SUBSTRATE INTERACTION AND SUB-CELLULAR LOCALIZATION IN MAP KINASE PATHWAYS

APPROVED BY SUPERVISORY COMMITTEE

_____
Melanie H. Cobb, Ph.D.

_____
Michael A. White, Ph.D.

_____
John D. Minna, M.D.

_____
Kristen Lynch, Ph.D.

To my Mom and Dad

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By

AARATI RANGANATHAN

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Aarati Ranganathan, Ph.D.

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Supervising Professor: Melanie H. Cobb, Ph.D.

Protein kinase cascades control responses to extracellular cues by transmitting signals throughout the cell. Prominent among multifunctional enzymes in kinase cascades are the mitogen-activated protein kinases (MAPKs). Among the various MAPKs identified, the extracellular signal-regulated kinases, ERK2 and ERK5 are two closely related enzymes that have overlapping functions in a number of cellular pathways. Sub-cellular localization and specificity towards substrates are two mechanisms of controlling the

function of an enzyme in the cell. My dissertation discusses the insights we have gained into both these regulated processes through our studies on ERK2 and ERK5.

Sub-cellular localization of ERK2 is a tightly regulated process. The current model for sub-cellular localization of ERK2 suggests that there is continuous nuclear-cytoplasmic shuttling of the free pool of ERK2. Anchoring of ERK2 in the different compartments of the cell plays a critical role in determining its location. Entry of inactive ERK2 into the nucleus has been reported to occur by an energy- and carrier-independent mechanism. However, export of inactive ERK2 and import of active ERK2 in intact cells seem to occur by an active process. The mechanisms governing these processes have not been investigated. We have used an *in vitro* permeabilized-cell reconstitution assay in HeLa and BJ fibroblast cells to explore the mechanism of GFP-ERK2 export and His_6-tagged thiophosphorylated ERK2 import. Our results identify more levels of regulation within this model.

The ERK5 pathway is triggered in response to various stimuli including growth factors and cellular stresses. Compared to other MAPKs, little is known about ERK5 substrate specificity. Our lab had shown previously that ERK5 is capable of stimulating nuclear factor-κB (NF-κB). Our data suggested that this function might be attributed in part to ribosomal protein S6 kinase (known as RSK or p90RSK), which was activated by coexpression with ERK5 and a constitutively active form of its MAP2K, MEK5DD. Here we demonstrate that RSK, among the first known substrates of the ERK1/2 MAPKs, is also directly phosphorylated and activated by ERK5. We have used RSK to explore the basis of substrate recognition by ERK5.

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LIST OF ABBREVIATIONS

aa	amino acids
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Mono Phosphate
CD	Common Docking
CRM1	Chromosome Region Maintainence 1
CTD	C-terminal Domain
D-domain	Docking Domain
EC	Endothelial Cell
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-regulated Kinase
GAP	GTPase-activating Protein
G-CSF	Granulocyte-Colony Stimulating Factor
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
GST	Glutathione S-transferase
GTP	Guanosine 5'-Triphosphate
HA	Hemagglutinin
HME	Human Mammary Epithelial
JNK	c-Jun N-Terminal Kinase
kap	Karyopherin
KSR	Kinase Suppressor of Ras
LMB	Leptomycin B
MAPK	Mitogen-activated Protein Kinase
MEF	Myocyte-Enhancement Factor
MEK	MAPK-ERK Kinase
MEKK	MEK Kinase
MKP	MAP Kinase Phosphatase
MNK	MAPK-interacting Kinase
MSK	Mitogen and Stress-activated Kinase
NES	Nuclear Export Sequence
NFκB	Nuclear Factor κB
NGF	Nerve Growth Factor
NLS	Nuclear Localization Sequence
NPC	Nuclear Pore Complex
NTD	N-terminal Domain
NTF2	Nuclear Transport Factor-2
PB1p	Phox and Bem 1p
PEA-15	Phosphoprotein Enriched in Astrocytes-15kDa
PKC	Protein Kinase C
Ran-BP	Ran Binding Protein
RCC1	Regulator of Chromosome Condensation

RNAi	RNA interference
RSK	Ribosomal S6 Kinase
SGK	Serum and Glucocorticoid-induced protein kinase
TB	Transport Buffer
Thio-P	Thiophosphorylated
TNF	Tumor Necrosis Factor
TRITC	Tetramethylrhodamine B Isothiocyanate
WGA	Wheat Germ Agglutinin
WNK	With No Lysine (K)

CHAPTER I

Background

MAP Kinases

Mitogen-activated protein kinases (MAPK) are integral components of the signaling processes in a cell that control physiologic responses to external stimuli. They regulate various cellular events including proliferation, differentiation and cell death. When activated, the MAPKs phosphorylate a variety of substrates from transcription factors, cytoskeletal proteins to other protein kinases present in different sub-cellular locations. The MAPK family in mammals includes the extracellular regulated protein kinases 1 and 2 (ERK1 and ERK2), three members of the c-jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) sub-family, four members of the p38 sub-family, ERK5 and some other less characterized members (for instance ERK3, ERK7) (1).

MAPKs form the last step in a three-tier module (MAP3K-MAP2K-MAPK), the prototype of which is the budding yeast pheromone-induced mating pathway (2) (fig.1.1). The MAP3Ks are multi-functional enzymes that contribute towards integrating signals from different extracellular cues. Specificity in signaling within a module is achieved by the MAP2Ks, which activate specific MAPKs. Further, the three kinases in a cascade are often held together in a complex by scaffolding proteins that also provide for specificity in response.

One distinctive feature of MAP2Ks is that they are dual-specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues of the TXY motif, which is unique to members of the MAPK family. Phosphorylation of both residues is required for maximal activation of the MAPKs and this also makes them substrates for all three major

classes of protein phosphatases; serine/threonine phosphatases, tyrosine phosphatases and the dual specificity phosphatases. MAPKs themselves are serine/threonine kinases that most often require a proline residue at the +1 position of the minimal consensus site that is phosphorylated by them (S/TP) (3). Besides this, specificity of the MAPKs towards substrate is determined by the presence of docking domains on substrates that interact at high affinities with substrate-interaction sites on the MAPKs.

The projects detailed in this dissertation involve two of these kinase cascades, the ERK1/2 and ERK5 pathways. These will be discussed in greater detail below.

ERK1/2

Characteristics

ERK1 and ERK2 were originally identified as protein kinases that phosphorylate microtubule-associated protein 2 (MAP 2) (7). Shortly thereafter they were also identified as myelin basic protein (MBP) kinases (4,5). They are activated in response to a variety of stimuli including insulin (6,7) and nerve growth factor (NGF) (8). These kinases are ~85% identical to each other, 43 and 41 kDa in size and ubiquitously expressed in tissues, at concentrations ranging between 0.1-0.5 μM where they are most abundant (9). ERK2 has some over-lapping functions with ERK1 but is also non-redundant for others, as exemplified by knock-out studies in mouse for the two isoforms. Three independent groups reported the generation of ERK2-/- mice simultaneously (10-12). Yao et al and Saba-El-Leil et al demonstrated that the ERK2 null mice die between embryonic days 6.5 to 8.5 with defects in mesoderm differentiation and trophoblast development respectively. ERK1 was detected in these embryos but could not compensate for ERK2 at these stages of development (11,12). Hatano et al observed lethality of ERK2-/- mice only at E11.5

displaying defects in placental development (10). Survival of ERK2-/- embryos for longer time in the study by Hatano and colleagues can be explained by leaky expression of the protein. These studies indicate the essential requirement of ERK2 through various stages of embryonic development. Mice lacking ERK1 are viable but with defects in thymocyte maturation (13).

ERK2 structure – Understanding the basis of activation and function

The structures for both the unphosphorylated and phosphorylated forms of ERK2 have been solved (14,15) (fig 1.2). This reveals that the catalytic core of ERK2 is composed of two domains with the active site at their interface. Primarily, the N-terminal domain consists of β-strands while the C-terminal domain is composed of α-helices.

Both domains possess key residues involved in catalysis. The phosphoryl groups of adenosine triphosphate (ATP) bind to a lysine residue (K52) in the N-terminus and mutation of this residue renders the enzyme inactive. The sites for activation of ERK2 (T183 and Y185) are present on a surface loop, L12, in the C-terminal domain called the activation loop or phosphorylation lip. ERK2 is phosphorylated at both these residues in a non-processive manner (Y185 before T183) (16) and conformational changes brought about by these phosphorylations contribute towards recognition of the consensus site on the substrates.

A defined substrate interaction region is present in αD helix of the C-terminal domain and comprises negatively charged residues (D316 and D319) called the CD domain, near a hydrophobic groove (17,18). The hydrophobic residues in the αD helix (Y314 and Y315) have been shown to play a role in the interaction of ERK2 with its MAP2K, MAP-ERK kinase 1 (MEK1) (19). Similar interactions of substrates and

upstream activators in the same or adjacent regions of a MAPK have been defined for p38 (20). This study identified a hydrophobic groove distinct from, but close to, the CD domain that is essential for docking of p38 onto peptides derived from its substrate and activator. Mutational analysis revealed the greater significance of this hydrophobic groove compared to the acidic residues of the CD domain in the interaction of p38 with its docking site. Similar docking sites, as that for p38, exist for ERK2 on substrates (discussed in detail later) and the hydrophobic groove on p38, identified by Chang and colleagues, are also conserved in ERK2. This suggests a potential for involvement of this hydrophobic groove in interactions of ERK2 with substrates. Interaction of ERK2 with substrates and upstream kinases is likely to involve multiple stabilizing interactions. In support of this, a α-helical insert of ~50 residues called the MAPK insert has also been found on ERK2 that binds to MEK1 (21).

ERK2 dimerizes in the phosphorylated form (15). The residues L333, L336, L341 and L344 in the C-terminal domain contribute to the formation of a leucine-zipper along the interface of the dimer. Mutation of these residues combined with charge reversal of one residue in an ion pair creates a dimerization-deficient form of ERK2.

Upstream components of the ERK1/2 cascade

Immediate upstream activators of ERK1/2 are the MAP2Ks, MEK1/2 (also called MKK1/2) (22-25). Similar to ERK1/2, the MEK1/2 knock-out studies reveal a more significant role for MEK1 in cells than for MEK2 (26,27). MEK1 -/- mice die at embryonic day 10.5 with placental defects similar to those in ERK2 -/- mice whereas MEK2 -/- mice are viable, fertile and grossly normal. MEK1 activity is increased >1000 fold by phosphorylation of two residues, Ser217 and Ser221 (9,28). Mutations of these

sites to acidic residues combined with a small deletion at the N-terminus of the protein yields a constitutively-active enzyme, often called MEK1R4F (29). A number of pharmacological inhibitors have been developed that block the function of MEK1/2 i.e. PD 98059, U0126 and PD184352 (30-32). Since, MEK1/2 specifically activates ERK1/2 among the MAPKs, MEK1R4F and these inhibitors have been used extensively to attribute different biological events in the cell to the MEK1/2-ERK1/2 pathway. The crystal structure of MEK1 and MEK2 bound to the analogs of the inhibitor PD184352 have been solved (33). These structures confirm previous reports that suggested a non-competitive binding of the inhibitors with ATP on MEK1/2 and identify a novel binding pocket for the inhibitor adjacent to the ATP binding site that locks MEK1/2 proteins in an inactive conformation. Because of the non-competitive inhibition displayed by these inhibitors they have been assumed to be very selective for MEK1/2. More recently, these inhibitors were found to inhibit another MAP2K, MEK5 and the implications of this finding will be discussed later. Two regions on MEK1 that play a role in its interaction with ERK2 have been identified by deletion and mutational analysis. These include the docking domain (D-domain) at the N-terminus and a proline-rich insert in the C-terminus of the kinase domain (17,34,35). The D-domain, which is also present in a number of ERK1/2 substrates, will be discussed in detail below.

Further upstream in the cascade, is the MAP3K, Raf, which activates MEK1/2 by dual phosphorylation on the Ser/Thr residues mentioned above (36,37). There are three Raf isoforms, A-Raf, B-Raf and c-Raf (Raf-1). As opposed to the A-Raf and B-Raf isoforms, Raf-1 is distributed in most cell-types and hence, has been most extensively

studied. Raf-1 integrates signals from multiple upstream activators and is activated by interaction with the small GTPase Ras and by phosphorylation (38-41).

Activation of the ERK1/2 pathway

The ERK1/2 pathway is activated in response to a wide variety of stimuli; growth factors that stimulate tyrosine kinase receptors; ligands that activate G-protein coupled receptors (GPCRs) and hormones that work through nuclear receptors (1,9)(Fig. 1.3). Tyrosine kinase receptors and nuclear receptors share quite a few of the upstream components that activate the ERK1/2 pathway. Stimulation of these receptors leads to recruitment of Ras-activating guanine nucleotide exchange factors (GEFs), which activate the small GTPase Ras. Ras interacts with Raf and activates it. Various levels of regulation exist within this pathway. GPCRs are more versatile and activate ERK1/2 pathway through different mechanisms depending on the specific class of GPCR involved (1).

In response to stimulation, ERK1/2 control various cellular processes including proliferation, growth and survival. ERK1/2 have been implicated in the abnormal proliferation and transformation of many cell types (42,43). The involvement of ERK1/2 has also been shown in cell-cycle progression and glucose homeostasis (1).

Scaffolds

Organized complexes of the proteins in the ERK1/2 cascade are formed through interaction of the components with non-catalytic proteins that serve as scaffolds. This kind of organization provides for efficiency and specificity in signaling between closely related MAPK family members and spatial and temporal regulation of stimulus-

dependent responses (44). The prototype scaffold molecule is Ste5 in yeast that binds all three components of the MAPK module of the pheromone-induced mating pathway and is required for their proper functioning in the cell (45). A number of scaffolding molecules have been identified and their contribution to the ERK1/2 pathway are still under investigation. One well-studied scaffold is the kinase suppressor of Ras (KSR), which was identified to be critical for Ras signaling in *C. elegans* and the fruit fly, *Drosophila* (46,47). There are two KSR genes in mammals that bind Raf-1, MEK1/2 and ERK1/2 and mediate signaling in response to certain stimuli (48).

Inactivators of the ERK1/2 pathway

As mentioned before, all three major classes of protein phosphatases inactivate ERK1/2. One sub-class of the dual-specificity phosphatase is the MAP kinase phosphatase (MKP) family that specifically dephosphorylates MAPKs. MKP1 is localized to the nucleus and has been shown to dephosphorylate ERKs, JNKs and p38 family members (49). MKP3 seems to be more specific and only dephosphorylates ERKs in the cytoplasm (50). These MKPs have been implicated in anchoring of ERK2 in the different cellular compartments and will be discussed later.

ERK1/2 substrate interactions

One of the first substrates identified for ERK1/2 is the protein kinase, p90 ribosomal S6 kinase (p90RSK) (51). A detailed section on p90RSK follows later. A number of other cytoplasmic and nuclear targets for ERK1/2 have been identified; some of which are shared between two MAPK pathways. These include the RSK-related kinases; mitogen and stress activated kinases (MSKs), MAPK-interacting kinases (MNK1 and 2) and some ternary complex transcription factors (1,52,53). MSK1 is a

nuclear protein required for the mitogen stimulated phosphorylation of CREB through the ERK1/2 pathway (54) while MNK 1 and 2 are required for the inducible and basal phosphorylation of eucaryotic translation initiation factor - 4E (eIF- 4E) respectively (55). Fig 1.3 summarizes some of the well-characterized substrates of the MAPK pathway.

The substrates make tight contacts with ERK1/2 through their docking domains, which provide specificity between the closely related MAPK members. Three motifs have been defined, D-domain, FXF and LAQRR. The D-domain is closely related to the delta motif in c-jun (the first substrate docking site identified in a MAPK (56)) and consists of a stretch of basic and hydrophobic residues conforming to the consensus $(R/K)_{1-3}X_{2-4}\varphi X\varphi$ (φ – hydrophobic residue). The D-domain interacts with the αD helix of ERK2. The D-domain is present on many ERK1/2 substrates, Elk-1 and c-fos; on the upstream activators MEK1/2; and on the phosphatase, MKP-3. Recently, the function of the D-domain in relation to MEK1-ERK2 signaling within cells has been investigated (57). Grewal et al confirm earlier reports that an intact D-domain in MEK1/2 is required for ERK1/2 phosphorylation and cytoplasmic anchoring. Further, they show that the constitutively-active form of MEK1/2 lacking the D-domain has substantially reduced ability to cause transformation and focus formation of NIH3T3 cells. Subtle variations in D-domain sequence allow recognition by different MAPK family members. In contrast, the FXF motif (58-60) seems to be present only on ERK1/2 substrates. FXF has been found on substrates, Elk-1, Lin-1 and c-fos; the phosphatase, MKP-1; and the scaffold, KSR. Some proteins contain both the D-domain and the FXF motif providing cooperative interactions with ERK1/2. Recently, some key residues in a hydrophobic pocket on

ERK2 have been identified that are required for binding to the FXF motif containing Elk-1 peptide (61). The targeting motif LAQRR (62,63) is related to the D-domain and is found on ERK1/2 substrates RSK, MSK and MNK. Variations of this sequence, found on some MSK and MNK family members, are recognized by other MAPKs (9).

A schematic overview of the MEK1/2-ERK1/2 signaling in the cell is represented in figure 1.3.

Sub-cellular Localization of ERK2

Significance

ERK2 performs pleiotropic functions within the cell. Because ERK2 substrates are distributed throughout the cell (64-67), regulation of the sub-cellular localization of ERK2 in response to different stimuli is required for generating appropriate physiological outcomes. In the appropriate contexts, ERK2 activation and subsequent nuclear localization induces differentiation of PC12 cells (43,68-70), transformation of NIH3T3 cells (43,69) and cell-cycle progression and gene expression (71) while from its cytoplasmic localization it plays a role in regulating cytoskeletal organization (72) and cell motility (73).

Role of ERK2 Activation

Originally, Chen et al observed that inactive ERK2 is present in the cytoplasm of resting cells (64) and upon stimulation ERK2 moves to the nucleus (64,74,75). Activation of ERK2 seems to be sufficient for nuclear accumulation (76-78) and in support of this, Lenormand and colleagues have seen reduced nuclear accumulation of ERK2 when cells are treated with the MEK1 inhibitor PD98059 prior to stimulation with serum (79). However, activation of ERK2 does not seem to be required for nuclear entry as inactive ERK2 has been observed in the nucleus under endogenous conditions (71,77) and when

over-expressed (80,81). Also, a mutant form of ERK2 (ERK2 T183AY185F) that cannot be activated enters the nucleus when microinjected into rat fibroblast cells (76) or when over-expressed in NIH3T3 cells (82). Similarly, different studies have shown presence of the kinase-dead form of ERK2 (ERK2 K52R) in the nucleus (76,83,84). Finally, nuclear localization is not an obligatory consequence of ERK2 activation and presence of ERK2 in the cytoplasm following stimulation has been reported (70,83,85,86). Some of the above studies were performed before a specific phospho-ERK2 antibody (87) was available and hence, the fraction of ERK2 in the active and inactive states, under the different conditions in different sub-cellular locations, cannot be estimated in these studies. Nevertheless, the various studies demonstrate that, in order to elicit proper responses, the process of ERK2 sub-cellular localization must be under tight regulation.

ERK2 anchoring hypothesis

One mechanism of regulating movement of ERK2 within the cell is by specific anchoring of the protein in different compartments, in the active and/or inactive states. Evidence to support this hypothesis has accumulated over the past few years. One obvious candidate is the upstream activator, MEK1. MEK1 is a cytoplasmic protein (88) as a consequence of its N-terminal nuclear export sequence (NES). When ERK2 is co-expressed with wild-type MEK1 or an N-terminal peptide (containing the NES and the D-domain of MEK1), it is localized to the cytoplasm (80,81). Disruption of the interaction between MEK1 and ERK2 either by co-expression with the D-domain mutant of MEK1 (19) or by stimulation (80,81) allows nuclear localization. This suggests that MEK1 serves as an anchor for ERK2 and retains it in the cytoplasm in the inactive state. Most of these conclusions have been drawn from the behavior of over-expressed proteins,

which may not reflect the physiological phenomena. Recently, Burack et al have used FRET (fluorescence resonance energy transfer) to confirm that MEK1 interaction with ERK2 does reduce the free pool of ERK2 available to enter the nucleus (89). They also show that ERK2 is sequestered in the cytoplasm in a complex with MEK1 and this complex is not bound to any specific region or component in the cell but moves freely within the cytoplasm. The region on MEK1 that has been identified as the cytoplasmic retention sequence, for ERK2, is the D-domain, a common ERK2 interacting motif in a number of proteins. Hence, it is conceivable that other D-domain containing proteins also serve as anchors for ERK2 in the different compartments of the cell. One probable candidate is MKP-3; when over-expressed MKP-3 retains ERK2 in the cytoplasm (71). Recently, the anti-apoptotic protein, phosphoprotein enriched in astrocytes-15 (PEA-15) has been shown to prevent nuclear entry of inactive ERK2 in *in vitro* reconstitution assays (90). Besides these anchors for inactive ERK2, one study has shown binding of ERK2 to the microtubule cytoskeleton even after stimulation, proposing a mechanism for cytoplasmic targeting of active ERK2 (85). Few nuclear anchors for ERK2 have been proposed. Two studies from the Ahn lab reported interaction of ERK2 with nuclear components, topoisomerase IIα and kinetochores (91,92). Lenormand and colleagues had implicated newly synthesized proteins in nuclear anchoring of ERK2, after stimulation with growth factors (79). More recently, a number of immediate-early gene-encoded transcription factors expressed as a result of prolonged ERK2 activation have been shown to bind active ERK2 and retain it in the nucleus (93,94). Finally, two candidates for anchoring inactive ERK2 in the nucleus have been proposed i.e. vanishin, a novel death-effector domain protein (95) and the nuclear phosphatases, MKP1/2 (77).

The picture that emerges is that of a dynamic protein that is continuously shuttling between discrete pools in the nuclear and cytoplasmic compartments of the cell. What is known about the mechanism of transport of ERK2 across the nuclear envelope will be discussed after an introduction to the transport apparatus.

Nuclear Pore Complex

Structure

Transport of molecules between two of the major compartments of a eucaryotic cell, the nucleus and the cytoplasm, is achieved through large supra-molecular transport channels that span the double membrane nuclear envelope. This transport channel, called the nuclear pore complex (NPC), is about 60-125 Mda in size in vertebrates and is comprised of 500-1000 protein molecules (96). Recently, major strides have been made in understanding the fine details of the NPC structure through use of cryo-electron tomography (97). The NPC has an eight-fold symmetry and consists of a cylindrical central channel (~ 10 nm in diameter), connected to the rim through spokes. The channel is sandwiched between 2 rings; a cytoplasmic ring from which eight fibrils extend into the cytoplasm and a nuclear ring from which eight filaments extend that are attached at the end with another ring forming a basket-like structure called the nuclear basket (98). The NPC is a highly dynamic structure (97,99) made of at least 30 different proteins called nucleoporins, some of which are constantly moving in and out. Nucleoporins are large proteins, ~ 100 kDa in size, natively unfolded with minimal secondary structure and highly flexible. Transport through the NPC is blocked by use of wheat germ agglutinin (WGA), a lectin that binds the O-linked N-acetyl glucosamine sugar modifications of the nucleoporins (100). Hence, this reagent has been used extensively to study the

mechanism of transport of macromolecules across the NPC (101,102). About a third of the nucleoporins have a hydrophobic stretch of repeats (FG, FXFG or GLFG) linked by hydrophilic, charged residues (103). The nucleoporins containing these repeats are present on the cytoplasmic fibrils, along the central channel and in the nuclear filaments, contributing a total of >10,000 FG repeats per NPC.

Transport through the NPC

The classical transport machinery in a cell is represented in figure1.4. Ions, small molecules and proteins <20 kDa in size passively diffuse through the aqueous central channel in the NPC (104). Proteins larger than 20 kDa in size, macromolecular complexes of ribosomes and RNA are transported by facilitated diffusion, in most cases with the help of transport factors (karyopherins) that directly interact with the FG repeats on the nucleoporins (105-108). The directionality of movement through the NPC is governed in most cases by a gradient of the small GTPase, Ran across the nuclear envelope (109,110). Ran is mostly in the GDP-bound form in the cytoplasm and in the GTP-bound state in the nucleus. Depending on the direction of movement, binding of cargo to karyopherins is stabilized by the specific nucleotide-bound form of Ran. Karyopherins mediating import (importins) bind tightly to their cargo in the presence of Ran-GDP (106,111) and dock onto the cytoplasmic face of the NPC near its periphery (112). The complex then enters the nucleus through the NPC. GTP is exchanged for GDP on Ran in the nucleoplasm and affinity of the importin for its cargo decreases; the cargo is released. Import involving karyopherins and Ran requires energy in the form of GTP only for the terminal step of the process i.e. release of cargo (113). In the nucleus, the karyopherins mediating export (exportins) bind their cargo only in the presence of Ran-

GTP (114) and move out through the NPC. On the cytoplasmic side, Ran-GTP is hydrolyzed and cargo is released by the exportin. Again, GTP hydrolysis is not required for the export process itself but for the release of cargo into the cytoplasm (115,116). Much work has been accomplished in this field by using permeabilized cells as model systems for transport across the nuclear envelope (117).

Ran

Ran was originally identified as a member of the small GTPase Ras superfamily in a screen using a cDNA-library from a teratocarcinoma cell line (118). It is an essential gene that is conserved in all eucaryotes. The protein product is 25 kDa in size and its function in the cell has been best studied in nuclear-cytoplasmic transport (see above). Besides this, Ran plays important roles in other cellular processes including cell-cycle progression and spindle assembly, post-mitotic nuclear envelope assembly and growth and DNA replication (119). Most functions of Ran seem to require a concentration gradient of the protein in the cell. This gradient is maintained by the function of two proteins. The first of these is a Ran guanine nucleotide exchange factor (GEF), regulator of chromosome condensation (RCC1). RCC1 is present in the nucleus bound to chromatin (120) and catalyzes the exchange of GDP for GTP (121), maintaining Ran in the GTP-bound state in the nucleus. The second important protein is a Ran-GTPase activating protein, RanGAP1, present free in the cytoplasm as a 70 kDa protein or primarily bound to cytoplasmic filaments of the NPC in a modified form (122,123). RanGAP1 induces hydrolysis of Ran-GTP (124), maintaining Ran in the GDP-bound form in the cytoplasm. Two other proteins that stimulate GTP hydrolysis include Ran binding protein 1 (Ran BP1), a cytosolic protein (125) and the nucleoporin, Ran BP2.

Ran BP2 is present on cytoplasmic fibrils and binds Ran-GTP and RanGAP1 (123). To accomplish its function in nucleo-cytoplasmic transport, Ran is continuously shuttled between the two compartments by two karyopherins; nuclear transport factor-2 (p10/NTF2), an importin (126,127) and chromosome region maintainence-1 (CRM1), an exportin (128,129).

Karyopherins – Structure and Function

Importins

A number of studies simultaneously identified the role of members from two families of karyopherins in nuclear import, karyopherinα (kapα, importinα) and karyopherinβ (kapβ, importin β) (107,130-133). These karyopherins recognize and bind a wide variety of cargo molecules through different sequences called nuclear localization sequences or NLS (134). Originally, classic NLS were identified in the SV-40 Large T-Antigen (consensus $(K/R)_{4-6}$) (135,136) and in nucleoplasmin (a bi-partite sequence with consensus $(K/R)_2X_{10-12}(K/R)_3$) (137). Later, a number of non-classical NLS i.e M9 of heterogenous nuclear ribonucleoprotein A1, hnRNPA1 (138), KNS of hnRNPK (139), HNS of HuR, an RNA-binding protein (140), all of which function as bi-directional signals for import and export; ankyrin repeats of IκBα (141) and others were identified. Not all proteins that have a recognizable NLS are localized to the nucleus (142) and not all classic NLS sequences are functional (143). NLS activity may be regulated by phosphorylation of residues near the NLS (143) or by signal masking (134). A number of proteins that are known to shuttle between the nucleus and cytoplasm have no recognizable NLS (144). Hence, much work is needed in the field to identify novel NLS. The Kapβ family members bind directly to cargo along with Ran and the nucleoporins mediating import. To date, about 20 evolutionarily conserved Kapβ proteins have been

identified in humans. These kapβ proteins are 95-145 kDa in size, with ~20% sequence identity (145). Given the apparent dissimilarity in the cargo recognition sequence (NLS), the discovery of such a small number of kapβ family members to transport a vast array of macromolecules is surprising. Structural studies have revealed multiple binding sites on kapβ1 for different cargo indicating one means of circumventing this issue (146-148). Another means is by interaction of kapβ with kapα which acts as an adaptor for >100 NLS containing proteins (149). Structural data accumulated over the past 5 years have provided insight into the functioning of these proteins. The crystal structure of kapα bound to the NLS containing SV-40 Large T-Antigen peptide (150) revealed a super-helix consisting of ten armadillo, ARM repeats (first identified in Drosophila segment polarity gene armadillo) on kapα that serve as the binding groove for the NLS peptide. Other studies have identified the structural basis for binding of kapβ family members to NLS-containing cargo and Ran (146,151). These studies show that Kapβ consists of a number of HEAT (named for the proteins in which this helix-loop-helix motif was originally identified – Huntington, elongation factor EF-3, A subunit of protein phosphatase, PP2A and TOR1, target of rapamycin) repeats that serve as binding regions for Ran at the N-terminus and cargo at the C-terminus. Cingolani and colleagues solved the structure of kapβ1 bound to kapα and identified the N-terminal Arg-rich domain of kapα as the Importin-beta binding domain, IBB (152). Further, Kobe et al showed that the N-terminal positive residues of kapα also bound its ARM repeats intra-molecularly and identified it as an auto-inhibitory domain (153). Finally, Bayliss et al identified a region on kapβ1 that binds FXFG repeats on nucleoporins (154). Functions of kapα and kapβ in cellular processes other than nucleo-cytoplasmic transport span the same repertoire as

Ran, indicating their cooperative activity in these pathways i.e. microtubule assembly and mitosis (155,156), nuclear envelope biogenesis and NPC assembly (157,158).

Exportins

All exportins identified, to date, belong to the kapβ family. One extensively studied exportin is CRM1 that was originally characterized for its function in chromosome maintainence in *S. pombe* (159). Later, it was identified as a nuclear transporter in yeast (160) and found to play a role in NES-mediated export of cargo in mammalian cells (80,128,129,161,162). To date, the NES identified for CRM1 have conformed to a consensus Leu-rich sequence $LX_{1-3}LX_{2-3}LXL$, such as have been found in the protein kinase inhibitor, PKI (163), human immunodeficiency virus Rev protein (164) and the NF-κB inhibitor, IκBα (165). Proteins that do not posssess an NES can be exported by binding to other NES containing proteins. An example of this piggy-back export is that of the C-subunit of protein kinase A, PKA in complex with PKI (166). Most of the studies implicating CRM1 in nuclear export have made use of the anti-fungal antibiotic, Leptomycin B (162,167). Besides arresting cells in the G1 and G2 phases of the cell cycle, LMB also specifically inhibits CRM1 function (168) by binding covalently to Cys-529 (169,170). CRM1 is most similar to kapβ at its N-terminus. Recent structural data obtained on CRM1 identified HEAT repeats, Ran-binding loop and NES-cargo interaction site; comparison of the Ran binding loop of CRM1 with different importin β family members predicts a mechanistic basis for the different affinities of kaps towards substrate in the presence of Ran-GTP (171). Considering that the importin family is so large, it is surprising that export of a diverse array of proteins is mediated by a single exportin, CRM1. Hence, a number of studies in the recent past have focused on identifying novel exportins which include CAS (Cse-1) for kapα (172,173), exportin t for

t-RNA (174), exportin 5 for micro-RNA (175-177), exportin 4 for eucaryotic translation initiation factor 5A (eIF-5A) (178), exportin 6 for profilin-actin complexes (179), exportin 7 (also called Ran BP16) for p50 Rho-GAP and 14-3-3σ (180) and calreticulin for the glucocorticoid hormone receptor (181,182). The NES identified by some of these exportins have been analyzed and they show variations from the CRM1 consensus. Much work still remains to be accomplished.

Mechanism of ERK2 transport through the NPC

ERK2 has no recognizable NLS or NES. Yet, as mentioned previously, the studies demonstrating regulated changes in sub-cellular localization of ERK2 indicate shuttling of the protein between the nuclear and cytoplasmic compartments of the cell. Khokhlatchev and colleagues have shown that unphosphorylated ERK2 injected into rat fibroblast cells enters the nucleus in 2 min and redistributes to the cytoplasm within 5-10 min (76). One study by the Nishida group reported that monomeric ERK2 enters the nucleus by passive diffusion (82). They showed nuclear accumulation of ERK2 even in the presence of WGA. Later, concurrent with Whitehurst et al, they reported that inactive ERK2 is imported through the NPC by an energy- and carrier-independent, facilitated mechanism (183,184). These studies used an *in vitro* permeabilized cell reconstitution assay system and GFP (green fluorescent protein)-tagged ERK2 to visualize nuclear accumulation. The discrepancy in earlier results (82) could have been because of the use of low concentrations of WGA that may not have been sufficient to block import. Further, Whitehurst et al and Matsubayashi et al showed that ERK2 requires access to nucleoporins to be imported and that ERK2 directly binds the FXFG repeats on different nucleoporins. Work from other groups on proteins, which lack the classical NLS, support

the existence of such a mechanism for import where direct access to the NPC mediates the process. This has been shown for kapβ (185), HIV-1 Vpr (186), β-catenin (187,188) and Smad 3 and 4 (189). Some studies suggest that the mechanism of import of phospho-ERK2 may be different from that of unphosphorylated ERK2. Khokhlatchev et al had shown that dimerization of active ERK2 enhances its import and a dimerization-deficient mutant of ERK2 did not accumulate in the nucleus, as well as the wild-type protein, under stimulatory conditions (76). Adachi and colleagues demonstrated that import of ERK2 dimer could occur by an active transport process (82). This suggests that dimerization and interaction with the classical import machinery may drive import of active ERK2. The mechanism of import of active ERK2 has not yet been investigated. One mechanism proposed for the export of ERK2 from the nucleus is by association with MEK1, which has an NES (190). Although MEK1 is a cytoplasmic protein, disrupting transport across the nuclear membrane has suggested that MEK1 shuttles between the nucleus and cytoplasm. MEK1 accumulates in the nucleus in experiments disrupting its NES (191) or in the presence of LMB (82). The disruption of MEK1 NES has also been shown to inhibit export of ERK2 that has been injected into the nucleus (190). This suggests that ERK2 and MEK1 are transported out of the nucleus as a complex. Inhibition of export of MEK1 and ERK2 by LMB implicates an active process mediated by CRM1. Yet recent reports based on live-cell imaging have indicated that very little MEK1 is present in the nucleus of unstimulated cells and this is not altered significantly upon stimulation (89). Whether MEK1 plays a significant role in ERK2 export from the nucleus is yet to be investigated. Also, Matsubayashi and colleagues have observed that ERK2 can be exported by an energy- and carrier-independent mechanism (184).

The first part of my dissertation deals with understanding some of the mechanisms governing transport of inactive and active ERK2 across the nuclear envelope.

ERK5

Identification

The MAPK ERK5 was discovered simultaneously by two separate groups, one used a PCR-based strategy (192) while the other pulled out ERK5 in a yeast two-hybrid screen using the upstream activator MEK5 as bait (193). ERK5 is also, aptly, called Big MAP Kinase 1 (BMK1), as it is the largest known MAPK measuring 815 amino acids (aa) in length and ~100 kD in size. ERK5 mRNA is expressed almost ubiquitously in tissues (except in liver) and is most abundant in the heart, skeletal muscle, lung and kidney. The core catalytic domain of ERK5, at the N-terminus, is about 50% identical to ERK2. Although like ERK1/2, ERK5 has the TEY dual phosphorylation motif at its activation site, a unique loop-12 structure and an unusually large C-terminus make it quite different from ERK1/2 (192). The primary sequence of the C-terminal 400 amino acids does not lend itself to quick analysis by comparison studies and determining the function of this region has been an uphill task. Some early experiments attribute the following roles to the C-terminus. It has been hypothesized that the C-terminal tail has an auto-inhibitory function as the activity of the catalytic domain but not the full-length protein towards its substrates can be enhanced several fold by V^{12} H-Ras (194). It has an NLS (aa 505-539) that is proposed to be required for the nuclear translocation of ERK5 (195). Also, the C-terminal tail seems to be required for the activation of its downstream targets myocyte-enhancement factors, MEF2C (195) and MEF2D (196). The C-terminus

is extensively auto-phosphorylated when ERK5 is in the active state (194) but a function for the autophosphorylation within cells remains to be determined. Finally, it has a proline-rich region (193) that may target it to the actin cytoskeleton or serve as a site for interaction with proteins containing SH3-binding motifs (197). This putative role has not been demonstrated.

Activation by MEK5

As for other MAPKs, upstream kinases display stringent specificity in activation of ERK5. The only known MEK that can interact with (193) and activate ERK5 is MEK5 (198). The activation has been shown *in vitro*, through phosphorylation of the catalytic domain of ERK5 (1) and of full-length ERK5 in cells (198) by using a constitutively active form of MEK5, MEK5DD. Mody and colleagues have performed a detailed analysis of the phosphorylation and activation of ERK5 by MEK5 *in vitro* (199). MEK5 preferentially phosphorylates T219 of ERK5 over Y221 in the TEY activation motif. The T219 mono-phosphorylated form of ERK5 retains 10% of the activity of the dual-phosphorylated form towards exogenous substrates and is equally active in auto-phosphorylation. In turn, ERK5 was found to phosphorylate MEK5 extensively *in vitro*, on an N-terminal region that resembles the D-domain in MEK1. The relevance of this phosphorylation in cells is yet to be determined. Yan and colleagues showed that the N-terminal residues 78-139 on ERK5 are required for its interaction with MEK5 (195).

Regulators of ERK5 pathway

ERK5 is activated by a diverse array of stimuli including growth factors and cellular stresses. The growth factors and proliferative stimuli include serum (198), epidermal growth factor (EGF) (200), nerve growth factor (NGF), lysophosphatidic acid

(LPA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (201), granulocyte colony-stimulating factor (G-CSF) (202), neurotrophic factors, NT3, NT4 and Brain-derived neurotrophic factor (BDNF) (203), insulin-like growth factor-1 (IGF-1) (204), neuregulins (NRGs) (205), phorbol 13-myristate 12-acetate (PMA) (206), interleukin-6 (IL-6) (207) and the hormone, gastrin (208). The stress agents include H_2O_2 and sorbitol (209), glucose (210), fluid shear stress (211) and we have seen activation of ERK5 with NaCl in human mammary epithelial (HME) cells. The activation of ERK5 by these stimuli may be Ras- dependent (201) or independent (200,205,207), Src- dependent (212) or independent (207) or mediated through protein-protein interactions between MEK5 and atypical protein kinase-C (PKC) isoforms (213). English et al have shown that the activation of ERK5 by Ras is independent of Raf1 catalytic activity but depends on the direct interaction between Raf1 and ERK5 (214). This mechanism of activation is distinct from the Ras-Raf activation of the ERK1/2 pathway. In addition to these mechanisms, GPCRs have been shown to activate ERK5 (215) via $G\alpha_q$ and $G\alpha_{12/13}$ families of heterotrimeric G proteins in a Ras- and Rho- independent manner (216). Pearson and colleagues reported the effects of increasing cAMP (cyclic adenosine 5'-monophosphate) concentrations on ERK5 in different cell types (217). They found that cAMP could increase or decrease the activity of ERK5 depending on cell type and cell context. Further, some protein phosphatases that act on other MAPKs have been found to inhibit ERK5 as well. These include the dual-specificity phosphatases MKP-1 and MKP-3 (201) and the tyrosine specific phosphatase PTP-SL (218). Two different studies have indicated negative feedback regulation of the ERK5 pathway by the ERK1/2 pathway (219,220). Recently, Zheng and colleagues showed an inhibitory interaction of ERK5

with 14-3-3β (221). Binding of the proteins was dependent on phosphorylation state of ERK5 and this interaction decreased the kinase activity of ERK5 stimulated by EGF or the constitutively active MEK5 form, MEK5DD.

Activators of the pathway immediately upstream of MEK5 include the MAP3Ks, MEKK3, MEKK2 and the Cot proto-oncogene product Tpl-2. MEKK3 has been shown to interact with MEK5 in yeast two-hybrid assays and in mammalian cells. It phosphorylates MEK5 *in vitro* and is required for EGF induced activation of ERK5 in HeLa cells (222). MEKK2 also interacts with MEK5 in yeast two-hybrid experiments and activates ERK5 to a greater extent than MEKK3, when transfected into HEK293 cells (223). MEKK2 and 3 are thought to mediate activation of ERK5 in response to different stimuli. Kinase inactive MEKK3 blocks signaling from EGF and H_2O_2 to ERK5 in Cos-7 and HEK 293 cells while kinase inactive MEKK2 blocks basal activity and signaling from T-cell receptor in D10T cells (223). Further, the MEK5-ERK5 pathway is activated by MEKK2, but not MEKK3, in mast cells in response to cross-linking of FcεRI receptors and is required for the consequent tumor necrosis factor-α (TNF-α) promoter activation (224). Recently, Xu et al have demonstrated that WNK1 (with no lysine (K)), a member of a unique family of protein kinases, lies upstream of MEKKs in the ERK5 cascade (225). WNK1 is required for the EGF-induced activation of ERK5 in an MEKK2/3 dependent manner. Finally, Tpl-2 activates the MEK5-ERK5 pathway when over expressed and this activation is required for Cot-induced cellular transformation (226). No direct phosphorylation or interaction between Tpl-2 and MEK5 has been shown.

Pharmacological Inhibitors of the MEK5-ERK5 pathway

The inhibitors of MEK1/2, PD98059 and U0126, also inhibit MEK5 with similar potencies (IC50 within 2-3X)(30,201,219) while PD184352 seems to be required at higher concentrations to inhibit MEK5 than MEK1/2 (IC50 >5X) (219). Even though this difference in IC50 of PD184352 is quite small, two independent groups have exploited this difference in delineating specific effects of the two pathways (220,227). They used low concentrations of the inhibitor (0.5μM - 1 μM) to show effects of ERK1/2, but not ERK5, on inhibition of cell proliferation in chinese hamster fibroblast cell line, CCl39 and on the chemotactic response in neutrophils. The effect of the inhibitor, PD184352 in these studies varies considerably with respect to its effect on MEK5 (in the study on neutrophils some inhibition was seen even at 2 μM); hence, whether the specific effects of the inhibitor on ERK1/2 vs. ERK5 pathway can be exploited for studies on different cell lines is yet to be investigated.

Involvement of ERK5 in diverse cellular processes

To date, the biological actions of ERK5 have been best understood in cell proliferative and developmental pathways. Because ERK5 is the only known substrate of MEK5, all cellular processes, in which MEK5 has been implicated, have been attributed to regulation by ERK5. Kato et al, first, described the requirement of ERK5 in EGF induced proliferation and cell-cycle progression (198). They used dominant-negative ERK5 to block proliferation of HeLa cells and prevent their entry into S-phase of the cell cycle. Later, Dong et al showed that G-CSF regulates proliferation and survival of BAF3 cells through activation of MEK5 and ERK5 (202). ERK5 knock-out studies in mice indicate an essential role for the ERK5 pathway in angiogenesis and vascular cell growth

(228). ERK5 acts as a positive and negative modulator of angiogenic signals. One mechanism of action is via inhibition of hypoxia inducible factor1α (HIF1α) activity, in response to hypoxia, which leads to a decrease in vascular endothelial growth factor (VEGF) expression and subsequent inhibition of endothelial cell (EC) migration (229). Also, proliferation of vascular smooth muscle cells requires ERK5 function (230). English and colleagues have shown a role for the MEK5-ERK5 pathway in cell growth control (214). Activation of this pathway is required for Raf-dependent cellular transformation and focus formation in NIH3T3 cells. NF-κB was identified as a possible downstream effector of this pathway in focus formation because MEK5-ERK5 activation was found to be sufficient for stimulation of NF-κB by Ras (231). ERK5 activation is also required for Src-mediated transformation and plays a role in the loss of actin stress fibres and disruption of the actin cytoskeleton in response to Src (232). ERK5 has been implicated in EGF-dependent branching morphogenesis of cells derived from renal epithelium (233). Neurotrophins have been shown to mediate retrograde signaling and survival response from distal axons to cell bodies via the MEK5-ERK5 pathway (234). A critical role for MEKK2-MEK5-ERK5 pathway in mast cell signaling in response to cross-linking of FcεRI receptors has been established and the resulting activation of ERK5 leads to TNF-α gene expression (224). Further, skeletal muscle differentiation requires activation of ERK5 that leads to upregulation of differentiation specific genes (235).

In addition to the role of ERK5 in normal cellular processes, some investigations have unraveled an involvement of ERK5 in pathological conditions. ERK5 is expressed in B-cells isolated from patients suffering from multiple myeloma (207) and is

constitutively activated in breast cancer cells over-expressing Erb B2 receptor (205). ERK5 activation is required for the proliferation of myeloma and breast cancer cell lines in response to IL-6 and neuregulins respectively. Cyclin D1 protein regulates the cell cycle and its deregulation is implicated in a wide variety of cancers. Mulloy et al have identified cyclin D1 as a target of the MEK5- ERK5 pathway and have shown that kinase activity of ERK5 is required for the induction of cyclin D1 in response to serum in some breast cancer cell lines (236). Recently, it has been reported that the oncogenic potential of Abl kinases expressed in leukemia cells is enhanced by their ability to increase the stability of ERK5 protein (237). MEK5 over-expression is seen in prostate cancer and is associated with poor survival (238). Inhibition of MEK5 signaling, by enhanced degradation of the protein, suppresses prostate cancer cell growth (239). MEK5 over expression has also been correlated with resistance to chemotherapy in breast cancer cell lines (240). Cardiac specific activation of MEK5 in transgenic mice has been shown to result in an eccentric type of cardiac hypertrophy that leads to dilated cardiomyopathy and sudden death. Also, constitutive activation of the MEK5-ERK5 pathway in cardiomyocytes induces hypertrophy in which the sarcomeres are assembled in a serial manner (241). Caroli's disease is characterized by congenital dilation of the intra-hepatic bile ducts and is associated with congenital hepatic fibrosis (CHF). MEK5 over-expression and activation of ERK5 has been reported to play a role in EGF-stimulated proliferation of cultured intra-hepatic biliary epithelial cells (BECs), a cell-line derived from rats serving as animal models for Caroli's disease (242).

ERK5 Substrates

ERK5 phosphorylates and activates three of the four members of the MEF2 family of transcription factors, MEF2A, MEF2C and MEF2D (198,243). MEF2C is phosphorylated by ERK5 at S^{387} and this phosphorylation is required for the serum and EGF-induced activation of MEF2C *in vivo*. The activation of MEF2C by ERK5 results in the increased transcription of the c-jun promoter. MEF2A and MEF2D isoforms are activated by ERK5 in response to EGF and their sites of phosphorylation *in vitro* and *in vivo* have been mapped. The direct interaction between different MEF2 isoforms and ERK5 has been shown *in vitro* and *in vivo* (244). Other ERK5 substrates identified include the ETS-domain transcription factor Sap 1a (201), serum and glucocorticoid-induced kinase, SGK1 (245) and connexin 43 (246). Phosphorylation of all above substrates by ERK5 has been shown *in vitro* and *in vivo*; although the functions of these phosphorylations have not been defined in every case. Direct interaction of ERK5 with SGK1 and connexin 43 has also been shown and the sites of phosphorylation for these proteins have been mapped. Another downstream target of ERK5, which may not be directly phosphorylated by ERK5, is the pro-apoptotic protein Bad (247).

The details of MEK5-ERK5 signaling cascade in the cell have been summarized in figure1.5.

MEK5

Two independent groups identified MEK5 in 1995 (193,248). ERK5 is the only known substrate for MEK5. Multiple splice variants of MEK5 exist including the α and β forms. The α form is larger (by ~89 aa), 50 kD in size and predominantly expressed in the liver and brain in a particulate fashion. The β isoform is about 40 kD in size, cytosolic

and ubiquitously distributed in tissues (248). MEK5α has been shown to directly stimulate ERK5 activity *in vitro* (199) while comparable data for MEK5β is lacking. Both MEK5α and β isoforms have been shown to co-immunoprecipitate with ERK5. This association is mutually exclusive and MEK5β has been shown to inhibit the EGF-mediated activation of ERK5 by MEK5α (249). This suggests that alternative splicing of MEK5 may serve as a mechanism for regulation of ERK5 activation. Some upstream activators of MEK5 have been identified (see above). MEK5 has a phox and Bem1p (PB1) domain that forms heterodimers with other PB1 containing proteins (250). Importantly, the MEK5 PB1 domain is required for its interaction with MEKK2 and 3 and subsequent activation (251). Besides this, the N-terminus of MEK5 has an atypical PKC-interaction domain (AID) that allows its activation by PKCζ (213).

MEKK2/3

MEKK2 and 3 were isolated using a cDNA cloning strategy by Blank et al in 1996. Both proteins are around 70 kD in size with 94% similarity in their C-terminal kinase domains. The N-terminal domains are regulatory in function and more diverse (252). MEKK2/3 respond to signals from growth factors, oxidative stress and hyper-osmolar conditions (222,253-255). Among the MAPKs, MEKKs 2 and 3 have been to shown to activate ERK1/2, JNK, p38 and ERK5 when over expressed (222,252,256,257). Preferences as to which MAPKs are activated by MEKK2 or MEKK3 exist; MEKK2 seems to prefer MEKs 4 and 5 causing activation of the JNK and ERK5 pathways while MEKK3 is more promiscuous and has been shown to target MEKs 1, 3, 6, 5 and 7 activating all four pathways to some extent. Other targets of MEKK2/3 signaling that have been identified include the inhibitor of IκB kinase, IKK (258,259) and PKC-related

kinase 2 (PRK 2) (260). A critical role for MEKK3 in NF-κB signaling has been defined using MEKK3-/- fibroblasts (259). Also, contrary to normal proliferative response of cells to MAPK cascade signaling, MEKK3 has been shown to cause cell-cycle arrest by blocking cyclin D1 expression in fibroblasts (261). MEKK2 seems to play an important role in cytokine production in mast cells via JNK activation (262) and causes delayed activation of NF-κB (263). Few regulators for MEKK2/3 have been identified. Osmosensing scaffold for MEKK3 (OSM) was identified as a scaffold for MEKK3 signaling from the GTPase Rac to p38 in response to sorbitol (255). MEKK2 interacts with the Lck-associated adapter/Rlk and Itk-binding protein, Lad/RIBP (223); MEKK3 interacts with PA28γ (264) and BRCA 1 (265); and both bind 14-3-3ζ and ε (266). The exact functions of these interactions are yet to be delineated. Two kinases have been shown to directly phosphorylate MEKK2/3. WNK1 phosphorylation of MEKK2 and 3 does not seem to alter their activity (225) and the relevance of this phosphorylation in cells is not yet known. SGK1 phosphorylates MEKK3 in cells and *in vitro* and this reduces the activity of MEKK3 towards one of its peptide substrates, MEK3 (267).

Sub-cellular localization of components of the ERK5 pathway

Localization of over-expressed and endogenous ERK5 has been analyzed in various cell types (195,198,207,218,237,268). Although seemingly contradictory, these studies primarily indicate a cell-type dependent distribution of ERK5 in the nucleus and cytoplasm. A more diffuse pattern of localization within resting cells changes into predominantly nuclear staining upon stimulation in most cell types. This is in line with identification of ERK5 substrates in both of these compartments of the cell. The C-terminal NLS containing region seems to be important for the activation-dependent

nuclear localization of ERK5 (269). Raviv et al report that MEK5 is present in the nucleus of HeLa and Rat-1 cells independent of the activation state of these cells (268). MEKK2 is localized to the cytosol in the resting state (268) and its association with golgi has been observed (270). Upon stimulation, MEKK2 translocates to different regions of the cell. Schaefer et al showed that MEKK2 concentrated at the T-cell/Antigen presenting cell interface upon antigen stimulation (271) and Raviv et al reported shuttling of MEKK2 between cytosol and the nucleus when stimulated with EGF (268).

Knock-out models of components in the ERK5 pathway

With the aim of understanding the specific role of the ERK5 pathway *in vivo*, knock-out mice models have been generated in all three tiers of the ERK5 MAPK cascade. In essence, all studies show very similar results and identify a role for ERK5 in embryonic angiogenesis and cardiac development. ERK5 -/- (228,272,273) embryos die as early as E9.5 indicating an indispensible role for ERK5 in embryogenesis even earlier than the requirement for the MAPK, p38. In order to distinguish the primary and secondary events resulting from gene disruption, Lee and colleagues have generated a conditional knock-out mouse model for ERK5 (274). This study confirms the requirement of ERK5 for endothelial cell (EC) survival in embryos and identifies its role in the maintainence of vascular integrity in adult mice. Also, tissue-specific ERK5 knock-out mice models reveal, surprisingly, that the ERK5 cascade is not crucial for the development and maintainence of most tissues other than EC (197). MEK5-/- mice suggest additional roles for MEK5 i.e. in normal development of the brain, besides EC survival (275). MEKK2-/- and MEKK3-/- mice (276) show differences in the function of these MEKKs. MEKK3-/- mice die early and display similar phenotypes as do mice with

disruptions in other ERK5 cascade components. MEKK2 null mice are viable but show enhanced T-cell proliferation upon stimulation. This is in agreement with studies implicating MEKK2 and not MEKK3 in T-cell function (223,271). Finally, one of the substrates of the ERK5 pathway, MEF2C, has also been knocked-out in two independent studies (277,278) with very similar results as for other upstream components. MEF2C is also a downstream target for the MAPK, p38, which is an essential gene. MEF2C-/- mice die at E9.5 with defects in the heart and with severely impaired vascular morphogenesis. But, p38α -/- mice die at E11.5-16.5, later than MEF2C-/- mice, as a result of placental abnormalities. The phenotype displayed by MEF2C-/- mice correlate better with defects seen in ERK5-/- mice and hence, it is likely that ERK5 has a more significant role to play in MEF2C activation, at least during early embryogenesis.

p90RSK

Discovery

p90RSK (also called MAPKAP K1) is a S/T protein kinase that was originally purified as a ribosomal subunit-6 (S6) kinase from *Xenopus* oocytes in 1986 (279). It belongs to the family of AGC kinases; three mammalian splice forms have been found (varying in size from 85 to 90 kD) that are encoded by separate genes (RSK1, 2 and3) (280).

Structure, Function and Activation

p90RSK is activated in response to various cellular stimuli including insulin (51), phorbol ester and changes in cAMP concentration (281), heat shock (282), T-cell receptor activation (283), growth hormone (284), ionizing radiation (IR) (285) and UV (286). Activation of p90RSK has consequences in cell proliferation, survival and cell cycle

regulation. Survival of cerebellar neurons mediated via transcriptional and non-transcriptional pathways requires p90RSK (287). Also in 1999, two groups showed the requirement of RSK1/2 for metaphase II arrest of meiosis in frog oocytes (288,289) but recently Dumont et al found that p90RSK was not required for the same activity in mouse oocytes (290).

p90RSK is one of the first substrates of ERK2 identified (51). Later, it was shown that specific isoforms of RSK (RSK1 and RSK2 but not RSK3) are activated by ERK2 *in vitro* (291-293). ERK1 and 2 have been found to interact with RSK *in vivo* (293-295). Depending on the cell type and method used, either only inactive or both active and inactive forms of ERK1 and 2 have been reported to interact with different RSK isoforms.

p90RSK has a unique and highly conserved feature in that it has two non-identical catalytic domains within the same polypeptide (296) (Figure 1.6). The N-terminal domain (NTD) is similar to p70S6K (60%)(297) and the C-terminal domain (CTD) is most related to CaM-activated protein kinases I and II, in structure and MNKs and MAPKAP kinase 2, in function (298). The NTD phosphorylates exogenous substrates while both the NTD and CTD are capable of autophosphorylation (299,300). The CTD is required for full activation of NTD and some of the regulatory phosphorylations on both the domains have been mapped (301). The specific motif recognized and phosphorylated by the two domains is different (298). ERK phosphorylates T^{573} in the CTD (292) and activates it. The CTD then autophosphorylates in the linker region (S^{380}) and activates the NTD (301). In addition, complete activation of p90RSK requires phosphorylation of the NTD by phosphoinositide-dependent protein kinase 1 (PDK1 (302,303). Interaction,

phosphorylation and activation of p90RSK by ERK2 require the C-terminal 33 aa, specifically residues 722-735 (62,63,304) which are highly conserved in all RSK isoforms and other RSK-related kinases. p90RSK activity is regulated by 2 autoinhibitory regions in the protein, N-terminal 43 aa (289) and C-terminal residues 697-712 (305). Deletion of either of these regions or the mutation, Y707A at the C-terminus leads to constitutive activation of the protein.

Downstream targets

Proteins directly downstream of p90RSK that are phosphorylated by it include c-fos (65), serum response factor (SRF) (306), Nur-77 (300), cAMP regulatory-element binding protein, CREB (307,308), Sos (309), IκBα (310), $p34^{cdc2}$ inhibitory kinase, Myt-1 (311), estrogen receptor (ER) (312), pro-apoptotic protein, BAD (287) and Na^+/H^+ exchanger isoform1, NHE1 (313).

Other MAPK pathways and p90RSK

To date, most of the functions of p90RSK have been attributed to activation of RSK by ERK1/2. Quite a few of these studies implicate ERK1/2 in p90RSK signaling as a consequence of inhibition by the pharmacological inhibitors of MEK1/2, PD98059 and U0126 (30,314). These inhibitors have now been shown to inhibit MEK5 also. Hence, some of the effects mediated by p90RSK may be due to activation of the MEK5-ERK5 pathway.

Some evidence for signaling to p90RSK via ERK1/2 independent pathways exists. Takeishi et al have shown that in perfused guinea pig hearts, ischemia activates p90RSK but not ERK1/2. Zhang et al have shown that in addition to ERK2, p90RSK co-immunoprecipitates with and is phosphorylated by JNK at S^{380} in response to UV (286).

ERK5 and p90RSK have been found to have some overlapping functions. Both are involved in the survival response in neurons (234,287) and both play a role in the regulation of NF-κB (231,310,315). ERK5 and p90RSK activities are stimulated by ischemia (316). ERK5 responds to signals from cAMP (217) and has been shown to play a role in signaling to the pro-apoptotic protein, Bad (247). CREB and Bad are direct substrates of p90RSK (287). This suggests a potential mechanism for regulation of these proteins by ERK5 through p90RSK. Finally, co-expression of MEK5DD, ERK5 and p90RSK in COS cells leads to significant phosphorylation and activation of p90RSK (231,234).

Interaction between these proteins or phosphorylation of p90RSK by ERK5 has not been demonstrated. The second part of my dissertation deals with establishing p90RSK as a direct substrate for ERK5 and characterizing their interaction.

Figure 1.1 MAP Kinase cascades

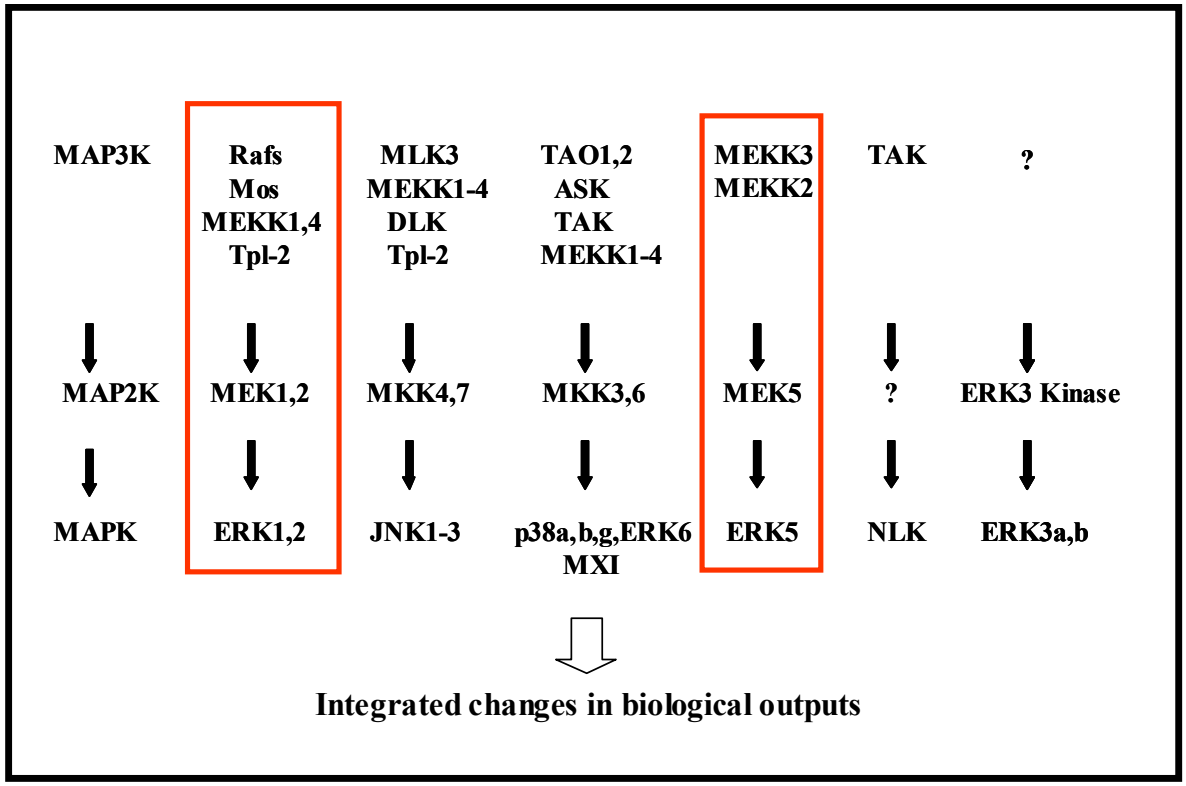

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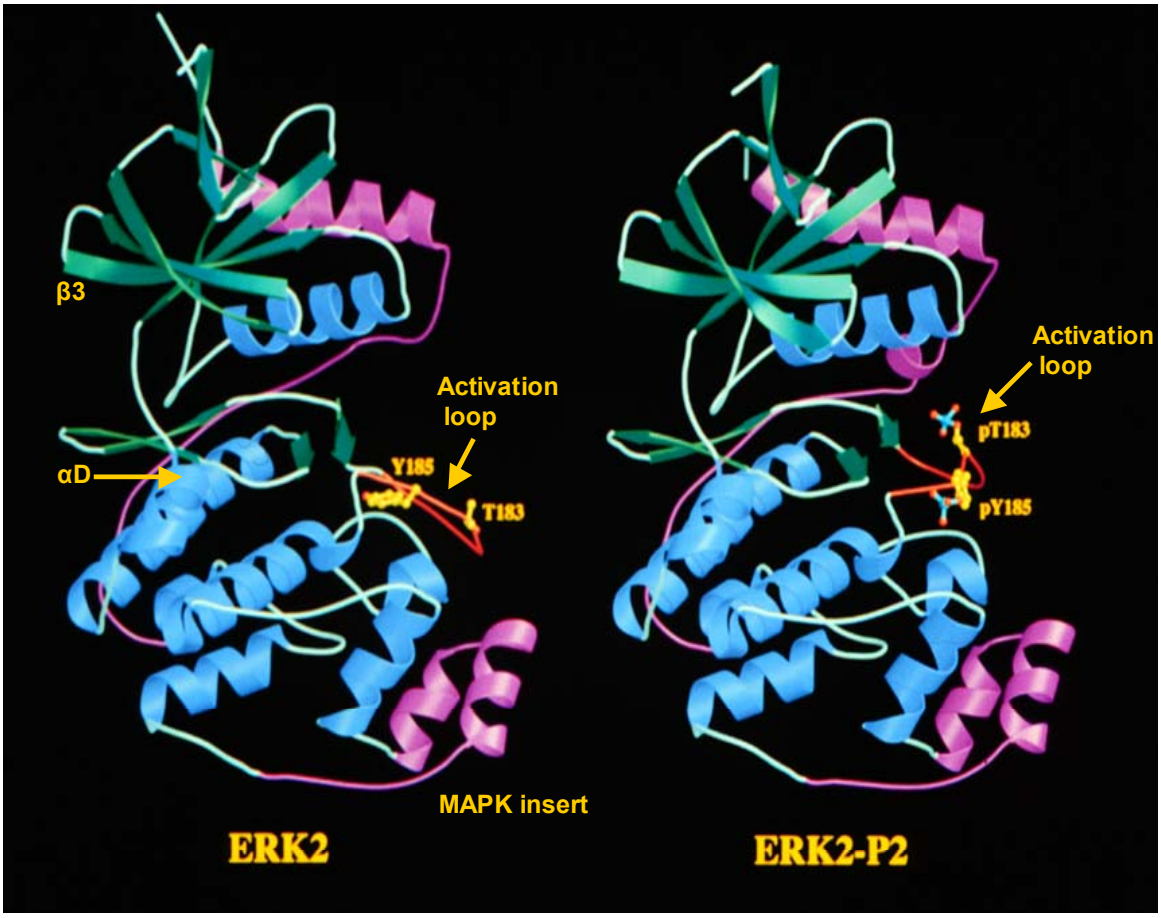
Figure 1.2 Comparison of the structures of unphosphorylated and phosphorylated forms of ERK2



Modified from Zhang F. and Canagarajah B.

Figure 1.3 Details of MEK1/2-ERK1/2 signaling in the cell

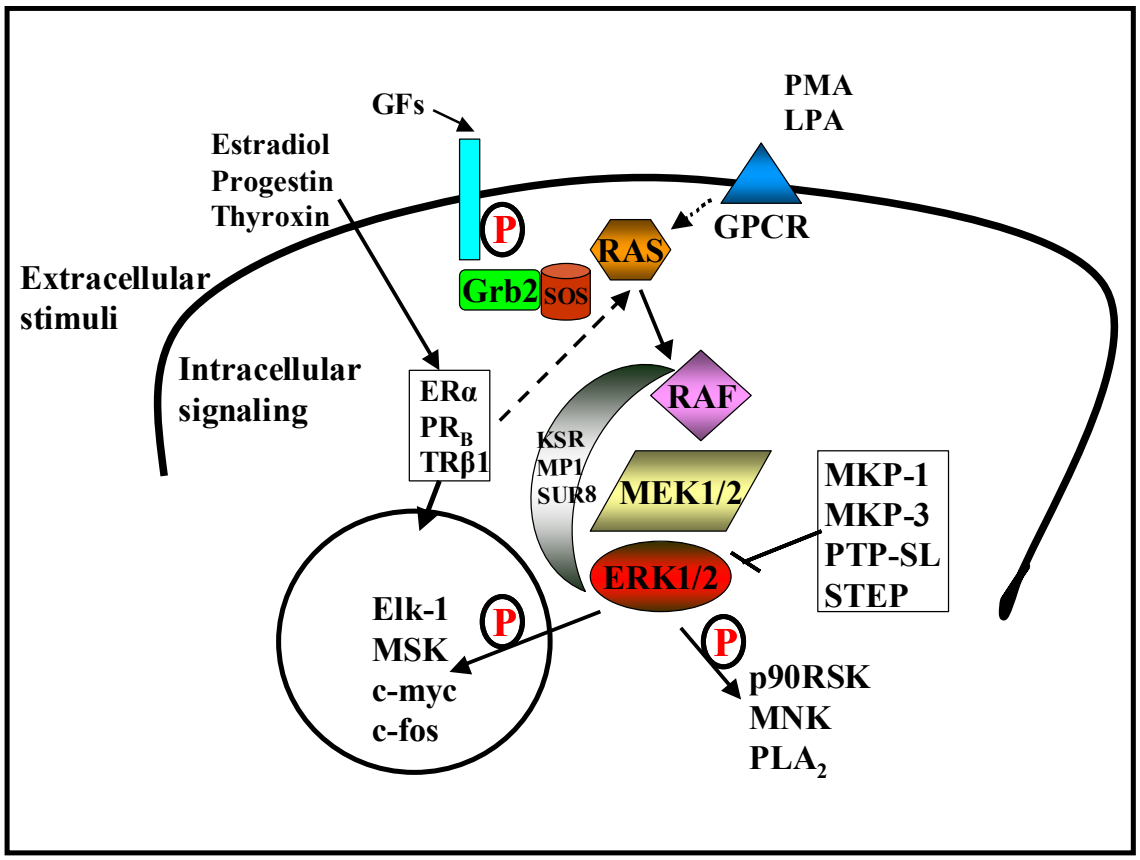
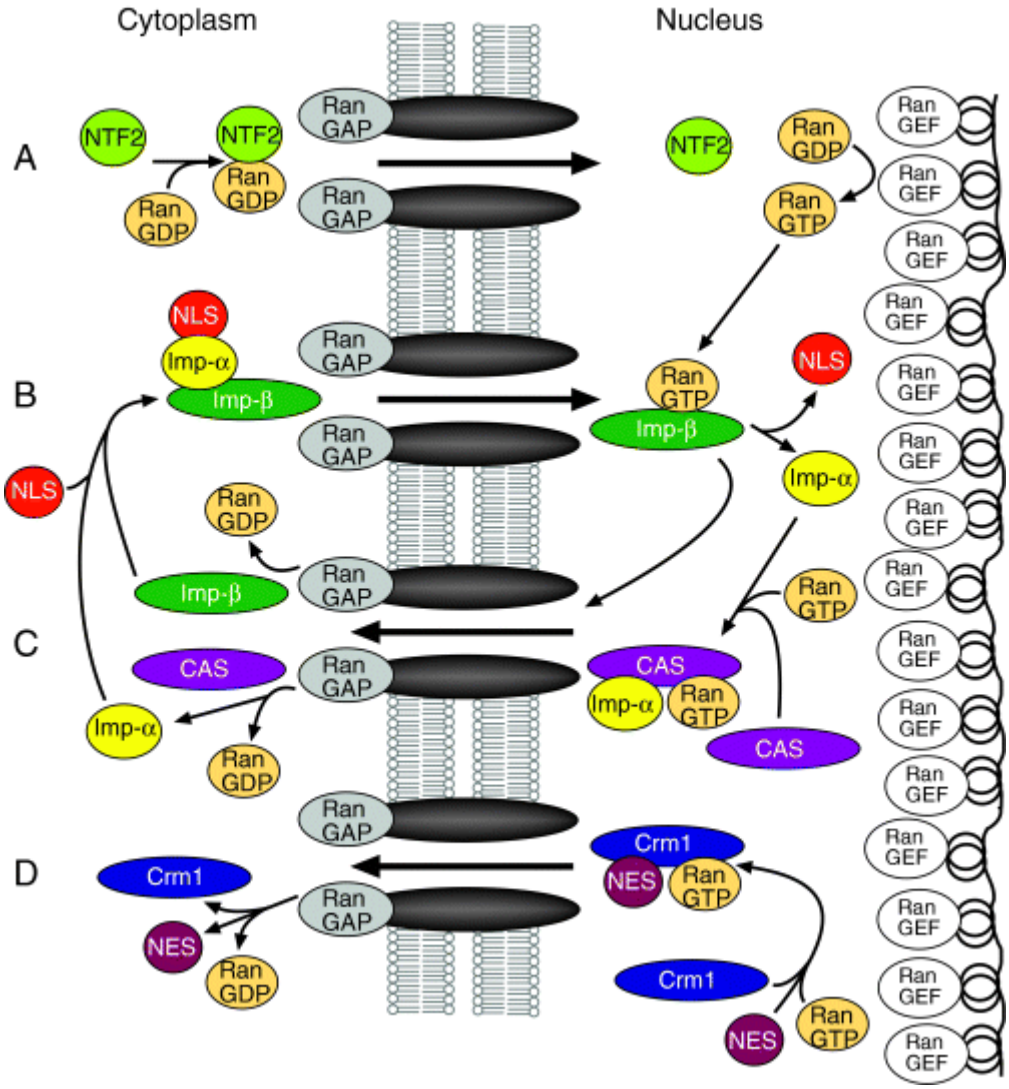


Figure 1.4 Nuclear-cytoplasmic transport of cargo through the NPC



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A. Nuclear Import of Ran-GDP by NTF2
B. Nuclear Import of NLS-cargo by importins
C. Nuclear Export of Importin α
D. Nuclear Export of NES-cargo

Figure 1.5 Details of MEK5-ERK5 signaling in the cell

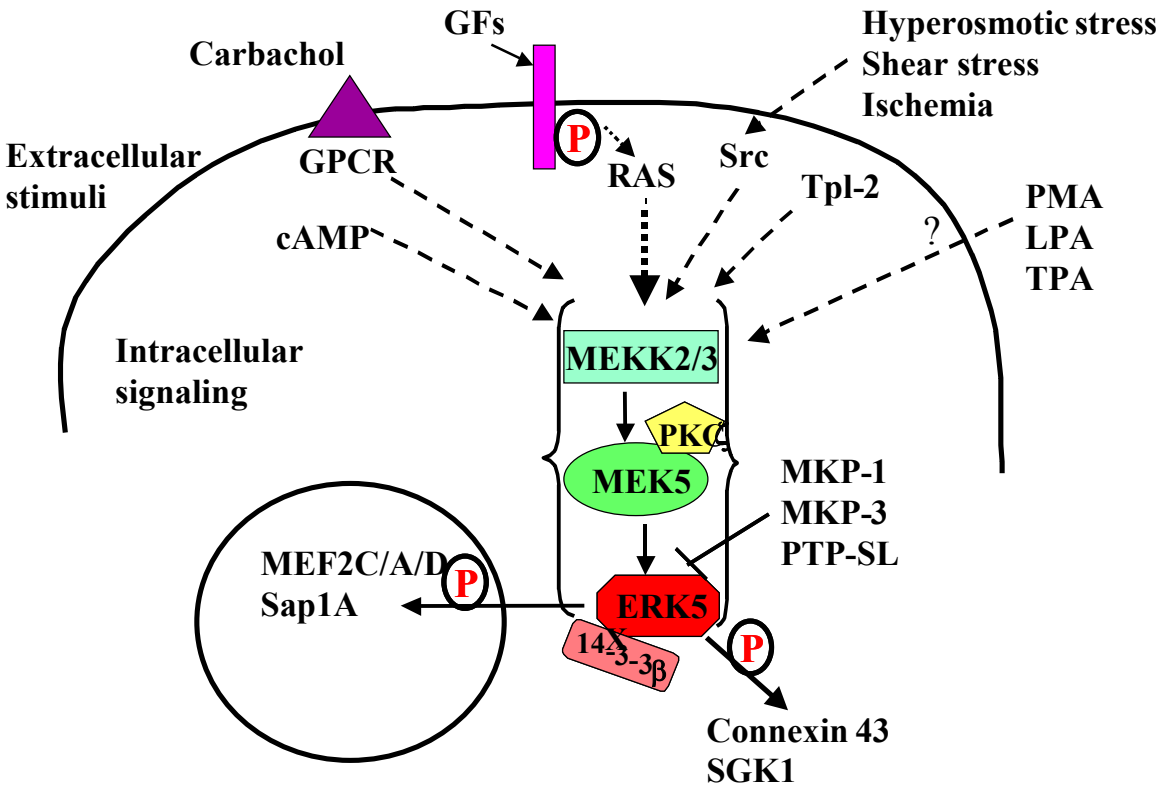
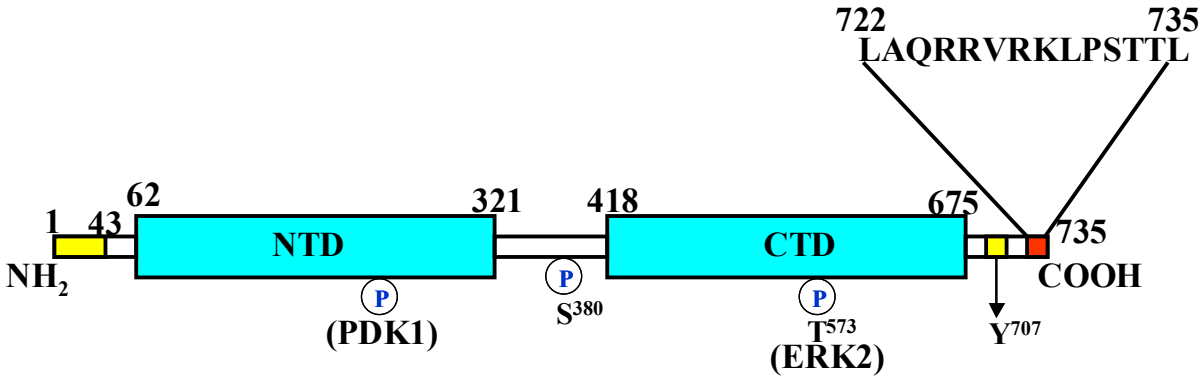


Figure 1.6 Schematic Representation of p90RSK



NTD - N-terminal kinase domain

CTD - C-terminal kinase domain

- auto-inhibitory region

- ERK2 docking region

CHAPTER II

Exploring the mechanism of ERK2 transport across the nuclear membrane

Abstract

Entry of inactive ERK2 into the nucleus has been reported to occur by an energy- and carrier-independent mechanism. However, export of inactive ERK2 and import of active ERK2 in intact cells seem to occur by an active process. The mechanisms governing these processes have not been investigated. We used an *in vitro* permeabilized-cell reconstitution assay in HeLa and BJ fibroblast cells to explore the mechanism of GFP-ERK2 export and His_6-tagged thiophosphorylated ERK2 import. We found that both processes can occur by an energy- and carrier-independent mechanism. Also, export of GFP-ERK2 occurs by two distinguishable mechanisms; the energy-dependent mechanism is mediated, at least, in part through the exportin, CRM1. Import of active ERK2 occurs predominantly through an energy-dependent active mechanism, which is enhanced by addition of exogenous transport factors. Further, some component in the HeLa cell cytosol inhibits import of active ERK2.

Introduction

Sub-cellular localization of ERK2 is a tightly regulated process. Originally, Chen et al observed that inactive ERK2 is present in the cytoplasm of resting cells and upon stimulation, a fraction of total ERK2 accumulates in the nucleus (64). Later, various studies reported that the phosphorylated and active form of ERK2 accumulates in the nucleus (76), (77), (78). Khokhlatchev and colleagues showed that microinjection of thio-phosphorylated ERK2 into the cytoplasm of REF52 cells results in its rapid translocation to the nucleus (76). In support of this, preventing the phosphorylation of ERK2, using the inhibitor PD98059, showed considerable reduction in nuclear localization of ERK2 (79). Hence, it was believed that activation of ERK2 plays a critical role in its entry into the nucleus. But various studies have also provided evidence for the presence of endogenous inactive ERK2 in the nucleus (71,77) and active ERK2 in the cytoplasm (83,85). Our laboratory has observed that microinjection of unphosphorylated ERK2 into the cytoplasm of fibroblast cells leads to its nuclear localization within 2 min and then, redistribution to the cytoplasm by 5-10 min (76). Others have reported that over-expressed inactive ERK2 accumulates in the nucleus (81,83). This suggests that nuclear localization of ERK2 can be uncoupled from its activation state. One mechanism by which unphosphorylated ERK2 enters the nucleus has been shown in import reconstitution assays (183,184). These studies report that inactive ERK2 enters the nucleus by an energy- and carrier-independent facilitated mechanism. More recently, Shaw and colleagues have used FRAP (Fluorescence recovery after photobleaching) to confirm the energy-independent movement of ERK2 into the nucleus (89). They also demonstrate that ERK2 interacts with MEK1 in the cytoplasm supporting the idea that

MEK1 is involved in cytoplasmic anchoring. Activation of the MEK1-ERK2 pathway releases ERK2 from this interaction and increases the free pool of ERK2 that is available to enter into the nucleus. This study implicates an important role for anchoring proteins in the localization of ERK2.

MEK1 is primarily localized in the cytoplasm of cells but can probably shuttle between the two compartments of the cell (82,191). Adachi et al showed that a mutant MEK1 protein, with disruption in the NES, caused nuclear retention of microinjected ERK2 while the wild-type MEK1 protein enhanced its export to the cytoplasm (190). Hence, they proposed that one mechanism by which inactive ERK2 can be exported from the nucleus is by association with MEK1. They also showed that Leptomycin B inhibited export of ERK2 from the nucleus suggesting that it is an energy-dependent process. Further, they hypothesized that since dually phosphorylated form of ERK2 does not bind MEK1 the active form of ERK2 is not exported from the nucleus. But recently, Shaw et al have shown that very little MEK1 is present in the nucleus during the resting state of the cell and that no significant translocation of MEK1 occurs into the nucleus upon stimulation (89). This would argue against the presence of sufficient quantities of MEK1 in the nucleus to drive ERK2 export.

Summarizing these studies, the current model for sub-cellular localization of ERK2 suggests that there is continuous nuclear-cytoplasmic shuttling of the free pool of ERK2. ERK2 can enter the nucleus in the active or inactive form and exits from the nucleus, at least, in the inactive form. Anchoring of ERK2 in the different compartments of the cell plays a critical role in determining its location.

We wanted to gain further insight into some aspects of the mechanism of ERK2 nuclear transport by analyzing import and export of ERK2 in reconstitution assays. Our results identify more levels of regulation within this model. We show that, perhaps unlike import, export of unphosphorylated ERK2 occurs by two separate processes, an energy-dependent and an energy-independent process. Again, unlike import of inactive ERK2, the import of the phosphorylated form of ERK2 occurs primarily by an energy-dependent mechanism. We also show that active ERK2 can be exported from the nucleus by a carrier- and energy-independent mechanism. Finally, we have identified some mutants of ERK2 that are impaired for nuclear import; these may be used in further studies to delineate the specific biological responses elicited by different sub-cellular localizations of ERK2.

Experimental Procedures

Constructs and Recombinant proteins – The His_6GFP-ERK2 plasmid and Thiophosphorylated (Thio-P) ERK2, Ran, p10/NTF2, karyopherin β2, karyopherin α1 and rhodamine-labeled NLS-BSA proteins were as described before (183). His_6GFP-ERK2 I196A/M197A, His_6GFP-ERK2 N199A/S200A, His_6GFP-ERK2 N199A/S200A/I207A and His_6GFP-ERK2 I196A/M197A/N199A/S200A/I207A were generated by site-directed mutagenesis using the Quikchange kit (Stratagene) according to the manufacturer's protocol. His_6GFP-ERK2 K52R, His_6GFP-ERK2 T183A, His_6GFP-ERK2 L4AH176E, His_6GFP-ERK2 D316AD319A, His_6GFP-ERK2 Y261N, His_6GFP-ERK2 Δ241-272 were as described before (90). All ERK2 proteins were purified as described in (76).

Cell Culture – HeLa cells and BJ fibroblasts {named after the patient from whom the cell-line was obtained and immortalized by insertion of h-TERT for maintainence in culture} were grown on coverslips for 24 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 100 units/ml penicillin/streptomycin at 37^{o}C under 10% CO_2. Where appropriate, cells were treated with 10-20 nM (5.5 - 11 ng/ml) leptomycin-B (LC Labs.) for 4-6 h prior to use in import assays. BJ cells were starved for 2 h in DMEM alone prior to use in import assays with Thio-P ERK2.

RNAi protocol – Mustafa Yazicioglu, a graduate student in our laboratory performed the CRM1 RNAi experiments according to a modified protocol (317) and I analyzed the cells in export reconstitution experiments. siRNA oligonucleotides were generated towards three different target sequences in the CRM1 DNA (CRM1-1 –

5’ATACGTTGTTGGCCTCATT 3’, CRM1-2 – 5’ATATGTTGTTGGTATCTGA 3’ , CRM1-3 – 5’TTACTCATCTGGATTATGT 3’) and synthesized at the UT-Southwestern core facility. Hela cells were grown on coverslips to 30-50% confluence in DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine (with no antibiotics). 100 nM CRM1-1 or CRM1-2 ds-RNA oligonucleotides were added and cells were grown in DMEM with 1% L- glutamine for 24 h. A second round of RNAi was performed using 100 nM CRM1-3 and cells were grown for an additional 12-24 h.

Import and Export Assays - Import assays were performed as described before (117) and export assays were according to a modified protocol (181). Cells were washed in transport buffer, TB {20 mM Hepes-KOH pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol-bis (β aminoethylether)-N,N,N’,N’tetraacetic acid (EGTA) and 2 mM dithiothreitol (DTT)} once unless otherwise specified and permeabilized with 70 μg/ml digitonin in TB for 5 min. Import reaction was performed in 40 μl reaction mix that contained the following components as specified in the figure legends; TB with 10 mg/ml bovine serum albumin (BSA), recombinant substrate {GFP-ERK2, Thio-P ERK2 or GFP-ERK2 mutants were used at 0.8 μM (50 μg/ml); tetramethylrhodamine B isothiocyanate (TRITC)-NLS-BSA was used at 0.14 μM (10 μg/ml)}, HeLa cell cytosol dialyzed against TB (2.5 mg/ml or as specified), energy (ATP-GTP regenerating system consisting of 1 mM ATP, 1 mM GTP, 5 mM phosphocreatine and 20 U/ml creatine phosphokinase), apyrase (4 U), Wheat Germ Agglutinin, WGA (0.5 mg/ml), kapα1 (0.5 μM), kapβ2 (0.25 μM), Ran (2 μM) and p10/NTF2 (0.4 μM). Import was performed for 15 min unless otherwise specified and the reaction was stopped with 250 μl TB. At this step, cells were fixed or used for export

assays. For the export phase, cells which had been pre-incubated with import substrate were transferred to 40 µl reaction mix which contained TB with 10 mg/ml BSA, cytosol, energy, WGA or apyrase at concentrations mentioned above as specified in the figure legends. Export was performed for 30 min unless otherwise specified. The reaction was terminated with 250 µl TB. Cells were fixed in 3% paraformaldehyde for 10 min and coverslips were mounted using polymount.

For indirect immunofluorescence of Thio-P ERK2, fixed cells were re-permeabilized in 0.5% Triton-X 100 for 10 min. Blocking was performed in PBTA (1X phosphate buffered saline (PBS), 0.1% Tween-20, 0.01 g/ml BSA) for 1 h at room temperature(RT); P-ERK (Sigma) primary antibody was used at 1:300 for 24 h at 4^{o}C and Alexa-546 anti-mouse secondary antibody (Molecular Probes) was used at 1:3000 for 1 h at RT. Washes between the incubations were performed with 1X PBS or 1X PBTA for 15 min at RT.

Flourescence Microscopy – GFP, TRITC and Alexa fluorophores were visualized by fluorescence microscopy using the Zeiss Axioshop 2-plus microscope and images were taken using Hamamatsu digital CCD camera (C4742-95). Exposures for all conditions within an experiment were kept the same and fluorescence intensity within the nucleus was quantified using the Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.).

Results

I. Understanding the mechanism of ERK2 export

To date what is known of the mechanism of ERK2 export from the nucleus presents an unclear picture. ERK2 has no identifiable NES that can be recognized by export factors, but a CRM1-dependent export process has been reported in cells (82,190). Recently Matsubayashi et al showed that ERK2 can be exported in the absence of transport factors and energy and hypothesized that export of ERK2 may not be mediated via CRM1 (184). We used an *in vitro* reconstituted assay system to analyze ERK2 export and possibly reconcile earlier findings.

GFP-ERK2 is exported from the nucleus through the NPC

An export assay was performed following import of a GFP-tagged fusion protein of ERK2 in permeabilized BJ fibroblast cells. Import of GFP-ERK2 into permeabilized cells has been reported to occur in an energy- and carrier-independent manner (183,184). Hence, 0.8 μM (50 μg/ml) GFP-ERK2, close to its intracellular concentration, was allowed to accumulate in the nucleus for 15 min without addition of cytosol or an ATP/GTP regenerating system (energy). The cells were, then, incubated for different times (15 min to 2 h) in transport buffer with (+) or without (-) cytosol and energy and the amount of ERK2 remaining in the nucleus was detected by fluorescence (fig. 2.1A). Export of GFP-ERK2 was observed at 15 min under both conditions and was nearly complete at 30 min. Quantification of fluorescence intensity in three independent experiments showed ~85% export by 30 min in the presence of cytosol and energy (fig. 2.1B). Similar behavior was observed in HeLa cells (data not shown). Rhodamine labeled NLS-BSA was used as the model substrate. It was imported in an energy- and cytosol-

dependent manner and was not exported under the conditions used even after 2 h (fig 2.1C and data not shown). To study export at shorter times, cells were incubated in export mix (in the presence or absence of cytosol and energy) from 2 min to 30 min (fig 2.2). GFP-ERK2 export was detected as early as 5 min.

GFP-ERK2 is 68 kD in size and is not expected to diffuse out of the intact nucleus. To test this assumption, we performed export of GFP-ERK2 in the presence of 0.5 mg/ml WGA. WGA blocks nuclear transport through the NPC by binding to nucleoporins (318) (fig. 2.3). Addition of WGA blocked export of GFP-ERK2. This confirms that GFP-ERK2 is being exported through the NPC and that our conditions of permeabilization do not grossly damage the nuclear membrane.

GFP-ERK2 export occurs by two distinguishable processes

As mentioned before, LMB increases the nuclear localization of ERK2 suggesting that it is actively exported from the nucleus in a CRM1-dependent manner in cells (190). In the above detailed experiments, we observed that GFP-ERK2 could be exported in the absence of cytosol and energy. But we also observed that export of GFP-ERK2 is enhanced in the presence of cytosol and energy indicating the existence of an energy-dependent mechanism for export of ERK2. The influence of cytosol and energy on export was more obvious after 15 min (fig 2.1).

To characterize the energy- and carrier-dependence of export further, we performed the export assay using GFP-ERK2 in BJ fibroblasts in the presence or absence of cytosolic factors or energy (fig 2.4). After 30 min, little fluorescence in the nucleus was detected when both cytosol and energy were present in the export mix (fig. 2.4 top panel, middle). The absence of energy, i.e. no exogenously added ATP/GTP regenerating

system or the inclusion of apyrase to scavenge nucleotides, inhibited export substantially (fig. 2.4 bottom panel). The absence of cytosolic factors (fig. 2.4 top panel right) also inhibited export, although to a lesser extent. We obtained similar results in HeLa cells (data not shown). Our results support the idea that export is a regulated process and occurs by, at least, two separate processes, an energy-dependent and an energy-independent process.

Export of GFP-ERK2 by the energy-dependent process is mediated by CRM1

CRM1 is the nuclear receptor that mediates energy-dependent transport of many cargo proteins from the nucleus (80,128,129,161,162). We wanted to examine the extent of involvement of CRM1 in GFP-ERK2 export from the nucleus. Towards this end, we knocked down the expression of CRM1 using RNA interference in HeLa cells. Mustafa Yazicioglu, a graduate student in our laboratory performed the RNAi experiments. Knockdown of CRM1 protein >75% was observed in all experiments by Western blotting (fig 2.5A). I used the cells, from which CRM1 had been knocked down, in export assays. Cells transfected with dsRNA targeting CRM1 or left untreated (labeled CRM1 RNAi (+) and Control (-) respectively) were used for export assays with GFP-ERK2, in the presence of cytosol and energy (fig. 2.5B). We observed inhibition of export in RNAi+ cells at 15 and 30 min. Quantification of the fluorescence intensity in the nucleus showed ~30% inhibition at 15 min and maximum of 45% at 30 min. CRM1 RNAi neither significantly altered the import of GFP-ERK2 (fig. 2.5B top panel) nor did it affect the import or export of NLS- BSA (fig. 2.5C). Export was also performed in the absence of cytosol and energy (fig. 2.5D). Little or no inhibition of export was evident under these

conditions. This is in agreement with mediation of carrier- and energy- dependent export by CRM1.

To confirm the involvement of CRM1 further, we performed export assays with GFP-ERK2 in HeLa cells treated with 10 or 20 nM (5.5 or 11 ng/ml) LMB for 4-6 h. We observed a maximum of 50% inhibition of export (with cytosol and energy) at 30 min after treatment with LMB (+LMB), compared to export assays lacking LMB (-LMB). Import and export carried out in the absence of cytosol and energy were minimally altered (fig 2.6A). NLS-BSA was used as the nuclear localized control and no effect of LMB on the localization of NLS-BSA was observed during the import or export process (fig. 2.6B).

In summary, we have shown that export of GFP-ERK2 occurs by a facilitated mechanism by two separate processes, one is energy- and carrier-dependent and the other independent of both. The energy-dependent process is mediated, at least in part, by CRM1. Furthermore, export of unphosphorylated ERK2 to the cytoplasm by both processes is rapid; significant export is seen within 5 min. This is in agreement with previous reports in intact cells (76).

II. Exploring the mechanism of nuclear transport of Active ERK2

Evidence for the mechanism of entry of active ERK2 into the nucleus is lacking. Whitehurst et al showed that the thio-phosphorylated (Thio-P) form of ERK2 competes for import with GFP-ERK2, under a carrier- and energy-independent condition, in a concentration-dependent manner (183). Also, in the same study, Thio-P ERK2 competed with NLS-BSA for entry into the nucleus, as did unphosphorylated ERK2. Although, these experiments suggest a similarity in the mechanism of import of unphosphorylated

and phosphorylated ERK2 forms, the existence of alternate processes cannot be ruled out. Nishida's group reported that GFP- ERK2 from *Xenopus* incubated with active MEK in an *in vitro* reconstitution assay was imported in the absence of transport factors and energy (184). The probability of dephosphorylation of ERK2 under the conditions employed for import in these assays leads to the question of contributions from unphosphorylated form of ERK2 to the observed result. Also, the effect of cytosolic factors and energy on the import of active ERK2 was not analyzed. To investigate the mechanism of entry of the active form of ERK2 into the nucleus, we used Thiophosphorylated (Thio-P) form of ERK2 in import reconstitution experiments. Thio-P ERK2 is a more stable form of the phosphorylated protein due to resistance to dephosphorylation (320) and may be better for analysis of active ERK2. Also, we used the phospho-ERK2 (P-ERK2) specific antibody for analysis by immunofluorescence further minimizing the chance of visualizing effects from unphosphorylated ERK2. We confirmed the specificity of the phospho-ERK antibody by loss of immunofluorescence upon serum starvation of cells and from its immunoreactive specificity seen on Western blots.

Active form of ERK2 can enter the nucleus in the absence of transport factors and energy via the NPC

BJ cells were permeabilized and incubated in the import mix for 20 min in the presence or absence of exogenously complemented transport factors (TF) or energy (fig. 2.7A). To reduce the levels of background endogenous phosphorylated ERK2, cells were either starved for 2 h prior to permeabilization or washed in three changes of transport buffer for 10 min each after permeabilization. Thio-P ERK2 import was visualized by

indirect immunofluorescence after incubation with P-ERK2 specific primary antibody and Alexa fluor secondary antibody. Thio-P ERK2 was imported without addition of transport factors or energy; although, uptake was significantly enhanced in the presence of these components. To show that, like ERK2, Thio-P ERK2 is imported via the nuclear pore, we performed the import assay in the presence of 0.5 mg/ml WGA. WGA blocked entry of Thio-P ERK2 into the nucleus (fig. 2.7A). Rhodamine-labeled NLS-BSA (used as control) was not imported in the absence of transport factors and energy or in the presence of WGA (fig 2.7B).

Import of Thio-P ERK2 occurs predominantly by an energy-dependent process

Since, we had observed an increase in Thio-P ERK2 import with addition of components of the classical import machinery, we wanted to further characterize the importance of these factors for import. First, we investigated whether the presence of energy affected nuclear uptake even in the absence of transport factors. BJ fibroblasts were incubated in transport buffer containing Thio-P ERK2 for 20 min with or without addition of energy (fig. 2.8A). We observed a dramatic increase in nuclear import of Thio-P ERK2 when energy was included in the import mix (+E Vs.-E). Quantification of fluorescence intensity in the nucleus indicated that, with the addition of an energy regenerating system, import in the absence of transport factors was very nearly equal to import in their presence (data not shown). We wanted to confirm that the increase in fluorescence intensity within the nucleus was not due to phosphorylation of endogenous ERK2 in the presence of energy. Hence, we performed the import assays in the presence of WGA that would block transport of Thio-P ERK2, but not of the components used in

the energy regenerating system into the nucleus. WGA blocked most of the fluorescence seen in the nucleus in the presence of energy (fig. 2.8A).

This suggests that, in addition to an energy- and carrier- independent import process, Thio-P ERK2 also utilizes an energy-dependent mechanism for nuclear translocation. From the difference in nuclear uptake detected, it can be hypothesized that the energy-dependent process is the predominant mechanism. This is different from what has been reported for ERK2 import.

Cytosol inhibits the import of P-ERK2

The requirement of transport factors for the import process can be satisfied in reconstitution experiments by two means; either by exogenous complementation with individual recombinant transport proteins or by addition of cytosol. With the intention of characterizing whether import occurs by a carrier-dependent mechanism, we performed import of Thio-P ERK2 in BJ cells with or without addition of cytosol or energy (fig. 2.8B). To our surprise, although we had seen significant import in the presence of recombinant transport factors, the addition of cytosol inhibited import of Thio-P ERK2 in the presence and absence of energy. To analyze this observation in further detail, we included energy and varied the final amount of cytosol (0.01 mg/ml to 2.5 mg/ml) in the import mix (fig. 2.9). As before, we saw substantial nuclear import in the presence of energy alone (0 mg/ml cytosol), compared to background levels of endogenous phospho-ERK2. Nuclear uptake of Thio-P ERK2 was enhanced in a dose-dependent manner up until 0.1 mg/ml cytosol in the import mix; then, import of Thio-P ERK2 started declining steadily until the entry of little or no Thio-P ERK2 was detected at >1 mg/ml cytosol.

Fluorescence intensity in the nucleus was quantified after subtracting background staining for endogenous phospho-ERK2.

Export of P-ERK2

We have demonstrated that, similar to its import, export of inactive ERK2 can occur by an energy- and carrier-independent mechanism (fig. 2.1A) and that P-ERK2 enters the nucleus, at least in part, by an energy-independent mechanism (fig. 2.7A, 2.8B). Hence, it is possible that P-ERK2 is exported from the nucleus by a similar mechanism. To test this, we allowed import of Thio-P ERK2 for 20 min in permeabilized BJ cells without addition of cytosol and energy. This was followed by an export assay in reaction mix containing transport buffer and BSA (with no added factors). We observed almost complete loss of fluorescence in the nucleus by 30 min (fig. 2.10).

In summary, we have shown that active ERK2 enters into the nucleus by two processes; one is energy-dependent and the other energy-independent. The energy-dependent process is most likely enhanced by the presence of transport factors. Both the processes of import utilize the NPC; most probably the energy-independent process of import is by a mechanism similar to that of inactive ERK2. Finally, we found that active ERK2 can be exported from the nucleus, at least, by an energy- and carrier- independent mechanism.

III Studies on some ERK2 mutants

Identification of residues important for import of inactive ERK2

It has been shown that import of unphosphorylated ERK2 occurs by a facilitated mechanism, probably by direct interaction with nucleoporins in the NPC (183,184). These studies reported direct binding of ERK2 with FXF containing region on two

different nucleoporins, Nup153 and Nup214. From structural considerations, Betsy Goldsmith predicted some residues on ERK2 that may be involved in its interaction with the FXF motif on nucleoporins. One way to study the requirement of these residues for binding to nucleoporins would be to analyze mutants of ERK2 in import assays. If these residues are involved, then, their import might be impaired. We generated mutants of GFP-ERK2 at these residues (GFP-ERK2 I196A/M197A, GFP-ERK2 N199A/S200A and GFP-ERK2 I207A) by site-directed mutagenesis. We then performed import assays with wild-type GFP-ERK2 and these mutants, in transport buffer, for 20 min (fig 2.11A). As expected, all the mutants were impaired for import. Quantification of fluorescence intensity indicated about 50% inhibition in nuclear entry for all mutants. We checked whether mutating more than one/two residues had a bigger impact on inhibition of import but additional mutations either did not show significant differences or were imported better (fig. 2.11B and data not shown).

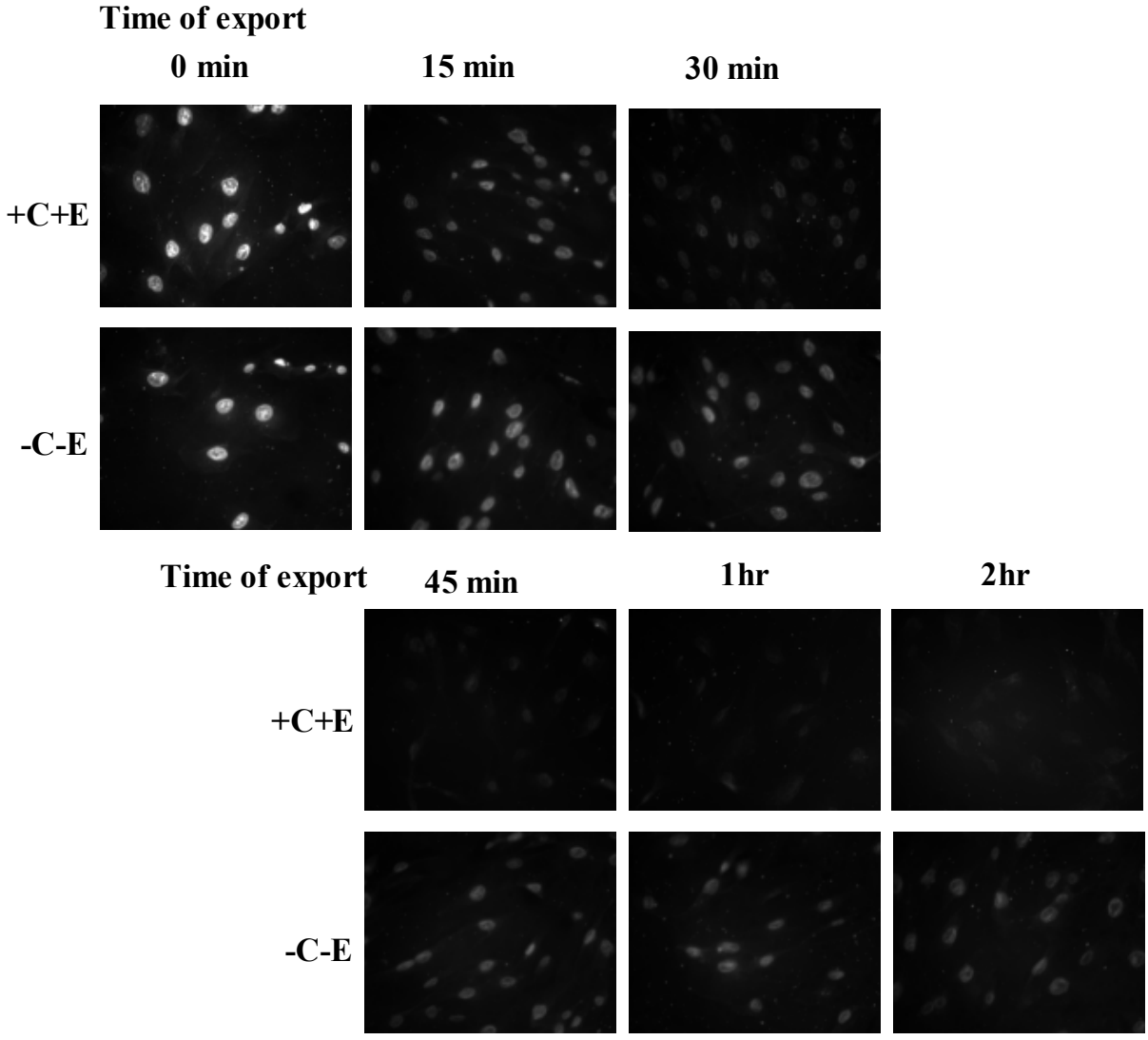
Import of different ERK2 mutants

Finally, we used a series of GFP-ERK2 mutants in import assays and compared their import to that of wild-type GFP-ERK2 to see if we could clarify some of the issues raised in different studies regarding different aspects of ERK2 nuclear localization. We performed import assays in BJ cells using dimerization-deficient mutant of GFP-ERK2 (L4H176E); GFP-ERK2 that cannot be activated (T183A); a constitutively inactive and hence, dominant negative form (GFP-ERK2 K52R); a MAPK insert mutant (GFP-ERK2 Δ241-272) and GFP-ERK2 Y261N mutant that are impaired for binding to MEK1 and the ERK2 cytoplasmic anchor protein, PEA-15; and finally, a common docking (CD) domain mutant (GFP-ERK2 D316AD319A) that is impaired for binding to

MEK1 and some substrates of ERK2 (fig. 2.12). The mutants were imported to varying extents at 15 min in the absence of transport factors and energy. GFP-ERK2 L4H176E, GFP-ERK2 T183A and GFP-ERK2 K52R were imported to almost the same degree as wild-type GFP-ERK2; GFP-ERK2 D316AD319A showed slight decrease in nuclear uptake; GFP-ERK2 Δ241-272 and GFP-ERK2 Y261N were severely impaired for import. To further characterize the extent to which GFP-ERK2 D316AD319A, GFP-ERK2 Δ241-272 and GFP-ERK2 Y261N were impaired, we compared import of these mutants with wild-type protein, in the presence or absence of cytosol and energy, from 5 min to 2 h (fig. 2.13). Import of wild-type ERK2 was obvious within 5 min while GFP-ERK2 Y261N and GFP-ERK2 D316AD319A were visible only after 10 min. GFP-ERK2 Y261N was imported better in the presence of cytosol and reached about 60% intensity of GFP-ERK2 wild-type protein. GFP-ERK2 D316AD319A was imported much better in the absence of cytosol and energy and about 75% uptake was observed. GFP-ERK2 Δ241-272 was most impaired under both conditions and was clearly visible only after 45 min in the presence of cytosol and energy. Quantification showed about 40% import at 2h under this condition and <20% import at all time-points in the absence of cytosol and energy. In summary, different ERK2 mutants defective, to varying extents, for import into the nucleus have been identified.

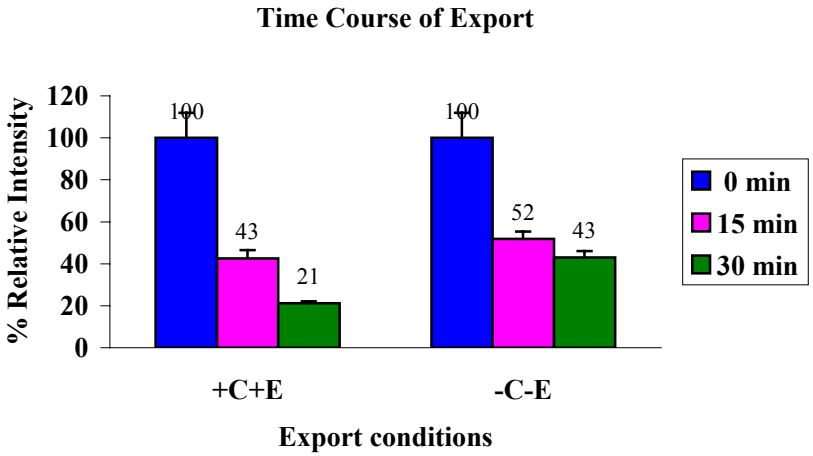
Figure 2.1 Export of GFP-ERK2 and NLS-BSA in BJ fibroblast cells

2.1A

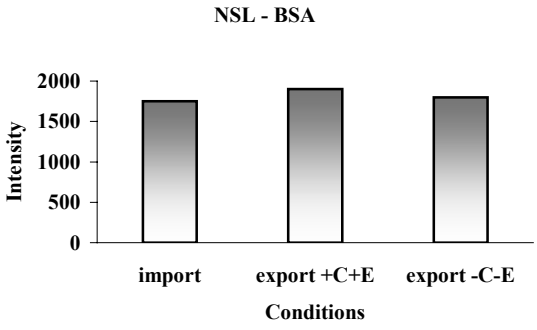


2.1A. Import of GFP-ERK2 was performed for 15 min in transport buffer with no added factors. For the export phase, cells were incubated in transport buffer alone (-C-E) or in transport buffer containing 2.5 mg/ml cytosol and energy (+C+E) for the indicated times. Each condition was performed in duplicate. Fluorescence data visualized after 24 hours is presented.

2.1B

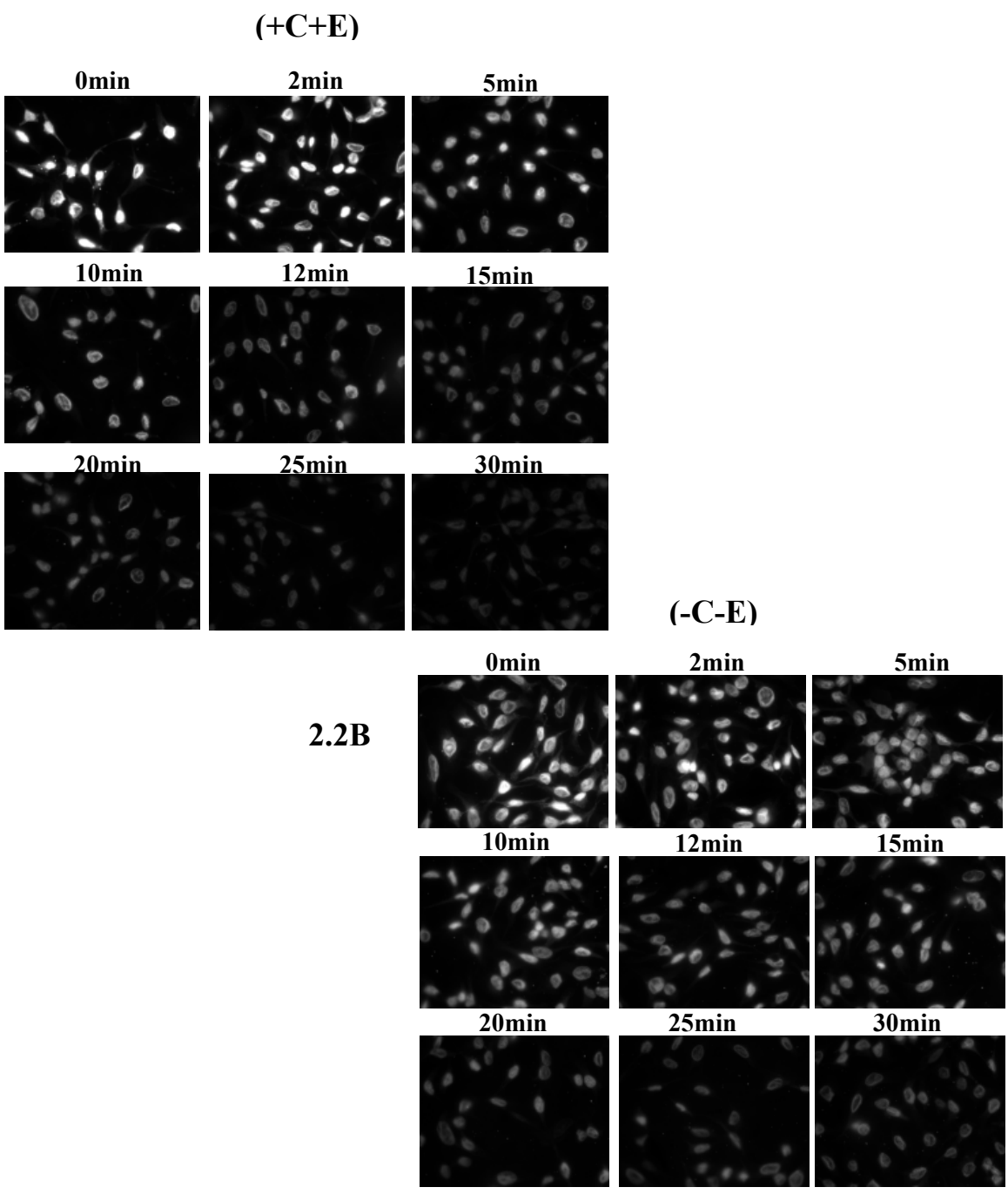


2.1C



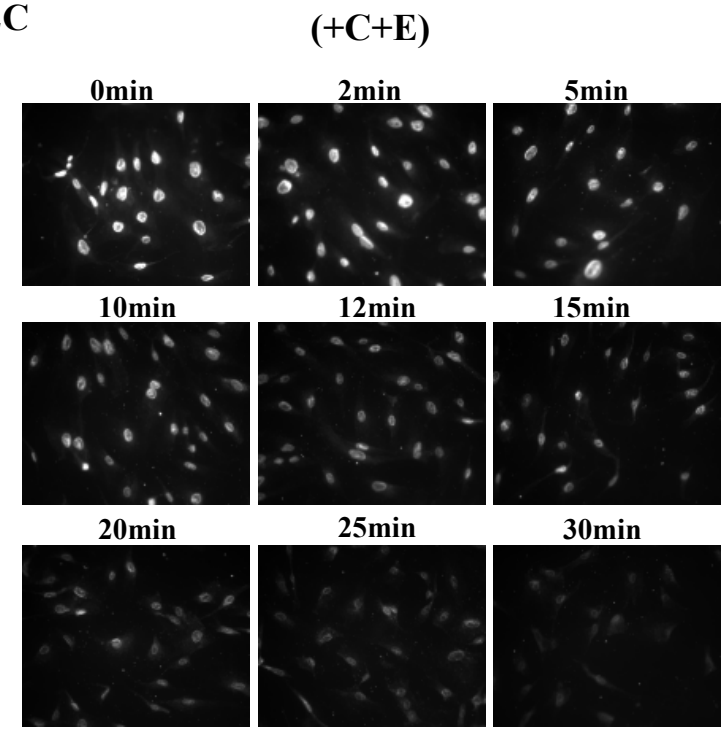
Fluorescence intensity of **2.1B** GFP-ERK2 and **2.1C** Rhodamine-labeled NLS-BSA quantified in BJ cells. Import was performed for 15 min in transport buffer containing cytosol and energy. Export was performed for the indicated times in the presence (+C+E) or absence (-C-E) of cytosol and energy.

Figure 2.2 Time course of export of GFP-ERK2 in HeLa and BJ cells

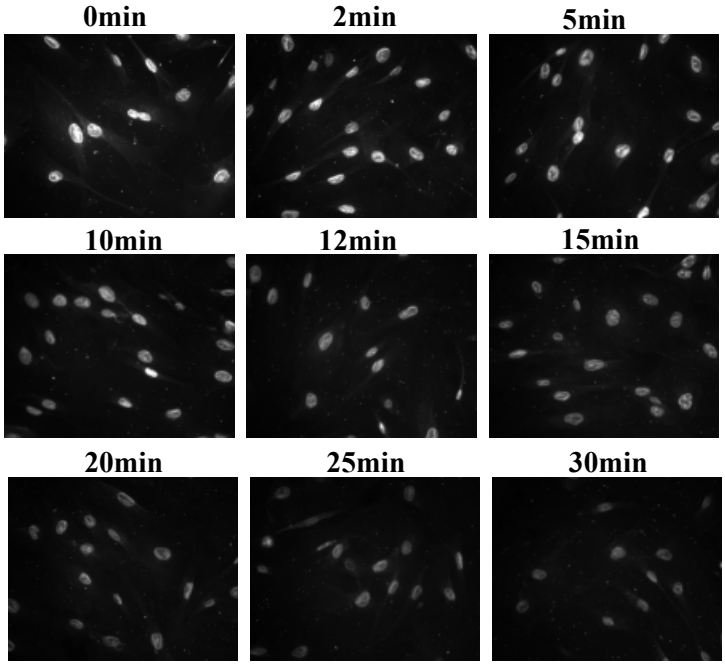


2.2A and B. Time course of GFP-ERK2 export in HeLa cells. Export assay was performed as in 2.1A. Each condition was done in duplicate. Fluorescence visualized is presented.

2.2C

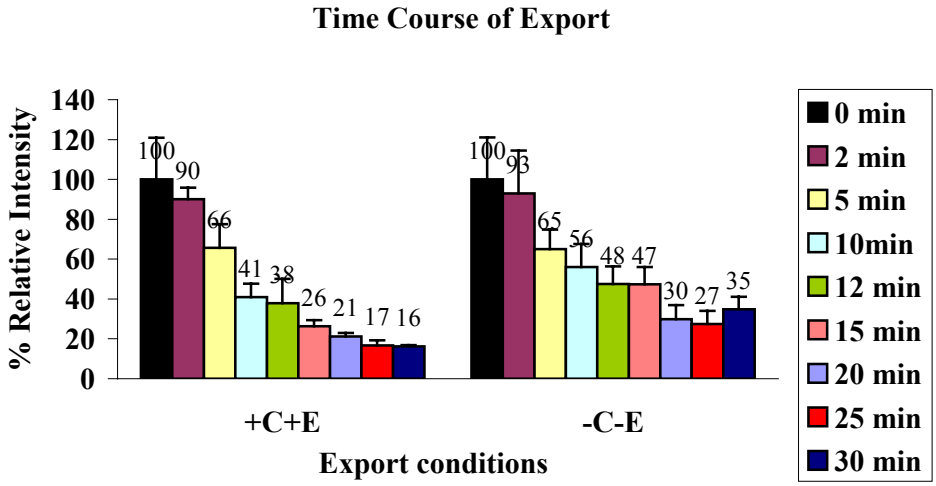


2.2D



2.2C and D. Time course of GFP-ERK2 export in BJ cells. Export assay was performed as in 2.1A. Each condition was done in duplicate. Fluorescence visualized after 24 h is presented.

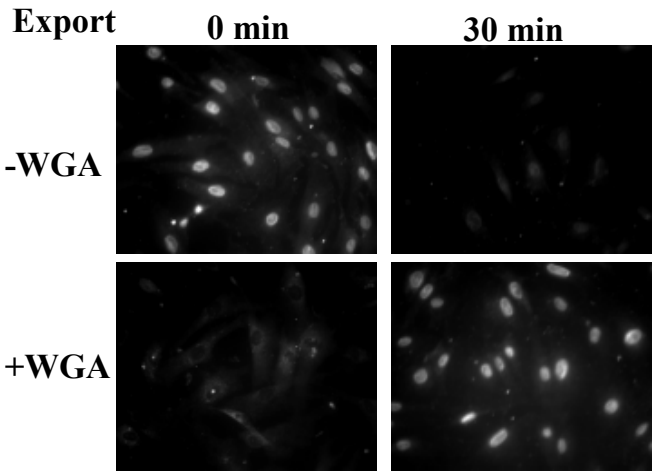
2.2E



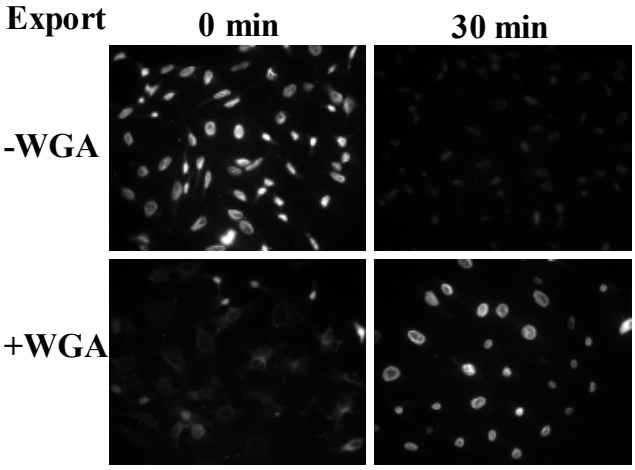
2.2E. Fluorescence intensities of GFP-ERK2 were quantified from two independent experiments. Import was performed for 15 min in transport buffer containing cytosol and energy. Export was performed in the presence (+C+E) or absence (-C-E) of cytosol and energy for the indicated times.

Figure 2.3 Export of GFP-ERK2 occurs through the Nuclear Pore Complex

2.3A

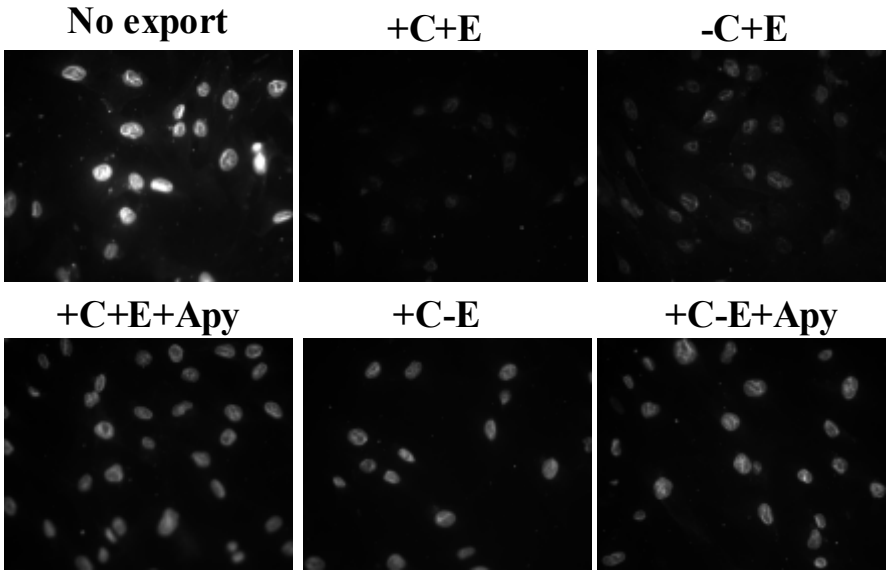


2.3B



2.3. Export of GFP-ERK2 in **A**. BJ cells and **B**. HeLa cells. Import of GFP-ERK2 was performed for 20 min in transport buffer with or without WGA. For the export phase, cells were incubated in transport buffer containing energy and 2.5 mg/ml cytosol in the presence or absence of WGA for 0 min or 30 min. Each condition was performed in duplicate. Fluorescence data visualized after 24 h is presented.

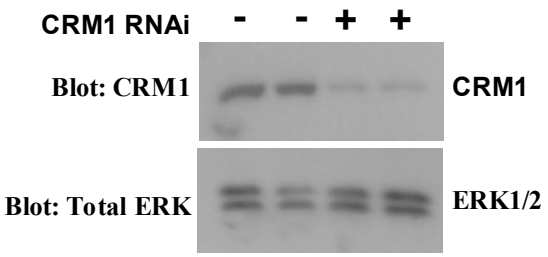
Figure 2.4 Export of GFP-ERK2 occurs by two distinguishable processes



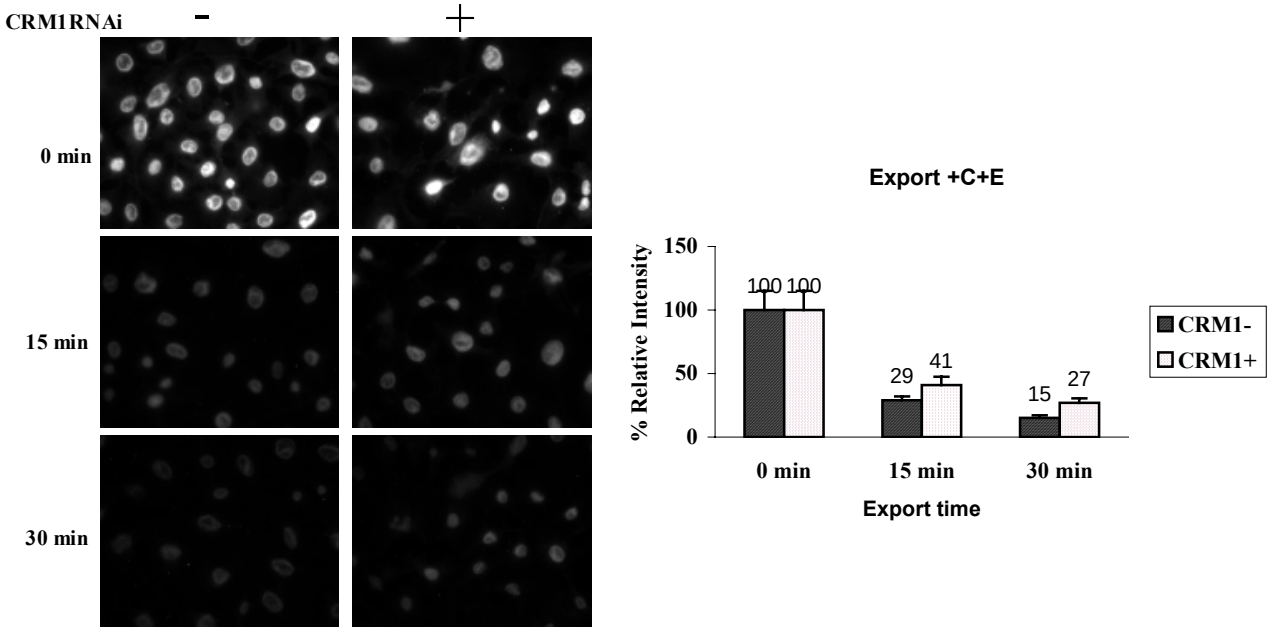
2.4. Import assays were performed in BJ cells with GFP-ERK2 for 15 min in transport buffer containing no added factors. For the export phase, cells were incubated for 30 min in transport buffer with cytosol and energy (+C+E), energy alone (-C+E), cytosol, energy and apyrase (+C+E+Apy), cytosol alone (+C-E) or cytosol and apyrase (+C-E+Apy). Each condition was performed in duplicate. Fluorescence data visualized after 24 h is presented.

Figure 2.5 Effect of CRM1 knock-down on the export of GFP-ERK2

2.5A

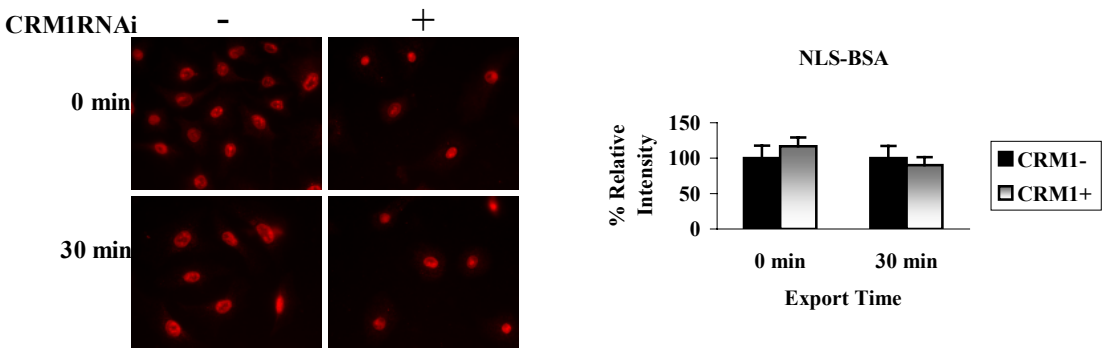


2.5B

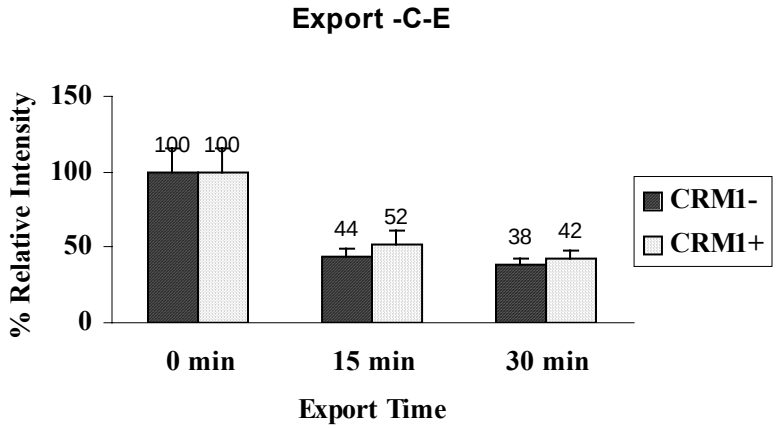


2.5A. Western blot showing knock-down of CRM1 protein (lanes marked +) compared to control (lanes marked -) in HeLa cell lysates. Total ERK blots are a control for specificity of knock-down and equal loading of protein. **2.5B**. Export assay of GFP-ERK2 in control (-) and CRM1 knock-down (+) HeLa cells. GFP-ERK2 was imported for 15 min in transport buffer without any added factors. Export was performed in the presence of cytosol and energy for the indicated times. Each condition was performed in duplicate. Fluorescence visualized and quantified from two independent experiments is presented.

2.5C



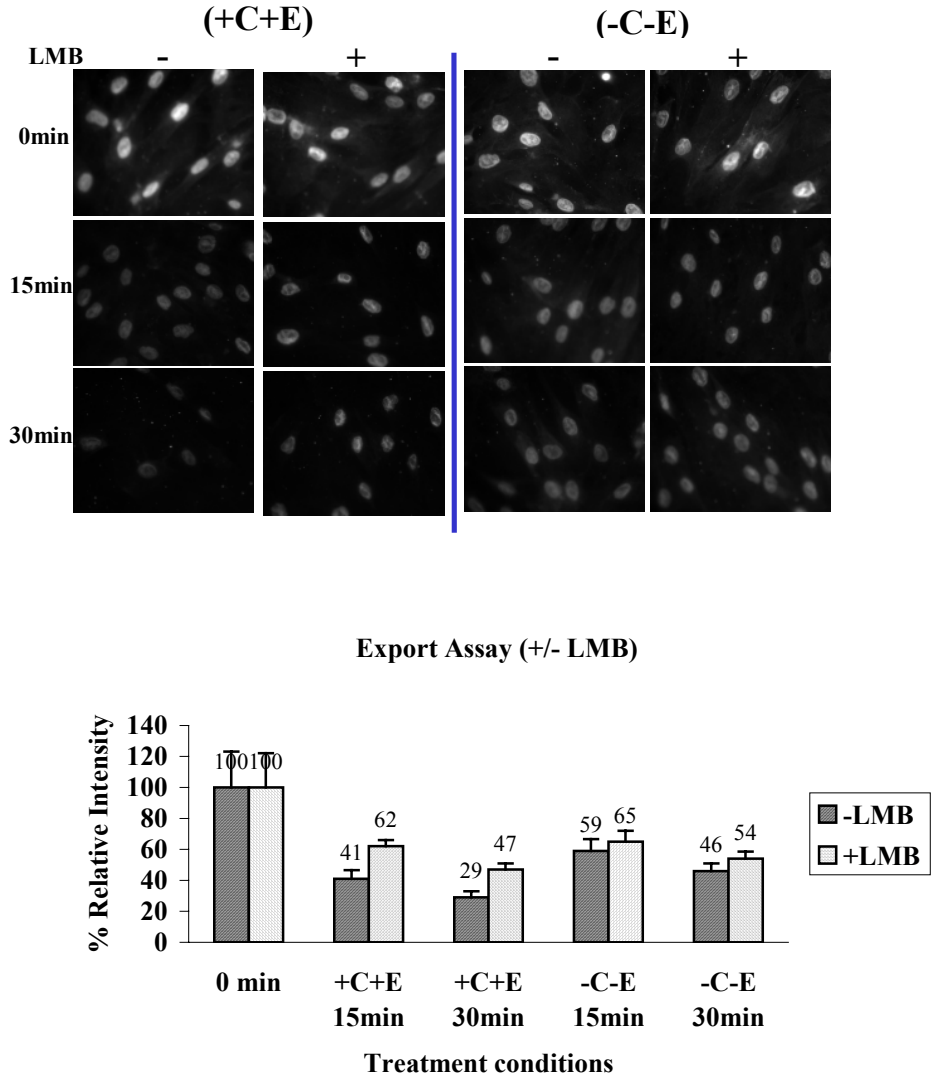
2.5D



2.5C Export assay of NLS-BSA in control (-) and CRM1 knock-down (+) HeLa cells. NLS-BSA was imported for 15 min in transport buffer with cytosol and energy. Export was performed in the presence of cytosol and energy for the indicated times. Flourescence visualized and the intensity quantified from two independent experiments is shown. **2.5D**. Flourescence intensity of GFP-ERK2 quantified from two independent experiments is presented. Import assay was same as in 2.5B. Export was performed in transport buffer without added factors for 0, 15 or 30 min.

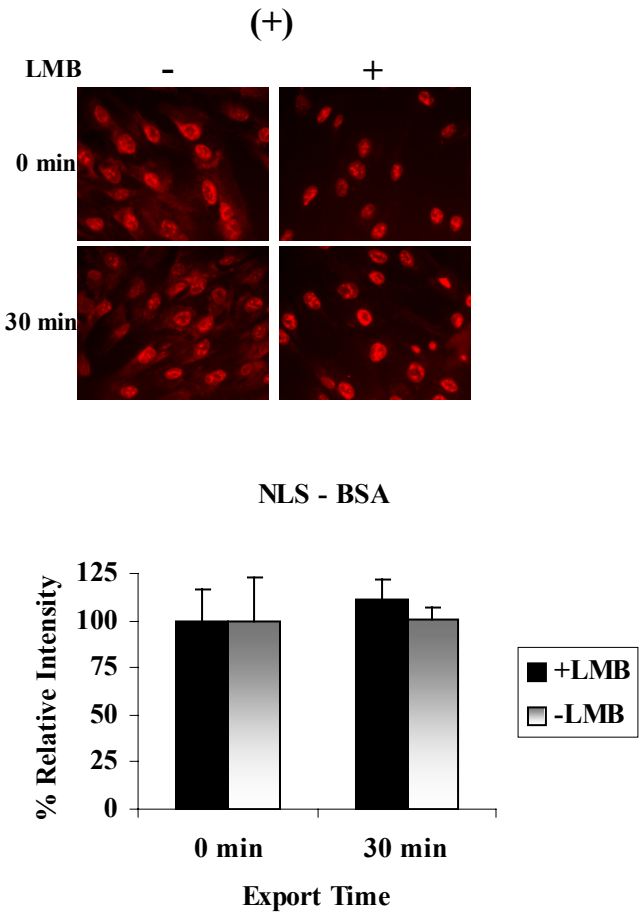
Figure 2.6 Effect of blocking CRM1 function on the export of GFP-ERK2

2.6A



2.6A. Export assay of GFP-ERK2 in control (-) and LMB treated (+) HeLa cells. HeLa cells and cytosol used in the export phase were treated with 20 nM LMB for 5 $^{1/2}$ h. Import was performed for 15 min in transport buffer. Export assay was performed in reaction mix either including cytosol and energy (+C+E) or not (-C-E) for the indicated times. Fluorescence visualized and quantified from two independent experiments is presented.

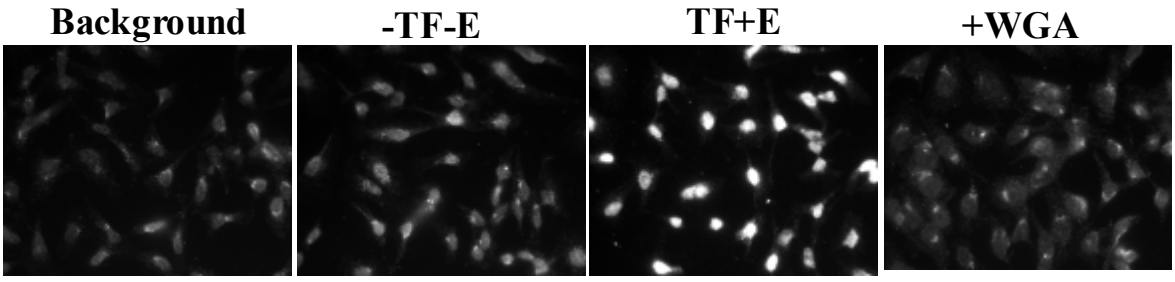
2.6B



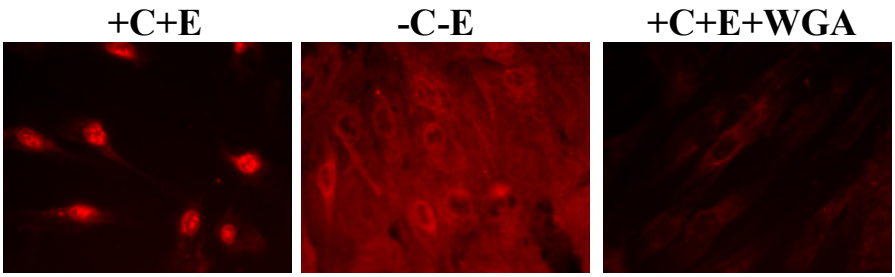
2.6B. Export assay of Rhodamine-labeled NLS-BSA in control (-) and LMB treated (+) HeLa cells. HeLa cells and cytosol used in the export phase were treated with 20nM LMB for 5 $^{1/2}$ h. Import and export were performed in transport buffer with cytosol and energy. Import was allowed for 15 min and export for the indicated times. Fluorescence visualized after 24 h and quantified data are presented.

Figure 2.7 Active form of ERK2 can enter the nucleus in the absence of transport factors and energy via the NPC

2.7A



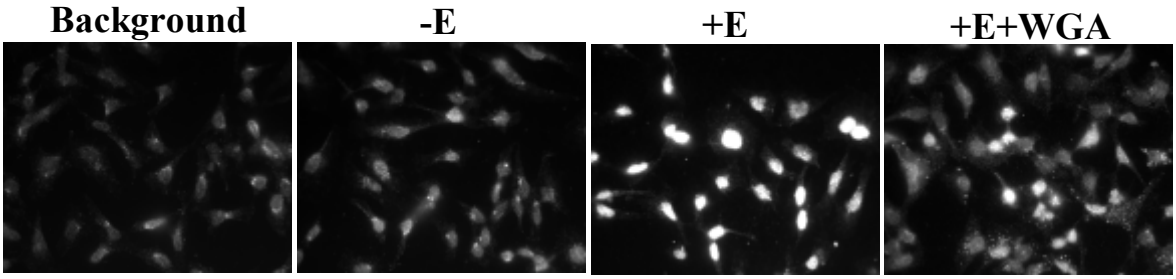
2.7B



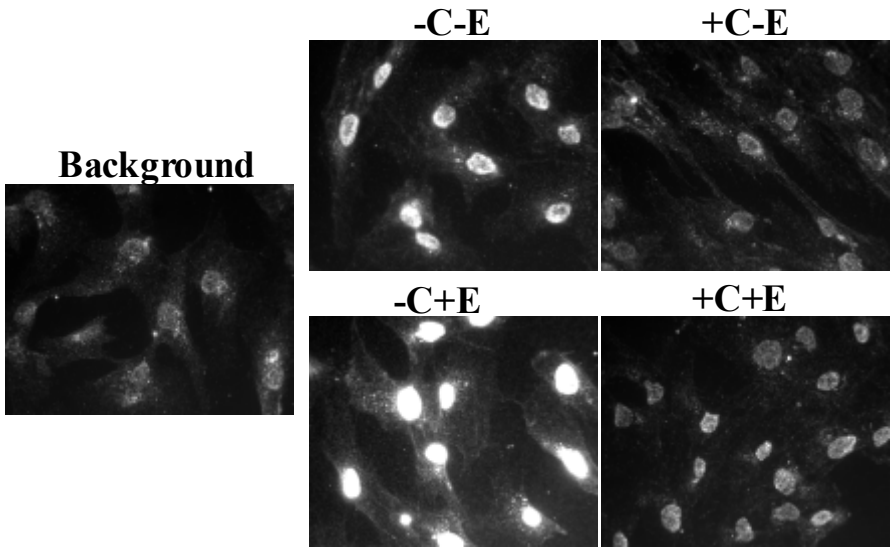
2.7A. Import of Thio-P ERK2 in BJ fibroblast cells. BJ cells were starved for 2 h (background) prior to performing the import assay. Import was allowed for 20 min in transport buffer alone (-TF-E), with transport factors and energy (+TF+E) or with WGA (+WGA). **2.7B**. Import of NLS-BSA in BJ fibroblast cells. Import was performed in the presence (+C+E) or absence (-C-E) of cytosol and energy or with cytosol, energy and WGA (+C+E+WGA) in the reaction mix. Each condition was performed in duplicate. Immunofluorescence visualized after 24 h is presented.

Figure 2.8 Import of Thio-P ERK2 in the presence of energy and cytosol

2.8A

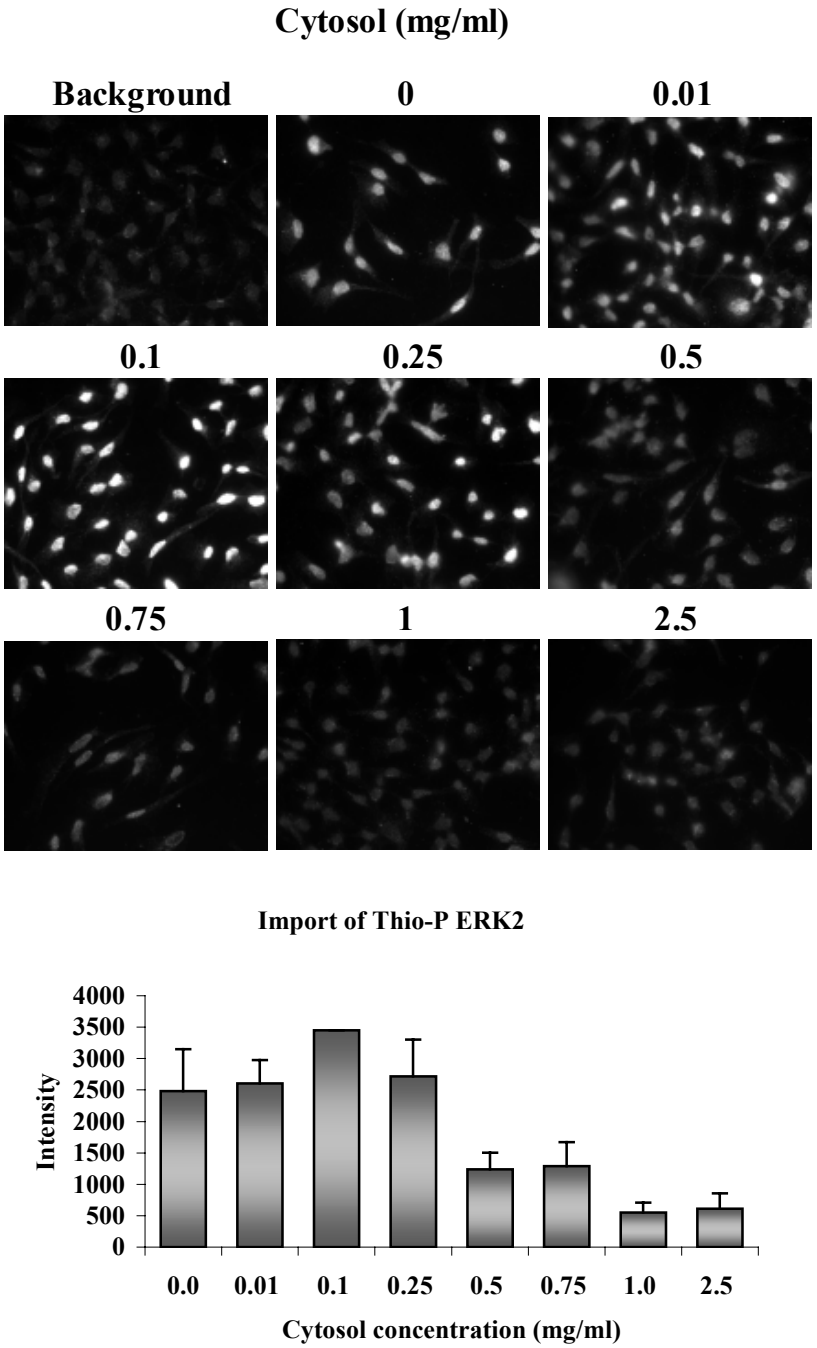


2.8B



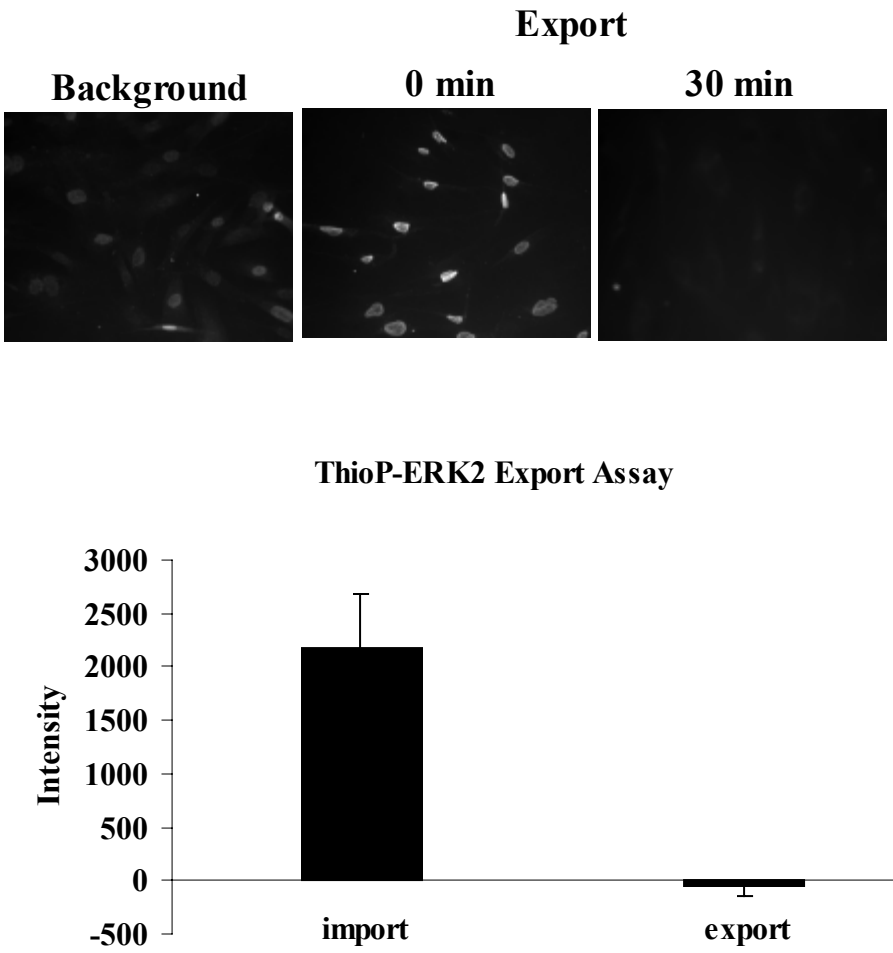
2.8A. Import of Thio-P ERK2 is enhanced in the presence of energy. BJ cells were starved for 2 h (background) prior to performing the import assay. Import was allowed for 20 min in the absence (-E) or presence (+E) of energy or with energy and WGA (+E+W). **2.8B**. Import of Thio-P ERK2 is inhibited in the presence of cytosol. BJ cells were washed three times in transport buffer after permeabilization. Import was carried out in the absence of cytosol and energy (-C-E) or presence of cytosol (+C-E), energy (-C+E) or both (+C+E). Each condition was performed in duplicate. Immunofluorescence visualized after 24 h is presented.

Figure 2.9 Thio-P ERK2 import is inhibited at higher concentrations of cytosol



2.9 BJ cells were starved for 2 h (background) prior to performing the import assay. Import of Thio-P ERK2 was performed for 20 min in transport buffer containing energy and the indicated concentrations of cytosol. Each condition was performed in duplicate. Immunofluorescence and quantified intensity data are presented.

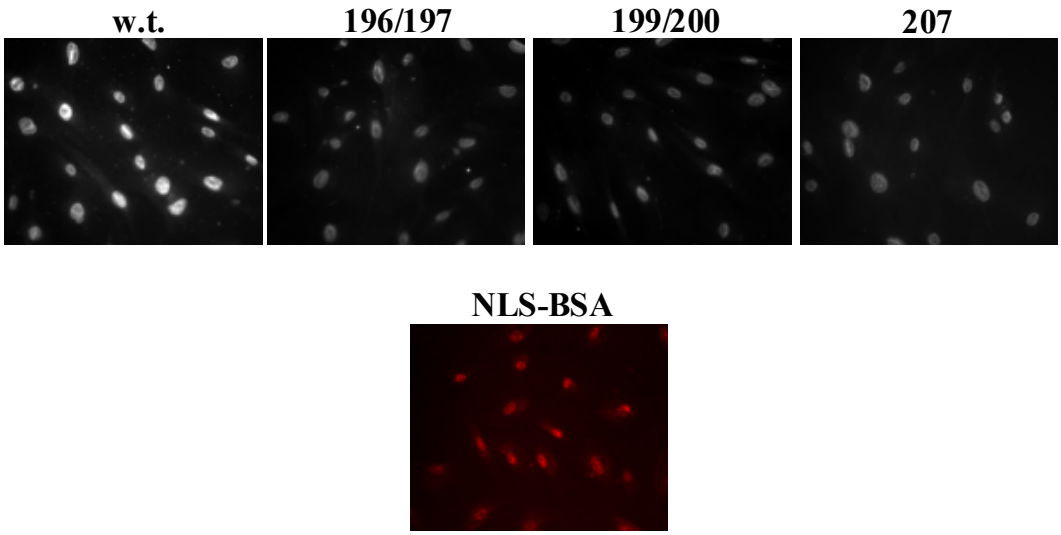
Figure 2.10 Export of Thio-P ERK2 can occur by a carrier- and energy-independent mechanism



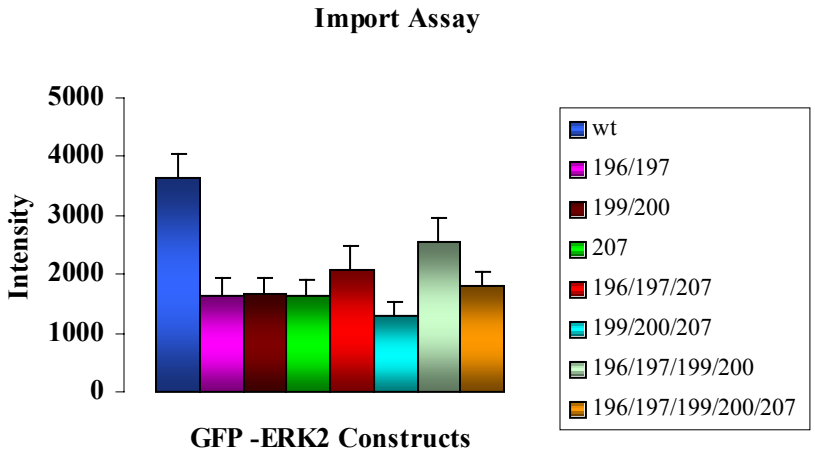
2.10 Thio-P ERK2 was imported into BJ fibroblast cells in transport buffer alone for 15 min (Export 0 min). Export was performed in the absence of transport factors and energy for 30 min. Immunofluorescence data visualized after 24 h is presented. Background indicates the immunofluorescence in BJ cells to which no exogenous Thio-P ERK2 has been added. Intensity was quantitated and background levels were subtracted. Quantitations are presented as a graph.

Figure 2.11 Import Assay of ERK2 mutants that are predicted to be impaired for binding to FXF motifs

2.11A

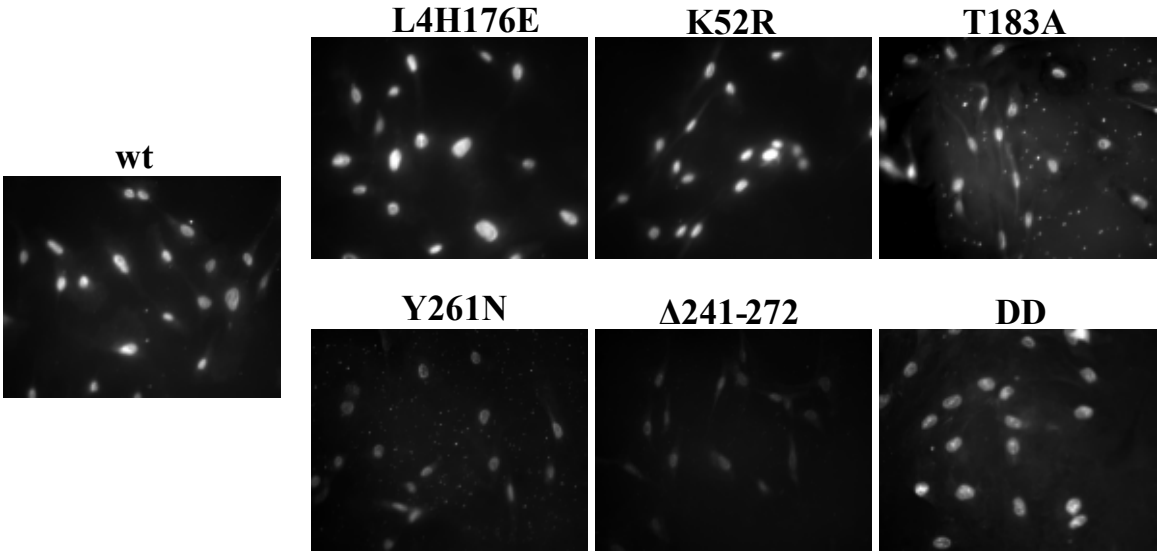


2.11B



2.11A Import of FXF binding mutants of GFP-ERK2 in BJ cells is impaired. Import was performed for 20 min in transport buffer with no added factors. Import of rhodamine-labeled NLS-BSA (as control) was performed in the presence of cytosol and energy for the same length of time. Each condition was performed in duplicate. Fluorescence data visualized after 24 h is presented. **2.11B** Intensity of fluorescence depicting import of GFP-ERK2 mutants was quantified from two independent experiments and is presented as a graph.

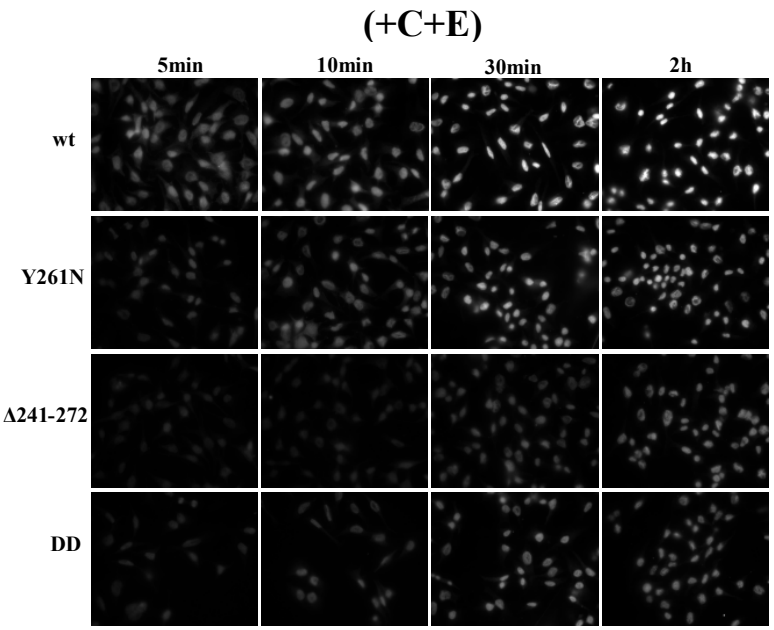
Figure 2.12 Import of various GFP-ERK2 mutants compared to that of wild-type protein



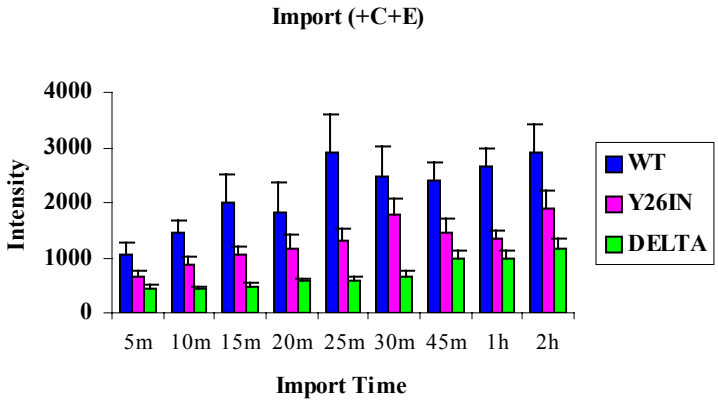
2.12 GFP-ERK2 wild-type (wt) protein and the different mutants of GFP-ERK2 i.e. L4H176E (dimerization-deficient mutant), K52R (an inactive form of GFP-ERK2), T183A (GFP-ERK2 that cannot be activated), Y261N, Δ241-272 and DD (mutants that are impaired for binding to MEK1) were imported for 15 min in transport buffer without any added factors in BJ cells. Fluorescence data visualized after 24 h is presented.

Figure 2.13 Time course of import of GFP-ERK2 mutants compared to wild-type protein

2.13A

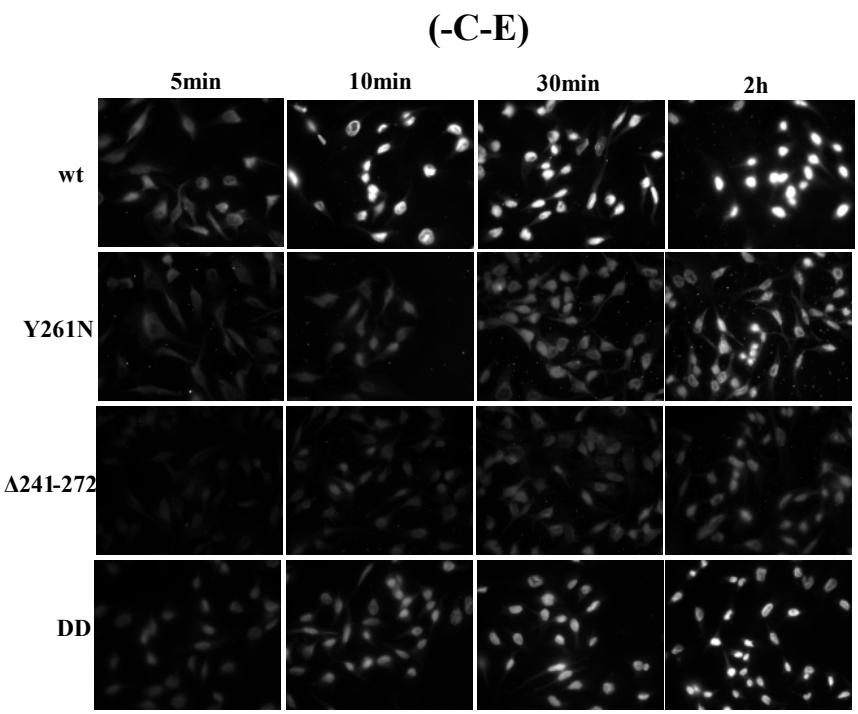


2.13B

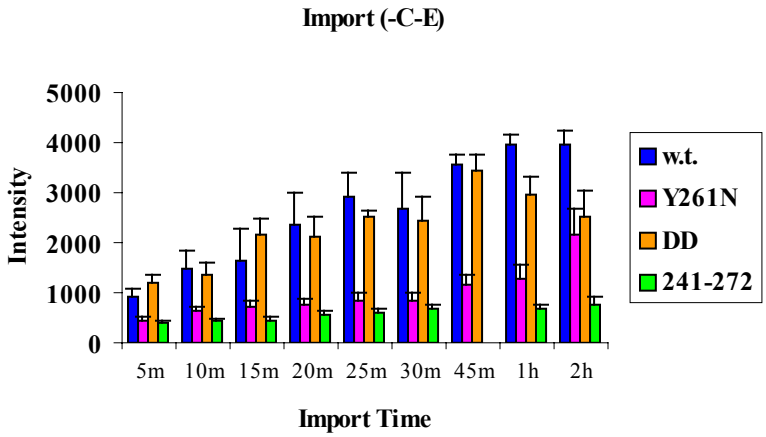


2.13A GFP-ERK2 wild-type protein (wt) and mutants that are impaired for binding to MEK1 (Y261N, Δ241-272 and DD) were imported for different times (from 5 min to 2 h) in transport buffer containing cytosol and energy (+C+E). Fluorescence visualized after 24 h is presented for some of the times. **2.13B** Fluorescence intensity within the nucleus was quantified for all times of import and a graphical representation of the same is shown. Each condition was performed in duplicate.

2.13C



2.13D



2.13C GFP-ERK2 wild-type protein (wt) and mutants that are impaired for binding to MEK1 (Y261N, Δ241-272 and DD) were imported for different times (from 5 min to 2h.) in transport buffer without any added factors (-C-E). Fluorescence visualized after 24 h is presented for some of the times. **2.13D** Fluorescence intensity within the nucleus was quantified for all times of import and a graphical representation of the same is shown. Each condition was performed in duplicate.

Discussion

In response to various cellular stimuli the MAPK, ERK2, elicits pleiotropic effects in the cell. In order to access its substrates in the nuclear and cytoplasmic compartments of the cell, ERK2 is continuously shuttled across the nuclear envelope. The mechanism governing transport of ERK2 through the NPC is slowly emerging. Most of the work done to understand these processes has involved use of over expressed mutant proteins that may not mimic the actual physiological phenomena or small molecule inhibitors that may block the function of unrelated components in the cell. Recently, our lab and others have successfully used an *in vitro* reconstitution assay system to delineate the mechanisms underlying inactive ERK2 import (183,184). This method provides for analyzing the requirement of individual cellular components for ERK2 nuclear transport and the relative contribution of each to the process. Here, we have used this system to address some of the outstanding issues governing export of inactive and active forms of ERK2 and import of active ERK2.

We were able to separate the export mechanism of unphosphorylated ERK2 into two distinct processes, an energy- and carrier-dependent process and an energy- and carrier-independent process. Both modes of transport occur through the NPC and hence, are facilitated. Using permeabilized cells it has been shown that the energy-independent import of inactive ERK2 requires access to nucleoporins (183,184) and import is probably mediated through direct interaction with them. It is tempting to assume a similar mechanism for export of inactive ERK2; for the component that occurs through the energy-independent process.

From RNAi experiments on the nuclear export receptor CRM1 and with use of the CRM1 inhibitor, LMB we conclude that the energy-dependent process of export is mediated through CRM1. Some of the export we observe after inhibition of CRM1 is probably due to the energy-independent process. However, blockade of CRM1 through RNAi or LMB did not completely eliminate the energy-dependent component of export. This raises the question, why does ablation of CRM1 only partially inhibit export? As mentioned before, other exportins, besides CRM1, have been identified that mediate the energy-dependent export process. So, one reason for partial inhibition of export could be that ERK2 is exported either by one of these exportins or by an, as yet, unidentified one. Second, there are technical concerns with the CRM1 knock-down and LMB inhibition experiments. On addition of CRM1 double strand RNA oligonucleotides, the cells begin to look unhealthy and start dying, as early as, at 36 h. Under these conditions, import and export of GFP-ERK2 may not be visible. The population of cells being monitored for differences in export may be the ones in which knock-down has not been very efficient. Also, in order to completely eliminate CRM1 in the assays, we tried to obtain HeLa cell cytosol from cells in which CRM1 expression had been knocked-down. We were unable to get adequately concentrated cytosol from these cells for use in these assays. The cytosol used in the export mix is from control HeLa cells and has not been depleted for CRM1. The amount of CRM1 present in the cytosol might be sufficient to mediate some energy-dependent export. LMB from different sources have been known to show varied effects on blocking CRM1 function (321). We have observed better inhibition of export with use of LMB from Sigma in our initial experiments.

Questions regarding details of the actual process are yet to be addressed. ERK2 has no recognizable NES and hence, may require binding to other NES containing proteins in order to be transported via the classical export machinery. One such protein is its upstream activator, MEK1. It has been shown that inactive ERK2 is weakly bound to MEK1 and may be exported to the cytoplasm as a result of this association (190) in a CRM1 dependent manner. This study used a NES-disrupted mutant of MEK1 to show reduced re-localization of ERK2 to the cytoplasm compared to that induced by wild-type MEK1, in intact cells. Other proteins that regulate the energy-dependent export phase of inactive ERK2, by a similar or different mechanism, may also exist. Alternatively, ERK2 may directly interact with CRM1 or karyopherin β family members bypassing the need for an NES. Interactions of MAPKs with transport factors have been reported before. Lorenzen et al showed binding between *Drosophila* ERK (D-ERK) and the homolog of importin7 (DIM-7) and Ferrigno et al found evidence, in yeast, for interaction between another MAPK family member, HOG1, the yeast p38 and a karyopherin β family member, NMD5 (322,323).

In our export assays, we also observed that, in the presence or absence of cytosol and energy, most of the export occurred within 30 min. The residual GFP-ERK2 remained in the nucleus even after 2 h. One reason for this could be the anchoring of ERK2 by nuclear proteins. Some studies have suggested this hypothesis and the following support this idea. Active ERK2 has been shown to bind kinetochores and topoisomerase II (91,92); nuclear retention of ERK2 seems to require newly synthesized protein anchors under activating conditions in a lung fibroblast cell line (79); and vanishin, a novel death-effector domain protein, binds ERK2 and when over-expressed

leads to nuclear accumulation of inactive ERK2 (95). The residual fluorescence observed in the presence of cytosol and energy at 30 min was ~50% less than that observed in their absence. It is possible that energy weakens binding of ERK2 to nuclear binding sites. It is less likely that an equilibrium between import and export phases accounts for the residual nuclear fluorescence because ERK2 exported from the nucleus quickly diffuses into the reaction mix and only very minimal cytosolic fluorescence is visualized in most experiments. We also observed substantially more export (>50% vs. 20%) in 30 min in the absence of cytosol and energy than that reported by Nishida and colleagues (184). We cannot account for this difference based on methodological issues.

In our attempt to understand the mechanism of phospho-ERK2 import, we found that, although an energy-independent process exists similar to that for unphosphorylated ERK2, in contrast, phospho-ERK2 is imported primarily by an energy-dependent mechanism. Energy-dependent import mediated via importins requires direct binding of cargo proteins through their NLS motif to the import factors (104,134). ERK2 does not contain an obvious NLS sequence. Hence, as for export, it is likely that the energy-dependent process of active ERK2 import is mediated by binding to transport factors or some other NLS containing protein/s. Modification of ERK2 through phosphorylation may allow tighter interaction with these proteins. This might explain previously observed enhanced nuclear accumulation of active ERK2 (64,74,75). It is also possible that phosphorylation unmasks an, as yet, unidentified NLS in ERK2.

We also found that cytosolic factors inhibit the import of phosphorylated ERK2. The reduced nuclear immunofluorescence observed is not due to enhanced export of phosphorylated ERK2 because we see accumulation of phospho-ERK2 when

recombinant transport factors are added. One simple explanation for the observed result could be the competition for import of phospho-ERK2 by unphosphorylated ERK2 in the cytosol. Our experiments show that transport factors enhance import of phospho-ERK2. In agreement with this, nuclear uptake increases with addition of low concentrations of cytosol. At this concentration, the amount of inactive ERK2 is not great enough to compete with active ERK2 for import. As the concentration of cytosol increases, a dose dependent decrease in import of active ERK2 is observed as a result of competition by inactive ERK2. In one preliminary experiment, I found that inactive ERK2 could compete for import of active ERK2.

A more interesting explanation would be that some regulatory component in cytosol, other than inactive ERK2 inhibits import of active ERK2. Reszka and colleagues, 1995 have shown the association of active ERK2 to microtubules in the cytoskeleton (85). It is likely that microtubules or other cytoskeletal components are in sufficient quantities in the exogenous cytosol added in these assays to sequester active ERK2 and prevent its entry into the nucleus. Some cytosolic anchor proteins for ERK2 have also been identified eg. Calponin (319) and PEA-15 (324). Whether these proteins are present in sufficient quantities in the cytosol to sequester ERK2 away from the nucleus needs to be investigated. If this second model is correct, then, the specific inhibition of phospho-ERK2 import observed in reconstitution assays has direct implication on biological outcomes regulated by discrete cellular pools of ERK2 in stimulated cells. ERK2 has substrates in both compartments of the cell. Its localization to the nucleus upon stimulation is not an obligatory event as was originally thought. Localization of active ERK2 is a regulated process that depends on cell-type, type of

stimulating ligand (Whitehurst AW. and Cobb MH. unpublished observation), sequestering molecules in the nucleus and cytoplasm (79,90) and probably interactions with scaffolds (325). The results from our experiment support the idea that cellular context of proteins is significant for their proper distribution within the sub-cellular compartments, in response to environmental signals.

Finally, it is possible that the inhibitory effect of cytosol on active ERK2 is due to indirect interactions of ERK2 held in a complex. The Thio-P ERK2 preparation used in these assays has some contaminating proteins. These proteins may bind Thio-P ERK2. The inhibition in import could be a result of one of these bound proteins being sequestered by cytosolic components.

Active ERK2 does not interact with MEK1 hence, it has been suggested that only the unphosphorylated form of ERK2 is exported. We have shown that phospho-ERK2 can be exported from the nucleus, at least, by an energy- and carrier independent mechanism. In addition to MEK1, other proteins may mediate export of ERK2 by the energy-dependent process. These proteins may also be capable of binding to the active form of ERK2. Whether an energy-dependent export process exists for active ERK2 needs to be investigated. A number of studies have reported nuclear accumulation of phospho-ERK2 in intact cells but its subsequent redistribution to the cytoplasm has not been recorded. We might be able to reconcile the contradictory results from *in vitro* and *in vivo* experiments by considering the implications of the study by Lenormand and colleagues in 1998 (79). They showed that nuclear accumulation of ERK2 in serum stimulated cells required neo-synthesis of nuclear anchor proteins. Probably, this phenomenon leads to anchoring of active ERK2 in the nucleus of intact cells. The *in vitro*

reconstitution system used in the export assay of Thio-P ERK2 does not provide the conditions for synthesis of new proteins. Hence, the active ERK2 that accumulates in the import phase is probably not anchored and can be exported within 30 min. Apart from this, we cannot over-look the fact that the loss of immunofluorescence visualized may actually be a reflection of the export of unphosphorylated ERK2 from the nucleus. Thiophosphorylation of a protein enhances its stability considerably yet K^+ ion can greatly enhance dethiophosphorylation (0.04 s-1 in the absence versus 0.54 s-1 in the presence of K^+) (320). The transport buffer used for reconstitution assays has 110 mM K^+ and may cause enhanced chemical de-thiophosphorylation of Thio-P ERK2. More rigorous analysis of the export process using active ERK2 is required to obtain a detailed picture.

We have identified some mutants of inactive ERK2 (in the region predicted to bind FXF motif on substrates) i.e. GFP-ERK2 I196A/M197A, GFP-ERK2 N199A/S200A and GFP-ERK2 I207A that are impaired for nuclear entry compared to the wild-type protein. The impaired nuclear transport of ERK2 mutants provides stronger evidence to support the notion that nuclear entry of ERK2 is mediated via interaction with nucleoporins. Wild-type ERK2 has been shown to interact directly with the FXF repeats of nucleoporins. Most probably, these ERK2 mutants bind nucleoporins less well. Their binding to nucleoporins has not been measured directly. Further, the extent of impairment of these ERK2 mutants (~50%) suggests that other regions of ERK2 may also play a role in nuclear transport. These mutants may be useful in future studies designed towards understanding different biological outcomes of activated ERK2, as a consequence of its different sub-cellular location.

It has been shown that a dimerization-deficient mutant of ERK2 accumulates in the nucleus less efficiently than the wild-type protein under stimulatory conditions (76), implicating a role for dimerization in nuclear localization of ERK2. We do not see a difference in reconstitution experiments. This suggests that dimerization of inactive ERK2 is not required for nuclear entry. Preliminary experiments showed no obvious impairment in the export of the dimerization mutant either. Although, more studies are required to confirm this observation, this suggests that more levels of regulation than those, thus far, identified in reconstitution assays may exist in cells. The unimpaired import of GFP-ERK2 K52R and GFP-ERK2 T183A is consistent with studies showing that neither activity nor activation of ERK2 is required for its nuclear uptake (76,83,84). The mutants impaired for binding to MEK1 GFP-ERK2 Δ241-272 and GFP-ERK2 Y261N (17,21) showed inhibition in nuclear import, which is not immediately intuitive. The region predicted to bind nucleoporins is located close to the MAPK insert and structural considerations may explain the reduced nuclear entry.

Future Directions

We have identified an energy-dependent process for export of inactive ERK2. The energy-dependent transport process is mediated via karyopherins and Ran and we have observed a modest enhancement in export of ERK2 in the presence of cytosol as compared to export in its absence. A more detailed analysis of the contribution of cytosolic factors to export of inactive ERK2 should be performed. First, export should be quantified in the presence of varying concentrations of cytosol. Then, the contribution of individual transport factors for the export process needs to be investigated. Reconstitution of energy-dependent export in permeabilized cells has been shown to require addition of recombinant CRM1 (where CRM1 mediated export is involved) and Ran and is greatly enhanced in the presence of RanBP1 (326). CRM1 binds the NES-cargo and Ran; Ran is required for directing export from the nuclear to the cytoplasmic side of the NPC and Ran BP1 stimulates GTP hydrolysis and release of cargo from the NPC. We can add these components individually or in combination to the export phase to restore energy-dependent export. Involvement of CRM1 in the export process may be investigated in further detail by adding increasing concentrations of recombinant CRM1 to the export phase.

In our attempts to identify a requirement for CRM1 in the export process, we were not able to obtain sufficient concentrations of cytosol from cells in which expression of CRM1 had been knocked-down, for use in export assays. As mentioned previously, we used cytosol from wild-type HeLa cells and the CRM1 present in this might have contributed to the observed export. Holaska et al have shown previously that CRM1 can be efficiently depleted from cytosol by treatment with phenyl-sepharose

(181). Cytosol obtained using this method may reveal a greater involvement of CRM1 on export of inactive ERK2 than what we have observed. Similarly, blocking CRM1 function by LMB showed only partial inhibition of export of ERK2 in the presence of energy. Previously, inactivation of CRM1 by N-ethyl maleimide has been exploited to study effects of CRM1 in export (181). Further, besides LMB, other inhibitors for CRM1, that block its function, have been identified recently i.e. Ratjadone-C and PKF050-68 (321,327). The effects of some of these inhibitors are reversible and may serve as better reagents to analyze CRM1 mediated export.

One other possible explanation for the incomplete inhibition of export observed upon blocking CRM1 mediated pathways could be that other exportins besides CRM1 are involved in the process. Comparing export of ERK2, in the presence of whole cell cytosol vs. recombinant CRM1 protein, may provide an insight into the contribution from other export factors. Although a difficult proposition, fractionation of cytosol may allow identification of such a component.

CRM1 has been shown to bind cargo proteins through their NES. As mentioned previously, since, ERK2 has no obvious NES, export of ERK2 mediated by CRM1 probably occurs through direct binding to CRM1 or through interaction with other NES containing proteins (adaptors). Direct binding of CRM1 to ERK2 may be analyzed by using recombinant proteins. Further, possibility of formation of a complex between CRM1, ERK2 and some other adaptor may be tested by co-immunoprecipitations of proteins from cell lysates.

A critical role for MEK1 has been implicated in ERK2 transport. Direct involvement of MEK1 in ERK2 transport across the nuclear envelope may be shown

using recombinant MEK1 protein in *in vitro* assays. Our preliminary investigations on the effect of MEK1 showed a cytoplasmic retention function for MEK1 in import reconstitution assays, as has been previously described in intact cells (80,81). Interpretation of results from these assays may be more difficult due to the added effects of phosphorylation of ERK2 by recombinant wild-type MEK1, in the presence of energy. Use of kinase-dead MEK1 that still retains the ability to shuttle between the nucleus and cytoplasm, in *in vitro* assays, may yield interpretable results. Also, whether MEK1 is required for ERK2 transport through the NPC may be determined by performing *in vitro* import and export assays in cells where expression of MEK1 has been knocked-down using RNAi.

We have shown that phosphorylated ERK2 enters the nucleus predominantly by an energy-dependent process. Again, detailed analysis of the contribution of energy and transport factors to the process is pending. The requirement of transport factors for this process has to be investigated in detail using individual recombinant proteins in *in vitro* assays. The energy dependence of the process may be further characterized by studying time-dependent import of active ERK2 in the presence of energy. To delineate the difference in mechanism of import between unphosphorylated and phosphorylated ERK2, detailed comparison of the import process in the presence of transport factors and energy of the two forms of ERK2, has to be undertaken. A difference in energy-dependent import of the active and inactive forms of ERK2 might explain the observed nuclear accumulation of ERK2 upon activation in most cell types. Like inactive ERK2, active ERK2 can also enter the nucleus by an energy- and carrier-independent process. This process is facilitated and occurs through the NPC. Whether the mechanism of entry for

the two forms is similar, in the energy-independent process, can be evidenced by competition assays. Increasing concentrations of recombinant inactive ERK2 may be used to compete for import of active ERK2. Preliminary experiments have indicated that inactive ERK2 competes with active ERK2 for entry into the nucleus. Direct binding of inactive ERK2 to the FG repeats on nucleoporins most likely mediates its import (183,184). Our import experiments with mutants of inactive ERK2, that are predicted to be impaired for binding to FG repeats on nucleoporins, further strengthen this notion. Yet, direct evidence for impairment in binding of these mutants with the FG repeats of nucleoporins, compared to wild-type ERK2, has to be demonstrated. Active form of ERK2 has also been shown to interact with substrates, like Elk1, through the FXF motif. The key residues in active ERK2 required for the interaction with FXF motif on substrates i.e. Elk1 peptide, have been identified in peptide protection assays by hydrogen exchange mass spectrometry (61). It is likely that the same residues of phosphorylated ERK2 interact with the FG repeats on nucleoporins. Comparison of import of Thio-P ERK2 mutants, that harbor mutations at these residues, with the wild-type Thio-P ERK2 protein will confirm whether these residues play a role in import of active ERK2. Further, this would suggest whether the mechanism for entry of the active form of ERK2 through the NPC is mediated by direct interaction with nucleoporins.

We noted an inhibition in import of phospho-ERK2 in the presence of cytosol. In order to analyze this effect without influence from transport factors in the cytosol, which enhance import in the presence of energy, import assays must be performed with active ERK2 in the absence of energy, with increasing concentrations of cytosol. To determine whether the observed effect is due to competition by unphosphorylated ERK2,

import assays of active ERK2 may be performed with cytosol depleted (using anti-ERK2 antibody) of ERK2. Further, the component/s in cytosol that inhibit import may be identified by fractionation techniques.

We have shown that export of phospho-ERK2 can occur by an energy- and carrier-independent mechanism. Whether an energy-dependent process of export exists for active ERK2, as for the inactive form, needs to be investigated. Also, export of active and inactive ERK2 under different conditions may be compared to gain further insight into the process of ERK2 nuclear transport. Understanding the mechanisms governing transport of ERK2 across the nuclear envelope will enhance our knowledge of how sub-cellular localization is regulated.

Chapter III

The MAPK ERK5 binds to and phosphorylates p90RSK

Abstract

We showed previously that p90 RSK was activated in cells expressing an activated mutant of MEK5, the kinase activator of the MAP kinase ERK5. Here we show that ERK5 directly activates RSK, and we have used RSK to explore the basis of substrate recognition by ERK5. ERK5 binds to RSK *in vitro* and in cells; activation of ERK5 weakens its binding to RSK. Based on the interactions of mutants, we found that, in ERK5 a cluster of negatively charged residues, often referred to as the common docking or CD site, near a hydrophobic docking groove (conserved in MAPKs) and the docking (D) domain of RSK are important for their association. In contrast, the long C-terminal tail of ERK5 is dispensable for the interaction. Stimulation of ERK5 but not p38 or the c-Jun N-terminal kinase is associated with activation of RSK in cells. *In vitro* phosphorylation of RSK by ERK5 increases its activity indicating that RSK is a direct substrate of ERK5. These data support the conclusion that ERK5, as well as ERK1/2, can promote activation of RSK in cells.

Introduction

Protein kinase cascades control responses to extracellular cues by transmitting signals throughout the cell. Prominent among multifunctional enzymes in kinase cascades are the mitogen-activated protein kinases (MAPKs) (1,3). MAPKs are activated via phosphorylation of tyrosine and threonine residues by highly selective MAPK kinases (MAP2Ks) also known as MKKs or MEKs (328,329). These dual specificity protein kinases are activated by MAPK kinase kinases (MAP3Ks). The active MAPKs subsequently phosphorylate a variety of substrates throughout cells including membrane and cytoskeletal proteins, downstream protein kinases, and transcription factors ((1,3).

Although MAPKs are activated by many ligands and stimuli, the extent of activation of individual MAPKs by a spectrum of agents differs. ERK5 is stimulated nearly equally well by growth factors and cellular stress (194,198,200,201,209,214). This is in contrast to the MAPKs ERK1/2, which are more responsive to growth factors, and the stress-activated protein kinases, JNKs and the p38 MAPKs, which are more responsive to cellular stress. The MAP2K that activates ERK5 is MEK5. MEK5 is present in two major splice forms that appear to have different functions (193,246,248). MEKK2 and MEKK3 are the best documented MAP3Ks that activate MEK5 (222-225,251); these MAP3Ks also have some ability to control the activities of JNKs and p38 MAPKs, which may in part account for the stress-sensitivity of this kinase pathway (330). ERK5 has also been linked to Raf, although Raf is not believed to be a MAP3K for this MAPK (214) and Src, which may in part account for the sensitivity of ERK5 to growth factors (211,254).

ERK5 substrates include the MADS box transcription factors MEF2A, C, and D (198,244), the Ets class transcription factor Sap1a (201), the serum and glucocorticoid responsive kinase, SGK1 (245) and the gap junction protein connexin 43 (246). The phosphorylation of MEF2 transcription factors may allow ERK5 to influence expression of c-Jun (226). Compared to other MAPKs, little is known about ERK5 substrate specificity. Although the number of identified substrates is smaller than for the other well-studied MAPKs, in its known exogenous substrates ERK5 phosphorylates Ser/Thr-Pro motifs typical of the MAPK family. But interestingly, some of the sites autophosphorylated by ERK5 and those phosphorylated on upstream kinase MEK5 do not conform to this specificity (199). ERK5 is larger than most MAPKs. It has a C-terminus of about 400 residues following its protein kinase domain (1,192), which has been proposed to be involved in recognition of substrates (196).

We previously reported that ERK5 is capable of stimulating nuclear factor-κB (NF-κB) (231). Our data suggested that this function might be attributed in part to ribosomal protein S6 kinase (known as RSK or p90RSK), which was activated by coexpression with ERK5 and a constitutively active form of its MAP2K, MEK5DD. Here we demonstrate that RSK, among the first known substrates of the ERK1/2 MAPKs, is also directly phosphorylated and activated by ERK5. We have used RSK to explore the basis of substrate recognition by ERK5.

Experimental Procedures

Plasmids and Reagents

The following plasmids were as described in the indicated references: pK3H-HA-RSK2, pK3H-HA-RSK2K100A, pK3H-HA-RSK2Y707A and pK3H-HA-RSK2 (1-729) (62,305); pcDNA3-Flag-ERK5 and pGEX-KG-GST-ERK5kin (194); pCMV5-HA-MEK5B, pCMV5-Myc-MEK5DD, and pCMV5-Myc-MEK5KM (214) and pCMV5-Myc-WNK1 (225). pCS3^{+}MT-Myc-MEK7DD (MEK7 – (332)), pCMV5-HA-MEK4DD (MEK4 – (333)), and the ERK5 mutants ERK5K83M, ERK5D363A, and ERK5D363AD366AE367A were generated by site-directed mutagenesis using the Quikchange kit (Stratagene) according to the manufacturer's protocol. All mutants were sequenced to confirm that no other changes had been introduced in the sequence.

The monoclonal HA antibody (12CA5) was from Berkeley Antibody Company and Roche. The anti-Flag antibody (M2 α-Flag) and rabbit polyclonal anti-ERK5 antibody for immunoblotting were from Sigma. Anti-ERK1/2 Y691 was as described (194). Goat polyclonal anti-ERK5 antibody used for immunoprecipitation, rabbit anti-RSK1, mouse anti-RSK2 and rabbit anti-p38 were from Santa Cruz Biotechnology.

Recombinant proteins

All GST fusion proteins were expressed in and purified from *E. coli* strain BL21DE3 using only minor adjustments to the standard protocol (334). Expression of GST-S6 was induced using 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 37^{o} C and that of GST-ERK5kin as described previously (194). Protein concentrations were estimated using serial dilutions of BSA as standards on gels stained

with Coomasie blue. Bing-e Xu generously provided GST-MEF2C (204-321). Histone 7S was purified as described (335).

Cell Culture, Transfection and Preparation of Cell Lysates

COS7 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 100 units/ml penicillin/streptomycin at 37^{o} C under 10% CO_2. COS7 cells were transfected at 50-80% confluence with the indicated plasmids using the Fugene6 reagent (Roche) according to the manufacturer's protocol. The ratio of Fugene6 reagent to DNA was maintained at 3:1 or 3:2. Cells were starved for 20-24 h prior to harvesting and lysates were prepared 48 h after transfection in buffer containing 40 mM Hepes pH 8.0, 150 mM NaCl, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EGTA, 100 mM sodium fluoride, 1 μg/ml pepstatin, 1 μg /ml leupeptin, 10 mM benzamidine and 1 mM dithiothreitol. Triton X-100 (0.5%) was included in the lysis buffer to prepare lysates for protein kinase assays. To analyze binding of endogenous proteins, HeLa cells grown to 80% confluence were starved for 2 h prior to treatment with 10 or 100 ng/ml EGF for 10 min and harvested into the lysis buffer above.

Immunoblotting

Proteins were separated on 8% polyacrylamide gels in sodium dodecyl sulfate and transferred electrophoretically onto nylon membranes for immunoblotting. Membranes were blocked in Tris-buffered saline with 1% Tween-20 (TBST) containing 1% milk and 2% BSA for 1 h or overnight. Primary antibodies were incubated in blocking buffer for 1 h or overnight. Anti-HA, anti-Flag, anti-ERK5 and anti-p38 antibodies were used at 1:1000, anti-RSK1 and anti-RSK2 were used at 1:400 and anti-ERK1/2 was used at

1:2500. Secondary antibody incubations were performed for 15 min at 1:1500. Blots were developed using enhanced chemiluminescence (ECL).

Immunoprecipitation

Lysates containing equal amounts of protein were precleared using 20 µl anti-Myc antibody and 30 µl protein A-Sepharose beads for 1 h or overnight. Immunoprecipitations were at 4^{o} C using anti-ERK5 antibody at 1:50, anti-HA antibody at 1:100 and anti-Flag antibody at 1:200. Lysates were incubated with the indicated antibody for 2 h for immune complex kinase assays and overnight for co-immunoprecipitation, prior to incubation with 30 µl protein A-sepharose beads for 2 h. Beads were washed 4X with buffer containing 25 mM Tris-HCl (pH 7.4), 1 M NaCl, 0.1% Triton X-100 and 0.1% deoxycholate and twice with 10 mM Hepes (pH 7.4). Proteins were released from beads by boiling in 20 µl H_2O+20 µl 5X electrophoresis sample buffer for 3 min at 100 ^{o} C.

Protein Kinase Assays

Immunoprecipitates were incubated in 30 µl of kinase buffer containing 10 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 50 µl ATP and [γ-^{32}P] ATP for 30 min at 30 ^{o}C. Histone 7S, GST-S6, GST-MEF2C (204-321), and over-expressed p90-RSK2 immunoprecipitated from COS7 cells were used as substrates. For coupled kinase assays, the first reaction mixture was sedimented for 2 min at 14,000 X g and the pellet was washed three times with 10 mM Hepes (pH 7.4). The beads were then incubated in kinase buffer with the second substrate for 30 min at 30 ^{o}C. Kinase reactions were terminated using 8 µl of 5X sample buffer and boiled at 100 ^{o}C for 3 min. Incorporation

of radioactive phosphate was detected by autoradiography and quantitated by liquid scintillation counting of excised bands.

In vitro Binding Assays

50 μg of recombinant GST-tagged protein or GST alone bound to glutathione-agarose beads was incubated with HeLa cell lysates at 4 ^{o}C for 2 h. Beads were washed 4X in 20 mM Tris (pH 7.5), 1 mM dithiothreitol, 1 mM EGTA, 0.1 M phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin. Proteins were released into 5X electrophoresis sample buffer by boiling at 100^{o}C for 3 min.

Results and Discussion

RSK activity is increased upon activation of ERK5 and ERK1/2, but not JNK or p38

A group of AGC protein kinases are substrates of MAPKs. RSK was the first of these identified as a substrate for ERK1/2 (51). Although RSK has been thought to be a substrate for ERK1/2 but not other MAPK family members, the related RSK-like kinases MSK1 and MSK2 are activated by two MAPKs, ERK1/2 and p38 (53). Previously we showed that RSK activity increased in cells coexpressing MEK5DD (an activated mutant of MEK5) along with ERK5 (231). This suggested that ERK5 might directly activate RSK. Therefore, to evaluate the ability of ERK5 and other MAPK family members to regulate RSK, we expressed HA-tagged RSK in cells with activators of four MAPKs: MEK1R4F to activate ERK1/2, MEK5DD and ERK5, MEK6DD to activate p38, and either MEK4DD or MEK7DD to activate JNK (Fig. 3.1A, B). RSK was immunoprecipitated and assayed using histone 7S as a substrate (335). RSK activity was stimulated by coexpression with activators of ERK1/2 and ERK5 but not by activators of p38 or JNK. These results confirm our earlier finding that activation of ERK5 leads to increased RSK activity. In addition, the lack of activation of RSK by p38 and JNK support a variety of studies that have failed to link RSK activation to these pathways (336).

ERK5 phosphorylates and activates RSK2 in vitro

To determine whether or not RSK was activated directly by ERK5, active Flag-ERK5 was immunoprecipitated from cells that had been cotransfected with MEK5DD. Kinase activity towards the substrate MEF2C and autophosphorylation confirmed that the protein was active (Fig. 3.2A). Active ERK5 also phosphorylated RSK2 that had been

immunoprecipitated from transfected cells using an anti-HA antibody (Fig. 3.2B). A catalytically defective mutant of ERK5 (ERK5KM) phosphorylated neither MEF2C nor RSK, supporting the conclusion that phosphorylation of RSK was catalyzed by ERK5, not some associated kinase (Fig. 3.2A, B). To determine the effect of phosphorylation by MAPKs on RSK activity, a coupled assay was performed using GST-S6 as RSK substrate. Phosphorylation of RSK2 by ERK5 *in vitro* increased its activity towards GST-S6 as much as 8-fold (Fig. 3.2C, E) as did ERK2 immunoprecipitated from cells (Fig. 3.2D). Although stimulation of p38 did not cause activation of RSK in cells, p38 did phosphorylate RSK and increase its activity *in vitro*. It was reported previously that JNK activates RSK3 *in vitro* (337), in spite of the fact that we do not find activation of RSK by coexpression with either MEK4DD or MEK7DD. These results indicate that multiple MAPKs have the capacity to recognize the activating sites of phosphorylation on RSK *in vitro*. Importantly, we find that the specificity in cell reconstitution experiments is greater than that *in vitro*, implying that factors in addition to substrate specificity determine the outputs from these kinase cascades.

ERK5 and RSK are associated in cells

In addition to substrate specificity, protein concentration, localization, and extended binding interactions determine the targets of protein kinases in cells. Extended binding interactions have been identified in MAPKs that facilitate the proper targeting of substrates to activators. RSK is known to bind tightly to ERK2 in cells. One possible explanation for the specificity noted in cell reconstitution experiments is that RSK not only binds stably to ERK1/2 but also to ERK5. Thus, we examined the potential association of RSK with ERK5. RSK was co-expressed with ERK5 with or without

MEK5DD. ERK5 immunoreactivity was found in the RSK immunoprecipitates (Fig. 3.3A), and RSK kinase activity assayed with GST-S6 was found in the ERK5 immunoprecipitates (Fig. 3.3B). RSK activity associated with ERK5 was increased in cells also expressing MEK5DD to activate ERK5. These findings support the idea that the specificity of activation of RSK by ERK5 arises from their interaction.

Some studies have shown that binding of RSK to ERK2 is lost once ERK2 is activated (295). Thus, we tested effects of ERK5 activators on the binding of RSK to ERK5. The ERK5 associated with HA-RSK was examined in cells co-transfected with a series of proteins that cause different extents of ERK5 activation; these included MEK5DD, MEKK3 (a MAP3K for ERK5), and WNK1, which is a weak ERK5 activator as a consequence of its MAP4K activity (225). Co-expression of wild type MEK5B, which does not promote ERK5 activation, was used as a control. ERK5 activation was detected by noting the amount of its slower mobility form by immunoblotting. Increased ERK5 activity was readily detected in cells transfected with MEKK3 and MEK5DD (Fig. 3.4). RSK was immunoprecipitated using the anti-HA antibody and the associated ERK5 was analyzed. Immunoprecipitation was also performed using the anti-ERK5 antibody and binding of RSK was analyzed. Activation of ERK5 generally decreased its binding to RSK (Fig. 3.4A, lanes 2 and 3, Fig.3.4B, lanes 2 and 4), consistent with the reduction noted in Fig. 3.3A. As a control, we examined the association of endogenous ERK2 with RSK expressed in COS cells with or without MEK1R4F. HA-RSK was immunoprecipitated and the precipitates were probed for endogenous ERK2 (Fig. 3.4C). As expected, binding of RSK to ERK2 was detected only if ERK2 was not activated by MEK1R4F.

Finally, we determined if endogenous ERK5 and RSK were associated in cells. Endogenous ERK5 was immunoprecipitated from HeLa cells. Both endogenous RSK1 and RSK2 were present in the ERK5 immunoprecipitates (Fig. 3.5A and 3.5B). In agreement with results above, activation of ERK5, in this case by EGF, appears to weaken their association, as it does with ERK2 and RSK. These studies show that endogenous RSK isoforms bind tightly enough to ERK5 to co-immunoprecipitate under a variety of conditions.

Mutation of catalytic residues influences ERK5-RSK binding

Because activation of ERK5 weakened its interaction with RSK, we characterized the interaction between catalytically inactive proteins in which the Lys required for phosphoryl transfer has been mutated. Inactive ERK5 (ERK5 K83M) was co-expressed with RSK2 in COS cells; either ERK5 (Fig. 3.6A) or RSK (Fig. 3.6B) was immunoprecipitated. The kinase-dead form of ERK5 bound to RSK better than did the wild type protein, consistent with the idea that residual ERK5 activity even in unstimulated cells weakens their association. We also used the comparable kinase-dead form of RSK2 (RSK2 K100A) and the constitutively active form of RSK2 (RSK2 Y707A) to determine the influence of RSK2 activity on its binding to ERK5. Both constitutively active RSK2 Y707A and the wild type protein bound ERK5, while RSK2 K100A showed a decreased interaction with ERK5 compared to either wild type RSK or RSK2 Y707A (Fig. 3.6C-E). The observation that kinase-dead RSK binds less well to ERK5 is surprising, because the D-domain binding site (Fig. 3.7) is at the C-terminus of RSK. In the case of ERK2-RSK interaction, autophosphorylation of RSK, following its activation, on a C-terminal site near the D domain promotes their dissociation (336).

Thus, the prediction might be that kinase-dead RSK would bind tighter to ERK5. The C-terminus is not presumed to be embedded in the folded structure; therefore, it is interesting that changes in the structure of the RSK catalytic core impact the interactions accessible to its C-terminus. The converse has been suggested from crystallographic analysis, i.e., that interaction of D domains with MAPKs influences MAPK structure; this is more easily rationalized from structural considerations (20). However, the current findings imply that the catalytic core of RSK communicates with its own D domain. Perhaps the reduced ability to bind ATP that is expected to occur upon mutation of K100 results in an altered conformation of the protein, one that functionally reduces accessibility of the D domain to its docking site.

The ERK5 kinase domain is sufficient for interaction with RSK1

MAPKs contain distinct binding sites for interaction with complementary motifs on substrates. The best studied of these regions of MAPKs is a cluster of negatively charged residues, often referred to as the common docking or CD site, near a hydrophobic docking groove (Fig. 3.7A) (17,20). This region lies across α-helix D almost on the opposite face relative to the kinase active site (20). This groove present in MAPKs binds to D motifs within substrates and a range of other interacting proteins (338). D-motifs on substrates contain basic and hydrophobic residues in either orientation that display remarkable selectivity in interacting with one or a subset of the MAPKs (Fig. 3.7B) (58,338). Previously, activation of RSK in cells was shown to be dependent on a reverse D-motif near its C-terminus that displays specificity for ERK1/2 but not p38 (62). Removal of the docking site prevented activation of truncated RSK in cells expressing it, in spite of the fact that truncated RSK was activated to an equivalent

extent as the wild type protein *in vitro*. Residues known to be involved in the common docking groove are conserved in ERK5 and the association of ERK5 with MEF2C has been shown to occur via this motif (339). Besides this, the C-terminal extension of ERK5 has been proposed as a site of substrate interactions (196).

To determine regions of ERK5 that are important for substrate interactions, we expressed the protein in two fragments, a catalytic core comprising the N-terminal half of the protein (1-409) and a C-terminal fragment of ~475 amino acids (339-816) as glutathione S-transferase (GST) fusion proteins (194). These fragments were used for *in vitro* pull-down assays with lysates from HeLa cells (Fig. 3.8A). The ERK5 kinase domain was sufficient for interaction with RSK1 and association decreased when HeLa cells were stimulated with EGF for 10 min. Although no interaction was detected with the C-terminal fragment, due to its poor stability, we cannot rule out the possibility that RSK also binds to this region of ERK5 (data not shown).

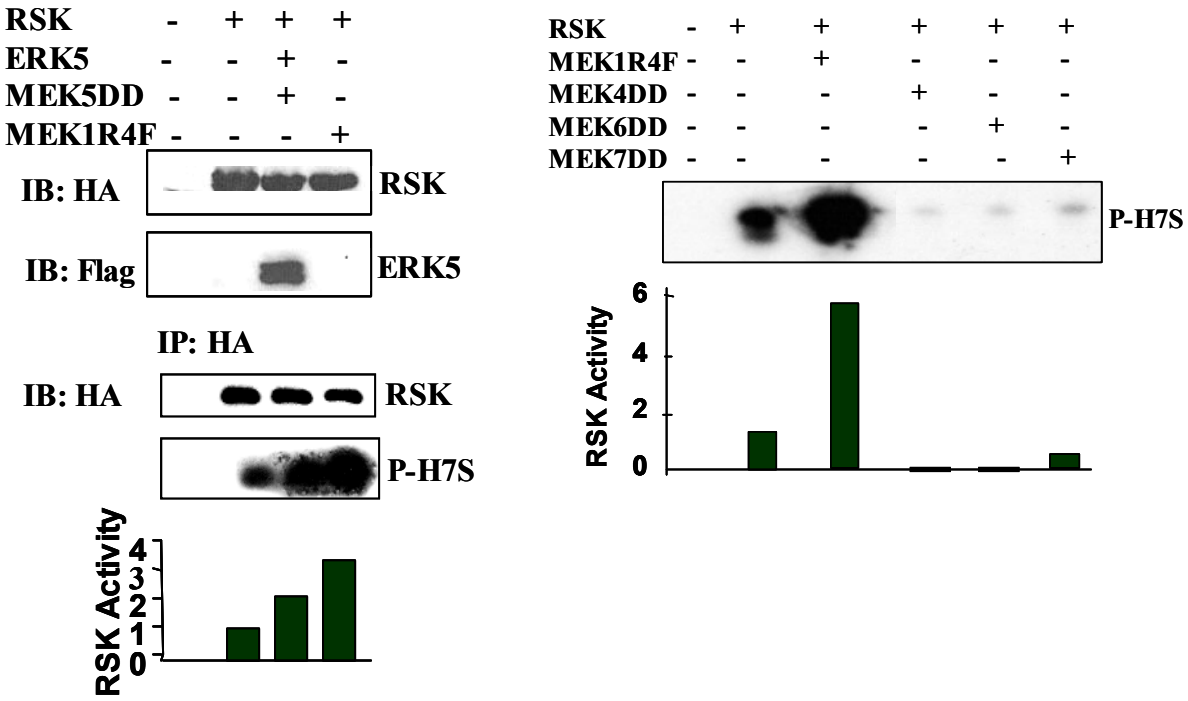
RSK binding occurs through its D domain interaction with the CD region of ERK5

We next determined if the common docking groove in the catalytic core of ERK5 is involved in the interaction by examining binding to ERK5 mutants in which acidic residues in the common docking site were replaced with alanine, ERK5D363A and ERK5D363AD366AE367A (Fig. 3.8B). EGF activated the mutant proteins in a manner comparable to wild type ERK5 based on reduced electrophoretic mobility (data not shown). We found that RSK bound less well to these ERK5 mutants. To confirm that the RSK D-domain was involved in this interaction, we examined binding of ERK5 to RSK2 lacking the C-terminal 11 amino acids (RSK2 (1-729)) (Fig. 3.8C). Immunoblotting for HA-RSK2 showed only a weak interaction of the RSK truncation

mutant with ERK5 compared to wild type RSK. The experiments with RSK truncations and ERK5 mutants strongly indicate that the interactions of ERK5 with substrates follow similar patterns to those established for other members of the MAPK family. Regions of extended substrate interactions, a docking groove on the kinase core structure binding to a D domain, have a major impact on cellular specificity of the family.

Figure 3.1 RSK activity is increased upon activation of ERK5 and ERK1/2, but not JNK or p38.

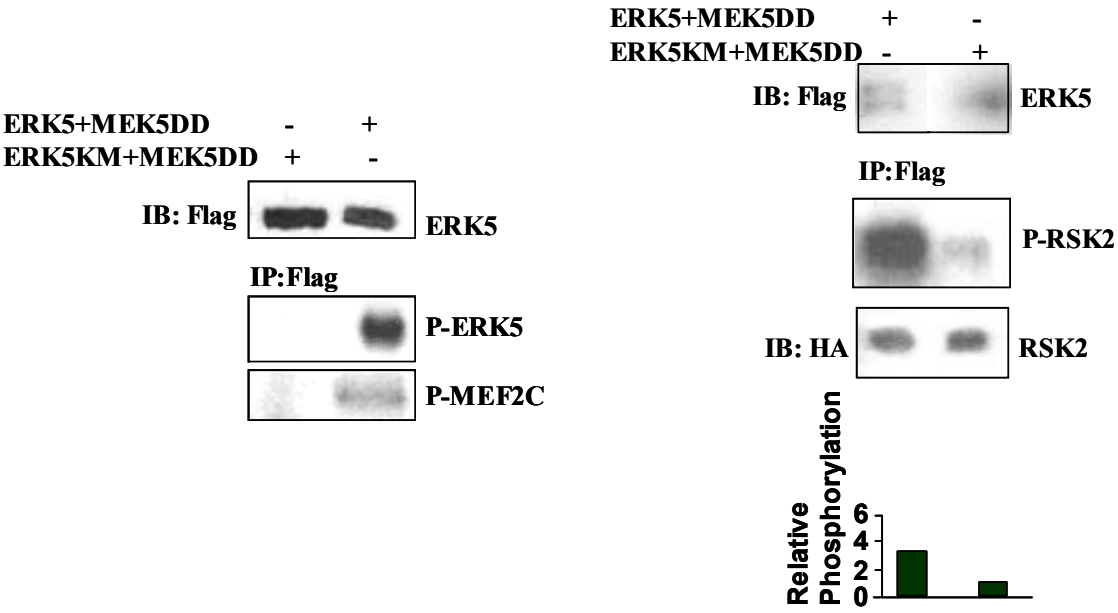
3.1A **3.1B**



A. pcDNA3-HA-RSK was transfected into COS cells alone (lane 2), with pcDNA-Flag-ERK5 and pCMV5-Myc-MEK5DD (lane 3), or with pCMV5-MEK1R4F (lane 4). RSK was immunoprecipitated with anti-HA for immune complex kinase assay with H7S as substrate. Immunoblots show amounts of proteins in cell lysates and immunoprecipitates. Autoradiogram shows phosphorylation of H7S. Data are also plotted as fold activation relative to basal activity of RSK alone. **B.** COS cells were co-transfected with pcDNA3-HA-RSK alone (lane 2) or with pCMV5-MEK1R4F (lane 3), pCMV5-HA-MEK4DD (lane 4), pSRα-HA-MEK6DD (lane 5), or pCS3^{+}MT-Myc-MEK7DD (lane 6). RSK activity was measured as in A. Autoradiogram shows phosphorylation of H7S. Data are shown as fold activation relative to basal activity of RSK.

Figure 3.2 ERK5 phosphorylates and activates RSK2 *in vitro*.

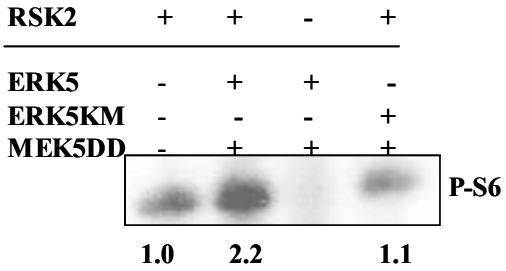
3.2A **3.2B**



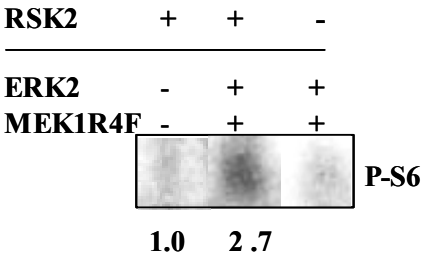
A. ERK5 constructs were tested for activity in kinase reactions using GST-MEF2C as substrate. pcDNA-Flag-ERK5K83M (lane 1) or pcDNA-Flag-ERK5 (lane 2) was co-transfected with pCMV5-Myc-MEK5DD in COS cells and ERK5 was immunoprecipitated using anti-Flag. Autoradiogram shows ERK5 autophosphorylation and phosphorylation of MEF2C by ERK5. Immunoblot to detect expression of ERK5 in lysates is also shown. **B**. pCMV5-Myc-MEK5DD was co-transfected into COS cells with pcDNA-Flag-ERK5 (lane1) or with pcDNA-Flag-ERK5K83M (lane 2). ERK5 was immunoprecipitated using anti-Flag and subjected to *in vitro* kinase assays using over-expressed RSK2 immunoprecipitated from COS cell lysates as substrate. Immunoblots detect expression of ERK5 in lysates and RSK2 in immunoprecipitates. Autoradiogram shows phosphorylation of RSK2 by ERK5. Data are also shown as fold phosphorylation relative to control.

Figure 3.2 (contd.) ERK5 phosphorylates and activates RSK2 *in vitro*.

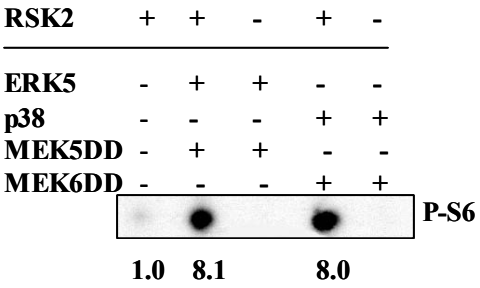
3.2C



3.2D



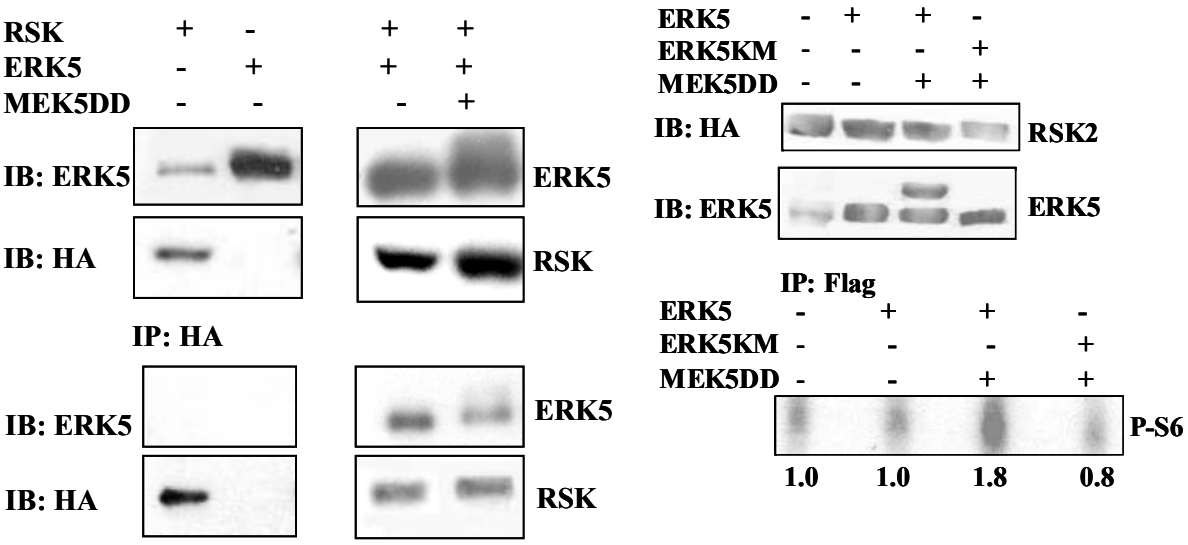
3.2E



C. pcDNA-Flag-ERK5 or pcDNA-Flag-ERK5K83M was co-transfected into COS cells with pCMV5-Myc-MEK5DD (lanes 2, 3 and 4). ERK5 was immunoprecipitated with anti-Flag and assayed using over-expressed RSK2 immunoprecipitated from COS cell lysates as substrate (lanes 2 and 4). RSK2 alone was used as a control (lane 1). Following phosphorylation of RSK2, a coupled assay was performed using GST-S6 as substrate. Autoradiogram shows phosphorylation of GST-S6 by RSK2. **D.** COS cells were co-transfected with pCMV5-MEK1R4F and pCEP4-HA-ERK2 (lanes 2 and 3). ERK2 was immunoprecipitated and coupled kinase assays were performed as in C. **E.** COS cells were co-transfected with pCMV5-Myc-MEK5DD and pcDNA-Flag-ERK5 (lanes 2 and 3) or with pSRα-HA-MEK6DD and pSRα-HA-p38 (lanes 4 and 5). ERK5 or p38 was immunoprecipitated and coupled kinase assay were performed as in C.

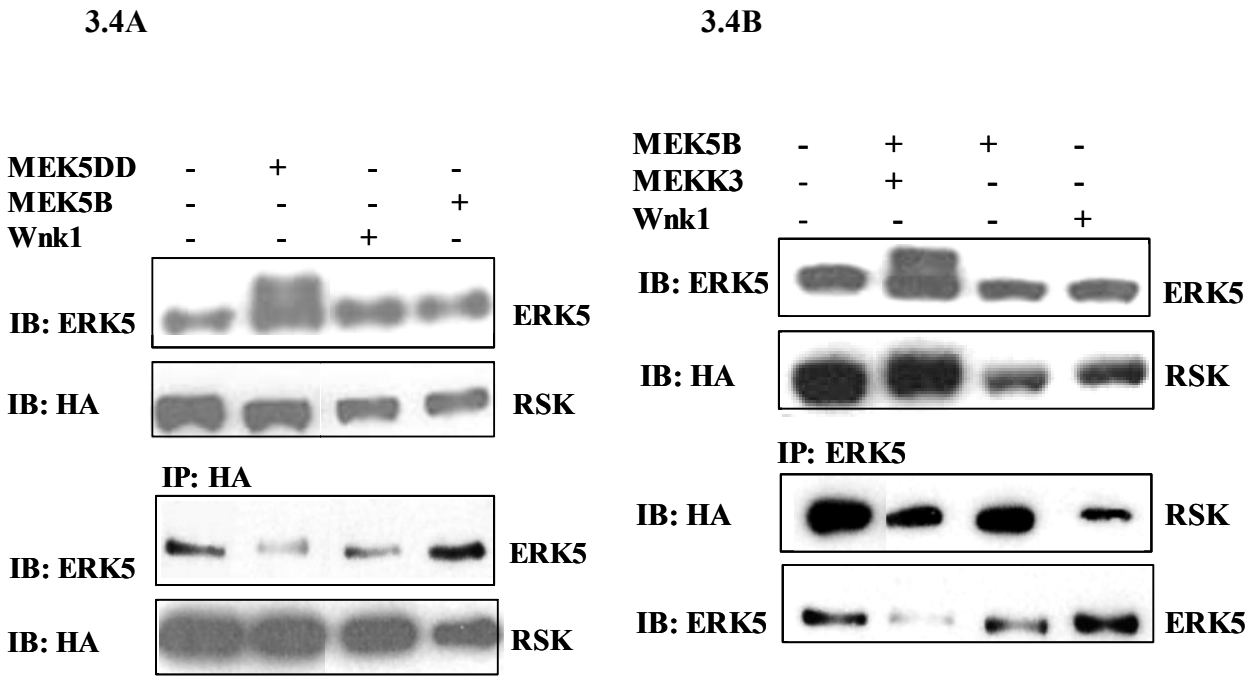
Figure 3.3 RSK associates with ERK5.

3.3A **3.3B**



A. COS cells were transfected with pcDNA-HA-RSK (lane 1), pcDNA-Flag-ERK5 (lane2), both (lane 3) or both with pCMV5-Myc-MEK5DD (lane 4). RSK was immunoprecipitated using anti-HA antibody and binding of ERK5 was analyzed by immunoblotting. Immunoblots of proteins in lysates are also shown. **B.** pK3H-RSK2 was transfected alone (lane1), with pcDNA-Flag-ERK5 (lane 2), with pcDNA-Flag-ERK5 and pCMV5-Myc-MEK5DD (lane 3) or with pcDNA-Flag-ERK5K83M and pCMV5-Myc-MEK5DD (lane 4). ERK5 was immunoprecipitated using anti-Flag and assayed using GST-S6 as substrate. Immunoblots of proteins in lysates are also shown. Autoradiogram shows phosphorylation of GST-S6 by RSK2.

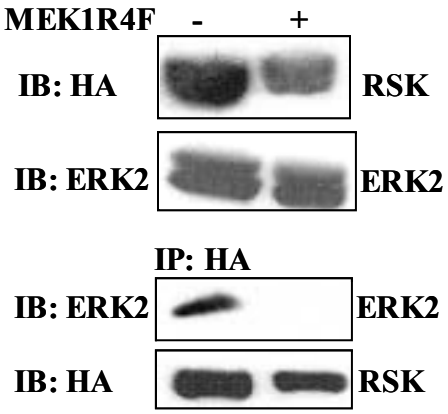
Figure 3.4 Association of over-expressed RSK and ERK5.



A. pcDNA-Flag-ERK5 and pcDNA-HA-RSK were co-transfected into COS cells alone (lane1), with pCMV5-Myc-MEK5DD (lane2), with pCMV5-Myc-WNK1 (lane 3), or with pCMV5-HA-MEK5B (lane 4). Immunoprecipitation was performed using anti-HA. Immunoblotting detected proteins in lysates and immunoprecipitates. **B**. pcDNA-Flag-ERK5 and pcDNA-HA-RSK were cotransfected into COS cells alone (lane1), with pCMV5-HA-MEK5B and pCMV5-HA-MEKK3 (lane 2), with pCMV5-HA-MEK5 (lane 3), or with pCMV5-Myc-WNK1 (lane 4). Immunoprecipitation was performed using anti-ERK5 and binding of RSK was analyzed. Immunoblots detect proteins in lysates and immunoprecipitates.

Figure 3.4 (contd.) Association of over-expressed RSK and ERK5.

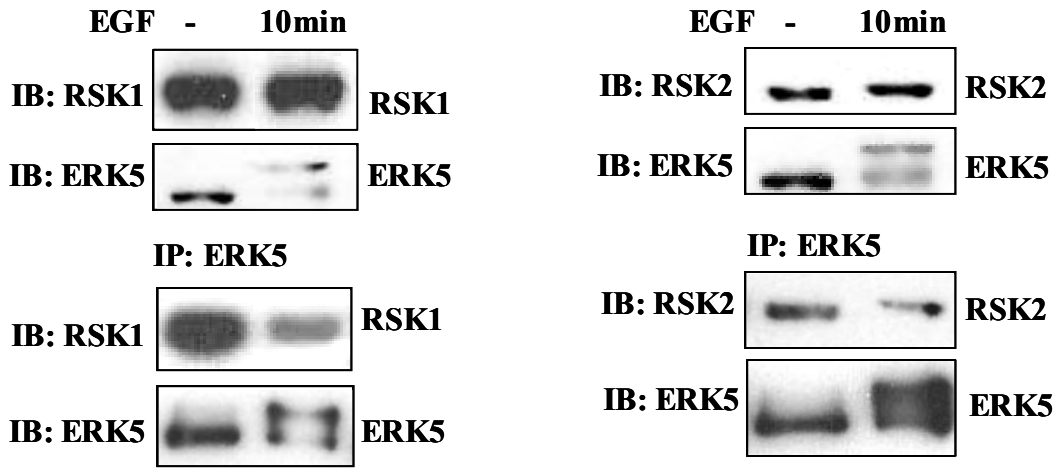
3.4C



C. COS cells were transfected with pcDNA-HA-RSK alone (lane1) or with pCMV5-MEK1R4F (lane2). RSK was immunoprecipitated with anti-HA and binding of endogenous ERK2 was analyzed by immunoblotting. Immunoblots are also shown for expression of over-expressed RSK and endogenous ERK2 in lysates.

Figure 3.5 Endogenous RSK and ERK5 proteins co-immunoprecipitate.

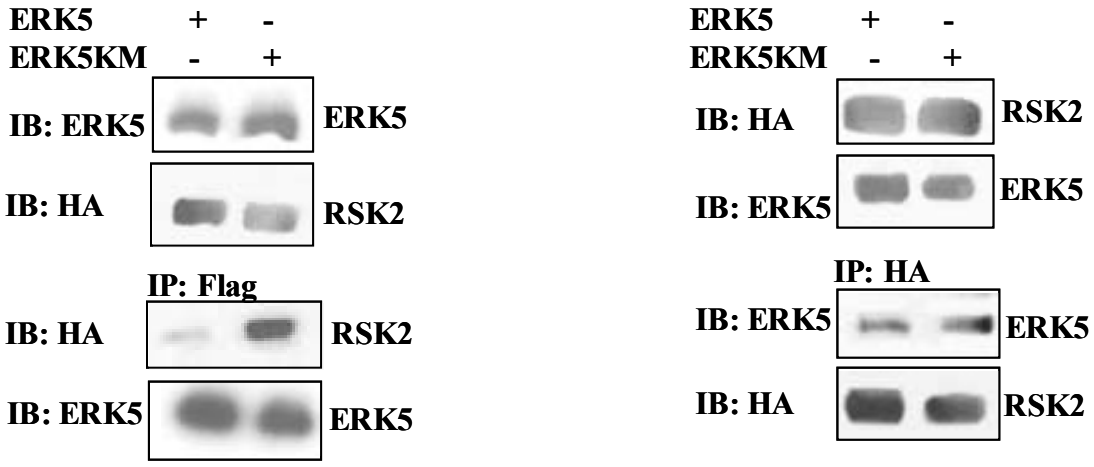
3.5A **3.5B**



ERK5 was immunoprecipitated from lysates of Hela cells either untreated or treated with 100 ng/ml EGF. Immunoblots show expression of proteins in lysates and detect co-immunoprecipitation of **A.** RSK1 and **B.** RSK2 with ERK5.

Figure 3.6 Interaction between ERK5 and RSK mutants.

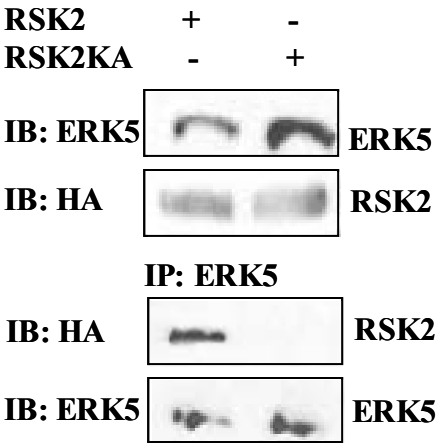
3.6A **3.6B**



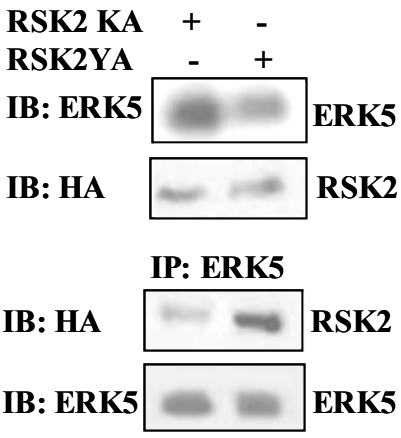
A. pK3H-HA-RSK2 was co-transfected into COS cells with pcDNA-Flag-ERK5 or pcDNA-Flag-ERK5K83M. ERK5 was immunoprecipitated with anti-Flag and binding of RSK2 was analyzed. **B**. RSK2 was immunoprecipitated from cells using anti-HA and binding of ERK5 was analyzed.

Figure 3.6 (contd.) Interaction between ERK5 and RSK mutants.

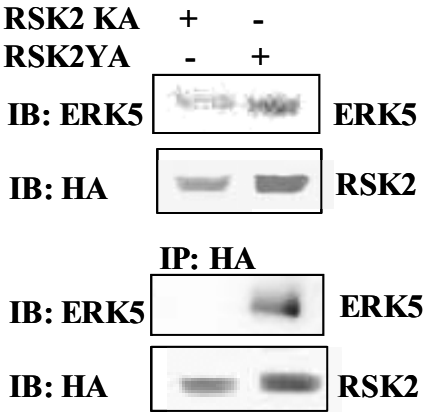
3.6C



3.6D



3.6E



(C, D, and E). pK3H-HA-RSK2, pK3H-HA-RSK2K100A or pK3H-HA-RSK2Y707A was co-transfected into COS cells with pcDNA-Flag-ERK5. **(C and D).** ERK5 protein was immunoprecipitated with anti-ERK5 and binding of RSK2 was analyzed. **(E).** RSK2 was immunoprecipitated using anti-HA and binding of ERK5 was analyzed. Immunoblots show expression of proteins in lysates and detect binding in immunoprecipitates.

Figure 3.7 Schematic representation of the CD domain on MAPKs and D-domain on substrates

3.7A

ERK2	**311**	**LEQYYDPS DEPI AE**	**324**
ERK5	**347**	**LAKYHDPDDEPDCP**	**361**

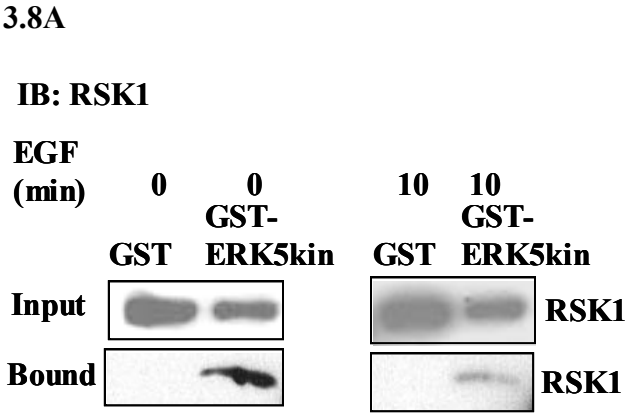
A. Alignment of CD domain in ERK2 and ERK5. The highlighted residues indicate the hydrophobic and negatively charged residues conserved between the two proteins. The underlined residues in ERK5 were mutated either singly or in combination to determine the importance of this motif.

3.7B

mRSK1	**712**	**L KPI ES SI LAQRRVK KLP - STTL**	**733**
mRSK2	**718**	**L EPVGR STLAQRRG I KKI TSTAL**	**740**
mRSK3	**686**	**L EPVLS SNLAQRRGMKRL TSTRL**	**708**
chkRSK	**731**	**L KPI ES SI LAQRRV KKL P - STTL**	**752**
hMSK1	**732**	**LQNVDK APLAKRRKMKKT STSTE**	**754**
mMSK2	**721**	**L KSVEN APLAKRRKQ KLR SA**	**740**
mMNK1	**383**	**L S PPS K SRLARRRA L AQAGRSRD**	**405**
mMNK2	**383**	**L S PPS Q SKLAQRRQR AS L SATPV**	**405**

B. Alignment of D-domains on MAPK substrates. The underlined residues are conserved in 6 of the 8 isoforms. Highlighted residues are conserved in all.

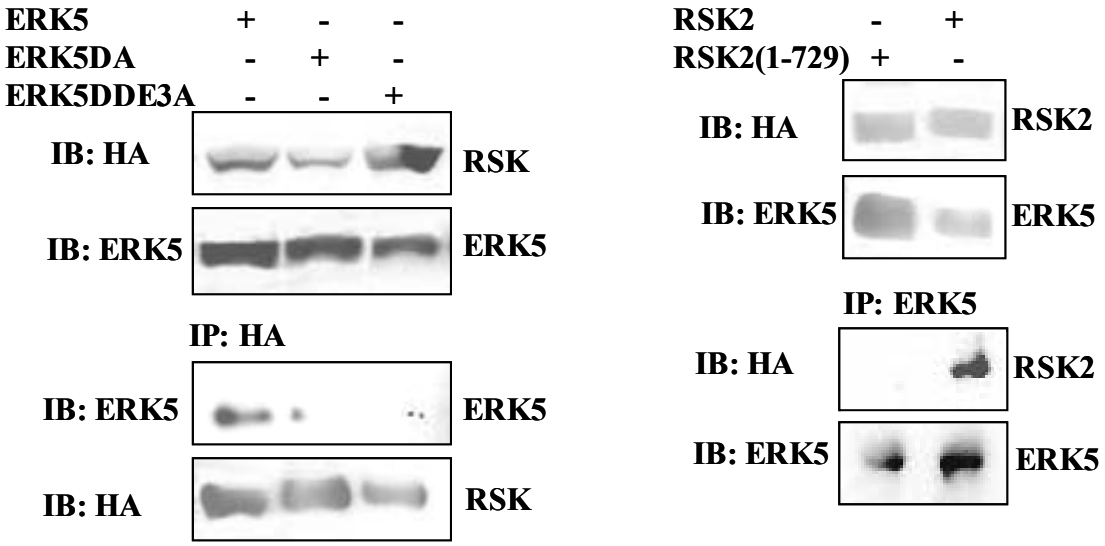
Figure 3.8 Identification of the regions of ERK5 and RSK that interact.



A. Interaction of endogenous RSK1 from Hela cells with GST-ERK5kin (1-409) bound to glutathione beads was analyzed by immunoblotting. Expression of RSK1 in Hela cell lysates is also shown.

Figure 3.8 (contd.) Identification of the regions of ERK5 and RSK that interact.

3.8B **3.8C**



B. pcDNA-Flag-ERK5, pcDNA-Flag-ERK5D363A or pcDNA-Flag-ERK5D363AD366AE367A was co-transfected into COS cells with pcDNA-HA-RSK. RSK was immunoprecipitated with anti-HA and binding of ERK5 was analyzed by immunoblotting. Expression of proteins in lysates is also shown. **C.** pcDNA-Flag-ERK5 was co-transfected into COS cells with pK3H-HA-RSK2 or pK3H-HA-RSK2(1-729). ERK5 was immunoprecipitated with anti-ERK5 and binding of RSK2 proteins was analyzed by immunoblotting. Expression of proteins in lysates is also shown.

Conclusions and Future Directions

We have shown that p90RSK is a direct substrate for ERK5 *in vitro*. Activation of the ERK5 pathway in cells is sufficient to activate p90RSK. We have established this by over-expressing the different components (MEK5DD, ERK5 and p90RSK) in COS cells. Over-expression of proteins, in excess of intracellular concentrations, can lead to events that seem physiological but may not actually reflect the normal *in vivo* processes. It is necessary to confirm the sufficiency and requirement of the ERK5 pathway for p90RSK activation under more physiologically relevant conditions. This might be achieved by specific activation or inhibition of the function of ERK5 within cells. In preliminary experiments designed to identify specific activators of ERK5 in cells, I used a variety of ligands to stimulate a primary cell line HME that has been immortalized with hTERT. Although, I identified some potent activators of the ERK5 pathway including sorbitol, NaCl and EGF, none were specific towards ERK5. I also tried knocking-down the expression of ERK5 using RNAi in HME cells but was only able to get an inconsistent 30% knock-down in these experiments. HME cells are not very amenable to transfection and probably use of a different cell-type like HeLa might yield better and consistent results. Also, more combinations of different dsRNA oligonucleotides for ERK5 and treatment conditions need to be attempted. Once knock-down of ERK5 is achieved, cells may be stimulated with EGF or some other appropriate ligand and the extent of p90RSK activation may be analyzed. This would indicate whether ERK5 is required for activation of p90RSK. As mentioned previously, the PD184352 inhibitor has been used at low concentrations to inhibit ERK1/2 pathway specifically (220,227). This inhibitor may be exploited in confirming the sufficiency of ERK5 to activate p90RSK in cells.

Our studies have a number of implications for inhibitor studies and further amplify our knowledge of pathway specificity and cross-talk. The well studied MEK1/2 inhibitors, notably PD98059 and U0126 have also been found to inhibit MEK5, upstream in the ERK5 pathway. Because RSK has been linked exclusively to the ERK1/2 pathway for 15 years, one of the means of validating the specificity of these inhibitors has been to examine the effect of the inhibitors on RSK activity as a selective read-out of ERK1/2 activation and inhibition. The previously known ERK5 substrates, such as MEF2A and Sap1a, are rather poor substrates for ERK1/2. Here we show that ERK5 also has the capacity to activate RSK. The susceptibility of the ERK5 pathway to blockade by these inhibitors suggests that in some conditions RSK may be under the control of ERK5 instead of or, more likely, in addition to ERK1/2.

Although we have shown an effect of stimulating ERK5 on p90RSK activation in cells, the physiological relevance of this phenomenon needs to be demonstrated. As mentioned before, ERK5 and p90RSK have been independently implicated in a number of over-lapping functions. The direct involvement of p90RSK downstream of ERK5 in these processes i.e. stimulation of NF-κB will further validate p90RSK as an ERK5 substrate. Since no specific activators for the ERK5 pathway have been identified as yet, selective stimulation of the ERK5 pathway can only be achieved using constitutively active mutants of the upstream activator MEK5. Studying the effects of over-expressing a dominant negative, kinase-inactive form of p90RSK (p90RSK K100A) (340) on NF-κB, under conditions that activate the ERK5 pathway might be one way of analyzing the physiologic relevance of activating p90RSK through ERK5.

In our attempts to identify regions on ERK5 that may play a role in interaction with p90RSK, we found that the kinase domain of ERK5 was sufficient for the binding. Further we found that the acidic residues at the C-terminus of the kinase domain (CD domain by sequence homology with ERK2), implicated in substrate interactions of other MAPKs, were involved. This does not rule out the possibility that regions outside of this motif are involved in substrate interactions. Various studies have shown the involvement of the N-terminus of MAPKs in docking interactions (341). Besides, ERK5 has a long C-terminal tail that may contribute towards substrate specificity. Involvement of other regions in interaction with p90RSK needs to be investigated.

RSK-like kinases MSK1 and MSK2 have been shown to be substrates of ERK1/2 and p38 MAPK pathways. With our identification of ERK5 as another upstream activator of RSK besides ERK1/2, it is conceivable that MSK1 and MSK2 are also ERK5 targets. Our preliminary results suggest that ERK5 has a limited capacity to activate MSK1 in cells, indicating it might do so under pathophysiological circumstances. Exploring the interaction of ERK5 with these related kinases may provide further insight into the specificity in substrate interactions of MAPKs. A number of substrates are shared between two MAPK pathways. Besides the protein kinases mentioned above, these include MNK1 and numerous transcriptional regulators such as ternary complex factors (1). The ability of multiple MAPK pathways to activate subsets of common substrates provides mechanisms for signal integration and amplification, and distinct active substrate localizations will likely have subtle but significant effects on outputs. Effects of ERK5 on RSK must now be factored into this complex picture. This additional upstream connection to RSK may lead to distinct localizations or greater activation under certain

conditions than might be caused by ERK1/2 alone. This will be an important area for future clarification.

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Vitae

Aarati Ranganathan was born in Madras, India on August 27, 1972. She graduated in 1995 from The Birla Institute of Technology and Science, Pilani, India with a Bachelors degree in Pharmacy and a Masters degree in Biological Sciences. She worked at the Tuberculosis Research Center, Madras, India until 1999. In August 2000, she enrolled in the Ph.D. program at the University of Texas Southwestern Medical Center, Dallas, TX. She lives in Flower Mound, Texas with her husband, Arun and daughter, Mithra.