SCAFFOLD-MEDIATED ORGANIZATION OF

SIGNAL TRANSDUCTION NETWORKS

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DEDICATION

Dedicated to my family

SCAFFOLD-MEDIATED ORGANIZATION OF SIGNAL TRANSDUCTION NETWORKS

by

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DISSERTATION

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The Raf-MEK-ERK kinase cascade is a highly conserved signal transduction module involved in cellular processes ranging from proliferation and differentiation to transformation and apoptosis, and is engaged in response to growth factors, cytokines, morphogens, and other extracellular stimuli. Given the ubiquitous nature of this signaling pathway, it is not clear how specialization with respect to various Raf-

dependent phenomena is acquired. In theory, functional specificity could be achieved by selectively coupling the core enzymatic components of a cascade to other regulatory pathways depending on the cellular context. Here, by using loss-of-function and partial loss-of-function mutant analysis, we demonstrate that Raf can participate in cell regulatory processes independently of its ability to activate MEK. We also show that scaffolding proteins can serve as specificity determinants in Ras/MAPK signaling, and may function to coordinate the activity of this cascade with additional regulatory pathways required for an appropriate biological response. Thus, we provide evidence for functional diversification both at the level of Raf substrates and at the level of molecular organization by accessory scaffolding molecules.

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ASK-1	Apoptosis-stimulated kinase 1
ATP	adenosine 5’-triphosphate
CNK	Connector Enhancer of KSR
CMV	cytomegalovirus
DAL-1	Deleted in adenocarcinoma of the lung 1
EGF	epidermal growth factor
ERK	Extracellular Regulated Kinase
FERM	4.1, ezrin, radixin, moesin
GEF	guanine nucleotide exchange factor
GAP	GTPase-activating protein
GDP	guanosine 5’-diphosphate
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine 5’-triphosphate
HA	hemagglutinin
HEK293	human embryonic kidney
HSP	Heat shock protein

JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
KSR	Kinase Suppressor of Ras
LPA	Lysophosphatidic acid
MAGUIN	MAGUK interacting protein
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen activated protein kinase
MEFs	Mouse embryonic fibroblasts
MEK	MAPK/ERK kinase
MEKK	MEK kinase
$MgCl_2$	magnesium chloride
MP-1	MEK partner 1
NaCl	sodium chloride
NF-κB	Nuclear factor kappa B
NGF	Neuronal growth factor
PBS	phosphate buffered saline
PC12	pheochromocytoma 12
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor

PDZ	PSD-95/DLG-1/ZO-1
PH	Pleckstrin homology
PI3K	Phosphatidyl inositol 3’ kinase
PKA	cAMP activated protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PSD-95	Post-synaptic density 95
PTB	Phosphotyrosine binding
RalGDS	Ral guanine nucleotide dissociation stimulator
Ras	rat sarcoma
RING	Really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
RSC	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase PCR
S2	Schneider L2
SAM	Sterile alpha motif

SDS	sodium dodecyl sulfate
SH2	Src homology 2
siRNA	short interfering RNA
Sos	Son of sevenless
SRF	Serum response factor
SRE	Serum response element
S-SCAM	Synaptic scaffolding molecule
Ste5	Sterile 5
Sur-8	Suppressor of Ras 8
C-TAK-1	Cdc25-activated kinase 1

CHAPTER ONE
Introduction

Molecular Organization in Signal Transduction

Eukaryotic cells in their natural environments are exposed to a diverse array of extracellular signals including growth factors, cytokines, and morphogens. Cell surface receptors receive these signals and communicate them to the interior of the cell, resulting in changes in cell behavior. Little is currently understood about how complex environments are appropriately interpreted at the cellular level to produce discrete responses.

Given the inherent complexity of orchestrating a large array of intracellular signaling pathways and networks, a cell is presented with a number of logistical problems that need to be resolved in order to achieve specificity and efficiency in signal transduction. First, parallel signal transduction pathways often utilize common catalytic components, therefore a strategy to avoid unproductive cross talk and maintain specificity may be required. Second, in order to achieve a rapid response following stimulation, it may be insufficient to rely on free diffusion rates to recruit cytosolic components of a cascade to their site of action. Third, allowing activated signal transduction molecules to

diffuse freely throughout the cell could potentially lead to de-regulated signal amplification, and may preclude the timely implementation of negative feedback control.

Solutions to these problems have been in many cases provided by the spatial restriction of signaling components through architectural organization of multi-protein complexes, as illustrated by the following examples. One mechanism that can minimize or eliminate cross talk is to physically sequester parallel signal transduction cascades away from each other. In *S. cerevisiae*, mating pheromones and osmotic stress both induce activation of the MEKK Ste11p. However, in the mating pathway, Ste11p activates the MEK Ste7p, which in turn activates the MAPK Fus3p, whereas in the osmotic stress pathway the homologous Ste11p/Pbs2p/Hog1p kinase module is engaged instead. It was demonstrated that in the mating pathway, the Ste11p/Ste7p/Fus3p module is assembled on the scaffolding protein Ste5p, while in the osmotic stress pathway, the scaffolding function is supplied by the MEK Pbs2p, which acts as both a scaffold and a kinase. Moreover, in the absence of Ste5p, activation of Ste11p leads to the stimulation of both pathways (van Drogen and Peter, 2002). Therefore, by utilizing distinct physical organization mechanisms, the two pathways downstream of Ste11p are able to give rise to appropriate physiological responses depending on the initial stimulus. Scaffolding proteins can also function to maximize signaling efficiency by pre-assembling dormant catalytic components. For example, in *Drosophila* photoreceptor cells, all components of

the phototransduction system downstream of rhodopsin and its target $G_q\alpha$ (i.e. PLCβ, PKC, TRP, CaM, and Ina-C) are constitutively assembled on the scaffolding protein Ina-D, which also targets this complex to a specific location in the cell. This arrangement allows for efficient activation of the TRP Ca^{2+} channel in response to rhodopsin stimulation, and for rapid deactivation through phosphorylation of TRP by PKC (Tsunoda et al., 1997). In addition, studies have demonstrated that Ina-D prevents signal amplification in this pathway, where activation of one rhodopsin molecule leads to the activation of one transducisome and one cluster of channels (Scott and Zuker, 1998).

Thus, spatial organization of signaling pathways is a critical component of cell regulation. One of the mechanisms of such organization appears to involve the assembly of signal transduction molecules on specialized scaffolds. Scaffolding proteins can be assigned distinct, yet often overlapping functions. The insulating function of scaffolds ensures specificity by preventing interaction with parallel signal transduction pathways, as in the case of Ste5p. The catalytic function serves to pre-localize components of a cascade in close proximity to each other, so as to enhance the efficiency of signaling, as in the case of Ina-D. Ina-D may also function to prevent deleterious signal amplification by assembling components of both the activation and the negative feedback regulation pathways on the same platform. Moreover, scaffolds are known to anchor receptors and other molecules at specified subcellular locations, such as apical or basolateral

membranes of epithelial cells, and pre- or post-synaptic densities of neurons (Bryant and Huwe, 2000; Dimitratos et al., 1999).

An additional aspect of cell regulation may involve coordinating multiple signaling pathways to achieve a particular outcome. For example, the intracellular domain of PDGFR can simultaneously bind multiple SH2 and PTB domain containing proteins, including PI3K, Ras-GAP, Shp-2, and PLCγ, possibly orchestrating diverse but related downstream signaling events (Burack et al., 2002). Similarly, insulin receptor substrate (IRS-1), which is associated with the insulin receptor (IR) cytoplasmic domain, recruits SH2 domain proteins PI3K, Grb-2, Shp-2, and Nck following tyrosine-phosphorylation by the IR kinase activity (Pawson and Scott, 1997). Work in the following chapters will include the functional characterization of a scaffolding protein that appears to be required both for specificity in MAPK signaling and for coordinating multiple regulatory pathways during neuronal differentiation.

Ras Regulation and Function

Ras was the first human transforming gene to be discovered, and is at the core of an extensively studied signal transduction network. Ras mediates diverse cellular phenomena, and its importance in human disease is underscored by the presence of activating Ras mutations in 20-30% of all human malignancies (Bos, 1988; Bos, 1989).

Although a great deal of understanding has been gained about Ras signaling over the past two decades, the critical role of spatial organization is becoming increasingly more apparent and is the focus of a significant proportion of Ras research today.

Ras belongs to a superfamily of 20–25 kDa monomeric GTPases known as small G-proteins. Ras is active in the GTP-bound state and inactivated upon hydrolysis of GTP to GDP. Binding of GTP causes a conformational change which allows Ras to interact with downstream effector molecules (Bourne et al., 1990). Ras binds guanine nucleotides very tightly, therefore its intrinsic rate of nucleotide exchange is very low. Guanine nucleotide exchange factors (GEFs) lower the affinity of GTP and GDP for Ras, which, due to the high GTP/GDP ratio in the cell, effectively results in GTP loading. The intrinsic rate of Ras GTP hydrolysis is also very slow, but is accelerated by several orders of magnitude by GTPase activating proteins (GAPs). 3D structure analysis shows that the GAP supplies an arginine residue to the active site of Ras, which appears to be required for stabilizing the transition state of the GTPase reaction (Geyer and Wittinghofer, 1997). Point mutations in Ras that prevent RasGAPs from stimulating Ras GTPase activity lead to constitutive activation of Ras (Krengel et al., 1990; Scheffzek et al., 1997; Tong et al., 1991).

The mechanism of Ras activation downstream of growth factor receptors was first outlined during the late 1980s and early 1990s. Upon stimulation, the cytoplasmic

domain of the receptor is tyrosine autophosphorylated, triggering recruitment to itself of the adaptor protein Grb2, which in turn recruits the RasGEF SOS. At the receptor, SOS is activated and subsequently stimulates activation of Ras. This molecular mechanism is highly conserved in animal species ranging from nematodes to flies to humans (Malumbres and Barbacid, 2003).

While in the active conformation, Ras can stimulate multiple downstream signaling pathways. All known downstream effectors of Ras bind to its effector domain, a region of 9 amino acids located in the amino-terminus. The best-studied Ras effectors include Raf family serine/threonine kinases, phophatidylinositol 3-kinase (PI3K), and RalGDS, a GEF for the small G-protein Ral (Shields et al., 2000). Recently, Impedes Mitogen signal Propagation (IMP), which is a RING-E3 ubiquitin ligase that acts as a threshold modulator in Raf/MEK signaling, was added to the list of Ras effectors (Matheny et al., 2004). Although many additional candidates have been proposed, none have been verified.

The initial discoveries that paved the way for Ras research date back to 1964 when Jennifer Harvey observed that preparations of murine leukemia virus from leukemic rats could induce sarcomas in newborn rodents (Harvey, 1964). In 1967, W. H. Kirsten made similar observations with murine erythroblastosis virus (Kirsten and Mayer, 1967). These viral strains were given the names Ha-MSV and Ki-MSV (murine sarcoma

virus). Subsequently, Ha-MSV and Ki-MSV were shown to contain rat sequences, which were named Ha-ras and Ki-ras (rat sarcoma) (Scolnick et al., 1973), and these sequences were found to be responsible for the transforming activities of Ha-MSV and Ki-MSV (Der et al., 1982; Parada et al., 1982; Parada and Weinberg, 1983; Santos et al., 1982). Shortly afterwards, N-Ras was isolated by a similar approach from human sarcoma and neuroblastoma cell lines (Hall et al., 1983; Shimizu et al., 1983). The three Ras genes code for individual members of the Ras gene family. In addition, K-Ras can exist as two alternate splice variants of the same transcript, K-Ras4a and K-Ras4b, differing only at the C-terminus.

Gene targeting studies in mice have revealed functional differences among Ras isoforms. Thus, K-Ras$^{-/-}$ embryos die during mid-gestation due to fetal liver defects and anemia (Johnson et al., 1997; Koera et al., 1997), while H-Ras$^{-/-}$ and N-Ras$^{-/-}$, as well as H-Ras$^{-/-}$/N-Ras$^{-/-}$, mice survive normally (Esteban et al., 2001; Umanoff et al., 1995). However, N-ras$^{-/-}$ mice exhibit defects in immune response and T-cell function (de Castro et al., 2003), whereas H-ras$^{-/-}$ mice have no obvious phenotype with the exception of decreased tumor formation after carcinogenic treatment (Ise et al., 2000). Although these studies are difficult to interpret in terms of functional differences at the molecular level, they clearly suggest unique physiological roles for H-Ras, K-Ras, and N-Ras.

Based on *in vitro* studies, Ras effectors do not exhibit significant differences in their affinity for individual Ras isoforms (Wolfman, 2001). However, selectivity with respect to downstream targets could potentially be conferred by isoform-specific subcellular compartmentalization and interaction with scaffolds or other molecules (the role of scaffolds in Ras signaling will be discussed in more detail later in this chapter). The N-terminal 85 amino acids of Ras, including the effector binding domain, are identical in all four isoforms, while the next segment of 80 amino acids shows some variability (85-90% identical). In contrast, Ras proteins are almost completely divergent in the C-terminal hypervariable region (HVR) consisting of 25 amino acids, with less than 15% identity. The HVR contains sequences that direct post-translational modifications and can regulate subcellular localization of the protein. The membrane anchor domain of HVR includes a CAAX motif, which is the target of farnesylation in all Ras isoforms, as well as sequences that direct palmitoylation in H-Ras, N-Ras and K-Ras4a. The K-Ras4b anchor domain is not palmitoylated, but instead contains a stretch of lysine residues. Just N-terminal to the anchor domain is the HVR linker domain, which in the case of H-Ras contains a sequence of 14 amino acids necessary for its regulated movement within plasma membrane microdomains. The targeting signals within the HVR are required for appropriate localization of Ras proteins to distinct regions of the plasma membrane (PM). For example, inactive H-Ras is highly enriched

in the specialized lipid raft structures known as caveolae, while K-Ras is excluded from them (Prior et al., 2001; Roy et al., 1999). Although H-Ras moves into the non-caveolae membrane upon activation, activated H-Ras and K-Ras still occupy distinct, yet poorly defined, PM microdomains (Hancock, 2003). Ras proteins can also be targeted to endomembrane structures such as ER, Golgi, and endosomes, as well as to mitochondria (Bivona and Philips, 2003; Wolfman, 2001). For example, active H-Ras appears to be partially localized to endosomes, unlike K-Ras, which is primarily on the plasma membrane (Jiang and Sorkin, 2002; Roy et al., 2002b). Also, both H-Ras and N-Ras are partially localized to the Golgi, whereas K-Ras is not (Hancock, 2003). Importantly, the quantity and kinetics of the Ras signal, as well as the choice of preferentially activated downstream targets, have been observed to vary depending on subcellular localization (Chiu et al., 2002; Hancock, 2003), which could therefore have a profound effect on the quality of the signal.

The functional consequences of differential subcellular compartmentalization are presently the subject of intense study, but it could, at least in part, form the basis for isoform-specific biological roles of Ras. In addition, there is evidence suggesting that discrete pools of a given Ras isoform could also be functionally unique. For example, N-Ras on the plasma membrane is found complexed with Raf-1 and PKCε and is involved in mitogen-induced MAPK activation (Hamilton et al., 2001). However, N-Ras is also

found in the mitochondria, where it s believed to deliver a steady state mitogen-independent cell survival signal, although the direct target of mitochondrial N-Ras is not known (Rebollo et al., 1999; Wolfman and Wolfman, 2000). Thus, distinct Ras pools can be differentially regulated and have unique functional outputs, possibly depending on the accessibility of various upstream activators and downstream targets. This again illustrates how spatial organization could impart functional diversity on a system containing "redundant" components.

Raf/MEK/ERK Kinase Module

The Raf/MEK/ERK cascade is the most extensively characterized of the Ras effector pathways. It has been implicated in proliferation, differentiation, transformation, apoptosis and other processes (Pearson et al., 2001). Raf-1 was the first downstream effector of Ras to be identified, and belongs to a family of serine/threonine kinases that also includes A-Raf and B-Raf (Malumbres and Barbacid, 2003). The Raf family kinases are composed of an N-terminal regulatory domain, which includes two conserved regions, CR1 and CR2, and a C-terminal kinase domain (CR3). Removing the regulatory domain from any of the Raf isoforms results in a constitutively activated kinase (Chong et al., 2003).

The mechanism of Raf-1 activation by Ras is still not completely understood, but involves recruitment of Raf-1 to the inner leaflet of the plasma membrane by activated Ras. The Raf-1/Ras interaction is mediated by amino acids 51-131 of Raf-1, referred to as the Ras-binding domain (RBD), and by the cysteine-rich domain (CRD), both of which are found within CR1 (Dhillon and Kolch, 2002). At the membrane, Raf-1 undergoes a number of activating phosphorylations by membrane-bound kinases, including on Y341, possibly by Src and/or Janus activated kinase (JAK), and on S338, by p21 activated kinases PAK1 and PAK3. In addition, S259 on Raf-1 is a target for inhibitory phosphorylation by Akt and PKA, and must be dephosphorylated by phosphatases PP1 or PP2A to allow mitogenic activation of Raf-1 (Chong et al., 2003; Dhillon and Kolch, 2002; O'Neill and Kolch, 2004). This complicated process leads to a conformational change in Raf-1, whereby the regulatory domain dissociates from the catalytic domain, resulting in an activated kinase.

Regulation of A-Raf activity is not well characterized, but is believed to be similar to that of Raf-1, since A-Raf has all the critical regulatory phosphorylation sites that are found in Raf-1 (Marais et al., 1997). On the other hand, regulation of B-Raf is different in several respects. For example, S445 in B-Raf, which corresponds to S338 in Raf-1, is constitutively phosphorylated, and D448 in B-Raf (Y341 in Raf-1) is a phosphomimetic residue (Mason et al., 1999). Thus, B-Raf bypasses two regulatory

phosphorylation events that are critical for activation of Raf-1, which likely explains why B-Raf has a higher basal activity than Raf-1 and does not require phosphorylation by Src or JAK. Furthermore, whereas Raf-1 has one Akt inhibitory phosphorylation site (S259), B-Raf has two additional sites, S428 and T439, and all three residues must be dephosphorylated to bring about full activation (Chong et al., 2001; Guan et al., 2000). Finally, B-Raf can also be activated by Rap1, a Ras-related small GTP-binding protein (York et al., 1998).

Once fully activated, Raf-1 phosphorylates and activates the dual specificity kinases MEK1/2, which in turn activate the serine/threonine kinases ERK1/2. ERK1/2 have a number of nuclear targets, including transcription factors Elk-1, Myc and Sap, as well as cytosolic targets such as p90 ribosomal S6 kinase (RSK) family kinases (Pearson et al., 2001). The three Raf isoforms have slightly different activities towards MEK1/2. For example, A-Raf appears to be a weaker MEK activator than Raf-1 or B-Raf, and can only activate MEK1, whereas Raf-1 and B-Raf can activate both MEK1 and MEK2 (Chong et al., 2003).

Although MEK1/2 are the only known *bona fide* Raf substrates, there is an accumulation of evidence to suggest the existence of other Raf effectors. For example, activation of Raf, but not MEK or ERK, is sufficient to induce differentiation of H19-7 rat hippocampal neurons (Kuo et al., 1996); Raf-1-mediated activation of NF-κB is not

blocked by the MEK inhibitor PD98059 or by dominant negative MEK mutants (Baumann et al., 2000); in CCL39 cells, activation of p70 S6 kinase by Raf-1 is not inhibited by the expression of a dominant negative ERK mutant or the MAPK phosphatase MPK-1 (Lenormand et al., 1996); Ras- and Raf-induced down-regulation of high molecular weight non-muscle tropomyosin during NIH3T3 transformation is also MEK-independent (Janssen et al., 1998); in 3T3-L1 adipocytes, v-Raf induces differentiation without activating ERK or p90 RSK, and activated MEK1 suppresses insulin-induced differentiation, which is restored by PD98059 (Font de Mora et al., 1997; Porras et al., 1994; Porras and Santos, 1996); in cardiac myocytes, PD98059 does not inhibit, but rather potentiates induction of atrial natriuritic factor (ANF) by activated Raf-1 (Post et al., 1996).

Some indication of the existence of MEK-independent Raf-1 functions also comes from gene targeting studies in mice. Thus, Raf-$1^{-/-}$ mice die during embryogenesis due to widespread apoptosis, and Raf-$1^{-/-}$ mouse embryonic fibroblasts (MEFs) show increased susceptibility to apoptotic agents (Mikula et al., 2001). However, mitogen-induced ERK activation in Raf-$1^{-/-}$ MEFs appears to be normal, possibly due to compensation by B-Raf. These observations suggests that the anti-apoptotic function of Raf-1 is mediated independently of MEK/ERK signaling. A potential problem with this interpretation is that while Raf-1 is dispensable for ERK activation by growth factors in cell culture, it

could still be required for ERK activation by other stimuli that may be encountered *in vivo*. Interestingly, a number of candidate Raf-1 substrates have been reported, including cell cycle regulators retinoblastoma protein (Rb) and Cdc25, and apoptosis regulators MEKK1, RIP2 and Tvl-1 (Hindley and Kolch, 2002) (Fig. 1.1). The unequivocal validation of these and other Raf-1 phosphorylation substrates as *bona fide* Raf-1 effectors, however, is still lacking.

Some of the recent data also point to the possibility that Raf-1 may have kinase-independent functions. Thus, Raf-$1^{-/-}$ mice expressing a Raf-1(Y340F/Y341F) transgene appear to be normal (Huser et al., 2001). The Raf-1(Y340F/Y341F) mutant cannot be phosphorylated on Y340 and Y341 and is therefore not activated in response to mitogenic stimuli, at least in cultured cells. This is consistent with the observation that a kinase-inactive Raf-1 mutant retains the ability to inhibit apoptosis mediated by apoptosis-stimulated kinase 1 (ASK-1) (Chen et al., 2001). However, the Raf-1(Y340F/Y341F) mutant could still possess a residual amount of MEK kinase activity that is not experimentally detectable but is sufficient for mediating the physiological function of Raf-1. Nevertheless, the fact that in excess of forty protein interactions have been reported for Raf-1 makes it plausible to predict that Raf-1 could act as platform for assembling signaling complexes with diverse regulatory functions.

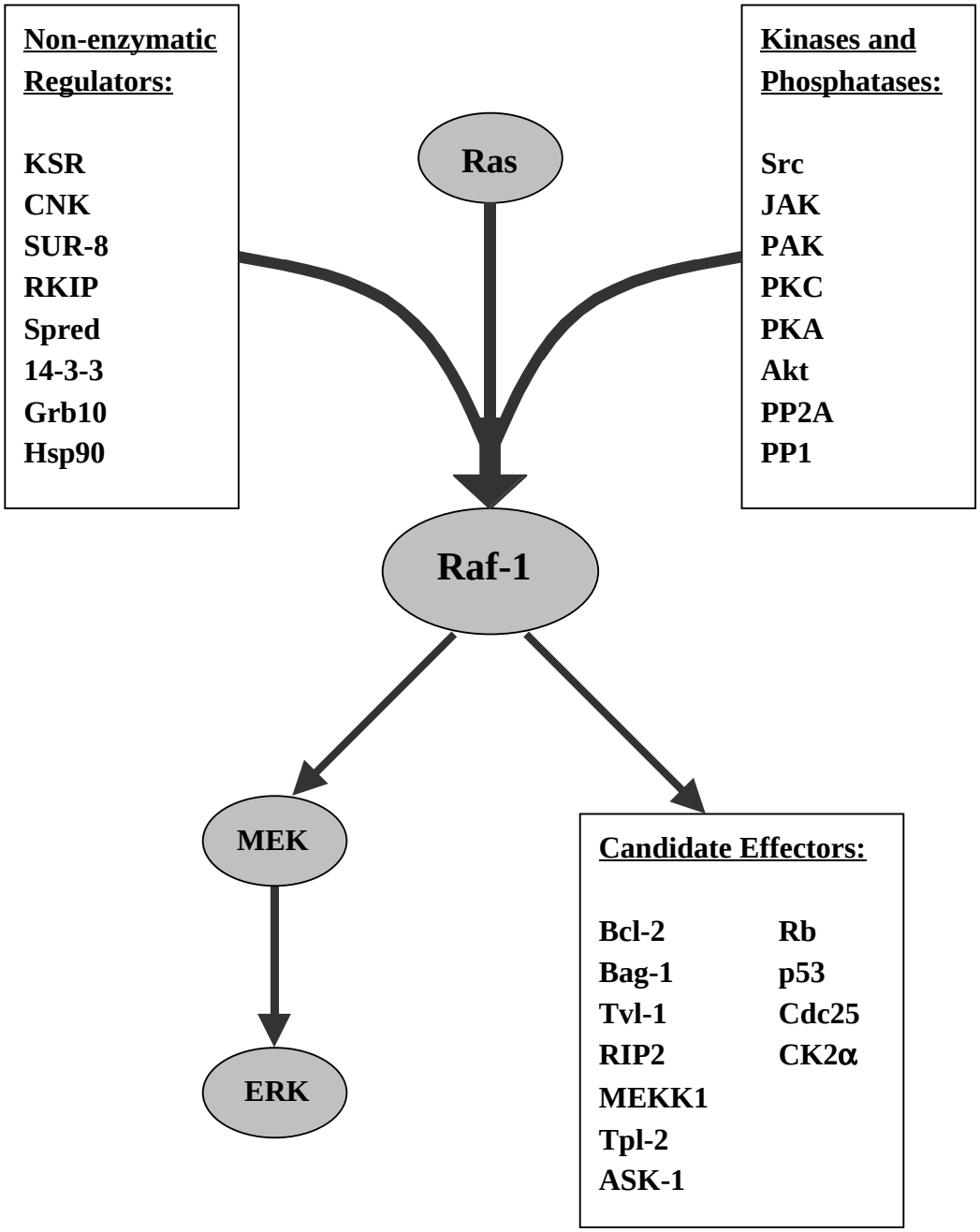


Figure 1.1. Regulators and effectors of Raf-1.

Chapter 2 will provide the clearest demonstration to date that some of the Raf-1 functions are mediated by downstream effectors other than MEK1/2 by employing a MEK-binding-defective mutant of Raf-1. In addition, Chapter 3 will describe a study which demonstrates that the contribution of Raf to regulating gene expression significantly exceeds its role in activating MEK, which could suggest both MEK-independent and kinase-independent functions for Raf.

Ras/MAPK Scaffolds.

An additional level of regulating Raf activity and Raf/MEK/ERK signaling appears to be provided by accessory proteins, several of which have been identified and to various extents characterized within the past decade. The initial discoveries in this area were made in *Drosophila* and *C. elegans* through the use of genetic screens for mutants that suppress an activated-Ras-induced phenotype. In *C.elegans*, over-stimulation of the *let-23/let-60/mpk-1* (EGFR/Ras/MAPK) pathway during development results in a multivulval phenotype, whereas under-stimulation results in a vulva-less phenotype (Moghal and Sternberg, 2003). In the *Drosophila* eye, excessive activation of the homologous Sev/Ras/Erk pathway results in a rough eye phenotype due to the presence of extra R7 cells. Insufficient stimulation of this pathway also results in rough eye, but in this case due to missing R7 cells (Raabe, 2000). By mutagenizing flies or

nematodes in an activated Ras genetic background and screening for revertants, suppressor mutations can be identified.

Such screens lead to the cloning of Suppressor of Ras-8 (SUR-8) in *C.elegans* (Sieburth et al., 1998), Kinase Suppressor of Ras (KSR) in both *C.elegans* and *Drosophila* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995), and Connector-Enhancer of KSR (CNK) in *Drosophila* (Therrien et al., 1998). According to the genetic epistasis analysis, SUR-8, KSR, and CNK are positive regulators of Ras/MAPK signaling that act upstream of Raf and downstream of or in parallel to Ras. Sequence analysis reveals that SUR-8 is made up exclusively of leucine-rich repeats (LRR), while CNK contains a sterile alpha motif (SAM), a PDZ domain, and a PH domain (Sieburth et al., 1998; Therrien et al., 1998) (Fig. 1.2). Since SUR-8 and CNK seem to consist exclusively of protein-protein or protein-lipid interaction domains, and lack any predicted catalytic domains, they were hypothesized to serve as linker or adaptor molecules. On the other hand, KSR was predicted to be a serine/threonine kinase based on sequence similarity to Raf family kinases. KSR contains conserved domains CA1 (unique to KSR proteins), CA2 (proline-rich), CA3 (cysteine-rich) and CA4 (serine/threonine-rich) in its N-terminal half, and a C-terminal putative kinase domain (CA5) (Roy and Therrien, 2002) (Fig. 1.2).

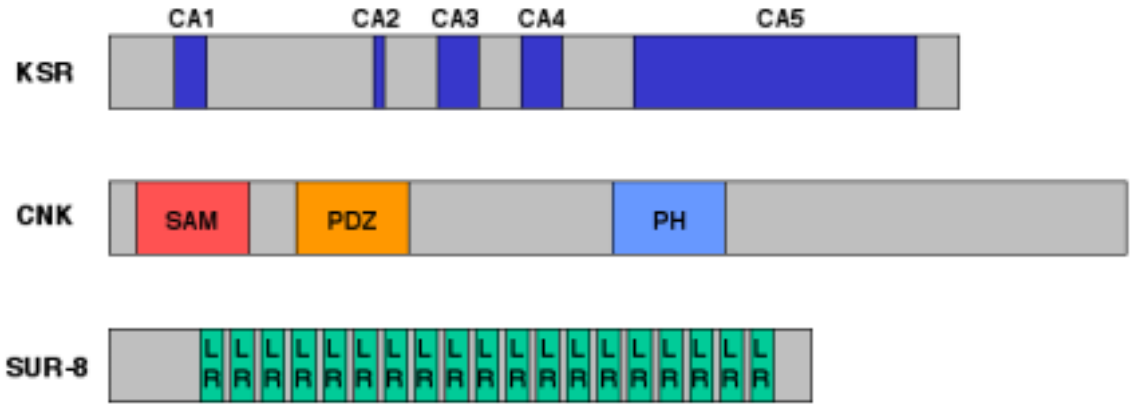


Figure 1.2. Domain structures of KSR, CNK, and Sur-8.

Biochemical studies of the single mammalian SUR-8 ortholog demonstrate that SUR-8 directly interacts with both Ras and Raf and, when present at low concentrations, can potentiate stimulus-dependent Ras-Raf interaction and ERK activation (Li et al., 2000). However, higher concentrations of SUR-8 are inhibitory to these events, which is consistent with SUR-8 being a scaffolding protein, since overexpression of a scaffold can lead to dilution of catalytic components, and would only potentiate the output of a cascade if the catalytic components are also overexpressed (Burack and Shaw, 2000). Interestingly, SUR-8 exhibits isoform-specificity in its constitutive interaction with Ras, as it selectively binds to K-Ras and N-Ras, but not to H-Ras, both in yeast two-hybrid and *in vitro* (Sieburth et al., 1998). This suggests that scaffolding proteins like SUR-8

could potentially direct the assembly of signaling complexes with Ras isoform-specific functional roles.

Despite the close sequence similarity between KSR and Raf, to date there has been no compelling demonstration that KSR functions as a kinase. Instead, it appears to play a scaffolding role in Ras/MAPK signaling, as illustrated by the following observations. KSR interacts with MEK1/2 constitutively and with ERK1/2 in a stimulus-dependent manner, and the interaction is direct in both cases (Cacace et al., 1999; Jacobs et al., 1999; Muller et al., 2000). KSR also binds to Raf, although this interaction is less well defined and seems to be constitutive in *Drosophila* S2 cells, but stimulus-induced in mammalian cells (Roy et al., 2002a; Therrien et al., 1996; Xing et al., 1997). Some of the loss-of-function KSR mutants identified in the *C. elegans* screens appear to be MEK-binding defective (Muller et al., 2000; Stewart et al., 1999). Additional interactors include 14-3-3 proteins (Xing et al., 1997), G-protein βγ (Bell et al., 1999), HSP70, HSP90, cdc37 (Stewart et al., 1999), and c-TAK-1 (Muller et al., 2001). Early overexpression studies demonstrated that the effect of KSR on Ras/MAPK signaling can vary depending on the amount of KSR expressed in the cell, where low levels enhance, and high levels inhibit, ERK-dependent signaling events (Cacace et al., 1999). In addition, when the predicted kinase domain of KSR is expressed by itself, it behaves as a dominant inhibitory mutant, blocking ERK activation (Stewart et al., 1999; Therrien et

al., 1996; Yu et al., 1998). This is contrary to what would be expected if KSR was a functional kinase, since Raf family kinases are constitutively activated when their amino-terminal regulatory domain is deleted (although it is theoretically possible that the kinase domain of KSR does not fold properly when separated from the regulatory domain). Finally, mutating a residue predicted to be required for kinase activity does not impair the function of *C. elegans* or *Drosophila* KSR (Roy et al., 2002a; Stewart et al., 1999).

A screen for mutations that modify a KSR-dependent rough eye phenotype in *Drosophila* identified Connector-Enhancer of KSR (CNK) (Therrien et al., 1998). The screen was performed in the genetic background of a KSR deletion mutant that lacks the N-terminal CA1-CA4 domains and is dominant inhibitory for Ras-dependent photoreceptor cell differentiation. As shown in this study, CNK interacts with the kinase domain of *Drosophila* Raf, is localized on the plasma membrane in regions of cell-cell contact, and is tyrosine phosphorylated in RTK-dependent manner. Homologues of CNK have been identified in mammalian species, as well as in *C. elegans*. *Rattus norvegicus* CNK2 (MAGUIN-1), which is expressed selectively in neuronal tissues, was isolated in a yeast two-hybrid screen with the synaptic scaffolding molecule S-SCAM, and subsequently shown to interact with the post-synaptic density proteins PSD-95 and Densin-180 (Ohtakara et al., 2002; Yao et al., 1999). By analogy to *Drosophila* CNK, CNK2 interacts with Raf-1 and is localized at the plasma membrane, for which the PH

domain is required (Yao et al., 2000). However, no interpretable functional data for CNK2 had been reported.

Another candidate Ras/MAPK scaffold, MEK Partner-1 (MP-1), was isolated in a yeast two-hybrid screen with MEK1 (Schaeffer et al., 1998), and has no sequence homology to any known proteins. MP-1 interacts with MEK1 and ERK1, but not with MEK2 or ERK2, *in vivo*, potentiates MEK1/ERK1 activation, and is localized to the cytoplasmic face of late endosomes through interaction with the adaptor protein p14 (Schaeffer et al., 1998; Wunderlich et al., 2001). Loss-of-function analysis using RNAi shows that MP-1 contributes to the sustained activation of MEK1 and ERK1 which occurs on late endosomes in response to EGF (Teis et al., 2002). These observations indicate that MP-1 assembles an isoform-selective MEK1/ERK1 signaling complex on late endosomes, and suggest the possibility that this complex could have a unique functional role in cell regulation.

Although genetic analysis of SUR-8, KSR, and CNK indicated that these gene products are required for Ras/MAPK dependent signaling events, and that their involvement is upstream of Raf and downstream of or in parallel to Ras, there had been no direct biochemical demonstration that SUR-8, KSR, and CNK are required for activation of Raf, MEK and ERK by upstream stimuli. In Chapter 4, such biochemical evidence is provided with respect to KSR and CNK in a *Drosophila* cell culture system.

In addition, Chapter 5 will include a functional analysis of mammalian CNK isoforms, demonstrating their role in regulating MAPK signaling and actin cytoskeleton dynamics.

CHAPTER TWO
Uncoupling Raf1 from MEK1/2 Impairs Only a Subset of Cellular Responses to Raf1 Activation

Abstract

The Raf family of serine/threonine protein kinases is intimately involved in the transmission of cell regulatory signals controlling proliferation and differentiation. The best characterized Raf substrates are MEK1 and MEK2. The activation of MEK1/2 by Raf is required to mediate many of the cellular responses to Raf activation, suggesting that MEK1/2 are the dominant Raf effector proteins. However, accumulating evidence suggests that there are additional Raf substrates, and that subsets of Raf-induced regulatory events are mediated independently of Raf activation of MEK1/2. To examine the possibility that there is bifurcation at the level of Raf in activation of MEK1/2-dependent and MEK1/2-independent cell regulatory events, we engineered a kinase active Raf1 variant (RafBXB(T481A)) with an amino-acid substitution that disrupts MEK1 binding. We find that disruption of MEK1 association uncouples Raf from activation of ERK1/2, induction of serum-response element-dependent gene expression, and induction of growth and morphological transformation. However, activation of NF-

κB dependent gene expression and induction of neurite differentiation were unimpaired. In addition, Raf-dependent activation of p90 RSK was only slightly impaired. These results support the hypothesis that Raf kinases utilize multiple downstream effectors to regulate distinct cellular activities.

Introduction

Cellular interpretation of growth regulatory signals requires functional grouping of molecules into signal transduction cascades. The Raf family of serine/threonine protein kinases is critically involved in this signal transduction process. Raf kinases were first discovered as gain of function mutants with the ability to induce growth and morphological transformation of established cell lines. Subsequently it was discovered that activation of cellular Raf proteins is a downstream response to growth factors and is required to link growth factor receptor signaling to activation of gene expression (Daum et al., 1994). Studies of genetic model systems have demonstrated that activation of Raf is an essential step in many growth and developmental programs, and studies of tumor model systems have demonstrated that Raf mediates transformation by many oncogenes (Naumann et al., 1997).

A major substrate of activated Raf is mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK1) (Daum et al., 1994). Upon

activation by Raf, MEK1 can in turn phosphorylate and activate the p42 and p44 MAP kinases (also known as extracellular ligand-regulated kinases, ERK1 and 2) (Daum et al., 1994). Activated ERKs phosphorylate a number of cytoplasmic and nuclear targets, including transcription factors that mediate growth factor regulation of gene expression. ERK activation is required for cellular transformation induced by oncogenic Ras and Raf (Khosravi-Far et al., 1995), and it has been reported that expression of constitutively activated MEK1 is sufficient to induce cellular transformation of immortalized fibroblasts (Cowley et al., 1994; Mansour et al., 1994).

These studies suggest that activation of MEK1, with subsequent ERK activation, is the primary event mediating cellular responses to activated Raf. However, several cellular responses to activated Raf have been characterized that appear to be independent of MEK or MAP kinase activation. For example, constitutively active variants of Raf but not MEK are sufficient to induce differentiation of hippocampal neuronal cells (Kuo et al., 1996). Expression of mutationally activated Raf1 in CCL 39 cells has been reported to result in an ERK1/2-independent activation of p70 S6 kinase, leading to increased translation of mRNAs with polypyrimidine tracts (Lenormand et al., 1996). Raf-mediated activation of NF-κB transcription factors may occur independently of MEK1/2 activation in Jurkat T cells (Baumann et al., 2000), and Raf activity promotes, whereas MEK1/2 activity inhibits, atrial natriuretic factor expression in cardiac myocytes (Jette and

Thorburn, 2000). Consistent with these observations, candidate Raf substrates in addition to MEK1 have been reported (Galaktionov et al., 1995; Lin et al., 1999; Wang et al., 1996).

To further explore the potential of Raf kinases to modulate cell regulatory pathways independently of MEK1/2 activation, we isolated a kinase active, MEK1/2 binding-defective Raf variant, RafBXB(T481A). RafBXB(T481A) can efficiently stimulate morphological changes in PC12 cells indicative of differentiation events, NF-κB-dependent gene expression, and activation of p90 ribosomal S6 kinase (RSK), despite severely impaired ERK1/2 activation.

Results

Isolation of a MEK association defective Raf variant.

We used the yeast two-hybrid protein interaction detection system to isolate Raf variants that are uncoupled from MEK1 and MEK2. cDNA encoding RafBXB, a constitutively active Raf1 variant with a deletion in the amino-terminal regulatory domain, was randomly mutagenized along its entire length by a polymerase chain reaction employing low fidelity *Taq* polymerase (Zhou et al., 1991). The polymerase chain reaction product was used to generate a yeast expression library encoding fusions of RafBXB to the LexA DNA binding domain. The resulting library was introduced into

a yeast two-hybrid reporter strain, together with MEK1 expressed as a GAL4 activation domain fusion, to isolate RafBXB variants that fail to associate with MEK1. In addition to various alterations that lead to expression of truncated products, we identified a substitution of alanine for threonine at position 481 as an alteration that inhibits association with MEK1 and MEK2 (Fig. 2.1A).

Thr481 is located in a loop (Knighton et al., 1991; Zhang et al., 1994) between conserved kinase subdomains VIB and VII as defined by Hanks et al. (1988). All known Raf kinase genes encode a threonine at this position except for *Drosophila raf* (*pole hole*), which encodes a serine. Expression of RafBXB but not RafBXB(T481A) in serum-starved HEK 293 cell culture results in detectable activation of endogenous Erk1 and Erk2 proteins suggesting an uncoupling of RafBXB(T481A) from endogenous MEK proteins (Fig. 2.1B). The T481A mutation does not appear to affect activity of the Raf kinase domain, as immunoprecipitated RafBXB and RafBXB(T481A) showed equivalent phosphorylation activity on saturating amounts of GST-MEK1K-M *in vitro* (Fig. 2.1C).

T481A uncouples activities that mediate RafBXB stimulation of neurite differentiation versus cellular transformation.

The use of dominant interfering MEK variants has defined MEK activation as a crucial step mediating oncogene induction of cellular transformation (Cowley et al.,

1994; Troppmair et al., 1994). Not surprisingly, the T481A substitution virtually eliminates RafBXB focus-forming activity in NIH 3T3 cells (Fig. 2.2). As in HEK 293 cells, RafBXB(T481A) expression does not result in detectable activation of ERK1/2 in NIH3T3 cells (data not shown). In contrast, despite defective ERK1/2 activation, RafBXB(T481A) retains the ability to induce formation of neurite-like extensions in PC12 cells (Fig. 2.3) to a similar extent as observed with RafBXB. Kinase-inactive RafBXB had no effect (data not shown). These observations suggest that Raf can positively modulate differentiation through a MEK-independent pathway.

T481A uncouples RafBXB regulation of SRE but not NF-κB-dependent gene expression.

RafBXB activates both ternary complex factor and NF-κB family transcription factors (Naumann et al., 1997). Raf activation of TCF is ERK1/2-dependent (Kortenjann et al., 1994), whereas some studies suggest that vRAF can activate NF-κB through MEK1/2- and ERK1/2-independent pathways (Baumann et al., 2000). Expression of RafBXB in quiescent NIH 3T3 cells is sufficient to induce both SRE- and NF-κB-coupled luciferase reporter constructs (Fig. 2.4). Consistent with defective ERK1/2 activation, RafBXB(T481A) expression results in poor activation of 3× SRE-Luc (Fig.

2.4A). In contrast, RafBXB(T481A) induces 2× NF-κB-Luc to a similar if not higher level than as observed with RafBXB (Fig. 2.4B).

The activation of NF-κB-dependent gene expression by Raf in HEK 293 cells has been reported to occur downstream of activation of HB-EGF expression (Troppmair et al., 1998). Raf activation of the HB-EGF promoter appears to occur through activation of MEK and ERK1/2 (McCarthy et al., 1997). However, results using Jurkat T-cells suggest that Raf activation of NF-κB can also occur through more direct mechanisms independent of ERK1/2 activation (Baumann et al., 2000). As shown in Fig. 2.5A, the T481A mutation eliminates the ability of RafBXB to stimulate gene expression from the HB-EGF promoter. This observation suggests that Raf can activate NF-κB in NIH 3T3 cells, as in Jurkat cells, through an HB-EGF-independent pathway.

p90 RSK is activated in response to active Raf and in some cell types RSK can phosphorylate inhibitor of κB on serine 32 (Ghoda et al., 1997; Schouten et al., 1997). This raises the possibility that RSK can contribute to Raf-mediated NF-κB activation. Interestingly, RafBXB(T481A) retains the ability to activate p90 RSK in NIH 3T3 cells (Fig. 2.5B). This results suggest that Raf may regulate p90 RSK through both MEK-dependent and MEK-independent pathways.

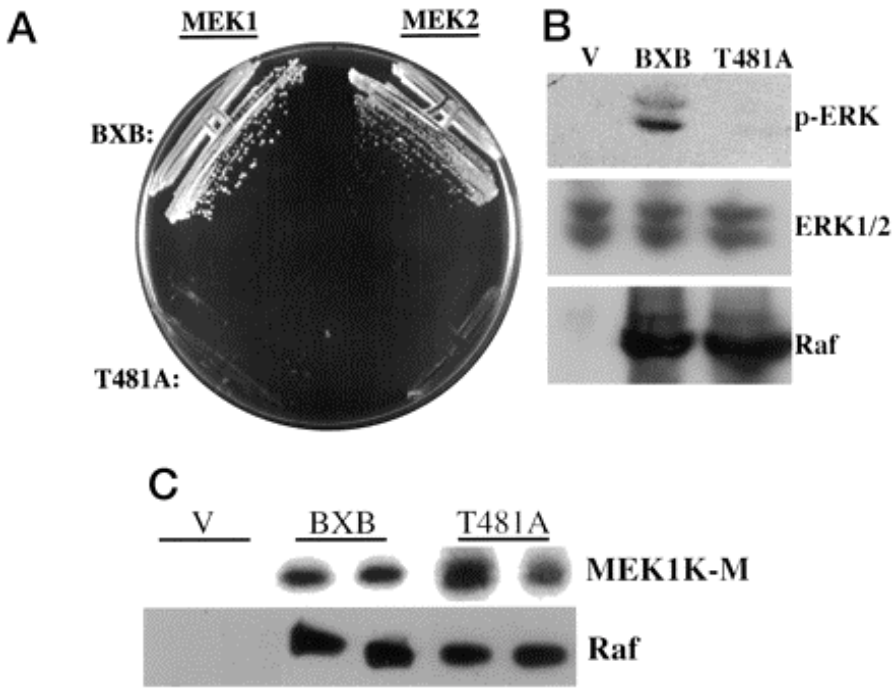


Figure 2.1. A single amino-acid substitution in Raf1 disrupts association with MEK1. **A)** L40 cells expressing the indicated fusion proteins were tested for the ability to grow on media lacking histidine. Growth on the selective plate indicates a positive two-hybrid interaction. **B)** HEK 293 cells were transiently transfected with the indicated constructs expressing myc-tagged RafBXB or myc-tagged RafBXB(T481A). Lysates from serum-starved cells were separated by SDS-PAGE and immunoblotted with the indicated antibodies to detect dually phosphorylated ERK1/2 (p-Erk1/2), total ERK1/2 and the expressed RafBXB variants. Similar results were obtained in repeated experiments. **C)** RafBXB and RafBXB(T481A) were immunoprecipitated from serum-starved transiently transfected HEK 293 cells using anti-myc monoclonal antibodies. Following extensive washing, the precipitates were added to in vitro kinase reactions with recombinant GST-MEK1K-M as substrate. A representative autoradiogram of one of three immune complex kinase assays performed in duplicate is shown (top panel). A portion of the precipitates were immunoblotted with anti-Raf antibodies to confirm that equivalent levels of Raf kinases were present in the reactions (bottom panel).

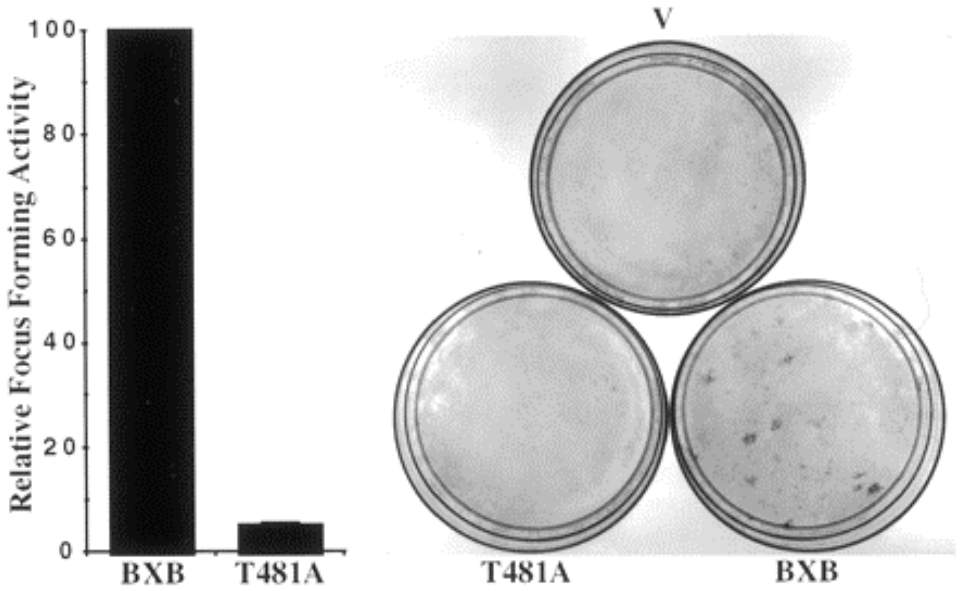


Figure 2.2. RafBXB(T481A) has severely impaired transformation activity. NIH 3T3 cells were transfected with constructs expressing the indicated proteins. Following 14 days of incubation in 5% serum, foci of growth and morphologically transformed cells were counted by microscopic observation. Transfections with MycPCDNA3-RafBXB typically resulted in 160 foci/ug DNA. Relative focus forming activity was determined from values obtained from three independent experiments performed in duplicate. Error bars represent standard error from the mean (SEM). Focus assay plates fixed and stained with geimsa, to reveal foci, are shown from a representative experiment.

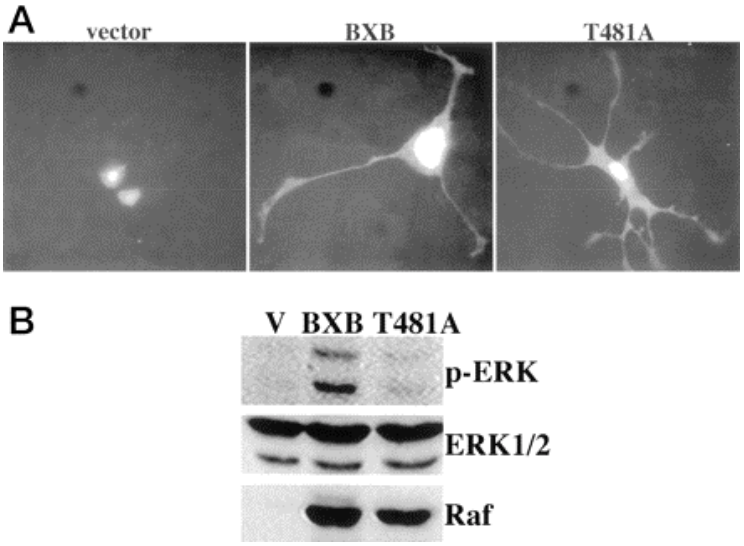


Figure 2.3. RafBXB(T481A) can stimulate PC12 cell differentiation in the absence of detectable ERK1/2 activation. **A)** PC12 cells were transfected with the indicated expression vectors together with pCEP4-GFP. 72 hours post-transfection, cells were fixed and visualized by GFP autofluorescence. Representative cells are shown in each panel. Similar numbers of "neurite-like" cells were observed in RafBXB and RafBXB(T481A) transfections. No "neurite-like" cells were observed in emtpy vector transfections. **B)** Levels of active ERK1 and ERK2 were assayed in serum-starved transiently transfected PC12 cells. Methods are as described in figure 2.1B.

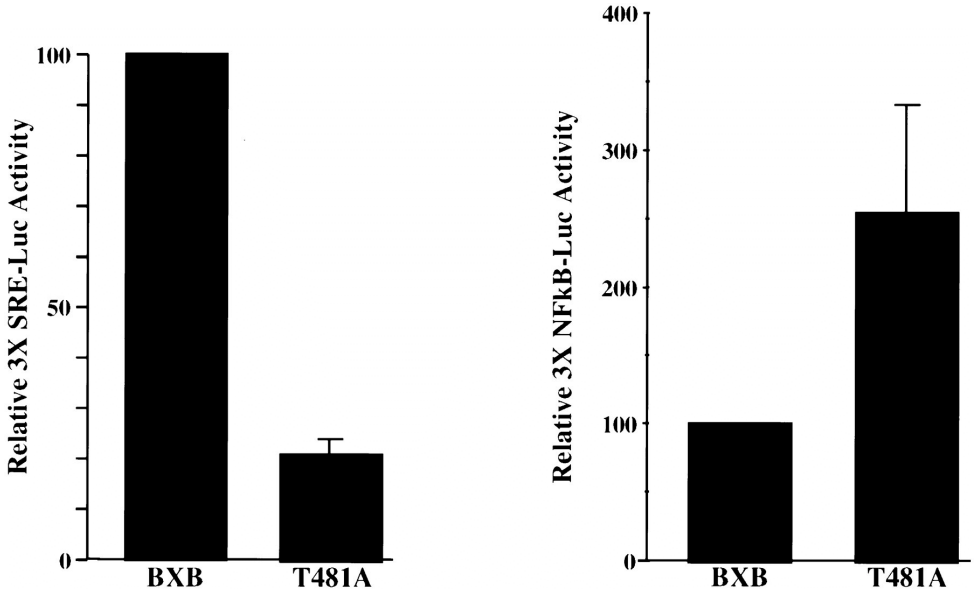


Figure 2.4. RafBXB(T481A) is uncoupled from regulation of SRE dependent but not NF-κB dependent gene expression. NIH 3T3 cells were transfected with mycPCDNA3, mycPCDNA3-RafBXB or mycPCDNA3-RafBXB(T481A) together with luciferase reporter constructs driven by three tandem copies of the c-Fos SRE (3X SRE-Luc) **(A)**, or two tandem copies of the NF-κB binding site from the κB promoter (2X NF-κB Luc) **(B)**. Relative luciferase activities were calculated by normalizing the fold reporter gene induction above empty vector to the values obtained with RafBXB, which were arbitrarily set at 100. RafBXB expression typically resulted in a more that 100-fold activation of 3X SRE-Luc and a 4-fold activation of 2X NF-κB Luc above empty vector controls. Error bars are the SEM from three independent experiments performed in duplicate.

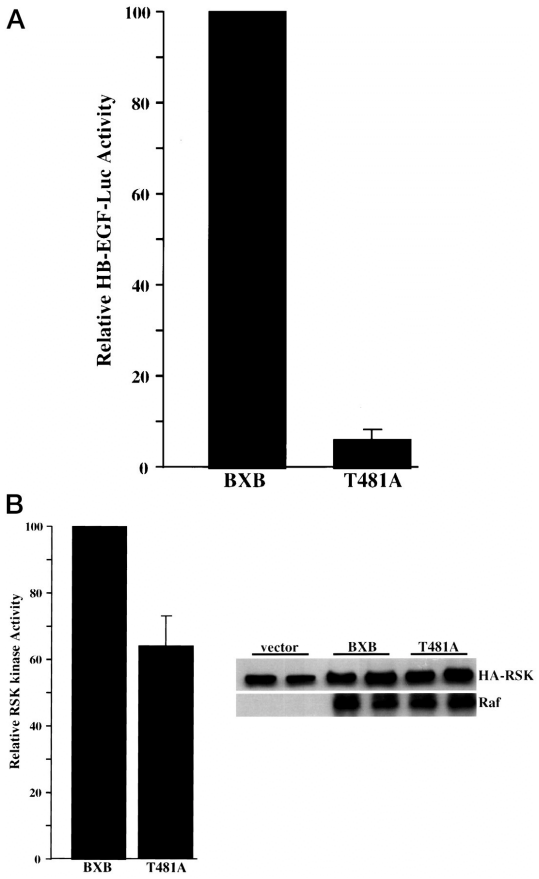


Figure 2.5. RafBXB(T481A) is uncoupled from regulation of the HB-EGF promoter, but can activate RSK. **A)** Experiments were performed as in figure 4 except that a luciferase reporter driven by a single copy of the murine HB-EGF promoter was used. RafBXB expression typically resulted in a 25-fold activation of the HB-EGF promoter above values obtained with empty vector. **B)** Lysates from NIH3T3 cells expressing the indicated constructs together with HA-tagged RSK were subjected to immonoprecipitation using anti-HA monoclonal antibody. To ensure that equivalent amounts of HA-RSK were immunoprecipitated, quantities of lysates containing equal amounts of RSK were used as assessed by anti-HA immunoblot. Immune complex kinase assays were performed using purified Histone 7S subunit. Kinase reactions were quantitated by measuring P32 incorporation into H7S and normalized to the activity observed with RafBXB. RafBXB expression typically resulted in a 10-fold elevation in RSK activity above that observed with empty vector. Bars represent the SEM of the average values of three independent experiments performed in duplicate (left panel). Anti-HA and anti-Raf1 immunoblots are shown from a representative experiment (right panel).

Discussion

Although it is clear that MEK1 and MEK2 are major substrates of Raf, accumulating observations suggest that Raf may regulate important cellular events independently of, or in parallel with, MEK1/2 activation (see the Introduction). To begin to explore this concept in detail, we have engineered a kinase-active Raf1 variant that is uncoupled from MEK1/2-dependent signaling events because of an amino acid substitution (T481A of human Raf1) that interferes with MEK1/2 binding. Expression of this Raf variant (RafBXB(T481A)) in cells revealed that it is uncoupled from activation of ERK1 and ERK2, cannot induce growth and morphological transformation, and cannot activate transcription from serum response elements or the HB-EGF promoter. In contrast RafBXB(T481A) retained the ability to induce neurite-like outgrowths in PC12 cells, activate transcription from NF-κB-dependent promoters, and induce the kinase activity of p90 RSK.

The above results suggest that RafBXB(T481A) can be used to define subsets of Raf-induced responses that are MEK-independent. Our observation that Raf activation of NF-κB can be uncoupled from MEK1/2 regulation is consistent with recent data examining NF-κB regulation in Jurkat T cells (Baumann et al., 2000). Our observations that RafBXB(T481A) mimics RafBXB effects on PC12 cell neurite differentiation and

p90 RSK activation were more unexpected. Experiments using dominant inhibitory variants of MEK1 and the pharmacological inhibitor PD98059 suggest that MEK activation is required for Raf-induced neurite differentiation and p90 RSK activation (Alessi et al., 1995; Cowley et al., 1994; Ghoda et al., 1997; Pang et al., 1995; Schouten et al., 1997). Our results suggest that although some extent of MEK1/2 activation may be required for these responses, activated Raf can contribute to neurite differentiation and RSK activation independently of MEK1/2 activation. It is important to note that dominant interfering MEK1/2 variants retain the ability to associate with Raf and therefore may block both MEK1/2-dependent and MEK1/2-independent Raf functions (Janssen et al., 1998). In addition, both of the widely used chemical inhibitors of MEK1/2, PD98059 and U0126, also inhibit at least one other MEK family member involved with Raf signaling, MEK5 (Kamakura et al., 1999).

The epistatic relationships of proteins functioning in mammalian signal transduction cascades are most often explored using combinations of constitutively active and dominant interfering variants of the components that contribute to regulation of the cascade. This approach has been highly successful in ordering the components of the Raf/MEK/ERK protein kinase cascade and for assessing the importance of this cascade in mediating mitogenic signals (English et al., 1999a). However, as a more sophisticated understanding of the molecular mechanisms of cell regulation develops, it is becoming

clear that many signal transduction proteins are multifunctional, introducing branch-points into what were once considered to be simple linear pathways of information flow (Pawson and Scott, 1997; Zuker and Ranganathan, 1999). In the context of complex regulatory networks, phenotypes observed with dominant interfering variants of signaling proteins can often be difficult to interpret because of association of the variants with multiple regulatory and effector proteins.

As all of the components that mediate Raf action in cells have not been identified, we currently can not rule out the formal possibility that RafBXB(T481A) regulation of neurite induction, NF-κB, and p90 RSK is mediated by activation of ERK1/2 to levels that are below the threshold of detection. Nevertheless, the dramatic uncoupling of RafBXB(T481A) from ERK1/2 activation, SRE activation, and focus formation is in stark contrast to the intact regulation of NF-κB, neurite formation, and p90 RSK activation. Further characterization of the mechanism of action of RafBXB(T481A) in cells, as well as the isolation of additional Raf variants that differentially uncouple association with Raf-binding proteins, will contribute to a better understanding of the complexities of cellular Raf kinase function.

Materials and Methods

Plasmids, Reagents and Expression of Recombinant Proteins. 3X SRE-Luc, pCEP4-GFP, pCH110βGal (Henry et al., 2000), 2X NF-κB-Luc (Frost et al., 2000), HB-EGF-Luc (Chen et al., 1995), and pCMV5-MEK1R4F (English et al., 1998) are as described elsewhere. PCEP4HA-RSK expresses full length avian RSK with an amino-terminal HA tag (gift from Megan Robinson). MycPCDNA3-rafBXB and MycPCDNA3-rafBXB(T481A) contain Raf1 cDNAs encoding amino acids 330-648 inserted as EcoRI/BamHI fragments into the EcoRI/BamHI sites of MycPCDNA3. For protein expression analysis, mouse anti-Myc (Cell Culture Center), mouse anti-HA (Berkeley Antibody Company), rabbit anti-Raf-1 (sc-133, Santa Cruz Biotechnology), rabbit anti-ERK1/2 (sc-93, Santa Cruz Biotechnology), and rabbit anti-active ERK1/2 (44-680, QCB/Biosource International) were used. Expression and purification of recombinant GST-MEK1K-M and Histone 7S subunits were performed by standard methods.

Library construction and screening. A library of randomly mutated cDNAs encoding RafBXB with an amino-terminal fusion to the LexA DNA-binding domain was constructed in the yeast expression vector pBTM116 using methods previously described (White et al., 1995). This library was screened for clones encoding fusion proteins that did not interact with a GAL4 activation domain/MEK1 fusion in the yeast reporter strain L40 (37) by standard techniques.

Cell culture and transfection assays. HEK293 cells were maintained as described (English et al., 1999a). Transfections were performed using calcium phosphate precipitation in 60mm plates. 2.5ug of either mycPCDNA3, mycPCDNA3rafBXB or mycPCDNA3rafBXB(T481A) were transfected as indicated. Lysates were prepared as described (English et al., 1999a) and immunoblotted or used for immune complex kinase assays where indicated. NIH3T3 cells were maintained as described (Mineo et al., 1997). Calcium phosphate precipitates were prepared using standard protocols. For 3X SRE-Luc assays 60mm dishes were transfected with 1ug of 3X-SRE-luc, 2ug of PRL-TK and 6ug of mycPCDNA3, mycPCDNA3rafBXB or mycPCDNA3rafBXB(T481A) as indicated. For 2X NF-κB-Luc assays 35mM dishes were transfected with 0.75ug of 2X-NF-κB-Luc, 1.0 ug of pCH110βGal, 0.2ug of pCMV-GFP and 6 ug of mycPCDNA3, mycPCDNA3rafBXB or mycPCDNA3rafBXB(T481A) as indicated. For HB-EGF-Luc assays 60mm plates were transfected with 1ug of HB-EGF-Luc, 2ug of PRL-TK and 1ug of mycPCDNA3, mycPCDNA3rafBXB or mycPCDNA3rafBXB(T481A) as indicated. 18-24 hours post-transfection precipitates were replaced with DMEM+0.5% calf serum. After 24 hours in low serum lysates were prepared in 200ul/plate for 35mm plates or 500ul/plate for 60mm plate with luciferase lysis buffer. Lysates were assayed for Firefly luciferase and Firefly renilla activity using a Dual Luciferase Assay kit (Promega) and the Turner Designs luminometer. Levels of reporter gene induction were calculated by

normalizing luciferase activity to either renilla or β-galactosidase activity. Focus assays were performed as described (Mineo et al., 1997). PC12 cells were maintained in RPMI 1640 with 5% horse serum and 10% FBS. Transfections were performed with calcium phosphate precipitates. To detect levels of active ERK1/2, 24 hours post-transfection cells were washed and incubated for an additional 16 hours in serum-free media and then lysed in sample buffer. Neurite extensions were visualized 72 hours post-transfection.

Immunoprecipitations and Kinase assays. Immunoprecipitations were performed as described (English et al., 1999b) using either anti-myc (myc-rafBXB and myc-rafBXB(T481A) or anti-HA (HA-RSK) antibodies. Kinase assays were performed as described (English et al., 1999b) using either GST-MEK1K-M or Histone 7S subunit as indicated.

CHAPTER THREE
Gene Expression Based Analysis of Signal Transduction Networks

Abstract

Raf proteins have a diverse array of binding partners, although the significance of many of these interactions is unknown. A point mutation in the Raf-1 catalytic domain (T481A) disrupts the Raf-1/MEK interaction when assayed by yeast two-hybrid. In mammalian cells RafBXB(T481A) is able to induce a subset of cellular responses to Raf-1 activation in the absence of detectable ERK activation, suggesting the existence of one or more MEK-independent Raf-1 effector pathways. To follow up on these observations, we used high density oligonucleotide microarray analysis coupled with RNAi to obtain a quantitative measure of the relative contributions of Ras, Raf, and Mek to the global pattern of gene expression in *Drosophila* S2 cells. Our results demonstrate that Ras and Mek have largely overlapping contributions to gene regulation, while that of Raf is very divergent, suggesting that Raf contributes to establishing regulatory control mechanisms in a manner that is distinct from, and in addition to, its role as a Mek activator.

Introduction

We began with a hypothesis that the global gene expression pattern, combined with inhibition of gene expression, could be used as an unbiased phenotype for studying the role of individual proteins in cell regulation. By comparing the effects of depletion of target proteins on the global pattern of gene expression, we hoped to derive information about the relationships of different target proteins to each other. The extent of relatedness between individual targets may be able to provide us with insight into the organization of signal transduction pathways and networks.

One way to approach this is to assay stimulus-dependent gene regulation. That is, only consider those genes that are induced or repressed in response to a stimulus, thereby restricting the analysis to a particular subset of genes. By analyzing the effect of inactivating various components of a signal transduction pathway that is engaged upon stimulation, we may be able to gain new insights into the organization of this pathway. Another approach is to examine the global pattern of stimulus-independent gene expression, thereby considering all the genes in the genome. Both approaches could potentially enable us to make comparisons between signaling molecules without prior knowledge about their role in cell regulation, and could also provide new information about well-characterized signaling pathways. Here, we have begun to apply these strategies to the analysis of Ras/MAPK signaling.

We performed our analysis in *Drosophila* S2 cells, as they are extremely amenable to RNAi and have previously been shown to respond to human insulin by activating the Ras/Raf/Mek/Erk, as well as the Ras/PI3K/AKT pathways (Clemens et al., 2000). In addition, the *Drosophila* genome codes for single isoforms of Ras (D-Ras1), Raf (D-Raf), and Mek (Dsor1). Therefore, we can rule out compensation due to isoform redundancy, which could potentially complicate data interpretation.

For gene expression analysis, we used Affymetrix high density oligonucleotide microarrays, which incorporate a number of features that enhance accuracy and reproducibility. On the Affymetrix *Drosophila* GeneChip®, each gene is represented by a probe set containing 16 probe pairs. Each of the 16 probe pairs consists of a perfect match (PM) and a single base mismatch (MM) oligonucleotide corresponding to a unique locus in the target gene expressed sequence. The Affymetrix MAS 5.0 algorithms take advantage of PM/MM information, as well as of the 16-fold redundancy, to derive a target-specific signal intensity value and a statistical Detection *p*-value for each probe set. During comparison analysis between two arrays, the corresponding probe pairs are individually compared to determine a Change *p*-value, which reflects the probability and direction of change, and a Signal Log Ratio, which is a log base 2 representation of the magnitude of change.

Results and Discussion

Drosophila Schneider L2 (S2) cells were incubated with dsRNA targeting Ras, Raf, Mek, or GFP (negative control) prior to being treated with insulin for 1 hour (Fig. 3.1A). The efficacy of RNAi was verified by assaying Ras, Raf, and Mek protein levels (Fig. 3.1B). Total RNA from S2 cells was isolated and subsequently used to generate cRNA for hybridization to Affymetrix GeneChip high density oligonucleotide arrays. Initially, the expression levels from each of the insulin-stimulated samples were compared to the unstimulated control using a pairwise comparison algorithm in MAS 5.0. We defined the insulin-responsive set of genes by setting the cut-off threshold at Signal Log Ratio of 1.0 (a 2-fold change) and were able to evaluate the effect of depleting Ras, Raf, or Mek on the profile of insulin-dependent gene regulation. The Signal Log Ratios were clustered using XCluster (http://genetics.stanford.edu/~sherlock/cluster.html), and the results were graphically visualized in Tree-View (http://rana.lbl.gov/EisenSoftware.htm), where red, green, and black colors signify increase, decrease, or no change, respectively. Some of the representative changes in gene expression were verified by semi-quantitative real-time PCR, with the rate of true positives being approximately 75% (data not shown). This is consistent with a recent report where the rate of true positives for MAS 5.0 was determined to be 80% (Rosati et al., 2004). In the same study, a program which utilizes the robust multiarray average

(RMA) procedure (Irizarry et al., 2003) called true positives at a rate of 100%. We are currently in the process of implementing the RMA program for our microarray data analysis.

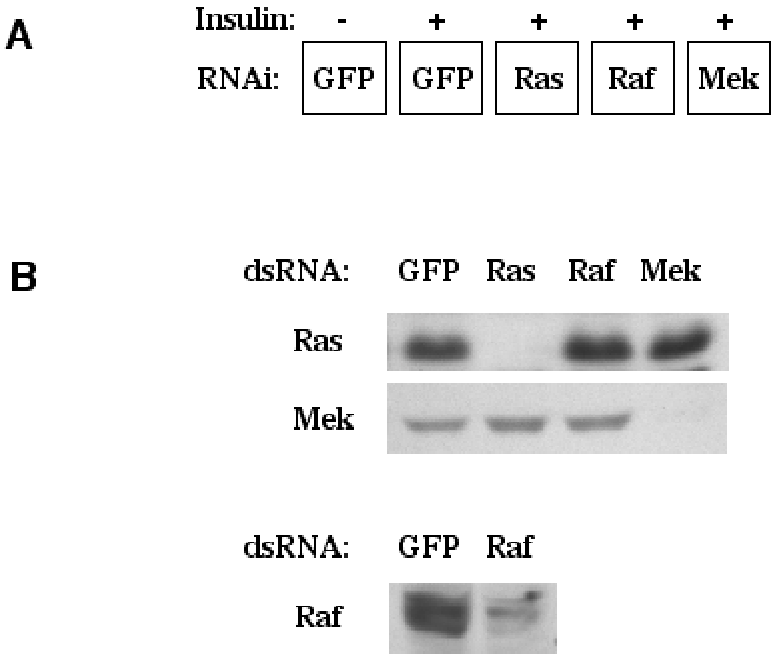


Figure 3.1. Experimental setup and knockdown verification. **A)** *Drosophila* S2 cells were treated with dsRNA targeting the expression Ras, Raf, Mek, or GFP (negative control) for 72 hours, and subsequently stimulated with 10 μg/ml human insulin for 1 hour, as indicated. **B)** Immunoblot analysis of Ras, Raf and Mek protein expression levels following dsRNA treatment.

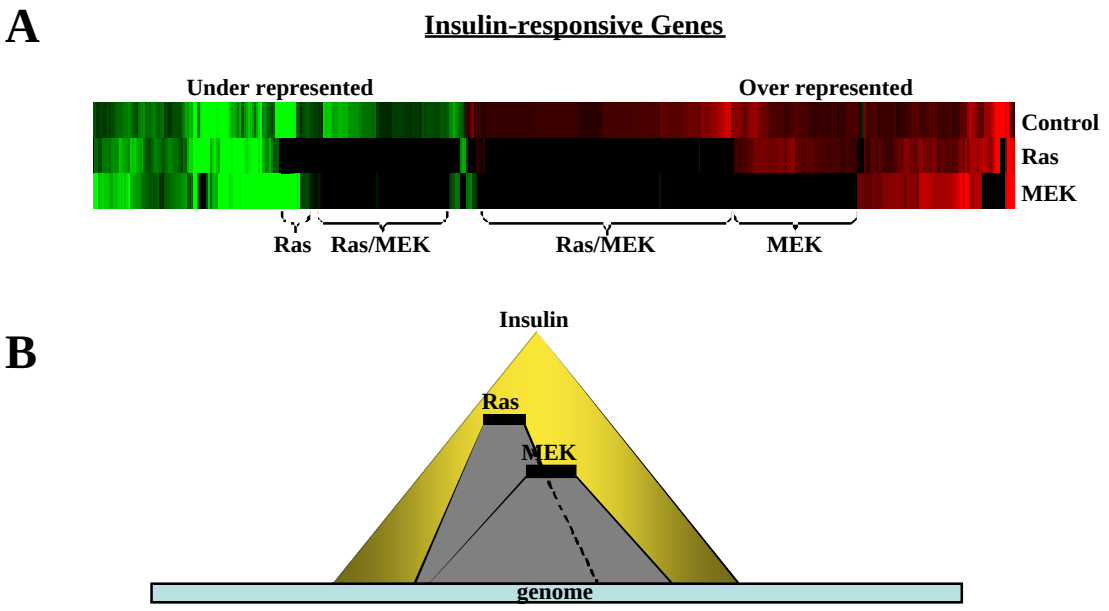


Figure 3.2. Relative contributions of Ras and Mek to insulin-regulated gene expression in S2 cells. **A)** Signal Log Ratios from pairwise comparisons of insulin-stimulated Ras, Mek and GFP samples to the unstimulated GFP sample were clustered using the XCluster algorithm and the results visualized in Tree-View. **B)** A schematic representation of the results in (A).

Figure 3.2A shows the clustering of insulin-regulated genes for GFP, Ras, and Mek knockdown arrays from one of two experiments. From this analysis, we can readily identify gene clusters that may reflect relationships among the components of insulin-mediated Ras/Raf/Mek/Erk signaling. For example, of the insulin-repressed genes, there is a subset which is dependent on both Ras and Mek (labeled “Ras/Mek”), as well as a subset dependent on Ras, but not on Mek (labeled “Ras”). These results are consistent with some of the known aspects of Ras signaling organization, in particular the ability of

Ras to regulate gene expression through activation of both Raf and PI3K (Junger et al., 2003). For the insulin-induced genes, in addition to the Ras/Mek-dependent subset, we also find a group of genes that are dependent on Mek, but not on Ras. This unexpected observation could reflect novel relationships within the Ras/MAPK signaling cascade, and warrants further investigation. A conceptual representation of these results is displayed in a shadow diagram of insulin-dependent gene regulation (Fig. 3.2B).

Surprisingly, depletion of Raf led to an almost complete abrogation of the insulin-response (data not shown). While this observation is non-intuitive in the context of a canonical Ras/MAPK signaling pathway, it is consistent with mounting evidence suggesting that Raf may function independently of its ability to activate Mek, either by phosphorylating other substrates, or by directing the assembly of multi-protein complexes in a kinase-independent manner (Hindley and Kolch, 2002) (see Chapter 2). However, such observations have so far not been reported for *Drosophila* Raf, and our result could indicate that a MEK kinase-independent role for Raf is conserved in *Drosophila*.

The divergent contribution of Raf to gene regulation, as compared to those of Ras and Mek, is also evident when we expand our analysis to include insulin non-responsive genes, which represent approximately 95% of the genome. We used the Signal Log Ratios obtained from pairwise comparisons of Ras, Raf, and Mek knockdown samples to the insulin-stimulated control as the basis for deriving a single numerical value for the

overall similarity between any two arrays. More specifically, the Signal Log Ratios from Ras/GFP, Raf/GFP, and Mek/GFP comparisons (all stimulated with insulin) for the entire *Drosophila* gene set were compared to each other (Ras to Raf, Ras to Mek, and Raf to Mek) by calculating the Pearson's correlation coefficient. Pearson's correlation coefficient is a measure of similarity between two arrays of values, where a coefficient of 1.0 represents perfect identity, and a coefficient of 0 represents absence of similarity. The averages of Pearson's correlation coefficients obtained in this manner from two independent experiments are shown in Figure 3.3. This analysis clearly illustrates a significant and reproducible difference in similarity among Ras, Raf, and Mek samples, such that the Ras/Mek pair exhibits a relatively high degree of similarity (~0.6), while those of the Ras/Raf and Raf/Mek pairs are much lower (~0.1). These results are consistent with a contribution of Raf to cell regulatory processes in *Drosophila* that extends beyond its role as a Mek activator.

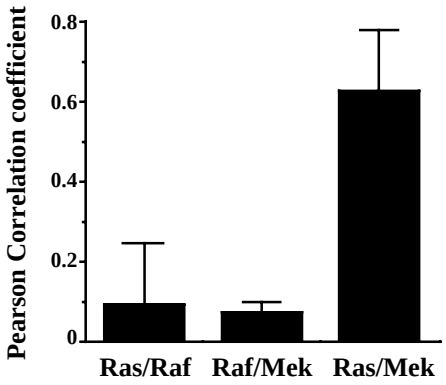


Figure 3.3. Comparison of the contributions of Raf, Ras and Mek to stimulus-independent gene regulation in S2 cells. Pearson's correlation coefficients for the indicated pairs were calculated from two independent experiments (see text for details).

One of the advantages of this type of approach to analyzing cell regulatory networks is its potential to uncover unexpected relationships among signal transduction molecules independently of pre-existing knowledge. The coupling of extracellular stimuli to discrete cellular events relies on complex networks of signal transduction pathways, and a systems-based approach, combined with the more traditional biochemical and genetic strategies, is likely to be needed in order to understand this complexity. By building extensive databases containing information about the relative proximities of proteins to each other with respect to gene regulation, we can begin to develop a broader understanding of cell regulatory mechanisms. Once a sufficiently

detailed database exists, “unknowns” can be mapped onto it, and inferences about their role in cell regulation can be made by evaluating their position relative to previously characterized components.

Materials and Methods

Tissue culture and transfection. Schneider S2 cells were purchased from Invitrogen and cultured in *Drosophila* Serum-Free media (Invitrogen) supplemented with 16.5 mM L-Glutamine (Invitrogen) and gentamycin (Sigma). Double-stranded RNA (dsRNA) was prepared using the MegaScript T7 In Vitro Transcription Kit (Ambion, Inc.) according to Clements et al. (2000). S2 cells were incubated with dsRNA for 72 hours prior to stimulation with 10 μg/ml recombinant human insulin (Sigma) for 1 hour.

RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and subsequently analyzed by gel electrophoresis.

Microarray analysis. RNA samples were submitted to either the UCSD or UT Southwestern microarray core facilities, where they were processed and hybridized to the Affymetrix *Drosophila* genome array chips (P/N 900335). Data analysis and pairwise array comparisons were performed using the Affymetrix MAS 5.0 software package. K-means hierarchical clustering based on Euclidean distance values was performed in the XCluster program (M. Eisen and G. Sherlock, Stanford University), which was obtained

from http://genetics.stanford.edu/~sherlock/cluster.html. The results of cluster analysis were displayed in Tree-View (M. Eisen, http://rana.lbl.gov/EisenSoftware.htm). Pearson's correlation coefficients were generated in Microsoft Excel.

Real-time PCR. Changes in the level of expression of a number of representative target genes were verified using a LightCycler apparatus (Roche).

Immunoblotting. S2 cells were lysed in sample buffer and analyzed by polyacrilamide gel electrophoresis. Relative protein expression levels were determined using antibodies against Ras (Pan-Ras Ab-3, Oncogene Research), Draf (gift from D.K. Morrison), and MEK1/2 (Cell Signaling).

CHAPTER FOUR
Critical Contribution of Linker Proteins to Raf Activation

Abstract

Genetic analysis of Ras signaling has unveiled the participation of non-enzymatic accessory proteins in signal transmission. These proteins, KSR, CNK, and Sur-8, can interact with multiple core components of the Ras/MAP kinase cascade and may contribute to the structural organization of this cascade. However, the precise biochemical nature of the contribution of these proteins to Ras signaling is currently unknown. Here we show directly that CNK and KSR are required for stimulus dependent Raf kinase activation. CNK is required for membrane recruitment of Raf, while KSR is likely required to couple Raf to upstream kinases. These results demonstrate that CNK and KSR are integral components of the cellular machinery mediating Raf activation.

Introduction

The mechanism by which Ras activation leads to activation of downstream effectors is only beginning to be understood. In the case of Raf kinases, activation by Ras

appears to involve a combination of membrane recruitment and other association-induced activity changes (Campbell et al., 1998). Observations that artificially membrane-targeted variants of Raf1 are constitutively active independently of Ras in transient transfection experiments, together with observations that active Ras can recruit Raf1 to the plasma membrane, have led to the current paradigm for Ras function. That is, Ras-GTP acts as "molecular flypaper" ensnaring effector molecules at the plasma membrane where they are subsequently activated by other partially characterized membrane-associated components (Leevers et al., 1994; Stokoe et al., 1994). However, some recent observations are inconsistent with this model. At least in the case of Raf1, Ras association makes an important contribution to activation of Raf1 kinase activity independently of membrane recruitment (Inouye et al., 2000; Mineo et al., 1997). In addition, the association of endogenous Raf1 kinases with the plasma membrane does not always correlate with the activity of the kinase or the mitogenic state of the cells (Liu et al., 1997). For example, Raf1, MEK1, and ERK1/2 can be found constitutively associated with the caveolar plasma membrane of primary human fibroblasts independently of the activation state of these proteins. In addition, these components can be activated in a mitogen-dependent fashion in purified caveolae (Liu et al., 1997) strongly suggesting that the Ras/MAP kinase cascade can exist as a coherent spatially organized signal transduction machine.

Adding to the complexity, a growing number of observations have implicated non-enzymatic accessory proteins in the regulation of the Ras-Raf-MAP kinase cascade. These include molecules such as KSR1, Sur-8, CNK, and MP-1. These proteins have characteristics suggestive of roles as scaffolding and or adapter proteins (reviewed in (Sternberg and Alberola-Ila, 1998). However it is still unknown whether any of these proteins directly participate in activation of the Raf/MAP kinase cascade, and, if so, whether they may function to localize kinases to sites of action, nucleate or stabilize activation complexes, enhance substrate recognition, alter kinetics of kinase activation, and/or restrict kinase specificity. Genetic epistasis analysis of KSR, CNK, and Sur-8 alleles in *Drosophila* or *Caenorhabditis elegans* places the function of all three proteins downstream of activated Ras and upstream of or in parallel with Raf (Kornfeld et al., 1995; Sieburth et al., 1998; Sundaram and Han, 1995; Therrien et al., 1995; Therrien et al., 1998). The direct consequence of KSR, CNK, or Sur-8 alleles on the biochemical activity of the Raf/Erk cascade has not been examined. However, biochemical analysis of mammalian orthologs of these genes suggest participation of these proteins in regulation of ERK kinases (Kolch, 2000). Sur-8 can interact with both Ras-GTP and Raf and can facilitate formation of functional Ras/Raf complexes when all three proteins are ectopically expressed, suggesting Sur-8 may function as an adapter for the Ras-GTP/Raf complex (Li et al., 2000; Sieburth et al., 1998). KSR can interact directly with MEK and

will inhibit ERK activation when overexpressed (Denouel-Galy et al., 1998; Joneson et al., 1998). However, low level expression of KSR can facilitate MEK and ERK activation (Therrien et al., 1996). These results, coupled with the observation that overexpressed KSR can associate with Raf in a Ras-dependent manner have led to the hypothesis that KSR may function as an adapter protein to facilitate Raf-MEK and/or MEK-ERK kinase-substrate interactions (Michaud et al., 1997; Sugimoto et al., 1998). MP1 was identified in two-hybrid screen as a MEK1-interacting protein and can simultaneously interact with both MEK1 and ERK1. Like KSR1, MP1 can inhibit or potentiate ERK activation depending upon levels of MP1 expression and may modulate the MEK-ERK interaction (Schaeffer et al., 1998).

The stoichiometry of scaffolds relative to the components they can assemble is likely to be strictly regulated. A surfeit of scaffold may disperse the very components that must function together to mediate a signal transduction cascade (Burack and Shaw, 2000; Levchenko et al., 2000). Therefore ectopic expression analysis is less than ideal for characterization of potential scaffold/adapter proteins and may be partially responsible for paradoxical or apparently contradictory observations of protein function (Burack and Shaw, 2000; Kolch, 2000; Morrison, 2001). For this reason, we sought a biochemically tractable model system in which to examine the consequences of loss-of-function of scaffolding proteins on regulation of the MAP kinase cascade.

Recently, it has been demonstrated that the *Drosophila* Schneider L2 cell line (S2) responds to insulin by activation of endogenous ERK-A (ERK2 ortholog) through activation of the canonical receptor-coupled Ras-Raf kinase cascade, just as has been previously characterized in mammalian cells. Together with the observation that S2 cells are extremely amenable to double-stranded RNA-mediated interference of gene expression (RNAi), thus allowing analysis of loss-of-function phenotypes, these cells become an ideal model system in which to characterize the contribution of accessory proteins to the regulation of the Ras/MAP kinase cascade (Clemens et al., 2000).

To directly assess the contribution of putative scaffold/adaptor proteins to regulation of the Ras/MAP kinase cascade, we examined the consequences of knocking down the expression of KSR1, CNK, Sur-8, and MP1 on stimulus-dependent activation of this cascade. We show here that both CNK and KSR are required for activation of ERK in response to insulin and phorbol ester. We demonstrate that the molecular level at which both CNK and KSR1 impact this cascade is directly at the Raf serine/threonine kinase. CNK and KSR are both required for activation of Raf but not Ras. Remarkably, CNK rather than Ras is primarily responsible for compartmentalization of a pool of Raf kinase at the plasma membrane. These results demonstrate that CNK and KSR are integral components of the cellular machinery that mediates Raf activation in response to active Ras.

Results and Discussion

To assess the contribution of putative scaffolding/adaptor proteins to ERK activation, S2 cells were treated with dsRNAs targeted to KSR-1, CNK, the *Drosophila* ortholog of Sur-8 (GenBankTM accession AE003717), and the *Drosophila* ortholog of MP1 (CG5110 accession AAF53620). As reported by Clemens et al. (2000), addition of double-stranded RNA to the culture media resulted in a robust and specific reduction in transcript levels for the targeted genes (Fig. 4.1). Targeting Sur-8 or MP1 had no detectable effect on activation of ERK (not shown), however, we found that both CNK and KSR are required for activation of ERK in response to insulin (Fig. 4.2A). AKT activation was unaffected by loss of KSR or CNK, demonstrating that insulin signaling was not generally inhibited (Fig. 4.2B). As expected, dsRNA directed against Ras also blocked ERK activation (Fig. 4.2A). In contrast to KSR and CNK, down-regulation of Ras partially reduces activation of AKT by insulin (Fig. 4.2B). This is consistent with published observations suggesting a contribution of Ras to activation of phosphatidylinositol 3-kinase (Marshall, 1996; Rodriguez-Viciana et al., 1994).

Genetic analysis of the developmental phenotypes induced upon hyperactivation of ERK suggests that KSR and CNK both act at the level of Raf or in parallel with Raf (Therrien et al., 1995; Therrien et al., 1998). However, the observation that KSR can

interact with MEK and ERK suggests that this protein may function as a linker to potentiate the MEK/ERK kinase-substrate interaction (Cacace et al., 1999; Denouel-Galy et al., 1998; Yu et al., 1998). As with ERK, we found insulin activation of MEK, as displayed with an anti-Ser(P)-217/221 MEK antibody, was inhibited by KSR or CNK dsRNAs (data not shown). To examine Raf activity, anti-dRAF immunoprecipitates were mixed with recombinant kinase-dead human MEK1 in *in vitro* kinase reactions. As shown in Fig. 4.3, both CNK and KSR are required for insulin activation of Raf kinase activity.

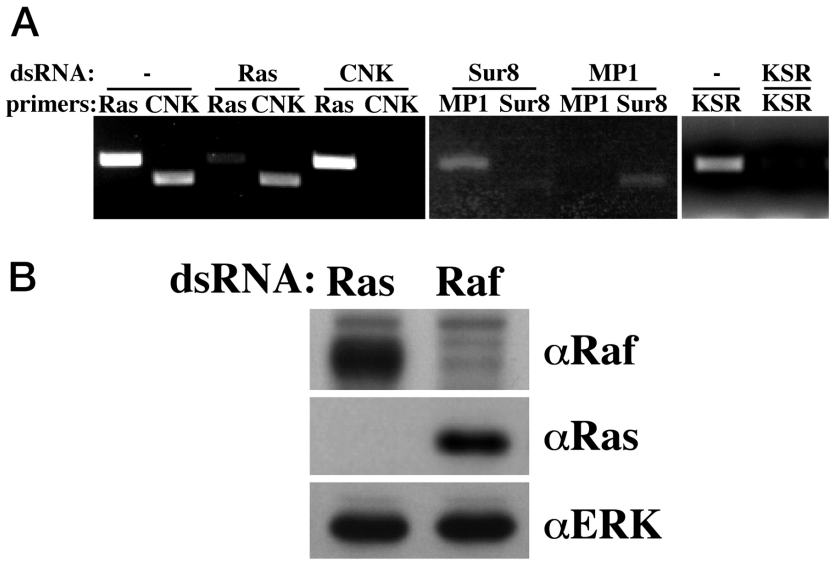


Fig. 4.1. RNA interference of gene expression. A, S2 cells (1×10^6) were plated in a 35-mm dish and treated with 5 μg/ml of the indicated dsRNA or left untreated. After 72 h, RNA was isolated and analyzed by reverse transcription-coupled PCR with the indicated transcript-specific primer pairs. B, whole cell lysates, from cells treated with Ras or Raf dsRNA as described above, were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

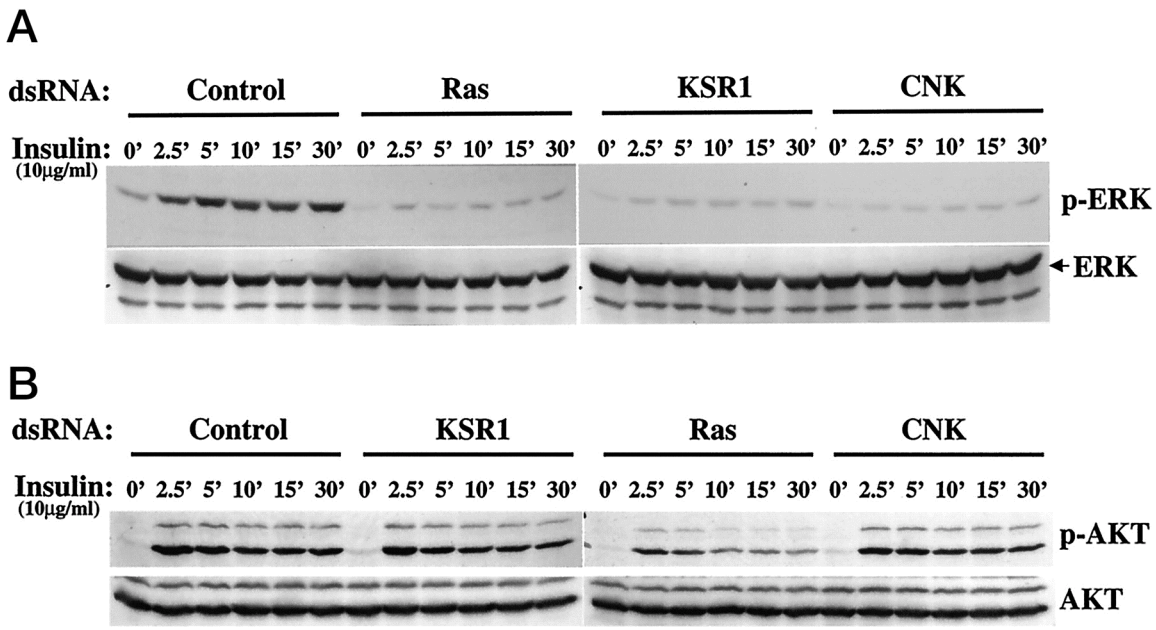


Figure 4.2. KSR and CNK are required for insulin-induced activation of ERK-A. Cells treated as described in the legend to Fig. 1 were stimulated with 10 μg/ml recombinant human insulin (Sigma) for the indicated times. Whole cell lysates were analyzed for levels of active ERK **(A)** or levels of active AKT **(B)** using activating phosphorylation site-specific antibodies. Similar results were obtained in three independent experiments.

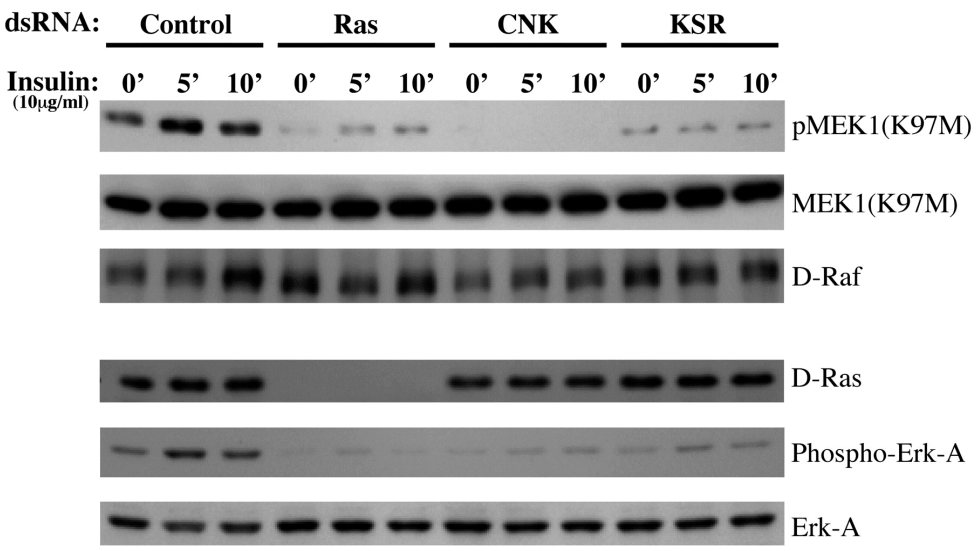


Figure 4.3. KSR and CNK are required for insulin-mediated activation of Raf. S2 cells were treated as in Fig. 1. Anti D-Raf immunoprecipitates were extensively washed, then incubated with recombinant His6-Mek1(K97M) for in vitro kinase reactions. Dually phosphorylated MEK1(K97M) was detected using anti-active MEK1 antibody. Relative substrate and D-Raf amounts present in the in vitro reactions are shown. Levels of phospho-ERK and Ras proteins present in the whole cell lysates from the same experiment are also shown. Similar results were obtained in two independent experiments.

The observation that Ras, but not CNK or KSR, contributes to activation of AKT in response to insulin suggests that insulin activation of Ras is not affected by down-regulation of CNK or KSR. This places the activity of KSR and CNK squarely at the level of Raf activation. Overexpressed CNK is enriched at sites of cell/cell contact potentially via PH domain-mediated interaction with phosphatidylinositol phosphates.

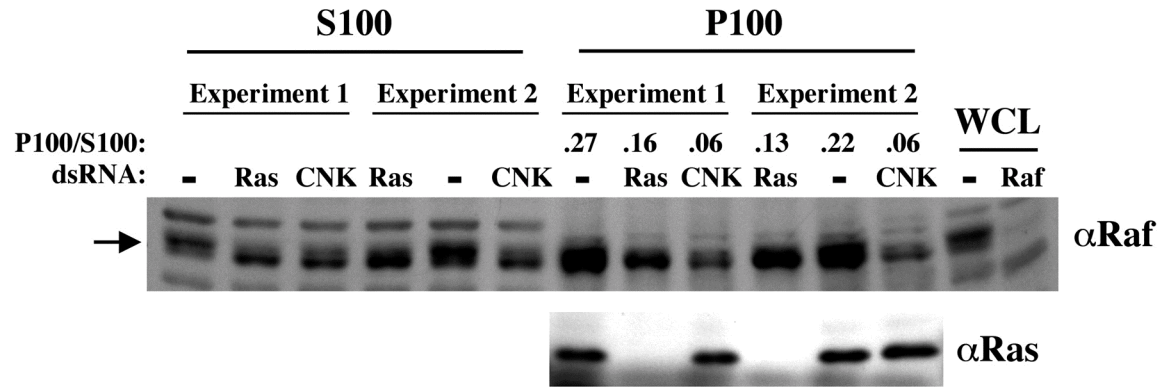


Figure 4.4. CNK is required for membrane localization of D-Raf. S2 cells (5×10^6) were treated for 72 h with or without 5 µg/ml of the indicated dsRNA. The cells were then disrupted by nitrogen cavitation at 600 psi. Cleared supernatant was centrifuged for 1 h at 100,000 × g. The levels of D-Raf and present in the membrane particulate fraction (p100) and soluble fraction (S100) are shown. The arrow indicates the bands corresponding to hypo- and hyperphosphorylated Raf. The last two lanes are whole cell lysates from S2 cells treated with the control or Raf dsRNAs. The Raf signal is greatly reduced by the Raf dsRNA. The levels of Ras present in the P100 fractions are shown in the bottom panel. No Ras signal was detected in the S100 fractions. Results from two independent experiments are shown. P100/S100 ratios were calculated as described under "Materials and Methods." Similar results were obtained in five independent experiments.

CNK can also associate with Raf when overexpressed in cells (Therrien et al., 1998). These observations hint that CNK may contribute to plasma membrane compartmentalization of Raf. Multiple studies suggest Raf must be targeted to the plasma membrane prior to activation (Kolch, 2000). Consistent with observations in mammalian cell culture systems, we find Raf protein both in the membrane particulate fraction (P100) and the soluble fraction (S100) of mechanically disrupted S2 cells. Down-regulation of CNK resulted in a dramatic reduction of Raf protein in the P100 fraction independently

of insulin stimulation (Fig. 4.4). Surprisingly, the presence of Raf in the P100 fraction is more dependent upon CNK than Ras. This result strongly suggests that the contribution of CNK to Raf activation is at least in part through appropriate compartmentalization of Raf proteins to the site of activation. In contrast, down-regulation of KSR had no detectable effect on Raf compartmentalization (data not shown).

To further elaborate a general requirement of KSR and CNK to mediate Raf activation, we screened for additional ERK stimuli in S2 cells. We found that ERK is activated in response to 1 μM phorbol 12-myristate 13-acetate (PMA). A body of literature suggests that PMA activation of ERK is mediated by PKC and Raf independently of Ras (de Vries-Smits et al., 1992; Hawes et al., 1995; Ueda et al., 1996; van Biesen et al., 1996). The majority of these studies employed dominant inhibitory Ras variants to exclude a role for Ras activity. However, recent studies using neutralizing Ras antibodies demonstrate that Ras is required for PMA activation of ERK (Chiloeches et al., 1999; Marais et al., 1998). Consistent with these later studies, we found that down-regulation of Ras blocks PMA activation of ERK (Fig. 4.5). Similarly, CNK and KSR are required for PMA-induced activation of ERK (Fig. 4.5).

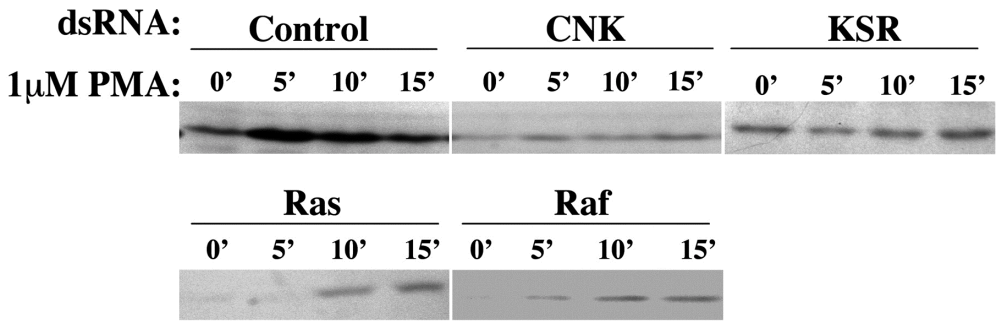


Figure 4.5. Ras, KSR, and CNK are required for PMA-induced activation of ERK-A. S2 cells were treated with dsRNAs as described in the legend to Fig. 4.1 and were subsequently stimulated with 1 μM PMA for the indicated times. Similar results were obtained in two independent experiments.

In summary, we have provided the first direct biochemical evidence that CNK and KSR are integral components of the cellular machinery required for Raf activation. Transfected epitope-tagged CNK, and a related mammalian protein MAGUIN (MAGUK-interacting protein), can both interact with Raf in cells suggesting that native CNK and Raf form complexes (Therrien et al., 1998; Yao et al., 2000). Both transfected CNK and MAGUIN partially localize to plasma membrane compartments (Therrien et al., 1998; Yao et al., 2000). These observations lead to the hypothesis that CNK may participate in regulating compartmentalization of Raf in cells. However, expression of MAGUIN was not sufficient to recruit Raf1 to the plasma membrane (Yao et al., 2000). Therefore, there have been no direct observations supporting this hypothesis. Here, we have directly

shown that inhibition of native CNK expression blocks native Raf activation, and prevents compartmentalization of Raf at the plasma membrane, suggesting the biochemical contribution of CNK to Raf activation is at least partially due to facilitation of appropriate cellular localization.

KSR contains a putative serine/threonine kinase domain (Therrien et al., 1995). One group has reported that immunoprecipitated KSR can phosphorylate Raf in vitro, which is compelling evidence for a biochemical relationship between Raf and KSR (Xing and Kolesnick, 2001). On the other hand, other groups find no evidence for an intrinsic kinase activity of KSR and contest the classification of this protein as a kinase (Morrison, 2001; Muller et al., 2000; Muller et al., 2001). Several reports do show that KSR immunoprecipitates from cells are tightly associated with kinases that can phosphorylate KSR itself, but these KSR-associated kinases reportedly do not utilize Raf1 as a substrate in vitro (Morrison, 2001; Muller et al., 2001). Our direct biochemical observation of the requirement of KSR for Raf activation is consistent with either the possibility that KSR is indeed a Raf kinase, or that it is a linker protein required to couple Raf to the upstream kinases that are responsible for activating phosphorylation events on Raf. Unlike CNK, KSR does not appear to be required to compartmentalize Raf at the plasma membrane (data not shown). Studies are currently underway to employ the system described here to identify critical kinases responsible for Raf activation.

Materials and Methods

Materials. Double-stranded RNA (dsRNA) was prepared and used according to Clemens *et al*. (2000). Schneider L2 (S2) cells were cultured in *Drosophila* serum-free media (Invitrogen) supplemented with 16.5 mM L-glutamine (Invitrogen) and 50 µg/ml gentamicin (Sigma). Total RNA was prepared using the High Pure RNA Isolation Kit (Roche Molecular Diagnostics). Reverse transcriptase-PCR was performed using Superscript First Strand Synthesis system for reverse transcriptase-PCR (Invitrogen). Antibodies against phospho-ERK and total ERK were purchased from Sigma (M5670, M8159). Anti-phospho-Akt antibody was from Cell Signaling (#9271). Antibody against *Drosophila* total Akt was a generous gift from Brian Hemmings. Draf and Dras antibodies were generous gifts from Deborah Morrison and Helmut Kramer, respectively. Antibodies against MEK1/2 and phospho-MEK1/2 were from Cell Signaling (#9122) and Sigma (I27-67).

Raf Kinase Assay. S2 cells were stimulated with 10 µg/ml human recombinant insulin for 0, 5, 10 min and immediately lysed in a modified RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 5 mM sodium orthovanadate, 25 mM glycerophosphate, 2 mM sodium pyrophosphate). After rotation for 20 min at 4 °C, the cell lysates were cleared by centrifugation at 17,000 × g for 15 min. From the cleared lysates, D-Raf was

immunoprecipitated with 2 µl of a polyclonal rabbit anti-D-Raf antibody. The immunoprecipitates were then washed three times in RIPA buffer (137 mM NaCl), two times in a high salt RIPA buffer (500 mM NaCl), and finally two times in 25 mM HEPES + 10 mM MgCl2. To the 20 µl of washed immunoprecipitates were added 30 µl of kinase reaction buffer (25 mM HEPES, pH 7.4, 10 mM MgCl2, 83 µM ATP, and 0.5 µg of recombinant His-MEK1N1(K97M)). After incubation for 30 min at 30°C, MEK1 phosphorylation was assayed using an antibody that specifically recognizes Ser(P)-217/221.

Cell Fractionation. S2 cells were resuspended in homogenization buffer (20 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM sodium orthovanadate, 25 mM glycerophosphate, 2 mM sodium pyrophosphate, 20 mM NaF) and incubated for 15 min on ice. The cells were then subject to nitrogen cavitation at 600 psi. Upon release of pressure, the disrupted cells were centrifuged at 17,000 × g for 5 min. The supernatant was then centrifuged for 1 h at 100,000 × g. P100 designates the pelleted material, whereas S100 designates the soluble supernatant. 1% of the total S100 and 10% of the total P100 fractions from each sample were loaded for Western analysis. Signal intensities from anti-Raf immunoblots were quantitated using an Alpha Inotech digital imaging system together with the Flouro-Chem software package (Scimetrics).

CHAPTER FIVE
CNK2 Couples NGF Signal Propagation to Multiple Regulatory Cascades Driving Differentiation.

Abstract

Neuronal precursor cells have the capacity to engage the Raf-MEK-ERK signal module to drive either of two distinctly different regulatory programs, proliferation or differentiation. This is, at least in part, a consequence of stimulus-specific shaping of the response of the kinase cascade. For example, the mitogen EGF induces a transient ERK activation, while the neurotrophin NGF induces prolonged ERK activation (Vaudry et al., 2002). Here we define a novel component of the regulatory machinery contributing to the selective integration of MAP kinase signaling with discreet biological responses. We show that the scaffold/adaptor protein CNK2/MAGUIN-1 is selectively required for NGF but not EGF induced ERK activation. In addition, CNK2 makes a separate essential contribution to the coupling of NGF signaling to membrane/cytoskeletal remodeling. One molecular component of this activity appears to be the membrane cytoskeleton remodeling protein and candidate tumor suppressor Band 4.1B/Dal-1.

Introduction

The Raf-MEK-ERK kinase cascade is a core signal transduction module implicated in the regulation of diverse biological processes, ranging from cellular proliferation and tumorigenesis, to differentiation and cell specialization (Pearson et al., 2001; Zhu et al., 2002). Little is currently understood about how the activity of this cascade is appropriately integrated with multiple regulatory pathways in order to contribute to discrete biological responses. One mechanism that may help confer signaling specificity to the Raf-MEK-ERK module is the generation of higher order molecular organization by scaffold proteins (Garrington and Johnson, 1999; Schaeffer and Weber, 1999; van Drogen and Peter, 2002). Candidates include the putative scaffolding or linker proteins Kinase Suppressor of Ras (KSR) (Kornfeld et al., 1995; Sundaram and Han, 1995) (Therrien et al., 1995), Connector-enhancer of KSR (CNK) (Therrien et al., 1998), Suppressor of Ras-8 (Sur-8) (Sieburth et al., 1998), and MEK Partner-1 (MP-1) (Schaeffer et al., 1998).

Kinase suppressor of Ras was initially identified as a suppressor of developmental defects induced by gain-of-function Ras mutant expression in *Drosophila* and *C. elegans*. KSR likely potentiates signal propagation by facilitating kinase/substrate interactions within the Raf-MEK-ERK cascade (Anselmo et al., 2002; Nguyen et al., 2002; Therrien et al., 1996). It is unknown if KSR selectively integrates ERK signaling with discrete

biological responses, or if it plays a general role in ERK activation. Connector-enhancer of KSR (CNK) was isolated in a *Drosophila* mutant screen for modifiers of the KSR mutant phenotype, and has similar consequences on MAP kinase signaling in *Drosophila* as KSR. *Drosophila* CNK contains SAM, PDZ, and PH domains, interacts with Raf, and is localized on the plasma membrane at regions of cell-cell contact (Therrien et al., 1998). Recently, it was demonstrated that both KSR and CNK are required for insulin-induced Ras-mediated activation of Raf, MEK, and ERK in *Drosophila* S2 cells, and that CNK is at least partly responsible for recruitment of Raf to the plasma membrane-enriched fraction of these cells (Anselmo et al., 2002). The human genome contains two predicted CNK orthologs, CNK1 and CNK2, which are 42% and 40% similar to *Drosophila* CNK, respectively. CNK1 has a broad expression profile, while CNK2 is restricted to neuronal tissues. *Rattus norvegicus* CNK2/MAGUIN-1, which is 99% identitical to human CNK2, interacts with synaptic density proteins PSD-95, S-SCAM , and Densin-180 (Ohtakara et al., 2002; Yao et al., 1999), and shows synaptic localization in cultured primary hippocampal neurons (Iida et al., 2002). Similarly to *Drosophila* CNK, rat CNK2 binds to the C-terminus of Raf-1, and is targeted to the plasma membrane by its PH domain (Yao et al., 2000).

Although CNK appears to be a necessary component of Ras-MAPK signaling at least in some cellular contexts in *Drosophila*, it is unknown if or in what capacity the

mammalian CNK isoforms may participate in this signaling pathway. Here we report that CNK2 plays an essential role in ERK kinase activation and neurite formation in PC12 cells. CNK2 selectively contributes to NGF-mediated ERK activation but is apparently dispensable for activation of this cascade by EGF. CNK2 also makes an ERK-independent contribution to NGF-induced neurite outgrowth, potentially through interaction with the membrane cytoskeleton organizing FERM domain protein 4.1B/DAL-1. Thus, CNK2 appears to enable receptor-selective coupling to the MAP kinase cascade and may function to integrate the activity of this cascade together with additional regulatory pathways required for an appropriate biological response to NGF.

Results

CNK2 is required for NGF-induced PC12 cell differentiation.

To reveal a potential contribution of CNK to Ras-Raf-MEK signaling in mammalian cells, we examined the consequences of inhibiting CNK2 expression in rat pheochromocytoma PC12 cells. This cell line is a well-characterized model of ERK-dependent neuronal differentiation, and is suited for both biochemical and morphological analysis of MAP kinase signaling (Vaudry et al., 2002). We first tested the effect of CNK2 inhibition on NGF-induced neurite outgrowth. Cells were transfected with siRNAs targeting CNK2 (CNK2-2313), together with GFP to mark the transfectants, and

induced to differentiate with NGF for 48 hours. As a positive control, we used siRNAs targeting the expression of the primary NGF receptor TrkA (Chao and Hempstead, 1995). SiRNA directed against human A-Raf, with no predicted target site in the rat genome, served as a negative control. SiRNAs targeting TrkA and CNK2 significantly inhibited NGF-induced PC12 cell differentiation (Figure 5.1A). Quantitation of transfected cells showed that a TrkA siRNA completely blocked formation of NGF-dependent neurite-like extensions, while CNK2 siRNA resulted in approximately 70% suppression (Figure 5.1B). Similar results were observed with a second independent siRNA sequence targeting CNK2 (CNK2-501), however, we found that a third siRNA (CNK2-881) was ineffective (data not shown). We assessed the relative activity and specificity of these siRNAs by targeting the expression of myc-tagged polypeptides corresponding to the amino and carboxyl terminal halves of CNK2. CNK2-2313 and CNK2-501 were both effective against their respective targets. However, CNK2-881, which did not suppress NGF-induced neurite formation, had no activity (Figure 5.1C). As transfection efficiencies rarely exceeded 30%, the consequence of CNK2 siRNA on endogenous CNK2 expression was assayed by RT-PCR following sorting of transfected (GFP positive) cells (Fig. 5.1D).

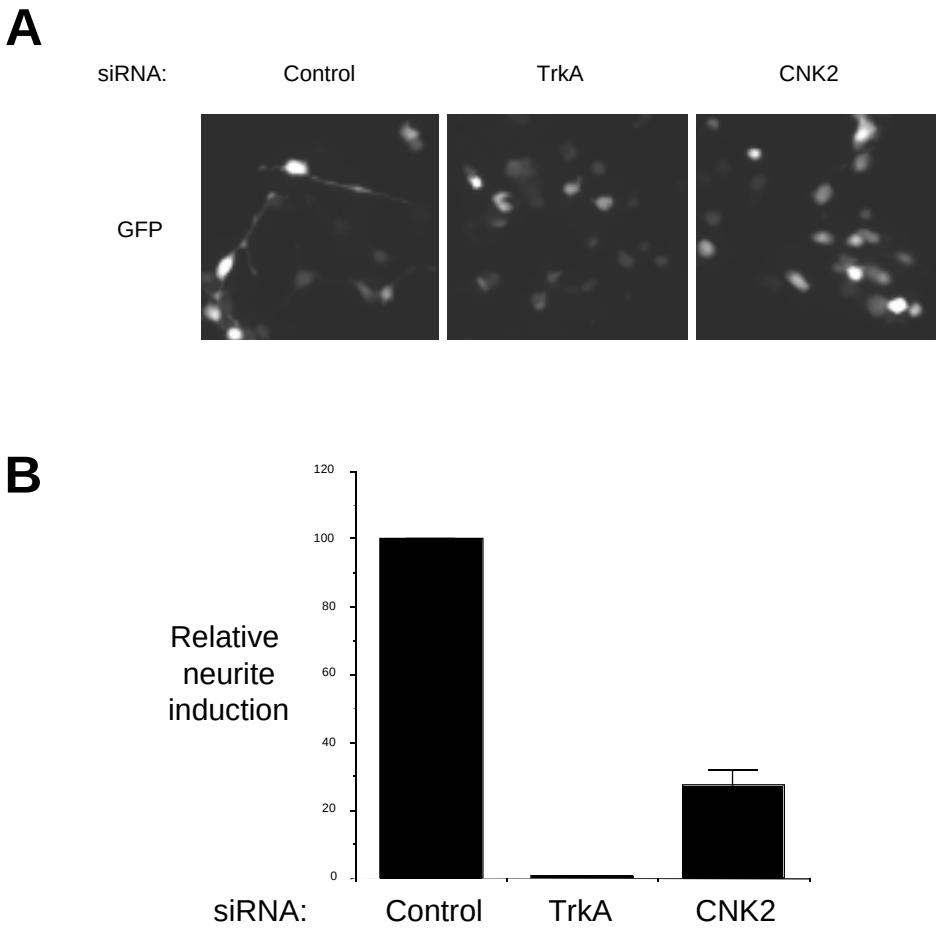
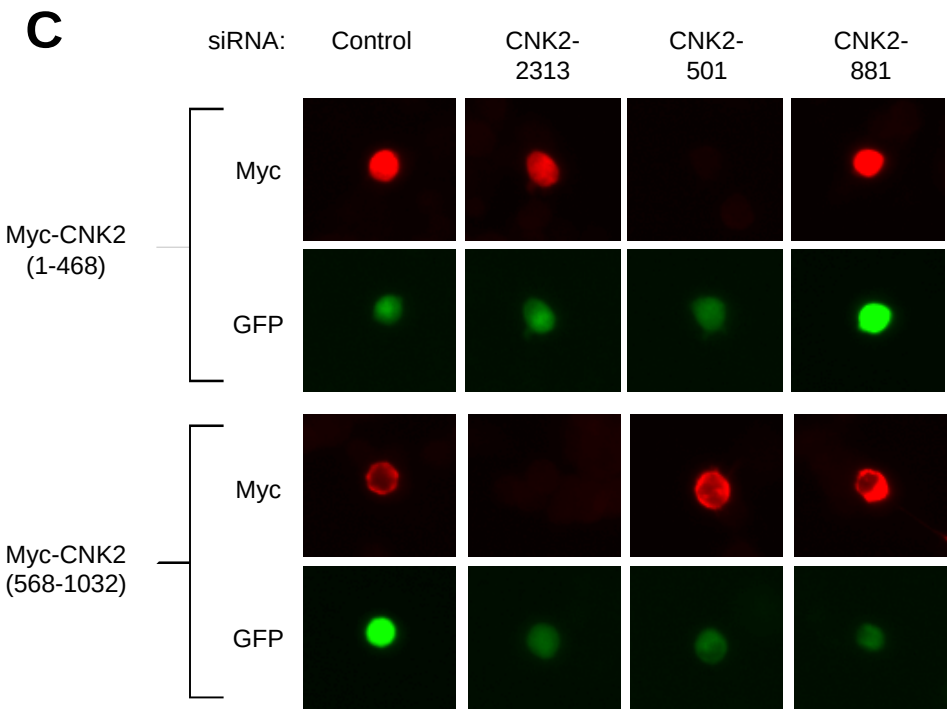


Figure 5.1 CNK2 is required for NGF-induced neurite outgrowth in PC12 cells. **A)** Cells were transfected with GFP together with the indicated siRNAs. Neurite formation was induced with 100 ng/ml NGF for 48 hours. **B)** The number of GFP-positive cells with neurites longer than one cell body was determined as a percentage of the total number of GFP-positive cells. The average of normalized results from three independent experiments is shown. Bars represent standard deviation.



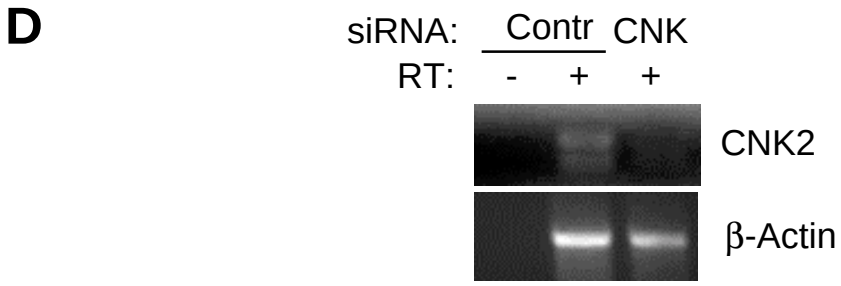
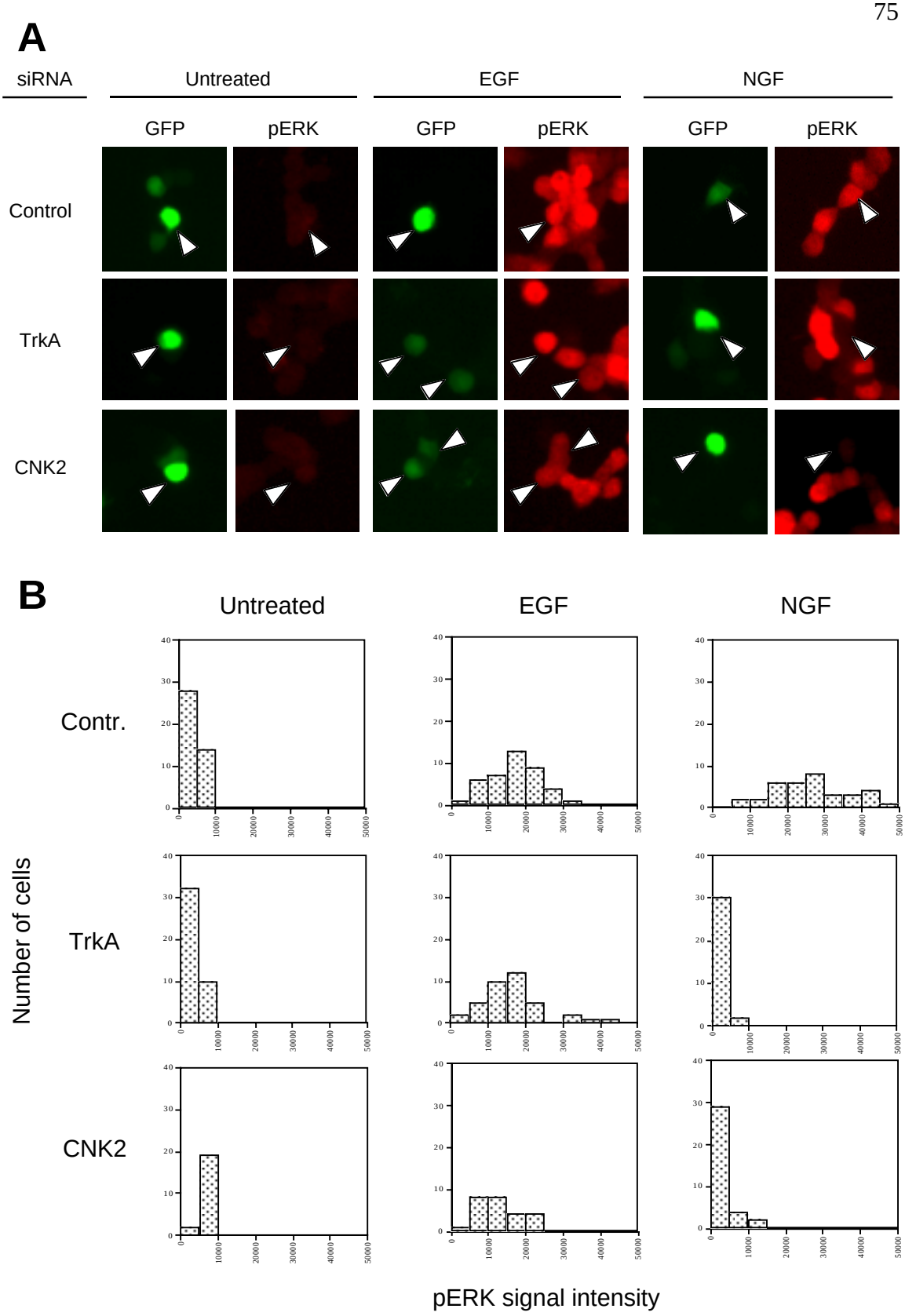


Figure 5.1 (cont.) **C)** PC12 cells were transfected with 0.8 µg pCINeo-Myc-CNK2(a.a.1-468) or pCINeo-Myc-CNK2(a.a.568-1032), together with 100 nM of the indicated siRNA and 0.1 µg pCEP4-GFP, and immunostained to detect Myc expression. SiRNA names indicate base pair positions in the CNK2 coding sequence. **D)** PC12 cells were co-transfected with siRNAs and GFP, and GFP-positive cells were sorted three days post-transfection. RNA was isolated and RT-PCR was performed to determine the abundance of CNK2 and β-actin transcripts. RT – reverse transcriptase.

CNK2 contributes to NGF but not EGF induced ERK activation.

To examine if suppression of neurite outgrowth by inhibition of CNK2 expression is correlated with an inhibition of NGF-mediated ERK activation, we measured the activity of endogenous ERK1/2 following ligand stimulation. ERK activation was examined at the single-cell level by immunofluorescent labeling using a phospho-ERK specific antibody. SiRNAs targeting either TrkA or CNK2 inhibited ERK activation in response to NGF treatment as compared to control siRNA. By contrast, EGF-mediated ERK activation was unaffected (Figure 5.2A). The distribution of phospho-ERK signal intensities was unaffected upon inhibition of CNK2 or TrkA in EGF-stimulated cells, but was clearly collapsed to a basal (uninduced) state upon inhibition of CNK2 or TrkA in NGF-stimulated cells (Figure 5.2B). These observations suggest that, in PC12 cells, CNK2 is selectively coupled to NGF versus EGF receptor signaling, at least with respect to stimulus-dependent ERK activation. Similar results were obtained by Western blot analysis of PC12 cell lysates (Figure 5.2C). The majority of the phospho-ERK signal in the presence of effective TrkA and CNK2 siRNAs on the Western blot is likely derived from untransfected cells.


A
siRNA
Untreated
EGF
NGF
GFP
pERK
GFP
pERK
GFP
pERK
Control
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CNK2
B
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EGF
NGF
Contr.
TrkA
CNK2
Number of cells
pERK signal intensity

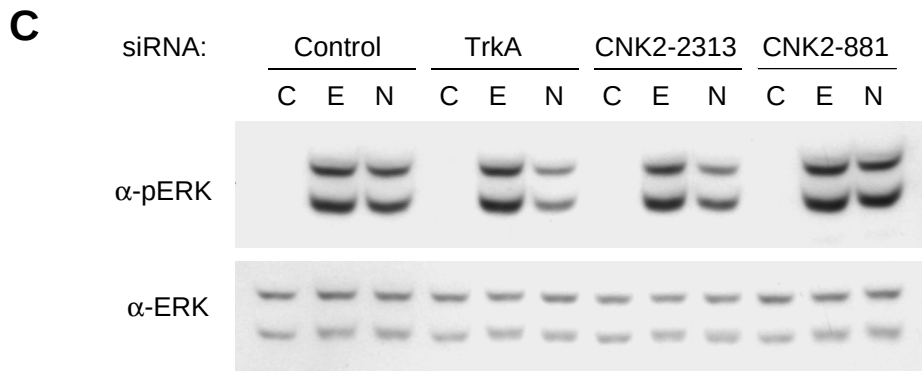

Figure 5.2 CNK2 is required for NGF-mediated ERK activation. *Previous page:* **A)** PC12 cells were transfected with the indicated siRNAs together with GFP, serum-starved for 18 hours, then stimulated for 5 minutes with EGF or NGF (100 ng/ml each). Following immunostaining with a phospho-ERK specific antibody, levels of activated ERK in the GFP-positive cells were evaluated. Arrows indicate transfected cells. **B)** Distribution of individual data points for phospho-ERK signal intensities from a representative experiment. Between 20 and 40 single-cell measurements per sample were collected using Open Lab 2.2.5 software. *Above:* **C)** Western blot analysis of lysates from PC12 cells that were transfected with the indicated siRNAs, starved, and stimulated with EGF or NGF.

ERK-independent contribution of CNK2 to PC12 cell differentiation.

Drosophila CNK has been placed upstream of Raf and downstream of or in parallel to Ras by genetic and biochemical analysis (Anselmo et al., 2002; Therrien et al., 1998). Therefore, we tested if activating the MAP kinase pathway at the level of Raf could bypass the requirement for CNK2 for neurite outgrowth. PC12 cells were transfected with RafBXB, a constitutively activated variant of Raf1 uncoupled from regulation by Ras, together with CNK2 siRNAs or control siRNAs. Unexpectedly,

CNK2 siRNA suppressed RafBXB-induced neurites by approximately 65% (Figure 5.3A), in the absence of inhibition of RafBXB-induced ERK activation (Figure 5.3B). This suggests that in addition to its contribution to NGF-stimulated ERK activation, there is another, distinct function of CNK2, which is required in concert with ERK activation for successful formation of neurite-like projections.

A Raf-MAP kinase independent function for *Drosophila* CNK has been suggested by the observation that transgenic expression of the amino-terminal half of CNK in the fly eye enhances an activated Ras-induced rough eye phenotype in an apparently MAP kinase-independent fashion (Therrien et al., 1999). When expressed in PC12 cells, we found that the orthologous molecule, mammalian CNK2(1-468) dominantly inhibits NGF-induced neurite outgrowth (Figure 5.4A). However, NGF-induced ERK activation was not affected (Figure 5.4B). We then evaluated the impact of CNK2(1-468) expression on RasV12, RafBXB, and MEKR4F induced neurite formation. As shown in Figure 5.4C, CNK2(1-468) potently suppressed the morphological response of PC12 cells to all three activated components of the Ras-MAP kinase pathway. CNK(1-468) did not interfere with ERK activation by RasV12 (data not shown). These results further indicate a role for CNK2 in PC12 cell differentiation distinct from its contribution to NGF-induced ERK activation.

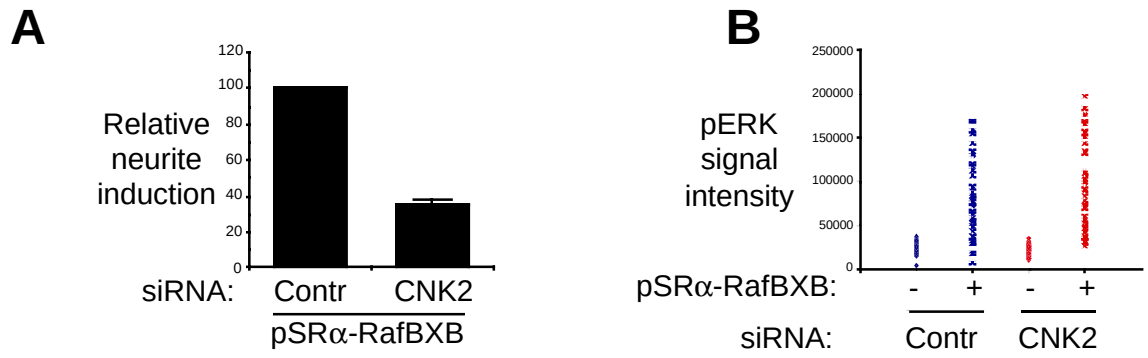

Figure 5.3 CNK2 is required for neurite induction by RafBXB. **A)** PC12 cells were transfected with pSRα-RafBXB together with the indicated siRNAs, and immunostained with anti-Raf-1 antibody to detect RafBXB expression. RafBXB-positive cells were scored for neurite outgrowth as in Figure 1. The average ± S.D. of normalized results from three experiments is shown. **B)** ERK activation in untransfected (♦) or RafBXB-expressing (×) cells was quantitated on a single-cell basis using a phospho-ERK-specific antibody. The individual raw signal intensities are shown.

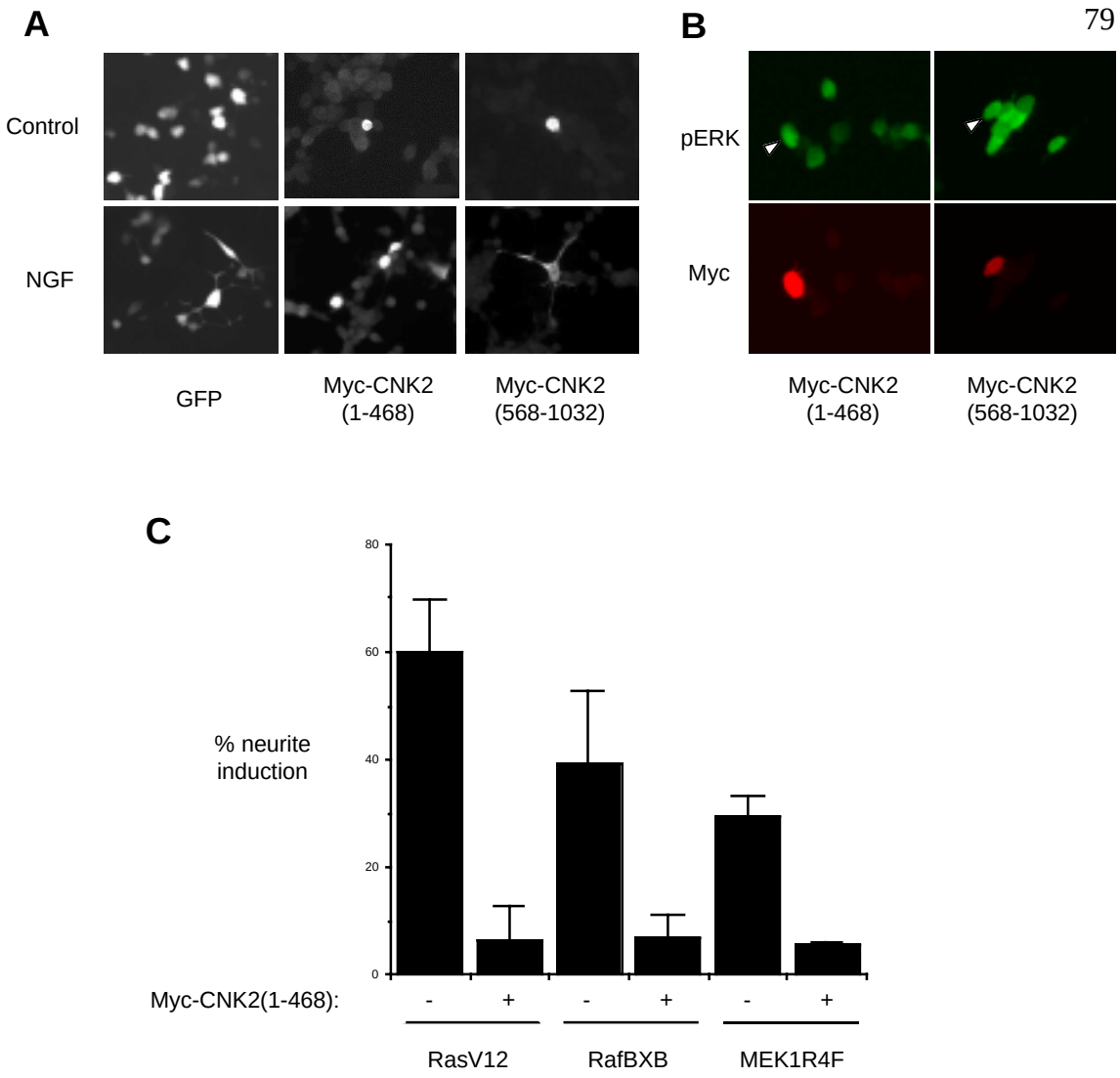

Figure 5.4 CNK2(a.a.1-468) inhibits NGF-induced neurite formation without inhibiting ERK activation. **A)** PC12 cells were transfected with pCEP4-GFP, pCIneo-myc-CNK2(a.a.1-468), or pCIneo-myc-CNK2(a.a.568-1032), then treated with NGF for 48 hours or left untreated. CNK2 expression was detected by immunostaining with anti-Myc antibody. **B)** PC12 cells transfected with pCIneo-myc-CNK2(a.a.1-468) or pCIneo-myc-CNK2(a.a.568-1032) were serum-starved, stimulated with 100 ng/ml NGF for 5 minutes, and immunostained with anti-phospho-ERK and anti-Myc antibodies. Arrows indicate transfected cells. **C)** PC12 cells were transfected with pDCR-RasV12, pSRα-RafBXB, or pCMV5-MEK1R4F alone or in combination with pCIneo-myc-CNK2(a.a.1-468). Following immunostaining to detect expression of the necessary constructs, cells were scored for the presence of neurites. The average ± S.D. of three independent experiments is shown.

Band 4.1B/DAL-1 interacts with CNK2 and is required for neurite induction

To begin to gain further understanding of CNK2 function, beyond MAP kinase signaling, we searched for novel molecular partners of CNK2. A yeast two-hybrid screen of a human placenta cDNA library identified the tumor suppressor DAL-1/band 4.1B (Tran et al., 1999) as a candidate CNK-interacting protein. DAL-1 is a member of the band 4.1 family of proteins that have been implicated in the regulation of actin cytoskeleton and plasma membrane organization (Hoover and Bryant, 2000; Sun et al., 2002). To validate this interaction in mammalian cells, we expressed DAL-1 together with myc-tagged amino-terminal and carboxy-terminal truncations of CNK2. As shown in Figure 5.5A, DAL-1 can selectively associate with CNK2(568-1032).

We next examined the contribution of DAL-1/4.1B to PC12 cell differentiation. Expression of 4.1B in PC12 cells has been reported previously (Parra et al., 2000), and was verified by PCR amplification from PC12 cDNA (data not shown). SiRNAs targeting 4.1B significantly inhibited NGF-induced neurite outgrowth as compared to control siRNAs (Figure 5.5B), without inhibiting NGF-mediated ERK activation (Fig. 5.5D). The activity of 4.1B siRNAs against endogenous 4.1B was verified by immunostaining (Fig. 5.5C).

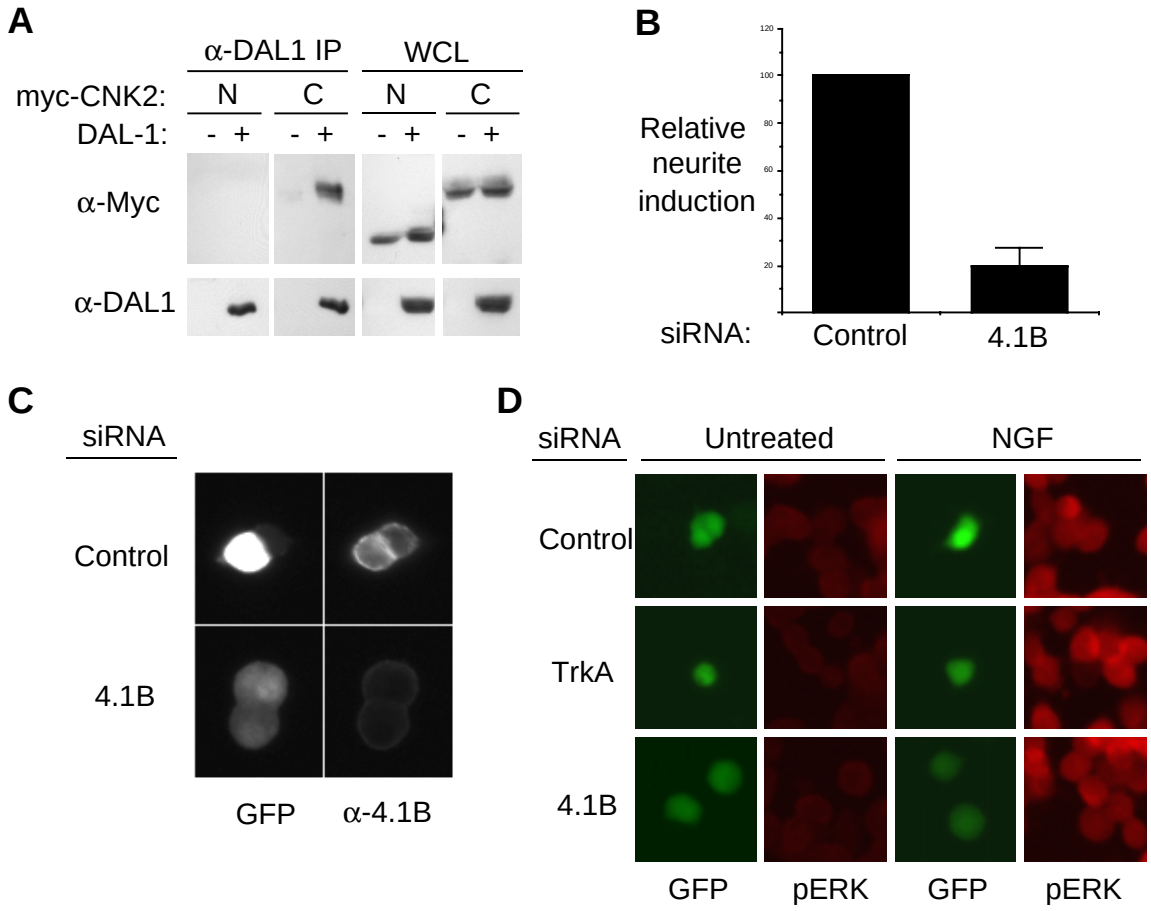

Figure 5.5 DAL-1 interacts with mammalian CNK isoforms and is required for neurite outgrowth. **A)** HEK293 cells were transfected with the indicated constructs, lysed, and immunoprecipitated with anti-DAL-1 antibody. N: CNK2(1-468), C: CNK2(568-1032). **B)** PC12 cells were transfected with GFP together with the indicated siRNAs, and GFP-positive cells were evaluated for NGF-induced neurite outgrowth. The average ± S.D. of normalized results from three independent experiments is shown. **C)** PC12 cells were co-transfected with siRNAs and GFP, and immunostained to detect the expression of endogenous protein 4.1B. **D)** PC12 cells were transfected with the indicated siRNAs together with GFP, serum-starved for 18 hours, then stimulated for 5 minutes with NGF (100 ng/ml). Following immunostaining with a phospho-ERK specific antibody, levels of activated ERK in the GFP-positive cells were evaluated. E) PC12 cells co-transfected with siRNAs and GFP, and immunostained to detect the expression of endogenous protein 4.1B.

CNK1 modulates actin cytoskeleton dynamics

The observations described above suggest that CNK2 may integrate MAP kinase activation with regulation of cytoskeleton/plasma membrane dynamics to mediate PC12 cell differentiation. CNK2 expression is restricted to neuronal cell types (Yao et al., 1999), while the closely related family member CNK1 has a broader expression profile (NCBI database analysis). As defined by RT-PCR, HeLa cells do not express CNK2, but do express CNK1 (data not shown and Figure 5.6A). We targeted the expression of CNK1 by RNAi to assess its contribution to mitogen-dependent MAP kinase signaling and regulation of actin-cytoskeletal morphology. Similar to our observations of CNK2 function in PC12 cells, CNK1 did not appear to be limiting for EGF-mediated activation of ERK in HeLa cells (Figure 5.6A). Nor could we reveal a contribution of CNK1 to the activation of MEK or ERK in response to PMA or LPA (data not shown). However, inhibition of CNK1 expression did lead to a striking alteration in the cortical actin cytoskeleton, correlating with a cobble-stone cell morphology very similar to that adopted by normal differentiated epithelial cells (Figure 5.6B). This observation suggests the intriguing possibility that CNK1 may participate in maintaining the epithelial to mesenchymal transition that occurs during oncogenic transformation.

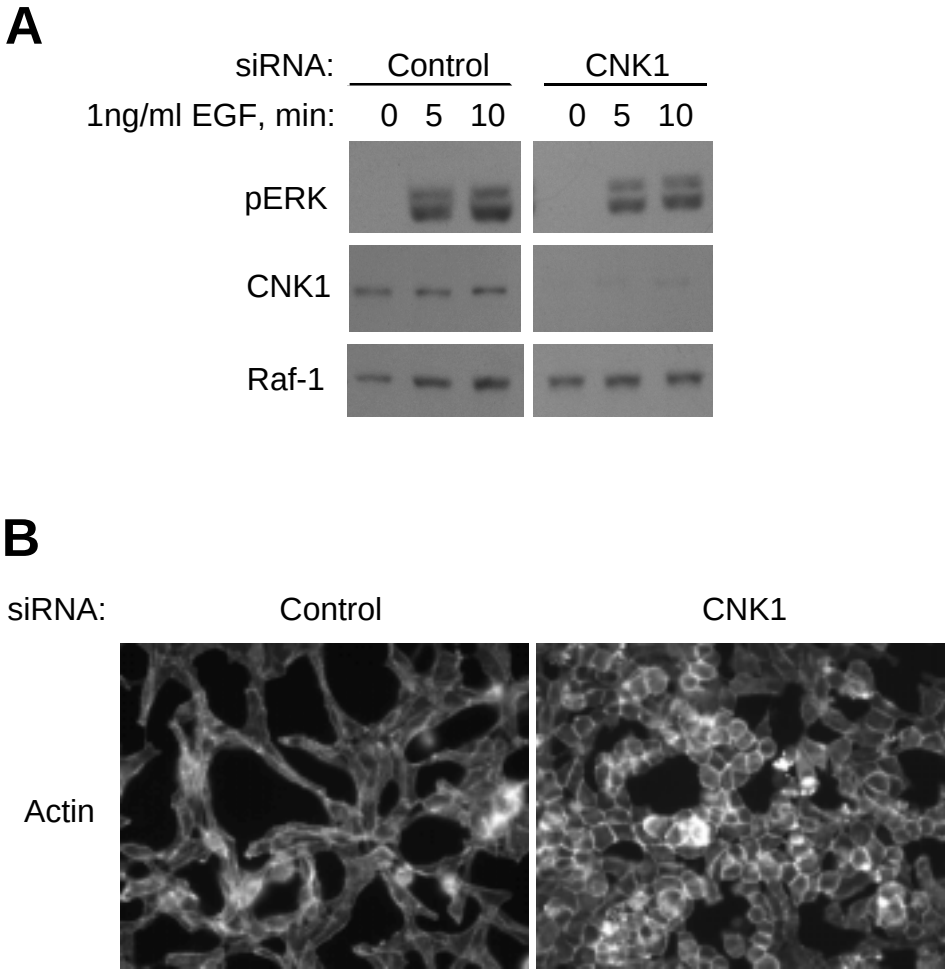

Figure 5.6 CNK1 is not limiting for EGF-induced ERK activation, but contributes to transformed morphology in HeLa cells. **A)** HeLa cells were transfected with control or CNK1 siRNAs, starved for 24 hours, and stimulated with 1 ng/ml EGF for the indicated times. Lysates were analyzed on a Western blot with the indicated antibodies. Raf-1 is shown as a loading control. **B)** HeLa cells were transfected with control or CNK1 siRNAs and labeled with rhodamine-phalloidin to detect F-actin.

Discussion

A great deal of information has been gathered about the participation of the Ras-MAPK signal transduction circuitry in the modulation and/or propagation of a broad range of dynamic cell regulatory events (Pearson et al., 2001). Attention is now necessarily becoming focused on understanding the molecular architecture that allows the core enzymatic components of this cascade to be shared among multiple regulatory pathways yet produce distinct biological outcomes in response to discreet inductive signals. There is accumulating evidence that control of the amplitude, duration, and/or subcellular compartmentalization of ERK activation are critical determinants of the biological response (Pearson et al., 2001). The discovery and characterization of non-catalytic accessory components that can modulate the assembly, activation and compartmentalization of MAP kinase cascades is beginning to reveal mechanisms that generate specificity in the coupling of MAP kinase activation to appropriate biological responses (Sternberg and Alberola-Ila, 1998).

CNK2 is a candidate component of molecular machinery that may be responsible for higher order functional assembly of the Raf-MAP kinase cascade. In neuronal precursor cells, two different ligands require coupling to activation of the Ras-MAP kinase cascade to produce distinct biological outcomes: NGF promotes G_0 cell cycle arrest and neuronal differentiation, while EGF promotes cell cycle progression (Kao et

al., 2001; Vaudry et al., 2002). We have used this system to evaluate the contribution of CNK2 to NGF and EGF signal propagation. Using RNAi-mediated loss-of-function analysis, we find that CNK2 is required for NGF-induced ERK activation and neurite outgrowth in PC12 cells. In contrast, EGF-induced ERK activation is independent of CNK2 expression. These observations demonstrate that the MAP Kinase cascade can be discretely coupled to distinct receptor tyrosine kinases, revealing a novel control mechanism that may contribute to the specificity of signal propagation or signal integration.

Further observations suggest that the participation of CNK2 in NGF signaling is not limited to facilitation of MAP kinase activation, such that CNK2 may be involved in coordinating MAP kinase signaling with additional regulatory pathways that participate in cellular differentiation. Expression of mutationally activated variants of Raf or MEK in PC12 cells is sufficient to drive ERK activation (Cowley et al., 1994) and this occurred independently of CNK2 expression, consistent with observations in *Drosophila* cells placing the contribution of CNK to ERK activation upstream of Raf (Anselmo et al., 2002; Therrien et al., 1998). However, despite robust ERK1/2 activation, we found that neurite formation induced by expression of activated Raf was dependent on CNK2 expression. Supporting evidence for a MAP kinase-independent role of CNK2 in neurite formation comes from the observation that the amino-terminal half of CNK2 can function

as a dominant inhibitor of neurite outgrowth without suppressing ERK activation mediated by NGF or by an activated mutant of Ras.

We have implicated protein 4.1B/DAL-1 as a candidate molecular connection between CNK2 and ERK-independent contributions to the development of NGF-induced neurite-like extensions. CNK2 associates with protein 4.1B, and protein 4.1B is required for generation of NGF-induced neurite-like extensions, but not ERK1/2 activation. Protein 4.1 isoforms belong to the larger FERM (4.1/ezrin/radixin/moesin) domain family of proteins, members of which participate in signal-induced modulation of the actin cytoskeleton (Bretscher et al., 2002). Protein 4.1B is found on the plasma membrane, predominantly enriched at cell-cell junctions (Tran et al., 1999). Band 4.1 proteins can function to stabilize plasma membrane cytoskeleton by promoting spectrin/actin association, and can be found in complexes together with transmembrane proteins, including glycophorin C, neurexins, and TSLC1, and membrane-associated guanylate kinases (MAGUKs), including p55, hDLG, and CASK (Hoover and Bryant, 2000; Subrahmanyam et al., 1991; Yageta et al., 2002). A 4.1/transmembrane protein/MAGUK ternary complex at the plasma membrane is suspected to participate in signal transduction events at sites of cell-cell contact (Hoover and Bryant, 2000). DAL-1 is a splice variant of 4.1B and was identified as a candidate tumor suppressor that is inactivated in approximately 60% of non-small cell lung carcinomas and sporadic meningiomas

(Gutmann et al., 2000; Tran et al., 1999), and in 56% of ductal carcinomas (Kittiniyom et al., 2001). Re-expression of DAL-1 in DAL-$1^{-/-}$ tumor cell lines inhibits proliferation, motility, and invasiveness, while promoting increased cell-cell and cell-substrate attachment (Charboneau et al., 2002; Gutmann et al., 2001; Tran et al., 1999). In *Drosophila* the band 4.1 homolog D4.1/Coracle is localized at septate junctions of epithelial cells, and is required for transepithelial barrier integrity (Fehon et al., 1994; Lamb et al., 1998). This localization pattern is similar to that described for *Drosophila* CNK (Therrien et al., 1998). Importantly, genetic interactions indicate that both Coracle and CNK function downstream of the activated EGF receptor (Fehon et al., 1994; Therrien et al., 1998). The molecular nature of the coupling of CNK2 to morphological differentiation remains to be determined, and likely will involve multiple protein/protein interactions. Clearly further work is warranted to evaluate the relationship between CNK2 and band 4.1, however, our observations in this study together with those described above suggest that a CNK2/4.1B complex may be one component that links NGF-signaling to the membrane cytoskeletal rearrangements required for neurite extension.

Additional evidence implicating CNK proteins in the regulation of cell morphology comes from our observations of the consequences of inhibiting CNK1 expression in an epithelial-derived cell line. We could find no evidence that CNK1

participates in MAP kinase signaling in HeLa cells, as it was not limiting for ERK activation in response to EGF, LPA, or PMA. We may have simply failed to identify an appropriate ligand that stimulates a CNK1-dependent receptor, or CNK1 may be uncoupled from this regulatory pathway. However, like CNK2 in PC12 cells, we found that CNK1 plays an important role in regulating cell morphology. Inhibiting CNK1 expression leads to the formation of extensive actin-rich cell-cell contacts that resemble junctions characteristic of normal epithelial cell monolayers. It remains to be determined if this response may be related to association of CNK1 with a band 4.1 family member.

In summary, the loss-of-function analysis presented here suggests that CNK2 is a key participant in NGF receptor-specific signal propagation through both selective coupling to ERK activation and integration with membrane/cytoskeletal remodeling. CNK proteins directly interact with Raf kinases and may therefore function as multivalent scaffolds for the Raf-MAP kinase cascade, providing spatial and/or kinetic integration of the activity of this cascade with additional regulatory pathways required for an appropriate biological response.

Materials and Methods

Cell culture and transfection. PC12 cells were cultured in RPMI1640 medium supplemented with 10% horse serum (heat-inactivated for 25 min. at 55C) and 5% FBS,

and transfected in LabTek 4-well chamber slides (Fisher Scientific) using Lipofectamine 2000 (Invitrogen). HEK293 cells and primary human foreskin fibroblasts were cultured in DMEM w/o sodium pyruvate (Gibco) with 10% FBS. HEK293 were transfected using Lipofectamine 2000. HeLa cells were cultured in DMEM supplemented with 10% FBS, and RNAi was performed using Oligofectamine (Invitrogen).

SiRNAs, plasmids, and antibodies. The following siRNA sequences were used (numbers in CNK1 and CNK2 oligo names refer to base pair positions in the respective coding sequences):

TrkA-FW, AUGUGGACAGAGGAGCAAATT;

TrkA-RV, UUUGCUCCUCUGUCCACAUTT;

CNK2-2313-FW, UCGAGACGCCACUGACAAGTT;

CNK2-2313-RV, CUUGUCAGUGGCGUCUCGATT;

CNK2-551-FW, CAACAAUUGUGCAACAGGATT;

CNK2-551-RV, UCCUGUUGCACAAUUGUUGTT;

CNK2-881-FW, AAGCGACCUCAGAGCAUGCTT;

CNK2-881-RV, GCAUGCUCUGAGGUCGCUUTT;

4.1B-FW, AGCAGAUUCGAAGUGGUGCTT;

4.1B-RV, GCACCACUUCGAAUCUGCUTT;

CNK1-805-FW, GAACAUGGUGAGGGAACUGTT;

CNK1-805-RV, CAGUUCCCUCACCAUGUUCTT;

MM-FW, CACCUAAUCCGUGGUUCAATT;

MM-RV, UUGAAGCACGGAUUAGGUGTT;

hA-Raf-FW, GACGCGACAUGUCAACAUCTT;

hA-Raf-RV, GAUGUUGACAUGUCGCGUCTT.

pCEP4-GFP (Henry et al., 2000), pDCR-RasV12 (White et al., 1995), pSRα-RafBXB (White et al., 1996), and pCMV5-MEK1R4F (English et al., 1998) are described elsewhere. pCIneo-myc-CNK2(a.a.1-468) and pCIneo-myc-CNK2(a.a. 568-1032) (Yao et al., 1999) were a gift from Yutaka Hata. pCDNA3-DAL-1(2A3) and DAL-1 antibody (Tran et al., 1999) were a gift from Irene Newsham.

Antibodies against c-Myc (A-14, 9E10), Raf-1 (C-20), MEK1 (H-8), and A-Raf (C-20) were purchased from Santa Cruz. Phospho-MEK, phospho-ERK, and ERK antibodies were from Sigma. Anti-MEK antibody was from Cell Signaling. Monoclonal antibodies against Raf-1 and CNK1 were from Transduction Laboratories. Antibody against rat 4.1B was purchased from Protein Express, Tokyo, Japan. v-H-Ras (Ab-2) was from Calbiochem, and rhodamine-phalloidin was from Molecular Probes.

RT-PCR. PC12 cells co-transfected with siRNAs and GFP were resuspended in RPMI with 0.5% horse serum three days after transfection, and GFP-positive cells were

collected by fluorescence-activated cell sorting (FACS). Total RNA was prepared using the High Pure RNA Isolation kit (Roche). Reverse transcriptase reaction was done using SuperScript First-Strand Synthesis System (Invitrogen). PCR amplification was performed using the following primers:

CNK2-FW2742, GGAGGAAGCCAGTCTGTCACC;

CNK2-RV, TTACACGTGTGTCTCAAT;

β-actin-FW, TGACGGGGTCACCCACACTGTGCCCATCTA;

β-actin-RV, CTAGAAGCATTTGCGGTGGACGATGGAGGG.

Neurite formation assay. PC12 cells were transfected with 100 nM siRNA and 0.1 μg pCEP4-GFP in antibiotic-free culture medium according to the Invitrogen protocol. 24 hours after transfection, neurite projections were induced with 100 ng/ml mouse NGF 2.5S (Alamone Labs) in low-serum medium (RPMI1640 supplemented with 0.5% horse serum). After 48 hours of NGF treatment, cells were fixed, coverslips were mounted with Gel/Mount (Biomeda), and GFP-positive cells were scored for the presence of neurites. For neurite induction by RasV12, RafBXB, or MEK1R4F, cells were processed as described above, but without NGF treatment, and immunostained with the appropriate antibodies.

Immunocytochemistry. PC12 or HeLa cells were fixed in 3.7% formaldehyde for 1 hour, washed with PBS, and permeabilized for 1 hour in blocking buffer (0.25% Triton X-100, 1% FCS, in PBS). Myc (A-14), v-H-Ras, Raf-1 (C-20), MEK-1 (H-8), and 4.1B antibodies and rhodamine-phalloidin were used at 1:80 dilution in blocking buffer. Phospho-ERK1/2 antibody was used at 1:100 dilution. Signal was detected with goat anti-mouse or goat anti-rabbit fluorescent-conjugated antibodies (Jackson ImmunoResearch) at 1:300 dilution. Images were collected using a Zeiss Axioplan 2 epifluorescence microscope and Open Lab 2.2.5 imaging software.

PC12 ERK activation assay. For ligand-induced ERK activation, cells were transfected with GFP and siRNAs and the medium was replaced with regular culture medium 24 hours post-transfection. Following an additional 24 hours, cells were incubated in low-serum medium for 18-24 hours, before being stimulated for 5 minutes with EGF (Calbiochem) or NGF (Alamone Labs). For RafBXB-induced ERK activation, 24 hours post-transfection cells were incubated in low-serum medium for 48 hours. After immunostaining and image acquisition, phospho-ERK signal intensities in GFP- or RafBXB-expressing cells were analyzed using Open Lab software.

Immunoprecipitation and western blotting. For co-immunoprecipitation, HEK293 cells were lysed 48 hours following transfection in modified RIPA buffer (20 mM Tris, pH 8.0, 137mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM

EDTA, 20 mM NaF, and protease inhibitors). Primary human foreskin fibroblasts were grown to full confluency in 10-cm culture dishes (one 10-cm dish per IP) and lysed in modified RIPA buffer. Lysates were rotated for 20 min at 4°C, cleared by centrifugation at 17,000 × g for 15 min, and immunoprecipitated overnight with anti-DAL-1 antibody. Immunoprecipitates were washed three times in lysis buffer and analyzed on a 10% agarose gel.

CHAPTER SIX
Conclusions and Future Directions

Conclusions

The basic functional and molecular organization of the canonical Ras/MAPK signal transduction pathway was elegantly outlined by using a combination of biochemical and genetic approaches over a decade ago. It has since become evident that the complexity of Ras/MAPK signaling greatly exceeds our initial reductionist view. For example, we have seen that Raf-1 is a multifunctional kinase, and that some of the consequences of Raf-1 activation are mediated independently of its ability to phosphorylate MEK. Also, the highly conserved and ubiquitous nature of this pathway may necessitate the deployment of additional control mechanisms that can allow the utilization of the Ras/MAPK module in diverse cellular processes. Ras/MAPK signaling can drive differentiation, proliferation, T-cell activation, and many other events, and specialization could be conferred by assembly of requisite modular components with the help of scaffolds.

Here, we have seen that while *Drosophila* KSR and CNK appear to function as obligate components in ERK signaling, their mammalian orthologs may have evolved to acquire pathway selectivity, as well as the ability to coordinate multiple cellular processes. Our results show that understanding how scaffolding proteins contribute to regulating MAPK signaling in particular, and signal transduction in general, is essential to understanding fundamental principles of cell regulation.

In summary, we have provided evidence for Mek-independent Raf functions, both through the use of a MEK-binding-defective, kinase-active mutant of Raf-1 in mammalian cells, and by analyzing the relative contributions of Ras, Raf and Mek to regulation of gene expression in *Drosophila*. The former suggests the existence of physiologically relevant Raf-1 substrates other than Mek, and the latter could suggest both new substrates and/or a kinase-independent scaffolding function for *Drosophila* Raf. We have also provided the first direct biochemical demonstration of the requirement for candidate scaffolding proteins KSR and CNK in stimulus-dependent MAPK signaling in *Drosophila*. In neuronal cells, we showed that mammalian CNK2 can act as a specificity determinant in MAPK signaling and may coordinate multiple regulatory pathways during neuritogenesis. Finally, we see that human CNK1 is involved in the control of actin cytoskeleton dynamics, but appears to be dispensable for regulation of ERK activity. The

following sections will outline some of the remaining questions regarding these observations, as well as potential ways to address them.

Future Directions

MEK kinase independent Raf functions. Given the accumulating evidence for Mek-independent functions of Raf-1 (Hindley and Kolch, 2002), it will be critical to identify the Raf-1 effectors mediating these functions. We have attempted a yeast two-hybrid screen with RafBXB-T481A as bait, but were unable to isolate novel interactors. However, there are already a number of proteins previously reported to interact with the kinase domain of Raf-1 in yeast two-hybrid, including Bcl-2 (Wang et al., 1996a), Bag-1 (Wang et al., 1996b), and Tvl-1 (Lin et al., 1999). Raf-1 mutants selectively impaired in binding to each of these proteins (yet still able to bind and activate MEK1/2) could be generated by the approach used in Chapter 2. The functional significance of these interactions can then be evaluated by testing the ability of each mutant to induce Raf-1 dependent cellular responses. To determine whether the functionally relevant interactors identified in this manner are potential Raf-1 substrates, kinase-inactive versions of the corresponding Raf-1 mutants could be used in parallel. The ability of Raf-1 to phosphorylate the candidate substrates could then be examined in an *in vitro* kinase assay.

Microarray analysis. As an immediate next step, the analysis of gene expression patterns coupled with RNAi in *Drosophila* tissue culture should be expanded to include additional components of Ras signaling. We can also translate this approach to mammalian cells. For example, as a starting point, it would be interesting to compare the isoform-specific contributions of Ras or Raf proteins to gene regulation.

Scaffolds and ligand specificity in ERK signaling. As demonstrated in Chapter 4, KSR and CNK are indispensable for insulin-dependent ERK activation in *Drosophila* S2 cells. However, it remains to be determined whether the requirement for these scaffolds in *Drosophila* MAPK signaling is general or context-specific, and in this respect, additional ligands and cell types should be tested. If KSR and CNK are non-selectively required for ERK signaling in *Drosophila*, it would suggest an evolutionary divergence in function, where mammalian CNK2 has acquired stimulus specificity. While we did not uncover a contribution of human CNK1 to ERK activation, it is possible that we have not identified a stimulus and cell type that rely on CNK1 in this process. Another possibility is that while CNK1 and CNK2 have both retained a role in cytoskeletal regulation, they have diverged with respect to regulation of ERK activity. In support of the latter, CNK1, unlike CNK2, does not appear to interact with Raf-1 (Therrien et al., 1998 and data not shown).

Regulation of actin morphology by CNK1. The ability of CNK1 to impact cellular morphology, in particular the organization of cortical actin filaments was demonstrated in Chapter 5. We have recently obtained evidence suggesting a role for CNK1 in regulating stress fiber formation, as CNK1 RNAi in HeLa cells is sufficient to induce a stress fiber phenotype (data not shown). Rho family small GTPases, including Rho, Rac and Cdc42, are key regulators of actin cytoskeleton dynamics (Hall, 1998). Since activation of Rho leads to stress fiber formation, we wanted to test whether CNK1 is involved in regulating Rho activity. However, siRNA-mediated depletion of CNK1, while able to enhance stress fiber formation, did not result in elevated Rho activation, as demonstrated in a GST-Rhotekin-RBD pull-down assay (data not shown). We are currently investigating whether CNK1 can contribute to actin depolymerization by regulating the actin filament severing enzyme cofilin. Phosphorylation of cofilin on Ser3 by LIM kinases inhibits its activity (Arber et al., 1998), which can be assayed by using phospho-Ser3-specific cofilin antibodies. Thus, if CNK1 knockdown results in increased cofilin phosphorylation on Ser3, it would suggest that CNK1 normally suppresses stress fiber formation by maintaining cofilin in the activated state.

In a recent report, CNK1 was shown to interact with Rho in a GTP-dependent manner, and to be important for SRF-mediated gene transcription, but not for stress fiber formation, in response to L63Rho, an activated Rho mutant (Jaffe et al., 2004). Based on

these findings, the authors propose that CNK1 is a downstream effector of Rho in regulation of gene transcription, but not in stress fiber formation. The lack of an observed effect of CNK1 depletion on L63Rho-mediated stress fiber induction appears to be inconsistent with our data. However, a simple explanation for this discrepancy could be that L63Rho induces a maximal stress fiber phenotype that cannot be further potentiated by the loss of CNK1. Furthermore, if CNK1 is downstream of Rho in SRF-mediated gene transcription, then it is possible that downregulating the SRF pathway by inhibiting CNK1 expression could result in some of the Rho signal being re-directed toward the stress fiber pathway. This could potentially explain the enhanced stress fiber formation induced by the loss of CNK1 in our experiments. Another possibility that would be consistent with these observations is that activated Rho prevents CNK1 from contributing to activation of cofilin, thereby leading to stress fiber induction.

Role of CNK2 in neurite formation. The results presented in Chapter 5 also indicate a possible role for CNK2 in regulation of actin dynamics. The requirement of CNK2 in neurite formation independently of its involvement in MAPK signaling and association of CNK2 with protein 4.1B could suggest that CNK2 participates in the reorganization of actin cytoskeleton that is necessary for growth cone formation (da Silva and Dotti, 2002). Another possibility is that CNK2 is involved in vesicle transport. Recently published

data indicates that both CNK1 and CNK2 interact with components of Ral signaling (Jaffe et al., 2004; Lanigan et al., 2003). The functional significance of these interactions is not known, but Ral has been implicated in the regulation of exocytosis (Moskalenko et al., 2002), which is required for neurite outgrowth (Tang, 2001). Interestingly, some of our preliminary data suggests that CNK1 may be required for Ral activation in response to EGF (data not shown). It will be important to conduct further investigation into the possible mechanisms by which CNK2 contributes to neurite formation.

CNK1 in cell motility. Cell migration is associated with dynamic assembly and breakdown of actin filaments (Ridley, 2001), and can be inhibited as a result of stress fiber stabilization (Vial et al., 2003). Consistent with this, we observe that depletion of CNK1 correlates with reduced cell motility (data not shown), which could have implications for a role of CNK1 in invasion and metastasis. Future tumorigenicity studies may shed some light on a potential requirement for CNK1 in these phenomena.

Scaffold-mediated assembly of multi-protein complexes. Since CNK proteins contain multiple protein-protein or protein-lipid interaction domains, it is possible that they could mediate the formation of multi-component complexes. To investigate this possibility, we could study the role of CNK proteins in complex formation between known components

of Ras signaling by using RNAi to inhibit CNK expression. The ability of KSR to mediate the assembly of a high molecular weight complex containing KSR, MEK, and ERK was clearly demonstrated by analysis of KSR1$^{-/-}$ MEFs (Nguyen et al., 2002). As a more unbiased approach, novel CNK-dependent components of a complex may be identified by immunoprecipitating the complex from cells treated with control or CNK siRNAs, followed by mass spectrometric analysis of differentially co-immunoprecipitating proteins. Mass spectrometry could also be a used to search for novel CNK interactors by analyzing proteins that co-immunoprecipitate with CNK.

CNK phosphorylation. Both *Drosophila* CNK and human CNK2 were shown to be tyrosine phosphorylated in RTK-dependent manner (Lanigan et al., 2003; Therrien et al., 1998). However, the functional significance of these phosphorylation events, and the kinases that are directly responsible, are currently unknown. In addition, it appears that the CNK1 protein could be subject to extensive constitutive phosphorylation, as it migrates at approximately 30 kDa higher than its predicted molecular weight of 78 kDa (data not shown). Such a pronounced shift due to phosphorylation is not common, but has been observed for at least some proteins (W.J. Snell, personal communication), and a phosphatase treatment assay should reveal whether this is the case for CNK1. CNK1 may also undergo stimulus-induced phosphorylation, as we can detect a slight increase in

its molecular weight in a stimulus-dependent manner (data not shown). It will be important to identity the kinases that phosphorylate CNK proteins, and to study the functional consequences of these phosphorylation events. Interestingly, ScanSite analysis (Obenauer et al., 2003) of CNK predicts a high probability consensus phosphorylation site for Cdk5, a serine/threonine kinase implicated in ERK-mediated neurite formation (Harada et al., 2001).

CNK isoforms. In addition to CNK1 and CNK2, mammalian genomes encode two truncated CNK homologs (Douziech et al., 2003). The amino-terminal homolog CNK3A contains SAM and PDZ domains, while the carboxy-terminal homolog CNK3B contains a PH domain. Both CNK3A and CNK3B have been mapped to the same chromosomal location (6q25.2), but are separated by ~100kb of DNA and do not share common sequences, suggesting that they represent two separate genes. Furthermore, the CNK3B polypeptide was recently isolated in a screen for phosphoinositide-binding proteins (Krugmann et al., 2002). There has been no functional data reported for CNK3A or CNK3B, but it is possible to imagine that they could act as decoy or dominant inhibitory CNK isoforms *in vivo*. For example, since the amino-terminus of CNK2 blocks neurite induction, it would be interesting to know whether CNK3A can function as a negative regulator of neuronal differentiation.

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