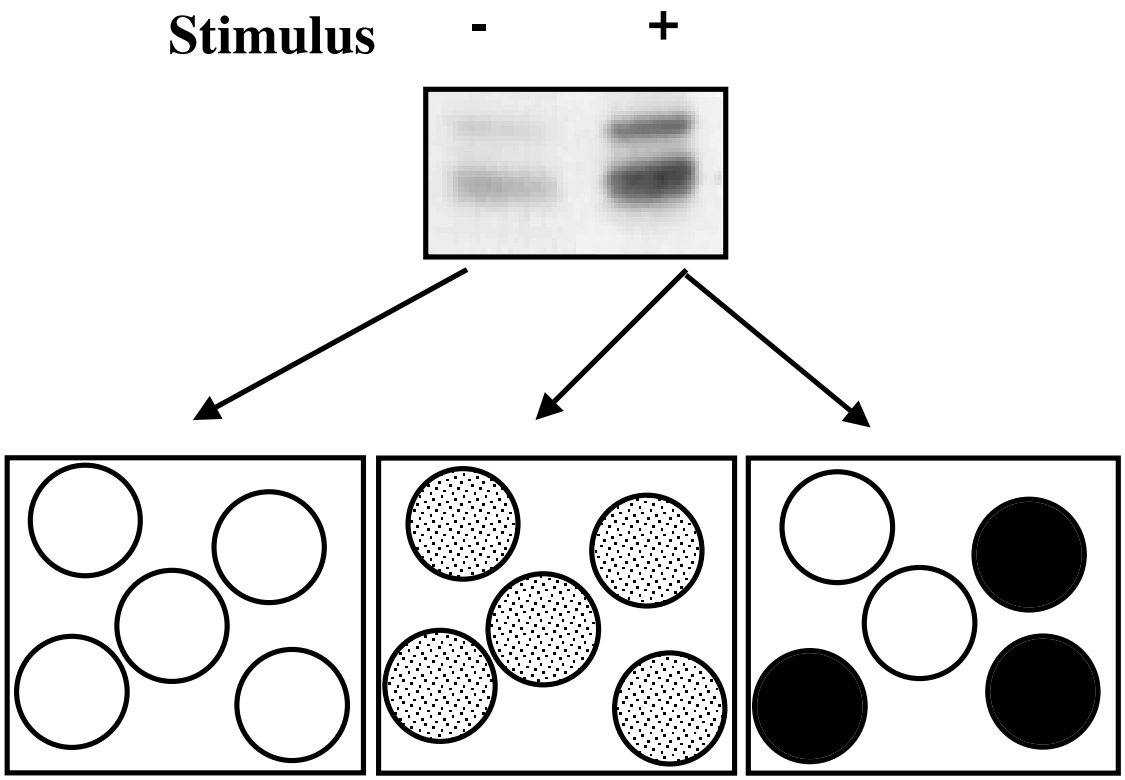


Figure 4-1. Schematic explanation of population analysis of ERK1/2 activation Circles represent cells and black represent dually phosphorylated ERK1/2. Western analysis of whole cell lysate of fibroblasts. In the unstimulated cells, ERK1/2 is in the unphosphorylated state. The western blot signal from stimulated cells cannot differentiate between a fraction of ERK1/2 in all cells becoming active and only a fraction of cells activating ERK1/2.

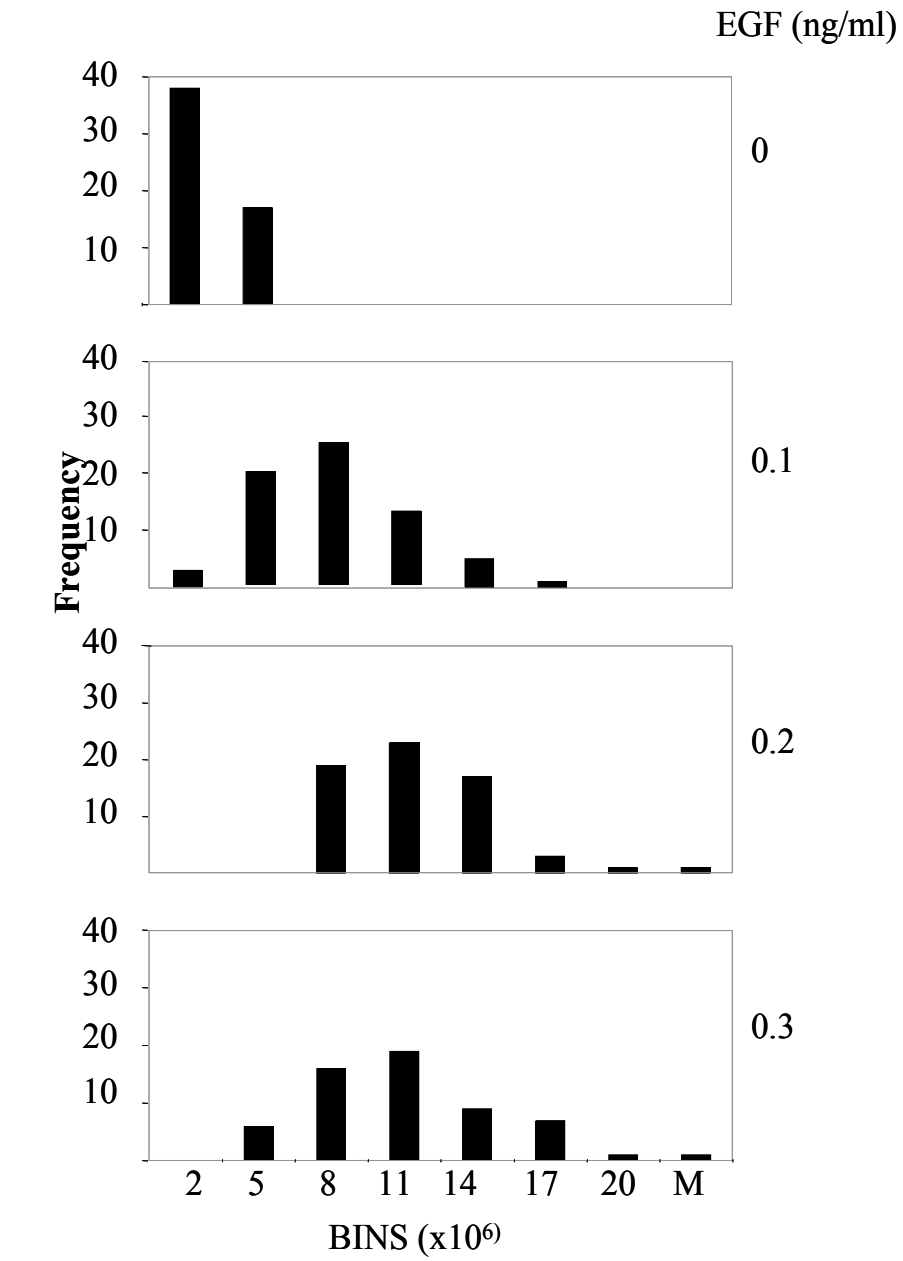
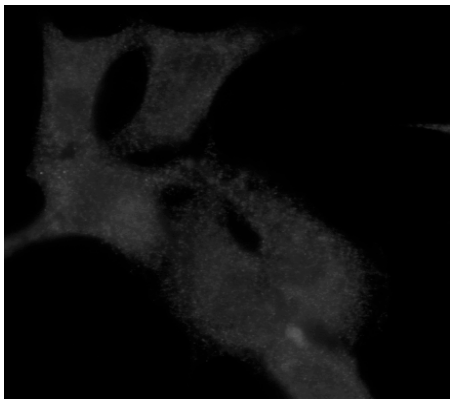


Figure 4-2. ERK1/2 activation in HeLa cell following EGF stimulation. A. Histograms of phosphorylated ERK1/2 immunofluorescent measurements of 50 HeLa cells stimulated with indicated amounts of EGF for 5 minutes. **B.** Phosphorylated ERK1/2 immunofluorescence staining of HeLa cells stimulate with indicated amounts of EGF for 5 minutes. **C.** Total and phosphorylated ERK1/2 immunblot analysis of starved HeLa cells stimulated with indicated amounts of EGF for 5 minutes.

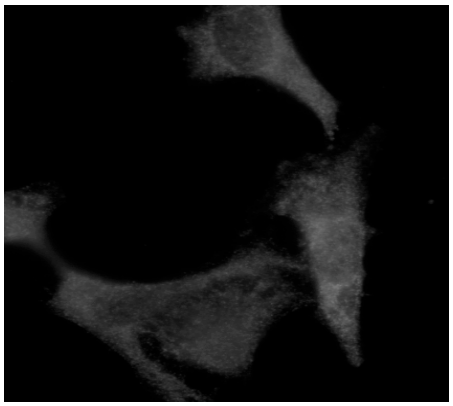
B.

EGF (ng/ml)

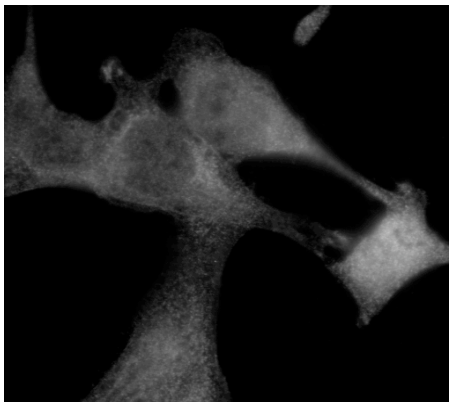
0



0.1



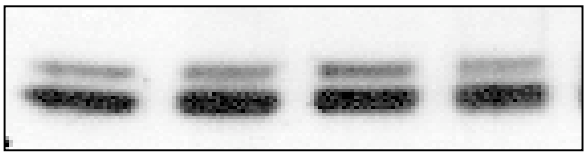
0.3



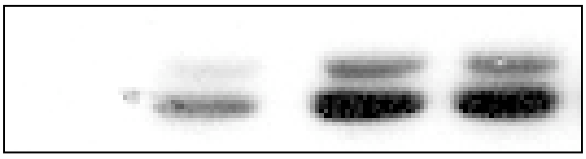
C.

EGF (ng/ml)

0 0.1 0.2 0.3



Total ERK1/2



P-ERK1/2

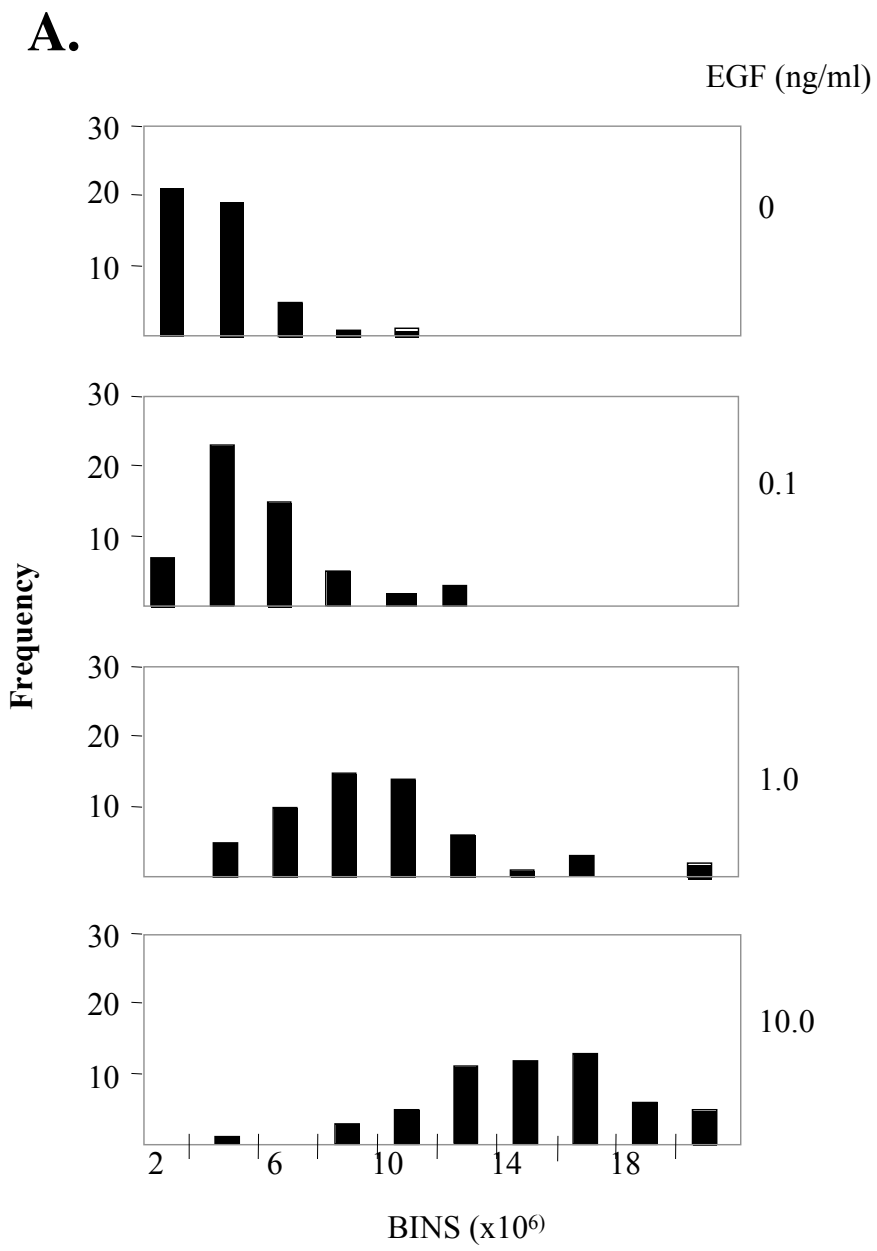
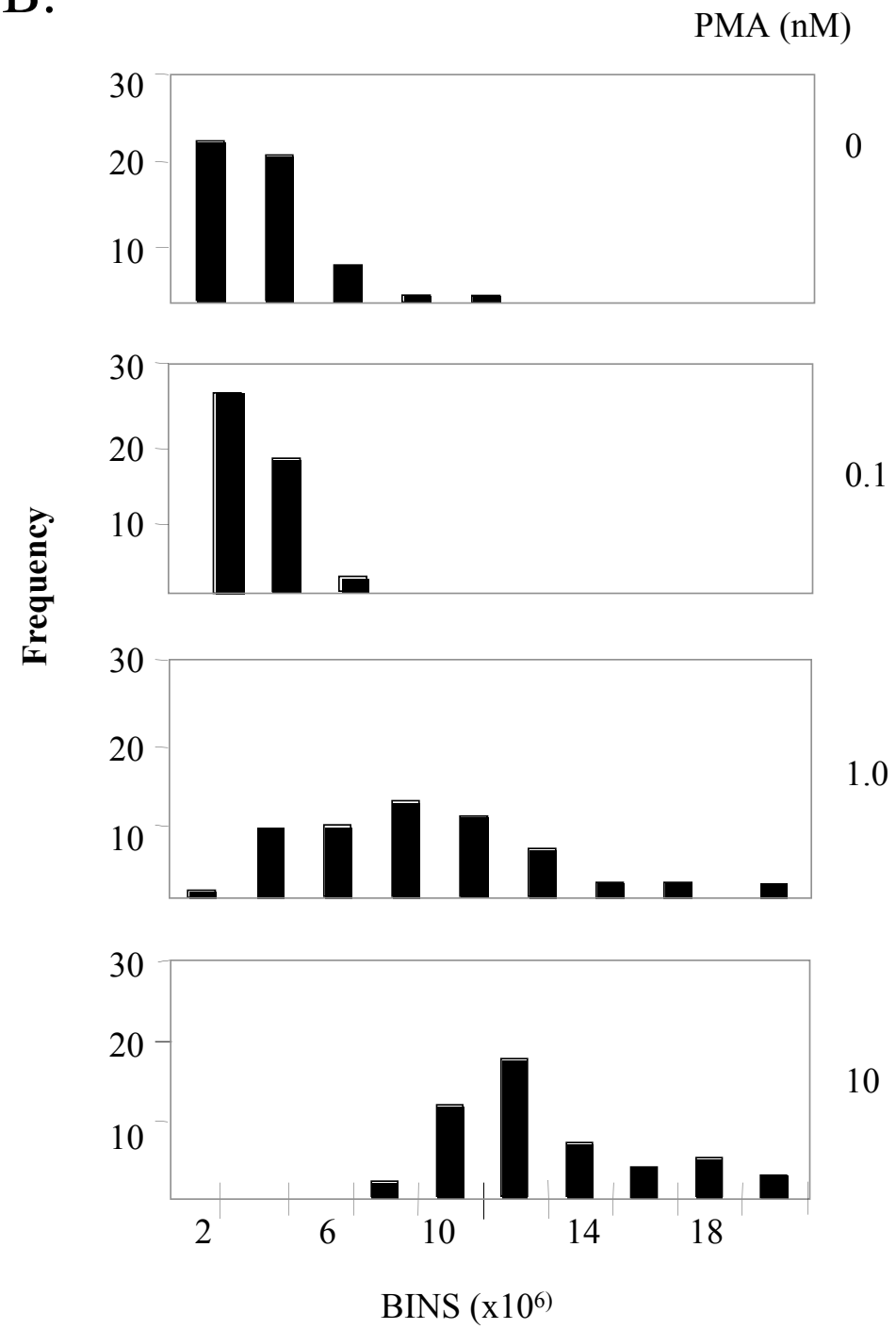


Figure 4-3. ERK1/2 activation in HFFs in response to EGF and PMA. A. &B. Histograms of phosphorylated ERK1/2 immunofluorescent measurements in HFFs following stimulation with indicated amounts of EGF and PMA for 5 minutes. For each concentration, 50 cells were measured.

B.



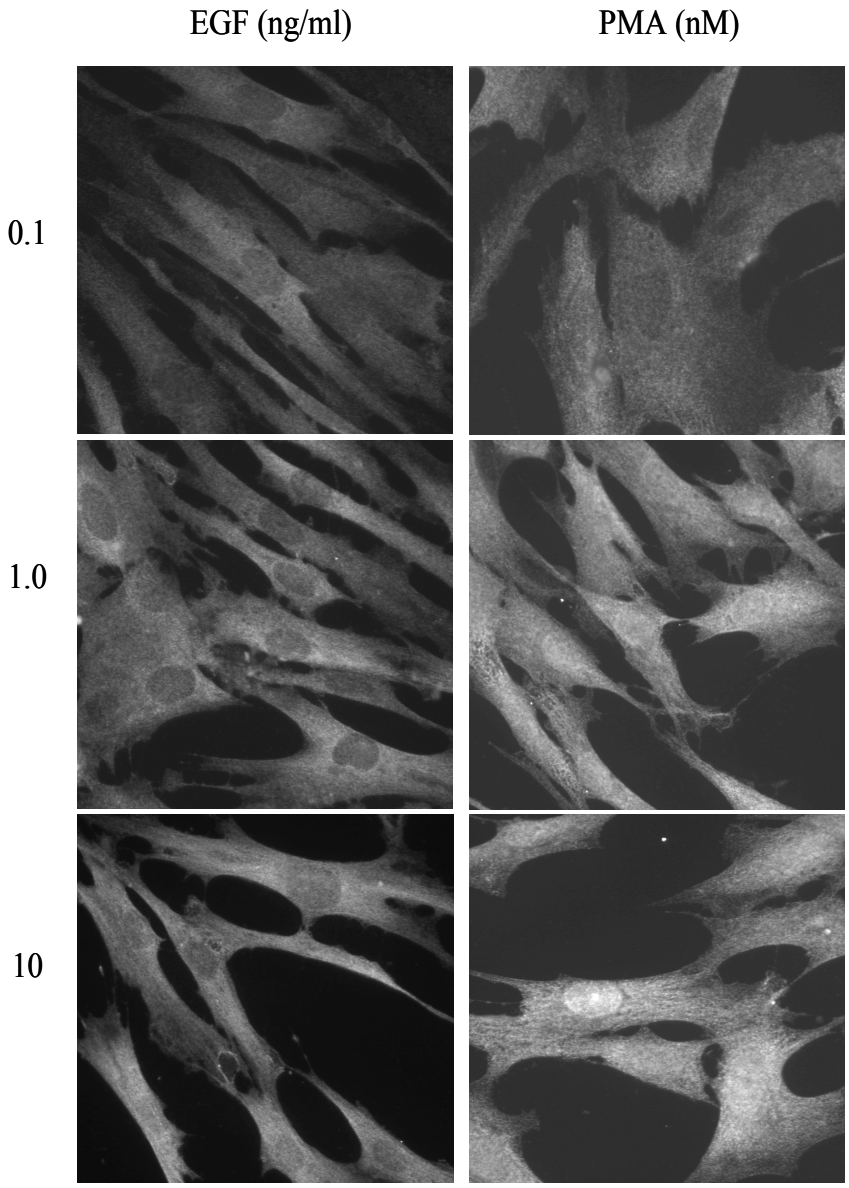


Figure 4-4. EGF does not induce nuclear localization of active ERK1/2. HFFs were stimulated for 5 minutes with indicated concentration of ligand.

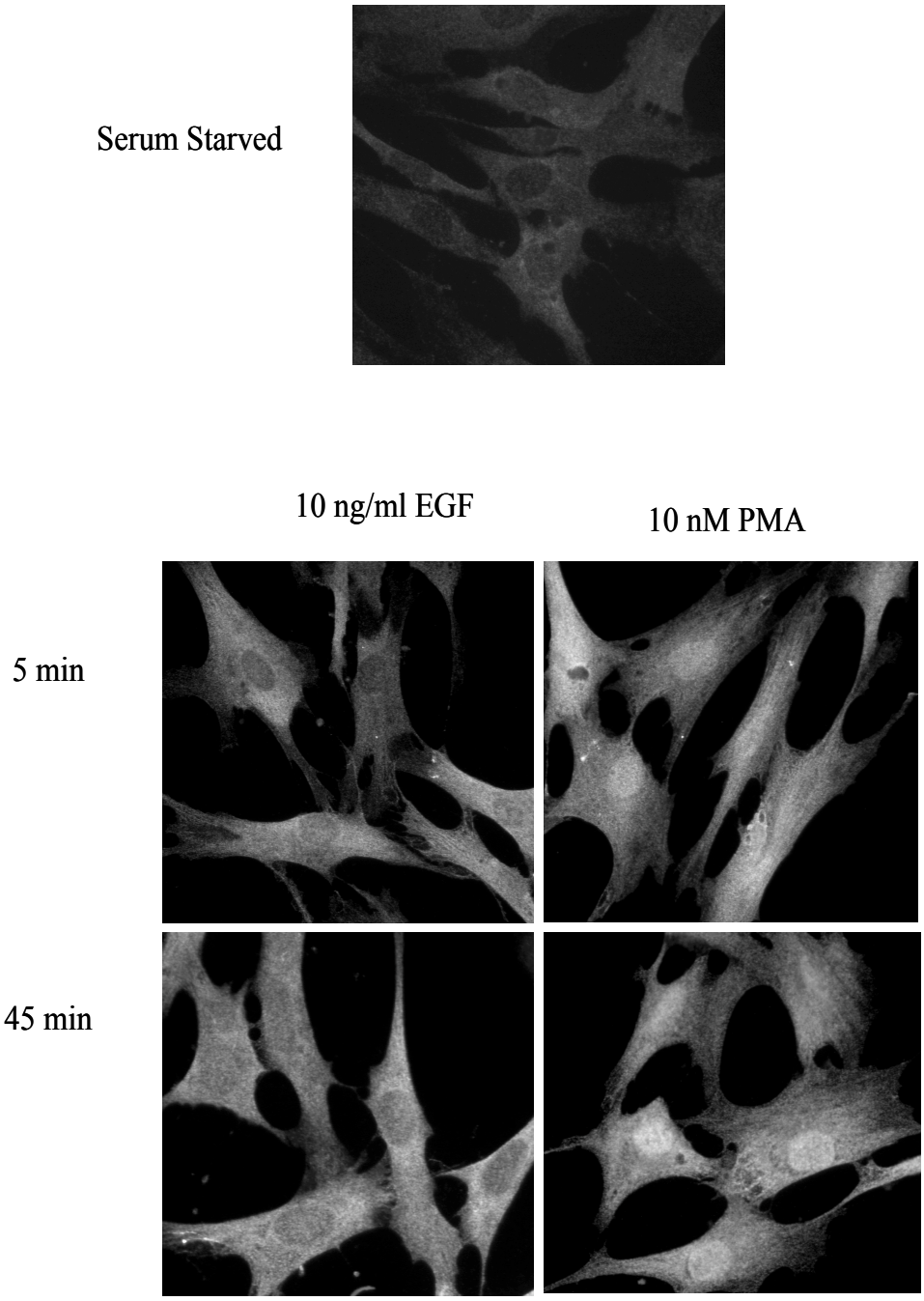


Figure 4-5. Phosphorylated ERK1/2 staining pattern in HFFs. Serum starved HFF's stimulated with 10 ng/ml of E1/2GF or 10 nM PMA for 5 and 45 minutes were stained with a phospho-specific ERK antibody.

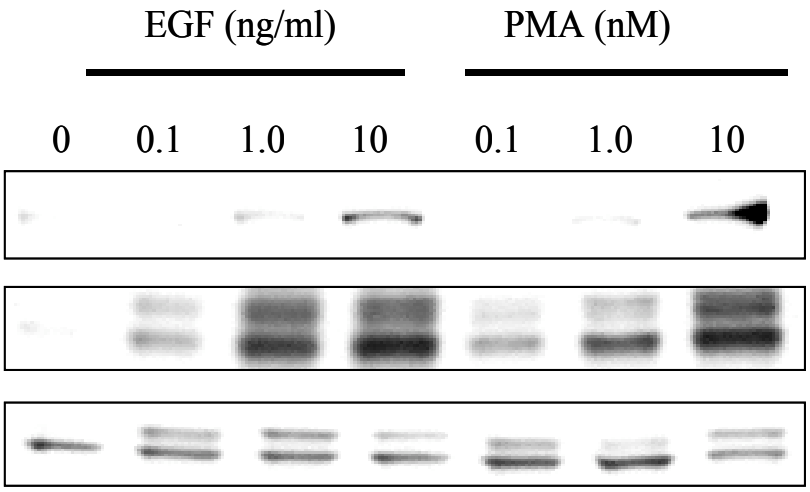
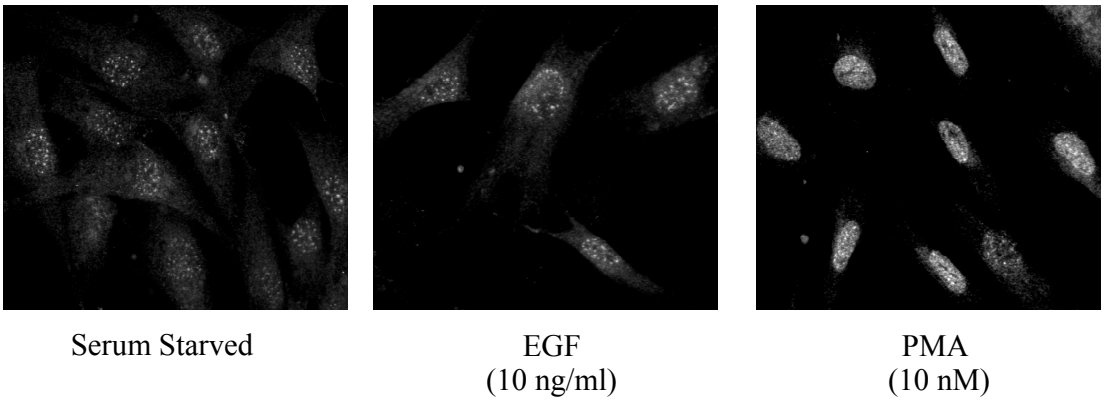


Figure 4-6. Phosphorylated p90RSK (Thr 573) status in response to doses of EGF and PMA. Phospho-ERK1/2, p90RSK and Total ERK1/2 immunoblot analysis of serum starved HFF's stimulated with indicated amounts of EGF and PMA for 5 minutes.

A.



B.

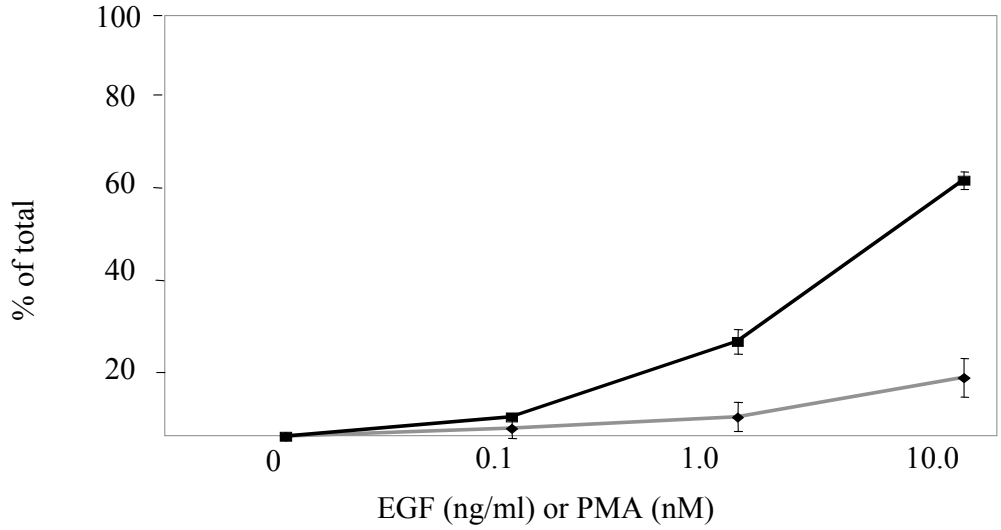
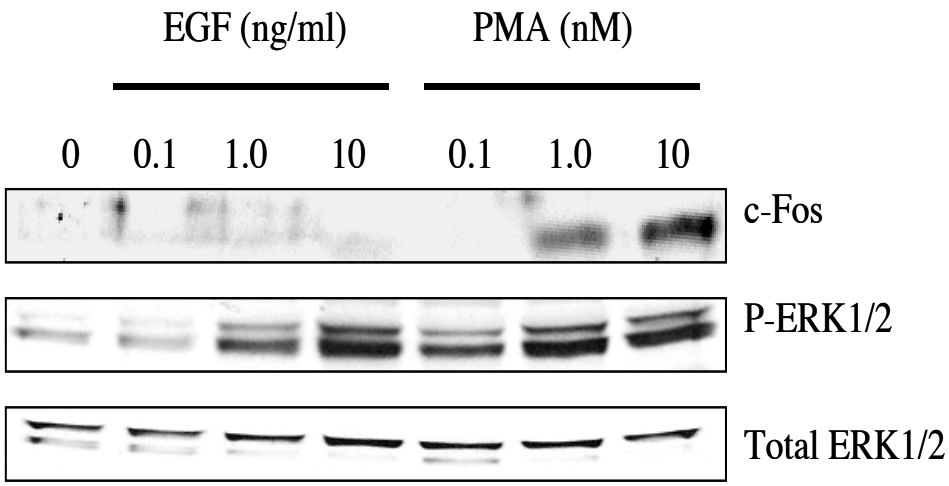


Figure 4-7. C-Fos expression in response to EGF and PMA. **A.** HFF's stimulated for 45 minutes with indicated amounts of EGF or PMA were immunostained with c-fos antibody. **B.** Quantitation of cells with c-fos expressed graphed as a percentage of total cells (average of 3 experiments) **C.** c-fos, phospho-ERK and total ERK immunoblots of lysates stimulated with indicated concentration of EGF and PMA for 45 minutes.

C.



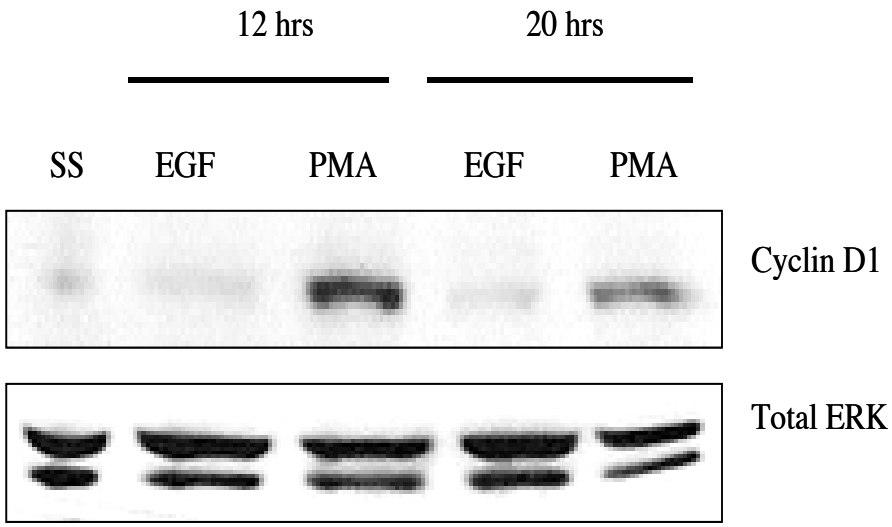


Figure 4-8 Cyclin D1 protein expression in response to EGF and PMA. HFF's were stimulated with 10 ng/ml EGF or 10 nM PMA for indicated times and lysates blotted for cyclin D1 and total ERK1/2 antibodies.

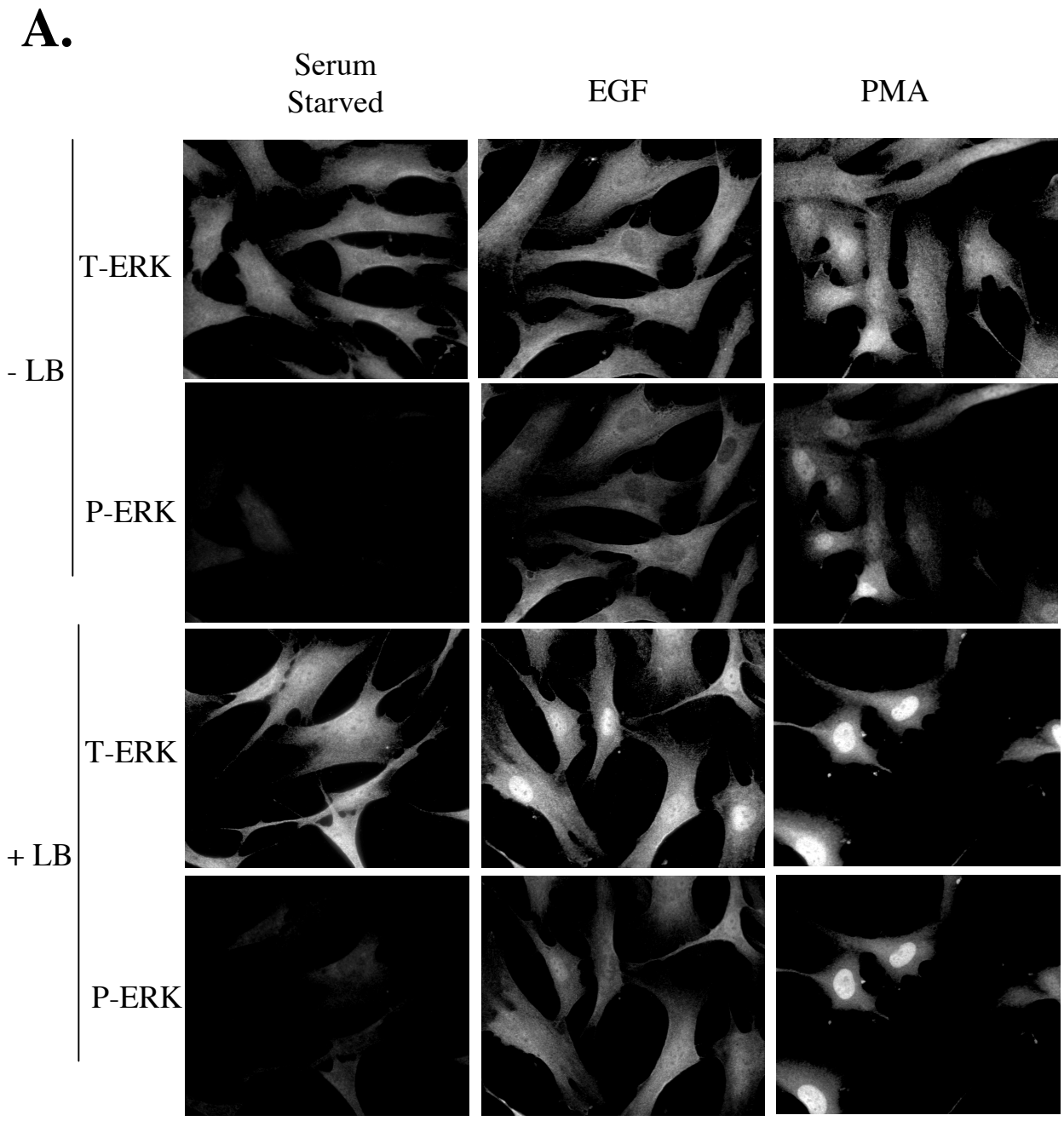
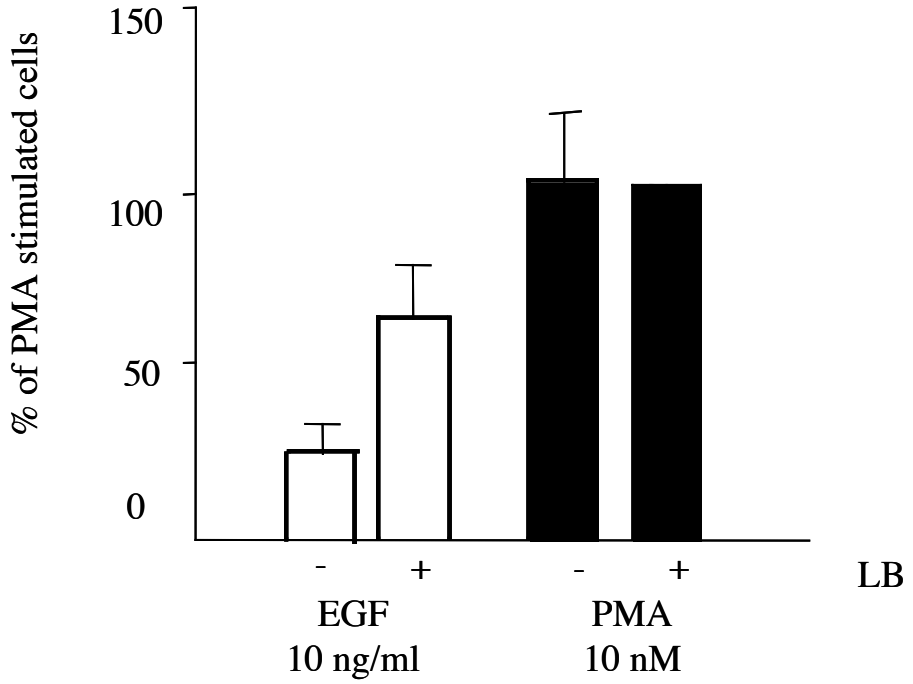
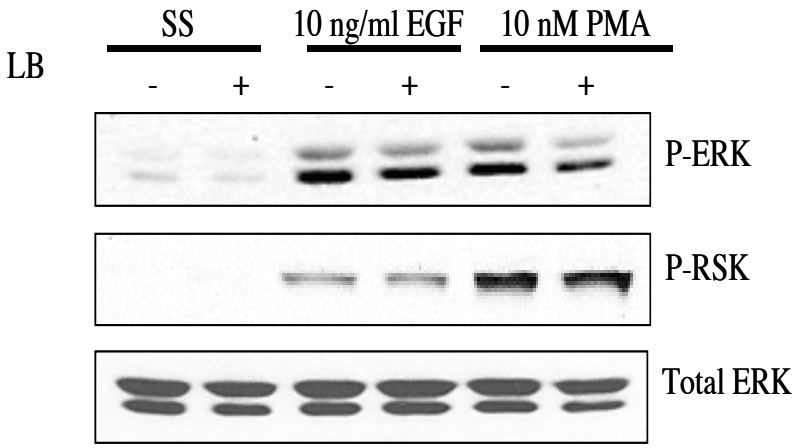


Figure 4-9. c-Fos activation is increased in HFFs treated with Leptomycin B and EGF. A. Cells were pre-treated with leptomycin B followed by stimulation with 10 ng/ml EGF or 10 nM PMA for 45 minutes. Cells were stained with phospho and total ERK antibodies. **B**. Cells were treated with leptomycin B (LB) and EGF or PMA as in A and the number of cells expressing c-fos quantitated (total of 50 cells for each sample). Graph represents the average of three experiments. **C**. Lysates from cells treated as described in A. were immunoblotted for phospho-ERK, phospho-p90RSK and total ERK. Leptomycin B (LB).

B.



C.



Discussion

Activation of ERK1/2 is a required step for the transduction of numerous cell surface signals. While the ligands that activate ERK1/2 and the phenotypic changes that follow have been well described, the mechanisms that regulate the function of the cascade remain obscure [1]. Here we examine two potential mechanisms, regulation of amplitude and localization, which could impact how this cascade transduces extracellular signals. We find that both of these properties can be regulated, either by the quantity or type of stimulus.

Previously, the ability of cells to activate a fraction of ERK1/2 has only been demonstrated in a population of cells. A correlation of different amplitudes of ERK1/2 activation with specific phenotypes has been difficult because it was not clear that individual cells were able activate different amounts of ERK1/2 [5, 179]. At the single cell level, we have demonstrated that intermediate activation states of ERK1/2 can be achieved and maintained for at least 45 minutes. Taken together, ours and previous studies indicate that different levels of ERK1/2 activation may direct specific cellular behaviors by selectively activating specific substrates. For example, integrin activation is known to stimulate ERK1/2 weakly, but this activation may be important for both survival and motility [51]. By examining substrates involved in these cellular programs, we may be able to establish the molecular mechanisms by which activation of the cascade leads to certain specific phenotypic changes but not others.

We find that the localization of active ERK1/2 to specific cellular compartments can be regulated by the activating ligand. The impact of these localization patterns on the activation of compartmentalized substrates indicates that stimulus-specific regulation of

ERK1/2 localization is important for directing discrete cellular behaviors. Previous reports have suggested that nuclear accumulation of ERK1/2 always occurs after activation [7, 86, 90]. While a low level of active ERK1/2 is present in the nucleus following EGF stimulation of HFFs, our data suggest the activation can be uncoupled from nuclear localization and that ERK1/2 activation is not a switch to induce nuclear localization. Instead, the discrete patterns of active ERK1/2 localization in response to specific stimuli suggest that additional mechanisms for regulating nuclear accumulation exist.

A number of mechanisms may be responsible for the cytoplasmic localization of ERK1/2 in EGF-stimulated HFFs. The nuclear entry of ERK1/2 in EGF-treated cells following the leptomycin B treatment, indicates that some ERK1/2 is entering the nucleus. Because the amount of nuclear ERK1/2 in EGF-stimulated cells treated with leptomycin B is less than that in PMA-stimulated cells exposed to leptomycin B, nuclear import may be the affected step. It is possible that less ERK1/2 is entering the nucleus due to an ERK1/2 cytoplasmic anchoring protein that is activated in response to EGF. A number of anchoring proteins have been identified that may target ERK1/2 to the cytoplasmic compartment. Calponin has been demonstrated to prevent nuclear entry in smooth muscle cells and PEA-15 can cytoplasmically sequester ERK2 in the cytoplasm, restricting the activation of nuclear targets and promoting integrin activation [174, 178]. However, these proteins have not been demonstrated to function in a stimulus-dependent manner. Perhaps sub-populations of ERK1/2 are targeted for activation by specific stimuli. In some cases, the relevant sub-population may be complexed to cytoplasmic anchors that prevent nuclear entry. Alternatively, cytoplasmic anchoring proteins may be

regulated by different cellular stimuli. In this case, the activation of a parallel pathway may induce the loss of association of ERK1/2 with a cytoplasmic anchor. In preliminary experiments, stimulation of cells first with EGF for 5 minutes followed by PMA, induced nuclear accumulation to a similar extent as PMA alone. This result suggests that a cytoplasmic anchoring protein has the capacity to sequester ERK1/2-stimulated by EGF. The presence of nuclear anchors only in PMA-stimulated cells could also be responsible for the altered patterns of localization in EGF- and PMA- stimulated cells. Pouyssegur has suggested that the synthesis of nuclear anchors may prevent nuclear export of ERK1/2 [7]. The identity of potential nuclear anchors nor evidence for their synthesis in a stimulus-dependent manner exists. In this case, PMA-stimulated ERK1/2 could be anchored by nuclear substrates while ERK1/2 in EGF-stimulated cells is exported rapidly. Clearly nuclear entry of ERK1/2 is far more complicated than a simple activation dependent mechanism. Our report reveals that additional mechanisms exist and that these mechanisms are important to induce stimulus-specific cellular changes.

Chapter 5: Conclusions and Future Directions

The proper subcellular localization of activated signaling proteins is required for efficient and specific target activation. Therefore, the regulated movement of either activated proteins or their substrates to the appropriate location at the right time is important for proper signal transmission. In many cases, the nature of the activating modification directs movement to a specific compartment. For example, covalent lipid modifications of proteins regulate membrane association [185]. Post translational modifications of proteins may induce conformational changes that reveal NLSs, which promote nuclear localization [186].

While it is well established that ERK1/2 undergo nuclear localization following phosphorylation, no targeting domains have been identified on the protein to explain a mechanism for this movement [86, 90]. Furthermore, previous work has presented the paradigm that once active, ERK1/2 move into the nucleus to phosphorylate substrates and induce phenotypic changes [4, 87, 90]. Despite the presence of ERK1/2 substrates in numerous cellular locations in addition to the nucleus, no evidence previously existed that activation of ERK1/2 could be uncoupled from nuclear localization.

To increase our understanding of the mechanisms that regulate compartmentalization of ERK1/2, the goals of my project were first to elucidate mechanisms for ERK2 nuclear import, second to examine how nuclear localization can be regulated by cytoplasmic anchoring proteins and finally to determine if activation of ERK1/2 can be uncoupled from nuclear translocation. My research has identified a mechanism in which ERK2 binds directly to nucleoporins for movement into the nucleus. The cytoplasmic anchoring protein, PEA-15, can inhibit the interaction between ERK2 and nucleoporins and thereby

prevent nuclear entry. My studies of endogenous ERK1/2 in primary fibroblasts revealed that over a sustained period of time ERK1/2 can localize to either the cytoplasm or nucleus depending on the activating stimulus. This finding is in contrast to numerous previous reports that nuclear accumulation of the kinase is an obligatory step following activation [89]. These studies also are the basis for future work exploring how ERK1/2 localization may be directed by the nature of the activating stimulus, thereby providing a regulatory mechanism for inducing appropriate phenotypic changes.

Previous studies of ERK2 nuclear import had suggested that the monomeric form of the kinase passively diffuses into the nucleus while the dimeric form enters by an undefined active mechanism [132]. To determine the specific requirements for non-passive movement across the nuclear pore complex, I used an in vitro import assay that allows the reconstitution of nuclear import using recombinant import substrates and factors (karyopherins, Ran etc.) in a defined system. This assay revealed that the only requirement for ERK2 nuclear entry is an interaction with nucleoporins. This mechanism indicates that movement across the nuclear pore is not exclusively regulated in the conventional, karyopherin mediated fashion, as it is for many signaling proteins.

Further studies will be required to determine the extent to which facilitated entry accounts for nuclear import of activated ERK1/2 in intact cells. An approach for evaluating the relative contribution of facilitated import as a mechanism for ERK2 entry is to prevent the interaction of ERK2 with nucleoporins and assess the impact on ERK2 localization. One strategy for inhibiting these interactions is to make ERK2 mutants that are unable to interact with FXF motifs; however, we have not yet identified the residues on ERK2 required for the nucleoporin interaction. It is presumed, perhaps incorrectly,

that this region on ERK2 is identical or overlapping with that which binds to substrates with FXF motifs. One method for identifying point mutants would be to use a library of ERK2 mutants as prey in a yeast two-hybrid screen with an FXF containing bait. This method has been used previously to identify Ras mutants that lose the ability to interact with effectors as well as ERK2 mutants that are unable to interact with MEK1 [40, 187]. We attempted to establish an interaction between ERK2 and FXF containing baits in yeast. We failed to find a bait for two-hybrid tests that included FXF motifs. It is possible that the interaction of ERK2 with the FXF motif is too weak to detect by this method.

One region that may contribute to FXF binding is the MAP kinase insert. GFP-ERK2 mutants lacking the insert did not accumulate to the same degree as wild-type GFP-ERK2 in the import assay. In a collaborative study with the Ginsberg laboratory, Y231 also was demonstrated to be important for the interaction between ERK2 and PEA-15 [188]. Because PEA-15 blocks ERK2 binding to nucleoporins in vitro, I examined the behavior of this mutant in the import assay. GFP-ERK Y231A accumulated in the nucleus as well as the wild type protein, suggesting the Y231 residue alone may not mediate an interaction with nucleoporins.

Analysis of the ERK2 structure with Betsy Goldsmith revealed a number of residues that could be involved in a binding pocket that interfaces with the MAP kinase insert. The region identified included residues Y261, L198, Y232, L235, I254, T179 and L182. The Y261 mutant has a slight import defect in the import assay as noted in Chapter 3. GFP-ERK2 Y232A did not have an import defect as assessed by our import assay. Because the interaction is relatively weak, mutation of a single residue may not be

sufficient to impair nuclear importation. Future experiments should employ combinations of these mutations to further elucidate the requirements of ERK2 for nucleoporin binding.

The majority of GFP-ERK2 used in the import assay is in the unphosphorylated state. Because ERK1/2 enters the nucleus following stimulation, it is important to determine if dually phosphorylated GFP-ERK2 is subject to the same entry mechanism or if an additional mechanism, not yet identified, contributes to the nuclear accumulation of ERK2-P2. In intact cells treated with PD98059, low levels of ERK1/2 are found in the nucleus [7]. This result suggests that some ERK1/2 are cycling into the nucleus even in resting cells, consistent with my import reconstitution studies. In stimulated cells, the amount of ERK1/2 found in the nucleus is much greater. This observation could be the result of a number of different possibilities for ERK1/2 movement. ERK1/2 may be continually entering the nucleus and rapidly exported, but when activated, ERK1/2 are retained in the nucleus by anchoring proteins. Alternatively, a large fraction of the cellular ERK1/2 may be anchored in the cytoplasm in the inactive state. Once active, ERK1/2 lose affinity for the anchoring protein and move into the nucleus.

One mechanism for differentiating these two possibilities is through experiments using fluorescence recovery after photobleaching (FRAP). BJ fibroblasts immortalized with h-TERT, the catalytic subunit of telomerase, would be a good cell type for these experiments because YFP-ERK2 is localized in the cytoplasm in resting cells when 1 g of DNA is transfected. Furthermore, BJ fibroblasts have the same localization patterns as HFFs in response to EGF and PMA. These cells can be maintained indefinitely as the h-TERT transgene allows them to bypass replicative senescence. In a FRAP experiment,

the nuclei of fibroblasts are bleached and the time for the recovery of a fluorescent signal measured. The time for recovery is an indirect measurement of the amount of YFP-ERK that is moving into the nucleus. By comparing the time for recovery in the PMA- stimulated and unstimulated state, one could determine if more ERK2 is moving into the nucleus in stimulated cells.

As described in Chapter 1, a genetic study in *D. melanogaster* suggested that a member of the karyopherin-β family binds to D-ERK [140]. While D-ERK may be regulated differently from mammalian ERK1/2, this result suggests the existence of an additional mechanism for nuclear import. Currently, no evidence exists for a receptor-mediated transport mechanism in mammalian cells. However, multiple mechanisms for entry have been demonstrated for other proteins. The RNA-translocating protein hnRNPK uses both a karyopherin and a nucleoporin-mediated method to move into the nucleus [189]. It is possible that many proteins, including ERK1/2, are imported into the nucleus by several redundant. The existence of multiple import methods could provide additional points for regulation of subcellular localization. The ability of ERK2 to bind to the *D. melanogaster* karyopherin, RANBP7, should be analyzed. Additionally, the behavior of ERK1/2 in cells where RANBP7 is removed by RNAi should be examined. The direct binding of ERK1/2 to a member of the karyopherin-b family would perhaps not be unexpected because the yeast homolog of p38, HOG1, also appears to bind a yeast form of the karyopherin-β family [190].

My experiments in the in vitro import assay suggest a different mechanism for cytoplasmic sequestration of ERK2 by PEA-15 than that proposed by Ginsberg and colleagues. In their model, PEA-15, which has a NES, passively diffuses into the nucleus,

binds active or inactive ERK2, and the resulting bipartite complex is rapidly exported in a CRM1-dependent and leptomycin B-sensitive manner [146]. The import assay used for my experiments contains neither Ran nor CRM1, but PEA-15 is still capable of preventing ERK2 from accumulating in the nucleus. It is possible that both of these mechanisms are responsible for PEA-15 induced cytoplasmic localization of ERK2. A more detailed understanding of the regulation of PEA-15 nuclear import is needed to determine how PEA-15 regulates ERK1/2 subcellular localization. My initial studies in the import assay suggested that PEA-15 nuclear entry is passive and slow. The addition of import factors or cytosol in combination with Ran and an energy regenerating system had little effect on nuclear accumulation of PEA-15. However, these studies employed PEA-15 antibodies that may cross-react with other proteins present in the permeabilized cells. Using dually-GFP tagged form of PEA-15, which is too large for passive diffusion, in the import assay will more clearly determine whether nuclear import of PEA-15 can be mediated by karyopherins. Understanding the PEA-15 import mechanism will provide more insight into how the localization of PEA-15 influences the localization and function of ERK2.

The inhibition of ERK2 nuclear entry and nucleoporin binding by PEA-15 suggests that anchoring proteins can regulate facilitated import of ERK2. Thus, perhaps it is the role of specific, non-catalytic proteins, such as PEA-15, to target active ERK1/2 to cytoplasmic substrates. Recently, PEA-15 has been demonstrated to bind to the cytoplasmic ERK1/2 substrate, RSK2 [191]. This finding suggests that PEA-15 may be capable of binding to both ERK1/2 and its substrates, thereby forming a complex of signaling proteins and increasing the efficiency of enzyme-substrate interactions in the

cytoplasm. Because PEA-15 is small, it may form dimers in order to bind to multiple proteins at once.

PEA-15 is phosphorylated by protein kinase C (PKC), which is activated in response to growth factors and phorbol esters, among other agents. It is not clear if PEA-15 phosphorylation is important for ERK1/2 binding. Mutation of the PKC sites to alanine had no effect on ERK2 binding [146]; however, it is possible that phosphorylation of PEA-15 by PKC may cause a conformational change that inhibits ERK2 binding. In this scenario, only when unphosphorylated can PEA-15 cytoplasmically anchor ERK1/2 in the cytoplasm. PEA-15 may therefore be a signal sensor that sequesters ERK1/2 in the cytoplasm unless PKC is also activated. To determine if changes in interactions and consequently ERK2 localization occur following phosphorylation of PEA-15, a dually phosphorylated form of PEA-15 should be used in in vitro binding and import assays.

An important question in understanding signaling networks is: how do signaling proteins, which are activated by a wide range of stimuli, induce discrete cellular behaviors? ERK1/2 are good model proteins for understanding mechanisms that induce specificity because they are activated by numerous stimuli and participate in a broad range of cellular behaviors. One well-described system for specificity is PC12 cells, in which the duration of ERK1/2 activation is different depending on whether the cells are stimulated with EGF or NGF. EGF stimulates a transient (2-5 minute) increase in activated ERK1/2 and proliferation of PC12 cells. NGF activates ERK1/2 in a prolonged manner (hours) and induces neurite outgrowth [4, 87]. The importance of the different durations was demonstrated in PC12 cells that undergo differentiation when expressing a

mutant EGF receptor that produces prolonged ERK1/2 activation [192]. Previously, nuclear localization was demonstrated only for NGF-stimulated cells [4, 129]., however, my preliminary results suggest that active ERK1/2 can localize to the nucleus in response to EGF, but only at very early (1-2 minute) time points. Previously, the earliest time point examined was 5 minutes [129]. Additional properties, including amplitude and subcellular localization of activated ERK1/2 may also contribute to stimulus-specific behavioral changes.

In mammalian cells, the amount of active ERK1/2 has been measured only on a population basis by western blotting or immunoprecipitation kinase assays. These experiments are not capable of differentiating between a group of cells exhibiting a graded response of ERK1/2 activation or a subset of cells maximally activating ERK1/2, while ERK1/2 are not activated in other cells. The differences can be evaluated only by observing individual cells as described for ERK2 in *Xenopus* oocytes. In this system, treatment of oocytes with a specific amount of progesterone activates all of the ERK2 in a cell. Below this specific concentration of progesterone, no ERK2 is activated [180]. My studies reveale that in HeLa and HFF cells, ERK1/2 are activated in a graded manner, unlike the *Xenopus* system described previously. This result is not surprising because oocytes are programmed to generate only one response [181]. Until that response is triggered, they are at rest. Somatic cells, on the other hand, must sense an array of input signals and generate a number of potential outputs.

Now that we know that different amounts of ERK1/2 can be activated, it will be important to determine if cellular outcomes are dependent on the quantity of ERK1/2 activated. For example, are low levels of active ERK1/2 capable of inducing some

cellular programs (e.g.,migration), but not others (e.g.,proliferation)? This question can be addressed at the molecular level by examining the activation of ERK1/2 substrates by different concentrations of ligand in intact cells. My preliminary study of PMA-induced cyclin D1 activation indicated that only PMA concentrations above 1 nM induce cyclin D1.

Additional ligands should be used in this assay to develop a clear understanding of different localization patterns in response to various ligands. I performed preliminary experiments with PDGF and LPA. ERK1/2 accumulated in the nucleus in response to these ligands, but at a lower level compared to PMA. Interestingly, c-Fos expression in LPA stimulated cells was intermediate compared to PMA and EGF stimulated cells, and was not affected by leptomycin B treatment. PDGF induced a similar nuclear accumulation pattern to LPA. I did not examine the changes in downstream effector responses to PDGF. The study of the duration, amplitude and subcellular localization of ERK1/2 upon activation by these ligands will be required. Collection of these data will help determine the mechanisms that distinct ligands employ to generate specific phenotypes.

As described previously, active ERK1/2 are thought to translocate to the nucleus following stimulation; no alternative localization patterns for the activated kinase had previously been described [7, 86]. Studies in primary cells indicate that nuclear accumulation of ERK1/2 may be uncoupled from activation under certain circumstances. This observation is important for two reasons: First, it suggests that nuclear accumulation is not an intrinsic property of active ERK1/2 but rather a property conferred on ERK1/2 by the signal inducing activation. Second, the activation of ERK1/2 is not

the only requirement for nuclear accumulation. Previous work has established that ERK1/2 can translocate to the nucleus following stimulation but the existence of cytoplasmic substrates indicates that active ERK1/2 are required in the cytoplasm. The correlation of subcellular localization of active ERK1/2 with the differential activation of c-Fos demonstrates that stimulus-dependent compartmentalization of active ERK1/2 has direct consequences for the specificity of effector activation and the subsequent cellular response.

A number of mechanisms may underlie the discrete subcellular localization patterns of active ERK1/2 in response to PMA and EGF. A cytoplasmic anchoring protein, such as PEA-15, may be present in HFFs, preventing nuclear entry. Alternatively, a nuclear anchoring protein could prevent PMA induced, active ERK1/2 from being exported. It is interesting to note that in EGF stimulated cells, some active ERK1/2 was found in the nucleus, but the majority was localized to the cytoplasm. This observation suggests the existence of discrete pools of ERK1/2, which may be differentially activated in response to specific stimuli. This idea has also been suggested from studies on MAP kinase scaffold proteins, which when absent, prevent maximal activation of the cascade [84, 179]. Perhaps, the pool of ERK1/2 activated by EGF is anchored in the cytoplasm by a scaffold protein or is bound to the cytoskeleton as suggested previously [126]. Studies using RNAi to selectively remove known MAP kinase scaffold proteins may indicate if any of these are required for the EGF induce selective localization of ERK1/2 in HFFs.

An additional implication from the work in primary cells is the requirement for ERK1/2 nuclear localization for the initiation of cellular programs. PMA induced c-Fos

and cyclin D1, while EGF did not. This result suggests that ERK1/2 must phosphorylate nuclear substrates to induce proliferative changes in fibroblasts. Previous experiments used artificial means, such as constitutively nuclear forms of the kinase or leptomycin B, to demonstrate a sufficiency of ERK1/2 nuclear localization for specific phenotypic changes. No studies have attempted to determine which cellular programs may be initiated by active ERK1/2 localized in the cytoplasm. By using ERK1/2 mutants that are unable to undergo nuclear entry, it should be possible to determine which phenotypic changes are initiated in the cytoplasm versus the nucleus, thus increasing our understanding of the impact of ERK1/2 localization on function. My studies indicate that ERK1/2 localization may be more complicated than the MEK anchoring hypothesis suggested previously and the further study of ERK1/2 localization is warranted.

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Vitae

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