REVEALING REGULATION AND ORGANIZATION OF SIGNALING NETWORKS BY SCAFFOLDING PROTEINS

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DEDICATION

To my parents and husband for the unending love and support.

REVEALING REGULATION AND ORGANIZATION OF SIGNALING NETWORKS BY SCAFFOLDING PROTEINS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas
February, 2006

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Michael White, for providing a productive scientific environment where I could learn the necessary critical thinking skills to become a scientist. I am also grateful to him for imparting his knowledge, time, and patience. I would also like to acknowledge both past and present White lab members, specifically Ron Bumiester and Anthony Anselmo for helping me get started on my project, as well as Kiran Kaur, not only for her invaluable knowledge, but also helping the lab to run smoothly. To my committee, Elliott Ross, Melanie Cobb, and Yingming Zhao, I would like to say thank you for your time and patience. And last but not least, I would like to thank my parents and husband for their unending love and support.

REVELAING REGULATION AND ORGANIZATION OF SIGNALING NETWORKS BY SCAFFOLDING PROTEINS

Publication No. _____

Jackie Thomas Swanik Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

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Understanding the molecular mechanism that controls how cells respond to their environment is of major biological significance. The RAS/RAF signal transduction pathway is a good model with which to investigate this question as the major catalytic components of the pathway have been identified and it has been shown to elicit a wide variety of cellular responses such as proliferation, migration, differentiation, and apoptosis. The pathway is a three-tiered MAPK kinase cascade consisting of RAF, MEK, and ERK. Although the pathway has been extensively studied, pathway regulation is not completely understood. Genetic studies aimed at understanding pathway regulation have identified candidate scaffolding proteins. We used transient loss of function analysis to assess the contribution of the scaffolding proteins, Suppressor of RAS-8 (Sur-8) and Kinase Suppressor of RAS (KSR), to ligand mediated RAS/RAF signal transduction pathway activation. We show that Sur-8 and KSR are integral components of the RAS/RAF signal transduction pathway in mammalian cells. In addition, they display ligand specific coupling in that Sur-8 is required for EGF induced MEK activation while KSR is involved in LPA mediated MEK activation.

Investigation of the molecular mechanism of action of Sur-8 and KSR found that Sur-8 is required for both EGF induced RAF-1 and B-RAF activation while KSR is involved in EGF induced RAF-1 activation. Additionally, Sur-8 contributes to RAF-1 localization as well as being associated with a RAF-1 activating kinase.

Futhermore, we found that KSR does not impact LPA induced MEK activation through either RAF-1 or B-RAF activation and even though it impacts EGF induced RAF-1 activation it is not a limiting component to EGF induced MEK activation.

In this study, we show that a function of scaffolding proteins in the RAS/RAF signal transduction pathway is to contribute to ligand specific coupling of MEK/ERK to distinct stimuli.

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PRIOR PUBLICATIONS

Anselmo, A. N., Bumeister, R., Thomas, J. M. & White, M. A. (2002) *J. Biol. Chem.* **277,** 5940-5943

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LIST OF ABBREVIATIONS

ATP	Adenosine 5' triphosphate
BME	β-Mercaptoethanol
BSA	Bovine Serum Albumin
C/EBP	CCAATT/Enhancer Binding Protein
CNK	Connector of KSR
CRIC	Conserved Region in CNK
DAG	Diacylglyerol
dsRNA	double stranded RNA
EGF	Epidermal Growth Factor
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FIST	FAS/FADD Interacting Serine/threonine kinase
GAP	GTPase activating proteins
GDP	Guanosine 5' diphosphate
GEF	Guanine Exchange Factors
GFP	Green Flourescent Protein
GPCR	G Protein Coupled Receptor
GTP	Guanosine 5' triphosphate
HEK293	Human Embryonic Kidney
HIPK3	Homeodomain Interacting Protein Kinase 3
KSR	Kinase Suppressor of Ras

LLR	Leucine Rich Repeat
LPA	Lysophosphatidic Acid
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MEF	Mouse Embryo Fibroblast
MITF	Micropthalmia Associated Transcription Factor
MLCK	Myosin Light Chain Kinase
MORG1	Mitogen-activated Protein Kinase Organizer 1
MP1	MEK Partner 1
NGF	Neurite Growth Factor
NRK	Normal Rat Kidney
PAK	p-21 Activated Kinase
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma 12
PDGF	Platelet Derived Growth Factor
PDZ	PSD-95, Dlg, ZO1
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3 Kinase
PKA	cAMP activated protein kinase
PKC	Protein Kinase C
PMA	phorbol 12-myristate 13-acetate
PPAR	Peroxisome Prolerator Activated Receptor

PP2A	Protein Phosphatase 2A
RAS	Rat Sarcoma
RNAi	RNA interference
RSK	p90 Ribosomal Protein S6 kinase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S2	Schneider L2 cells
SAM	sterile α-motif
SDS	Sodium Dodecyl Sulfate
SH3	Src-homology-3 (SH3)
siRNA	small interfering RNA
Sur-8	Suppressor of Ras-8

CHAPTER ONE
Introduction

Chapter 1: Introduction

Understanding the molecular mechanism that controls how cells respond to their environment is of major biological significance. In other words, understanding how growth factors, environmental stresses, and hormones elicit such cellular outcomes as proliferation, apoptosis, and differentiation can not only help us to treat disease states such as cancer, but also help to understand "normal" processes such as adipocyte differentiation and wound healing. The RAS/RAF signal transduction pathway is a good model pathway to help understand these questions since it is involved in eliciting a wide variety of cellular outcomes and the major catalytic components are identified. The RAS/RAF signal transduction pathway is activated by a multitude of factors including growth factors, integrins, and apoptotic agents as well as being errantly activated in ~30% of human cancers (1-4). This pathway consists of the small G-protein RAS and a three-tiered MAPK kinase cascade comprised of RAF, MEK, and ERK. ERK has several substrates, some of which are cytoplasmic such as p90 Ribosomal Protein S6 kinase (RSK) (5,6), caspase 9 (7), and myosin light chain kinase (MLCK) (8) while other targets are nuclear such as the transcription factors c-Fos, MYC, and ELK (9-12). (Figure 1.1)

RAS, in its active state, is bound to GTP while in its inactive state, is bound to GDP. Two types of proteins help regulate the GDP for GTP cycle- GTPase activating proteins (GAPs),

which inactivate RAS by increasing the rate of hydrolysis of GTP to GDP, and guanine exchange factors (GEFs) that help to facilitate the exchange of GDP to GTP. While this study focuses on the RAF arm of the RAS pathway, RAS has other downstream effectors such as phosphoinositide 3 kinase (PI3K) and Ral GEFs.

In mammalian cells there are three isoforms of RAF- A-RAF, B-RAF, and C-RAF (or RAF-1). They all share similar protein structure consisting of three domains - CR1, 2, and 3. CR1 contains the RAS binding domain while together CR1 and CR2 form the amino terminus autoinhibitory domain. CR3 is the kinase domain. (Figure 1.2) Most work to date has been performed with RAF-1. The most well studied aspects of RAF-1 activation can be broken down into three groups – 1) phosphorylation and dephosphorylation, 2) RAS binding, and 3) localization. In the inactive state RAF-1 is cytoplasmic and phosphorylated on S259. Upon stimulation S259 is dephosphorylated, S338 and Y341 are phosphorylated, RAF-1 binds to RAS, and it translocates to the plasma membrane (13-16). Other less well characterized sites that are phosphorylated upon stimulation include S43, T491, S494, and S621 (17-19). The kinases and phosphatase that regulated RAF-1 include Protein Phosphatase 2A (PP2A) (20) and AKT, or Protein Kinase B, which regulate S259 (21); p-21 activated kinase (PAK) which phosphorylates S338 (22,23), Src which phosphorylates Y341 (24) and Protein Kinase A (PKA) which phosphorylates S43 (20,21,24). S621, when phosphorylated, binds 14-3-3 and has been shown to be an activating autophosphorylation site (25,26). (Figure 1.2) A-RAF seems to be regulated similarly as RAF-1 (reviewed in (27).

B-RAF regulation is similar to RAF-1 in that upon stimulation it binds to RAS and translocates to the plasma membrane. However, regulation by phosphorylation differs

between the two isoforms. S364, the analogous site to S259 of RAF-1, is not phosphorylated in the inactive state. Also, the analogous site for S338 (S445) is constitutively phosphorylated on B-RAF. Whereas, the analogous site of Y341 of RAF-1 (D448) in B-RAF acts as a phosphomimetic. However, T598 and S601 of B-RAF are known to be regulated by phosphorylation. The analogous sites of RAF-1 are T491 and S494. (Figure 1.2)

Studies aimed at understanding RAS/RAF signal transduction pathway regulation have identified non-enzymatic components, or scaffolds. Regulation of a MAPK cascade by scaffolding proteins has been observed with Ste5 in yeast. In yeast, the high osmolarity response and mating response are both mediated by a MAPK cascade and share some of the same signaling components. The mating response involves Ste20 (a MAPKKKK), Ste11 (the MAPKKK), Ste7 (the MAPKK) and Fus3p (the MAPK) tethered together by Ste5, while the high osmolarity response is mediated by Ste20, Ste11, Hog1 (MAPK), but tethered by Pbs2 (figure 1.3). Park et al. showed that scaffolds are very specific for their outcomes in that if Ste5 and Pbs2 are artificially linked, then yeast stimulated with a mating pheromone (Ste5 pathway) initiate an osmotic response (Pbs2 pathway) (28).

No Ste5 homologue exists in mammalian cells although several scaffolding proteins involved in the MAPK pathway have been discovered, including Suppressor of Ras-8 (Sur-8), Kinase Suppressor of Ras (KSR), Connector of KSR (CNK), and MEK Partner 1 (MP1) (figure 1.3). Sur-8 was identified in two independent genetic screens in *C. elegans* that were investigating suppressors of an activated RAS/RAF signal transduction pathway (29,30). Sur-8 is composed of 19 consecutive leucine rich repeat (LRR) domains which are believed to be

involved in protein-protein interaction. The carboxy-terminus of Sur-8 binds to the amino terminus of RAS, but does not compete with RAF binding to RAS. Overexpression studies indicated that Sur-8 binds the carboxy-terminus of RAF (the kinase domain), away from the RAS-binding domain. Sur-8, a cytosolic protein, was found in all tissues examined.(29-31) (Figure 1.4)

KSR was identified using genetic screens in *Drosophila* and *C. elegans* studying suppressors of activated RAS. KSR has five recognizable domains, CA1-5, that were defined by homology between orthologs in flies, mice, and humans. CA1 is a unique domain, CA2 is a proline-rich domain, CA3 is a cysteine-rich zinc finger domain, CA4 is a serine/threonine rich region and CA5 is a putative kinase domain. However, the functionality of the kinase domain is questionable. KSR binds Gγ, 14-3-3, ERK, Hsp90, RAF, and MEK (32-34). (Figure 1.4) KSR is regulated both by localization and phosphorylation. Phosphorylation of KSR by c-TAK1 at S392, in serum starved conditions, helps to sequester KSR in the cytoplasm (35). Upon stimulation KSR is dephosphorylated on S392, and then recruited to the plasma membrane where it takes its cargo, MEK (36,37). KSR can also shuttle in and out of the nucleus. Nuclear shuttling of KSR is regulated by phosphorylation of KSR on T274 and S392 (38).

KSR knock-out mice are viable and normal though mouse embryo fibroblast (MEF) cells show a 50% reduction in ERK activation in response to Epidermal Growth Factor (EGF) and phorbol 12-myristate 13-acetate (PMA). These studies also indicate KSR is important for T-cell proliferation. Further, the reduction of KSR can slow breast tumor progression, and reduce the incidence of skin cancer (39,40).

CNK was discovered in a genetic screen looking for enhancers of the *Drosophila* rough eye phenotype caused by the kinase domain of KSR. It contains a sterile α-motif (SAM); conserved region in CNK (CRIC); PSD-95, Dlg, ZO1 (PDZ) domain; proline rich Src-homology-3 (SH3) binding sites; and a pleckstrin homology (PH) domain. CNK binds RAF. (41,42) (Figure 1.4)

MP1 was discovered in a yeast two hybrid screen investigating MEK1 binding partners and is known to bind to MEK and ERK. MP1 is reported to bind specifically to MEK1 and ERK1. (43) (Figure 1.4)

Even though these scaffolds have been genetically implicated in the RAS/RAF signal transduction pathway and have been reported to bind to pathway components, it is not known whether they are integral components that impact pathway activation and if so what their function may be. Proposed functions of scaffolds are to alter pathway activation kinetics, enhance pathway efficiency, and restrict kinase specificity.

In this study, we investigate the contribution of candidate scaffolds to RAS/RAF signal transduction pathway activation. We observed that Sur-8 contributes to EGF induced RAS/RAF pathway activation while KSR is involved in LPA mediated pathway activation, therefore a function of scaffolds may be to contribute to selective coupling of MEK/ERK to distinct stimuli.

Figure 1.1

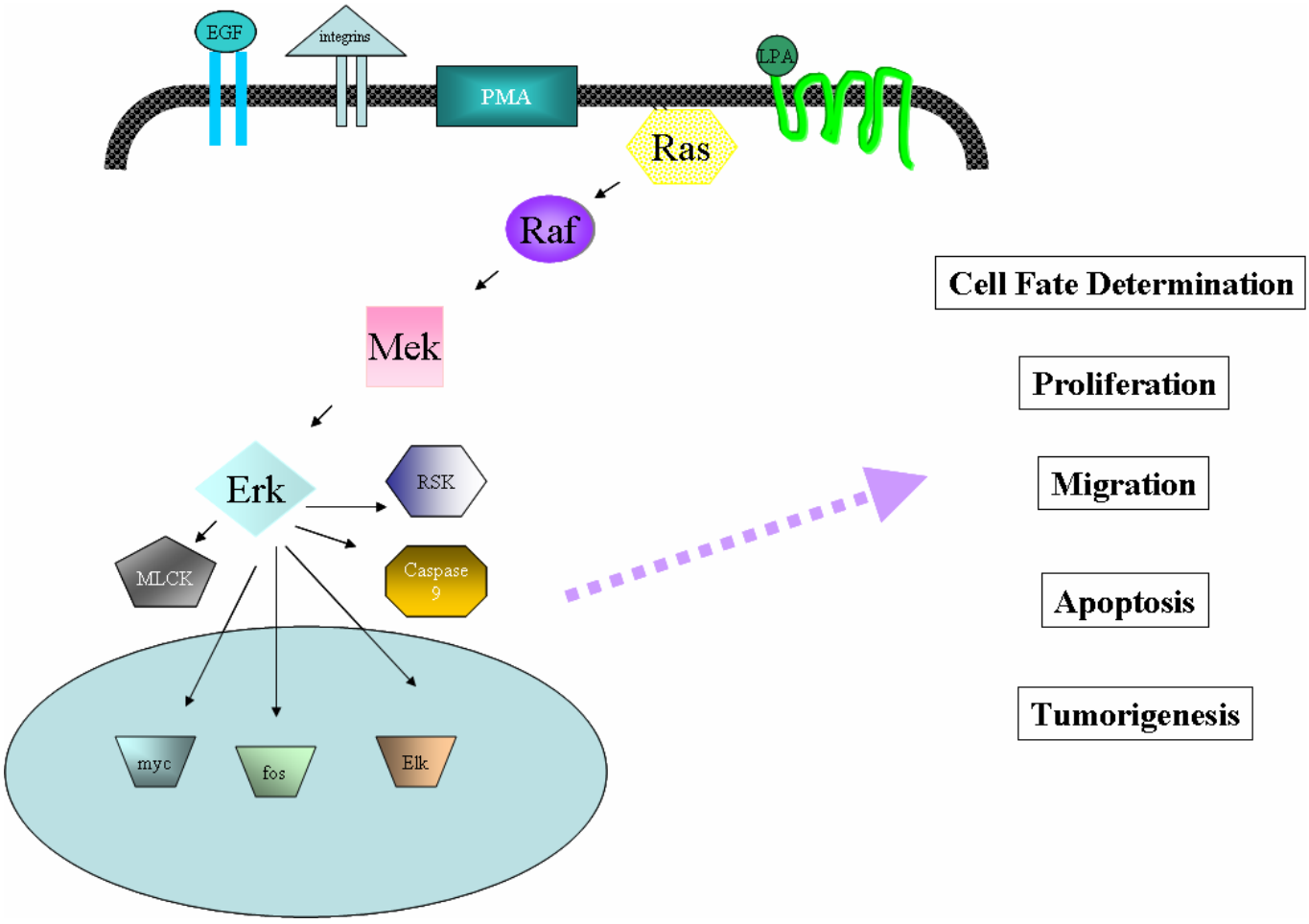
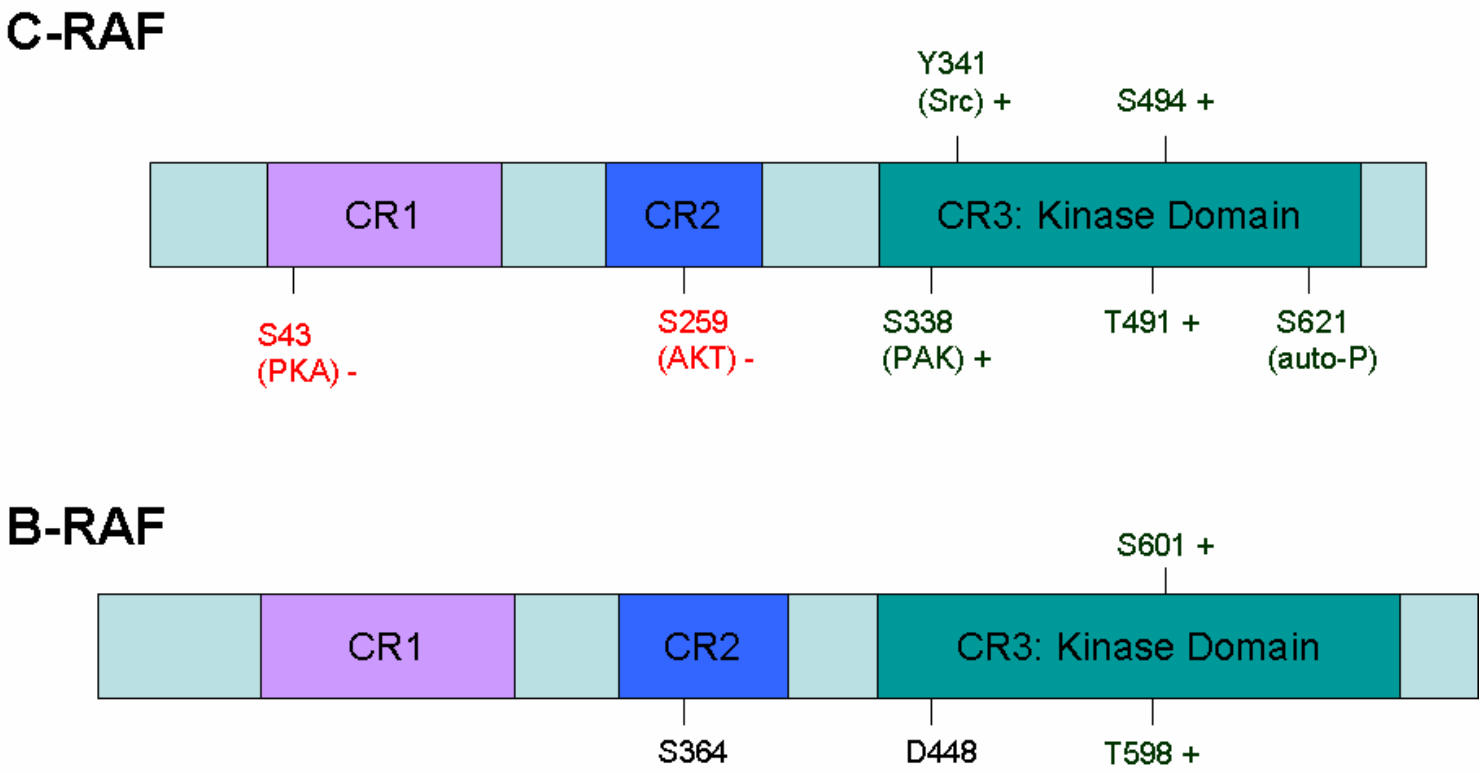


Figure 1.1 RAS/RAF signal transduction pathway. The RAS/RAF signal transduction pathway is activated by many factors including growth factor, integrins, and apoptic agents and mediates such cellular outcomes as cell fate determination, proliferation, migration, apoptosis, and tumorigenesis.

Figure 1.2



Structure of RAF. Raf is composed of 3 conserved regions- CR1, CR2, and CR3. Negative phosphorylation events are in red while positive phosphorylation events are in green. The kinase responsible for the phosphorylation is labeled in parenthesis. RAF-1 has two negative phosphorylation sites, S43 and S259. The positive phosphorylation sites on RAF-1 are S338, Y341, T491, S494, and S621. B-RAF has two positive phosphorylation sites, T598 and S601.

Figure 1.3

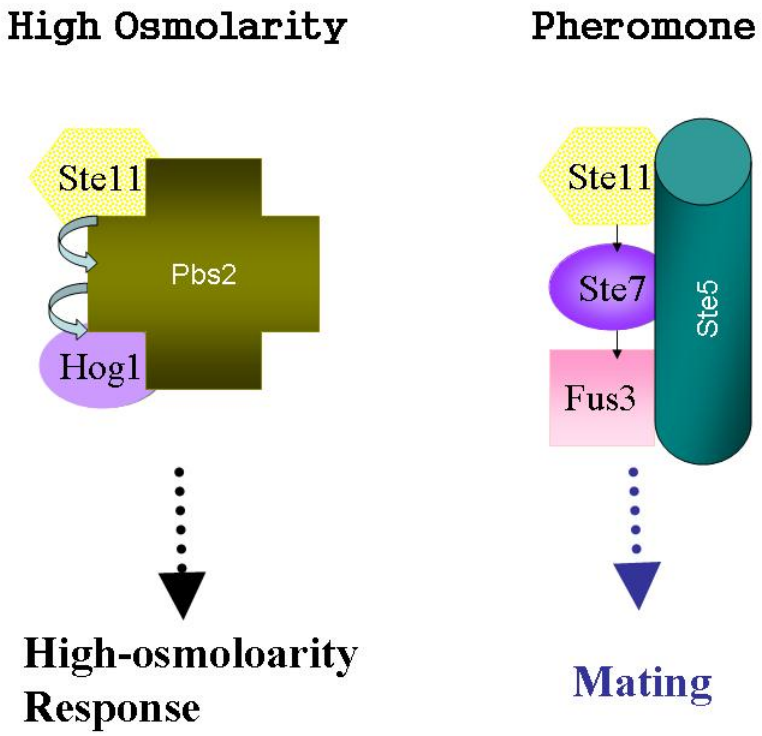


Figure 1.3 Yeast MAPK scaffolds. Pbs2 is the scaffold involved in the high osmolarity response. It connects Ste11 to Hog1. Ste5 is involved in the pheromone response and connects Ste11, Ste7, and Fus3.

Figure 1.4

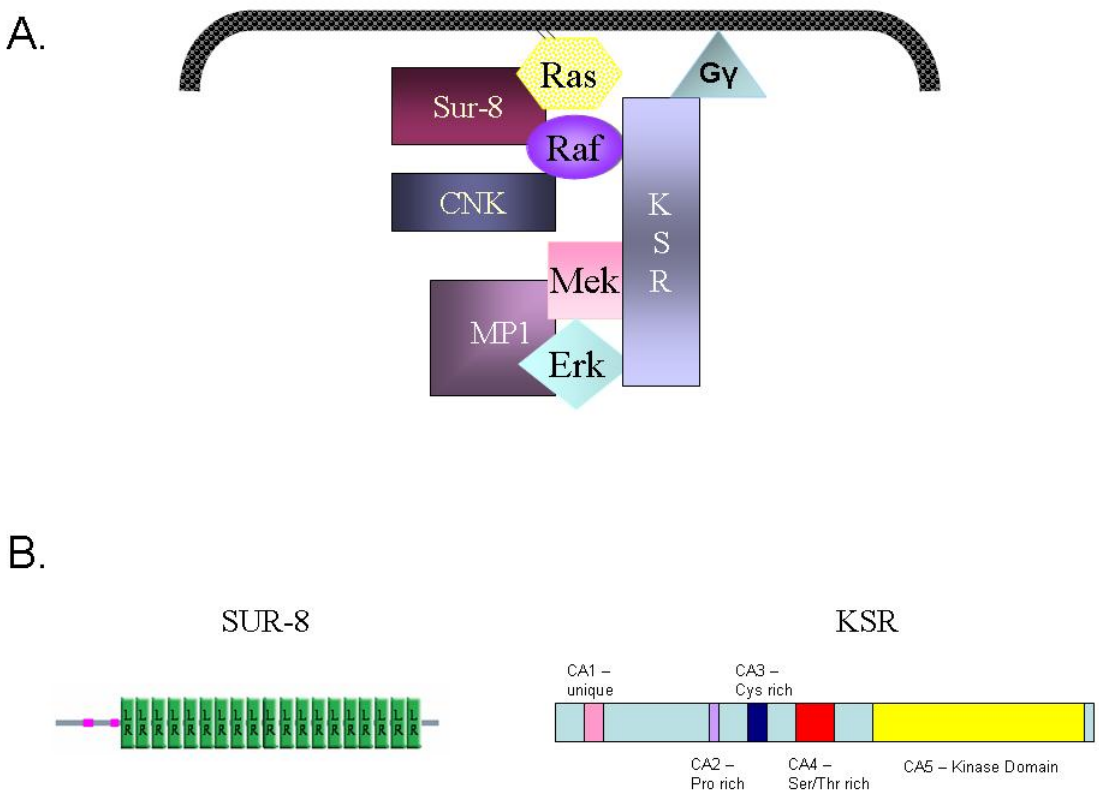


Figure 1.4 Mammalian MAPK scaffolds. A. Scaffold placement in MAPK pathway. Suppressor of RAS- 8 (Sur-8) binds to RAS and RAF. Connector of KSR (CNK) binds to RAF. Kinase Suppressor of RAS (KSR) binds to G_γ, RAF, MEK and ERK. MEK binding partner 1 (MP1) binds MEK and ERK. B. Domain structure of Sur-8 and KSR. Sur-8 is composed of approximately 19 leucine rich repeats (LRRs). KSR contain 5 domains – CA1-5. CA1 is a unique domain, CA2 is a proline rich domain, CA3 is a cysteine rich domain, CA4 is a serine/threonine rich region, and CA5 is a putative kinase domain.

CHAPTER TWO

Analysis of Scaffolding Proteins of the RAS/RAF signal transduction pathway in *Drosophila*

Abstract

The regulation of the RAS/RAF signal transduction pathway is an active area of research. Genetic screens have implicated candidate scaffolding proteins such as KSR, CNK, Sur-8, and MP1 as possible regulators. Here we use *Drosophila* S2 cells, which are amiable to RNAi, to understand the contribution of these proteins to ligand mediated RAS/RAF signal transduction pathway activation. We found that CNK and KSR are positive regulators of both insulin and PMA induced pathway activation, MP1 is a negative regulator of PMA induced pathway activation while Sur-8 is not a limiting factor for either insulin or PMA induced pathway activation.

Introduction

Although the RAS/RAF signal transduction pathway has been extensively studied and the major enzymatic components (RAS, RAF, MEK, and ERK) are known, pathway regulation is not completely understood. Genetic experiments have implicated scaffolds, or non-enzymatic components, specifically Kinase Suppressor of RAS (KSR), Connector of KSR

(CNK), MEK Partner 1 (MP1), and Suppressor of RAS – 8 (Sur-8), as regulators of the RAS/RAF signal transduction pathway.

We chose to investigate the role of scaffolds in RAS/RAF signal transduction pathway activation by RNAi. If the concentration of scaffolding proteins is critical, then overexpression systems can be problematic, often yielding conflicting results. If the purpose of scaffolds is to bring several members of the pathway together, then the stoichiometry is critical. Therefore, overexpressing a scaffolding protein may not "enhance" its physiological function, but may actually inhibit its function such that instead of bringing together various components of the pathway, too much scaffold is now keeping them apart. However, with a transient reduction of the concentration of the scaffold, the true physiological function may be better assessed.

This transient reduction is possible in *Drosophila* Schneider L2 (S2) cells, a cell culture model, with RNA interference (RNAi). Therefore, we used RNAi in S2 cells to knockdown KSR, CNK, MP1, and Sur-8 and assess their contribution to ligand mediated pathway activation. We found that in the context of PMA induced ERK activation that KSR and CNK are positive regulators, MP1 is a negative regulator, and Sur-8 is not a limiting factor.

Results

Our lab had determined that CNK and KSR were critical components for insulin induced ERK activation in *Drosophila* S2 cells (44); however I was interested in whether these

scaffolds were specific for a particular stimulus or whether they were required for ERK activation mediated by multiple stimuli. Therefore, I performed a ligand screen in *Drosophila* S2 cells in order to identify MAPK activators, other than insulin. Ligands tested were 1μM phorbol 12-myristate 13-acetate (PMA- PKC activator), forskolin (c-AMP dependent kinase activator), sorbitol (osmotic stress inducer), or insulin (as a positive control). These ligands have been shown to activate ERK in mammalian cells and we wanted to determine if they would activate *Drosophila* ERK (dERK). 1 $x10^6$ cells were plated and assays were performed after 72 hours. Assay conditions include either unstimulated, media removed and readded, fresh media added, cells treated with 50 μM of the MEK inhibitor PD 90859, or stimuluated with 1 μM PMA, 10μM forskolin, 500 nM sorbitol, or 10ug/ml insulin for five, ten, fifteen, or thirty minutes. Cells were lysed and immunoblots performed to examine ERK activation using anti-phospho ERK antibody. As shown in figure 2.1, there was slight basal ERK activation in the unstimulated cells. This activation was slightly increased when media was removed and replaced. Fresh media added to the cells did not activate ERK. Media controls were performed since media was removed from the cells, stimulus was added to the media and then stimulus containing media was added to the cells. It was uncertain whether the media on the cells had been conditioned – i.e. growth factors secreted into the media such that ERK activation could not be seen over background, and therefore new media needed to be added. However, readdition of the "conditioned" media did not cause significant ERK activation; therefore stimulus could be added to the "conditioned" media. PD 90859 inhibited the slight basal ERK activation. It appears that PMA displays a biphasic response, activating ERK at five and fifteen minutes

but not at ten and thirty minutes. This biphasic response was only seen when the cells were freshly thawed, but after culturing the cells, ERK activation was seen at five, ten, and fifteen minutes but never at thirty minutes. Neither forskolin nor sorbitol activates dERK at the concentration used. As expected, insulin gave robust activation of ERK at five minutes. Therefore, insulin and PMA are activators of dERK.

CNK, KSR, MP1, and Sur-8 was knockdowned down using RNAi and their contribution to PMA induced ERK activation was accessed. 1×10^6 cells were plated and 5 ug/ml of double stranded (dsRNA) targeted against the particular scaffold (CNK, KSR, MP1, or Sur-8) was added to the cells. 72 hours after dsRNA were added, cells were stimulated with 1μM PMA for five, ten, and fifteen minutes. As expected, dsRNA targeting RAF resulted in a decrease in PMA induced ERK activation. Knockdown of CNK and KSR also resulted in a dramatic decrease in ERK activation. In addition, RAS knockdown resulted in a reduction of PMA induced ERK activation. These results were seen at least three times. DREDD dsRNA, which does not contribute to the RAS/RAF signal transduction pathway, was used as a negative control. This suggest that RAF, RAS, CNK, and KSR are required components of PMA induced ERK activation.

When MP1 was knocked down, ERK signaling in response to PMA was increased over baseline, suggesting that MP1 acts as an inhibitor of PMA induced ERK activation. Knockdown of Sur-8 did not change ERK activation as compared to the control, suggesting that Sur-8 is not a limiting component in PMA induced ERK activation. RT-PCR was performed to verify Sur-8 knockdown (data not shown).

Discussion

Recently, non-enzymatic assessory proteins or scaffolds have been implicated as components participating in RAS/RAF signal transduction pathway activation. In this study we sought to determine whether these scaffolds were integral components of RAS/RAF signal transduction pathway activation. Since overexpression studies with these proteins had lead to conflicting results, we chose to perform loss of function studies in *Drosophila* S2 cells since they were amiable to RNAi.

Others in our lab had shown that both CNK and KSR are required for insulin induced ERK activation. However, I was interested in understanding whether the scaffolds would be specific for particular stimuli or whether they would impact RAS/RAF signal transduction pathway signaling mediated from multiple stimuli. Therefore, I performed a ligand screen in *Drosophila* S2 cells to determine what stimuli, other than insulin, would also activate ERK. PMA, forskolin, and sorbitol were tested. ERK was activated by addition of 1μM PMA, but not by addition of 10 μM forskolin or 500 μM sorbitol. Therefore further studies were carried out using PMA as the ERK activator.

We found that both CNK and KSR were required for PMA induced ERK activation as well as insulin induced ERK activation. PMA acts by mimicking diacylglycerol (DAG) thereby activating PKC whereas insulin acts through the insulin receptor, a tyrosine kinase receptor. Therefore, in *Drosophila,* the scaffolds are not restricted to a particular stimulus type or receptor type.

Even though MP1 was not limiting for insulin signaling, knockdown of MP1 displayed an increase in PMA induced ERK activation suggesting that MP1 acts as an inhibitor of PMA

induced ERK activation. As with insulin, reduction of Sur-8 does not impact PMA induced ERK activation, suggesting that Sur-8 is not a limiting factor for either PMA or insulin. Interestingly we found that reduction of RAS significantly decreased PMA induced ERK activation. There is controversy in the field as to whether or not RAS is required for PMA induced ERK activation. Studies using overexpressed dominant negative RAS suggested that RAS was not required for PMA induced ERK activation (45-48) while studies using neutralizing antibody suggest that RAS is a required component in ERK activation by PMA (49,50). Our results are consistent with those of the neutralizing antibody.

This study highlights the fact that scaffolding proteins can play a significant role directly at the level of RAS/RAF signal transduction pathway activation. Whereas CNK and KSR were required for both insulin and PMA induced ERK activation, MP1 is only involved in PMA mediated ERK activation while Sur-8 is not a limiting factor for either insulin or PMA mediated ERK activation.

Materials and Methods

Double stranded RNA (dsRNA) was prepared and used as previously described (51). Scheider 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 preparted using the High Pure RNA Isolation kit (Roche). Reverse transciptase –PCR (RT-PCR) was performed using Superscript first strand synthesis system for RT-PCR (Invitrogen). Antibodies against phospho-ERK and total ERK were purchased from Sigma (M5670, M8159).

Figure 2.1

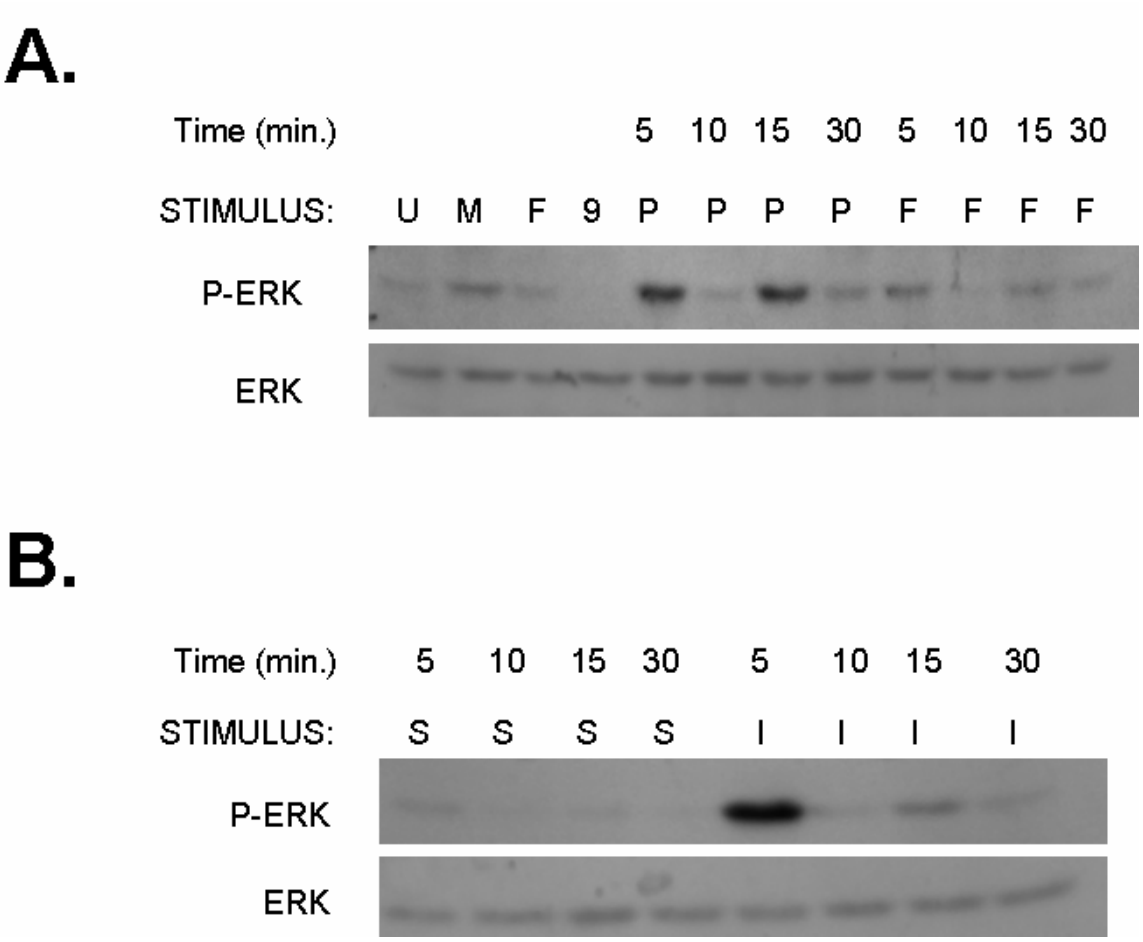


Figure 2.1 Ligand screen. A) S2 cells were unstimulated (U), media removed and readded (M), fresh media added (F), 50uM PD 90859 (9), or stimulated with 1 µM PMA (P), 10 µM forskolin, B) 500nM sorbitol (S), or 10ug/ml insulin (I) for the times indicated.

Figure 2.2

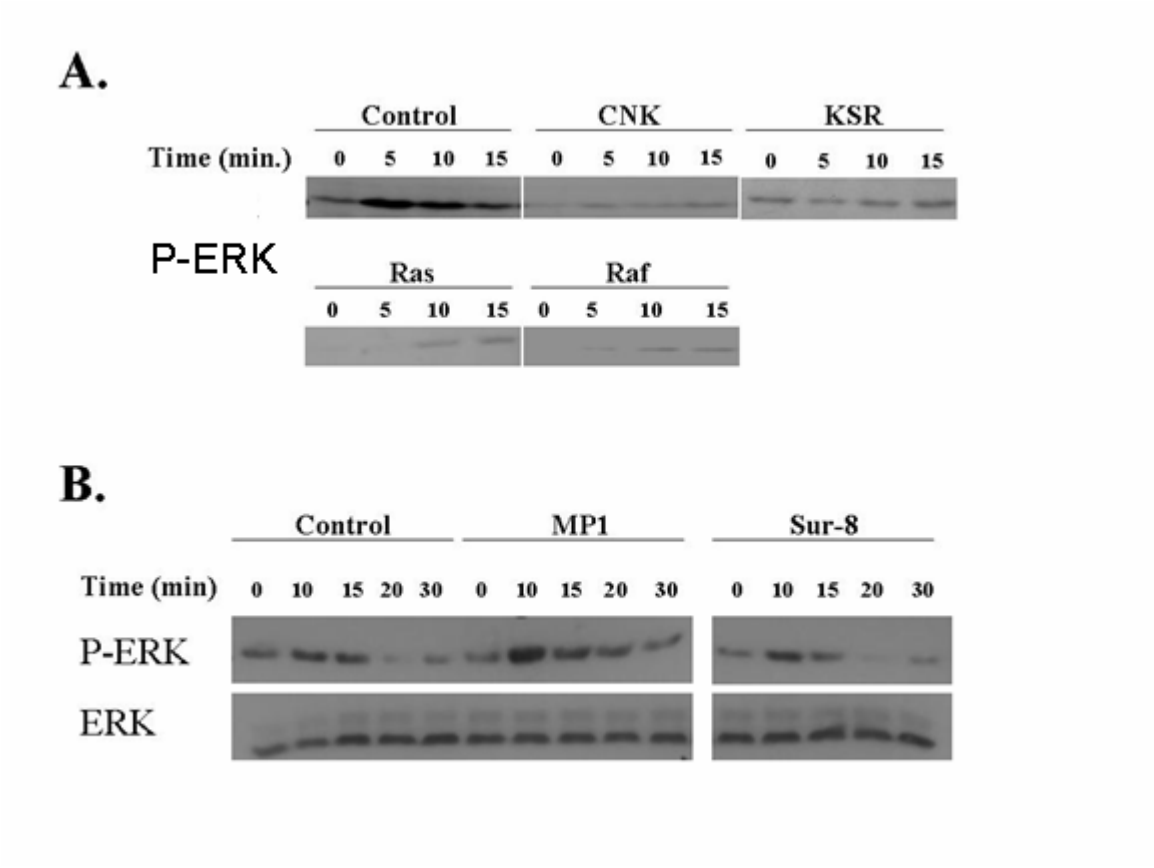


Figure 2.2 PMA Signaling in S2 cells. Cells were stimulated with 1µm PMA for the indicated times. Whole cell lysates from cells transfected with oligos targeting CNK, KSR, RAS, RAF (A) or MP1 and Sur-8 (B) were analyzed for levels of active ERK.

CHAPTER THREE

Sur-8 and KSR are involved in RAS/RAF signal transduction pathway activation

Abstract

The RAS/RAF signal transduction pathway is activated by a plethora of stimuli and mediates diverse cellular phenotypes as migration, proliferation, and differentiation. However, regulation of the RAS/RAF signal transduction pathway is not well understood. Studies aimed at understanding regulation of the RAS/RAF signal transduction pathway have uncovered scaffolding proteins. We used RNAi, in mammalian cells, to assess the contribution of the scaffolds, Sur-8 and KSR, to pathway activation. We found that not only are Sur-8 and KSR integral components of RAS/RAF signal transduction pathway activation, but that they contribute to selective coupling of RAS/RAF signal transduction pathway activation to distinct stimuli. More specifically, Sur-8 is required for EGF mediated RAS/RAF signal transduction pathway activation while KSR is involved in LPA induced RAS/RAF signal transduction pathway activation.

Introduction

The RAS/RAF signal transduction pathway is activated by an ever growing number of stimuli which culminates in various and often antagonistic cellular phenotypes such as proliferation and apoptosis. The pathway is a three tiered MAPK kinase cascade consisting of RAF (the apical kinase or MAPKKK), MEK (the middle kinase or MAPKK), and ERK (the terminal kinase or

MAPK). Upstream of the kinases is RAS, a small GTPase required for RAF activation. Downstream of ERK are several targets, some of which are cytoplasmic such as p90 Ribosomal Protein S6 Kinase (RSK) while others are nuclear such as the transcription factor c-Fos. Even though the core enzymatic components (RAS, RAF, MEK, and ERK) have been identified, the pathway regulation is not completely understood. Studies aimed at understanding pathway regulation have identified non-enzymatic components, or scaffolds. These scaffolds include Suppressor of Ras 8 (Sur-8) and Kinase Suppressor of Ras (KSR). Both Sur-8 and KSR, in genetic experiments, can rescue an activated RAS phenotype but not an activated RAF phenotype suggesting that they both function parallel or downstream of RAF. Sur-8 was identified in two independent genetic screens in *C. elegans* that were investigating suppressors of an activated RAS/RAF signal transduction pathway (29,30). Sur-8 is composed of nineteen leucine rich repeats (LRRs) which have been hypothesized to function in protein-protein interactions. It has been reported to bind RAS and RAF in a non-competitive manner (31).

KSR was identified using genetic screens in *Drosophila* and *C. elegans* which were studying suppressors of activated RAS (52-54). KSR has five recognizable domains, CA1-5 that were defined by homology between orthologs in flies, mice, and humans (53). CA1 is an unique domain, CA2 is a proline rich region, CA3 is a cysteine rich region, CA4 is a serine and threonine rich region while CA5 is a putative kinase domain. However, the functionality of the kinase domain is questionable (55-58). KSR is reported to bind several pathway components including RAF, MEK, and ERK (reviewed in (59).

Even though these scaffolds have been genetically implicated in the RAS/RAF signal transduction pathway and have been reported to bind to pathway components, it is not known whether they are integral components that impact pathway activation in mammalian cells, and if

so what their function may be. Proposed functions of scaffolds are to alter pathway activation kinetics, enhance pathway efficiency, and restrict kinase specificity.

The purpose of this study was to determine whether Sur-8 and KSR directly contribute to RAS/RAF signal transduction pathway activation in mammalian cells. We chose to use the technique of RNAi instead of overexpression in our studies. If the purpose of scaffolds is to bring several members of the pathway together, then stoichiometry is critical. Therefore, overexpressing a scaffold may not "enhance" pathway function, but may actually be inhibitory by separately sequestering, rather than assembling, pathway components. However, with a transient reduction of the concentration of the scaffold, the true physiological function may be better assessed.

In this study we found that Sur-8 and KSR are required for RAS/RAF signal transduction pathway activation. In addition, we found that Sur-8 contributes to EGF induced pathway activation while KSR is involved in LPA mediated pathway activation. Therefore, a scaffold may be essential to mediate responses to some extracellular stimuli while being dispensable for others. Hence, a role of scaffolds could be to link the RAS/RAF signal transduction pathway from a particular stimulus to an appropriate cellular phenotype.

Results

We wanted to determine whether Sur-8 and KSR were necessary for RAS/RAF signal transduction pathway activation in mammalian cells. We chose to activate the pathway with two

known pathway agonists, epidermal growth factor (EGF) and lysophosphatidic acid (LPA). The EGF receptor is a receptor tyrosine kinase (RTK) while the LPA receptor is a G protein coupled receptor (GPCR). A time course and dose curve was performed using both stimuli to determine the minimal effective dose and the time of peak activation. The stimulation conditions used, based on the time course and dose curve, were 1ng/ml EGF and 10 μM LPA both for five minutes (data not shown). RNAi was used to selectively knockdown KSR and Sur-8 and then investigate EGF and LPA pathway activation as assessed by MEK phosphorylation on S217 and S221. It has been shown that phosphorylation of MEK at these sites correlates with MEK activation (60-62). HeLa cells were transfected with control, Sur-8, or KSR directed oligos. The cells were serum starved overnight and then stimulated with either EGF or LPA. Whole cell lysates were analyzed for the presence of phosphorylated MEK. In control transfected cells, EGF stimulated MEK was phosphorylated to a comparable degree in control and KSR knockdown cells. However, in Sur-8 knockdown cells, there was a decrease in EGF induced MEK activation compared to control knockdown cells. (Figure 3.1A) Sur-8 knockdown had no effect on MEK phosphorylation in response to LPA. However, in KSR knockdown cells there was a decrease in LPA induced MEK phosphorylation as compared to control transfected cells. (Figure 3.1A) The results were repeated at least three times. The MEK phosphorylation was always decreased, but never ablated in either the EGF induced Sur-8 knockdown cells or the LPA induced KSR knockdown cells. These results suggest that Sur-8 and KSR may be involved in RAS pathway activation and that they may display ligand specificity in that Sur-8 was involved in EGF induced MEK activation while KSR contributed to LPA induced MEK activation.

To determine whether Sur-8's involvement in EGF induced MEK activation was HeLa specific, MEK activation was assessed after Sur-8 was knocked down in three other cell lines (human

primary pre-adipoctyes; BJ cells, a fibroblast cell line; and PC12 cells, a rat neuronal cell line) and MEK activation accessed. In serum starved conditions, MEK was not phosphorylated in any of the three cell lines nor in any of the transfection conditions - control, Sur-8, or KSR oligo transfected. In the human primary pre-adipoctyes, MEK was phosphorylated in the control transfected cells in response to 60ng/ml EGF. These cells were stimulated with 60ng/ml EGF since it was the concentration used to block adipocyte differentiation (discussed in chapter 5). The Sur-8 knockdown cells displayed a decrease in MEK activation in response to EGF (Figure 3.2A). There was no reduction in EGF induced MEK activation in the KSR knockdown cells as compared to the control cells. Although these data are consistent with the HeLa data, the experiment has only been performed once.

In the BJ cells, 1ng/ml EGF stimulated MEK phosphorylation in the control transfected cells. There was a comparable amount of MEK phosphorylation in the KSR knockdown cells in response to EGF. However, in the Sur-8 knockdown cells EGF induced MEK phosphorylation was reduced (Figure 3.2B). This result was repeated at least three times.

PC12 cells were stimulated with 100ng/ml EGF since this concentration produced the most reliable activation. MEK was phosphorylated in response to EGF in the control transfected cells. In the Sur-8 knockdown cells, MEK phosphorylation was slightly decreased in response to EGF (Figure 3.2C). MEK phosphorylation was similar in the EGF stimulated control and KSR knockdown cells. These results were repeated at least three times. Collectively, these results suggest that Sur-8's contribution to EGF induced RAS pathway activation is not HeLa specific.

All of the experiments mentioned were performed at one time point (five minutes) and one concentration (depending on the cell type). To determine if the inhibition of EGF induced MEK activation in Sur-8 knockdown cells was actually a shift in the timing of activation, a time course

of EGF was performed in control and Sur-8 knockdown HeLa cells. In the control cells, MEK was activated by five minutes and the MEK activation was sustained until fifteen minutes. By thirty minutes MEK activation was decreased and by an hour MEK activation was between the peak and baseline. In the Sur-8 knockdown cells, the same trend was seen with MEK being phosphorylated by five minutes with a plateau through fifteen minutes and a decline below the peak but above baseline by thirty and sixty minutes (Figure 3.3A). However, as shown in figure 3.1, when control and Sur-8 transfected cells were compared, the MEK activation in the Sur-8 knockdown cells was decreased. The trend was reproducible in that Sur-8 knockdown cells displayed reduced MEK phosphorylation for all time pointed tested. However there was variability in the time at which activation began to decline. In the experiment shown MEK phosphorylation started to decline after fifteen minutes while in the other two experiments MEK phosphorylation started to decline after ten minutes. Nevertheless, the time that MEK phosphorylation peaked and the time it started to decline was consistent between control and Sur-8 knockdown cells for each experiment. Therefore, the variability was not a Sur-8 dependent phenomenon.

Because there was some residual MEK activation in the Sur-8 knockdown cells, we wanted to determine whether or not activation of downstream components would be affected or if the residual MEK activation was sufficient to engage ERK substrate activation. To test this, we examined the transcription factor, c-Fos, which is an ERK substrate. As seen in figure 3.3A, c-Fos was induced after sixty minutes of EGF stimulation in the control transfected cells and this c-Fos induction was dramatically reduced in Sur-8 knockdown cells. These experiments were repeated three times. These data not only adds credence to the idea that Sur-8 is involved in EGF

induced MEK activation, but also suggest that reduction of Sur-8 expression negatively impacts downstream targets of the pathway.

To determine if Sur-8 influenced the efficiency of EGF induced MEK activation, a dose curve of EGF ranging from 20ng/ml - 100ng/ml was performed in HeLa cells that were control or Sur-8 oligo transfected (Figure 3.2B). MEK activation in control transfected cells appeared to increase at 20 and 40ng/ml EGF and was saturated at 60ng/ml EGF. This MEK activation plateaus through 100ng/ml. Knockdown of Sur-8 reduced MEK activation in response to all concentrations of EGF tested (even though the MEK phosphorylation appears slightly higher in 60ng/ml as compared to either 80 or 100ng/ml there is also more MEK protein in this lane). This experiment was repeated twice. These results suggest that Sur-8 is required even at saturating concentrations of ligand.

The residual MEK activation shown in figure 3.2 could be explained by two possibilities; the first is that the residual activity was from untransfected cells (since a population of cells was assayed) and the second possibility is that MEK is still activated to some degree despite limiting amounts of Sur-8. To distinguish between the two possibilities, HeLa cells were transfected with control or Sur-8 targeted oligos, serum starved overnight, and stimulated with 1ng/ml EGF for five minutes. ERK activation was measured on a per cell basis using immunofluorescent labeling with phospho-ERK specific antibody. The R.O.I. was used to manually determine the cells boundary which was used to obtain the total intensity of the phosphor-ERK signal per cell. The background was subtracted from the total intensity to obtain the amount of phospho-ERK signal in arbitrary units which was then plotted on a histogram using Cricket Graph. Over fifty cells for each condition were measured for each experiment. The graph depicts a representative experiment.

If the residual activation was the consequence of untransfected cells, then two peaks would be expected in the Sur-8 knockdown cells whereas if the residual activation was the result of partial MEK activation in a Sur-8 limiting environment, then a single peak left-shifted compared to the control, would be expected. In the control transfected serum starved cells, there was very little activation (Figure 3.4). It can also be noted that not all cells have the same amount of basal ERK activation. The majority of the cells displayed a distribution between zero and five million arbitrary units. When the control transfected cells were stimulated with 1ng/ml EGF, ERK activation was increased, producing signal broadly distributed between two and thirty-seven million arbitrary units. In the Sur-8 knockdown serum starved cells, there was little to no ERK activation, similar to the control transfected serum starved cells. In the Sur-8 knockdown EGF stimulated cells ERK activation was suppressed compared to the control. Importantly, the signal forms a single peak, suggesting that the majority of residual MEK activation seen in figure 3.1-figure 3.3 was not from untransfected cells. These results were seen three times. Therefore, Sur-8 is involved in the magnitude of pathway activation.

To determine if Sur-8 is involved in other receptor tyrosine kinase pathways (RTKs), Sur-8's contribution to Platelet Derived Growth Factor (PDGF) and Neurite Growth Factor (NGF) induced MEK activation was examined. HeLa cells were transfected with either control or Sur-8 oligos and stimulated with 50ng/ml PDGF for five minutes. The amount of basal MEK phosphorylation in the Sur-8 knockdown cells was comparable to the control transfected cells. There was a slight reduction in MEK phosphorylation in the PDGF stimulated Sur-8 knockdown cells. This experiment was conducted twice with similar results (Figure 3.5A).

PC12 cells were used to determine Sur-8's contribution to NGF induced MEK activation. Oligos targeted against CNK2 and TRKA were used as positive controls. CNK2 has been shown by

Bumeister et al. (63) to positively contribute to NGF induced MEK activation. Reduction of TRKA, the NGF receptor, should result in reduced MEK activation. In control transfected cells, stimulation with 100ng/ml NGF resulted in MEK phosphorylation. In the Sur-8, CNK2, and TRKA knockdown cells, the MEK phosphorylation in response to NGF was reduced as compared to the control transfected (Figure 3.5B). This experiment was conducted twice with similar results. MEK activation in response to NGF is required for neurite induction. Therefore, it would be plausible that Sur-8 would contribute to neurite induction. To test this, PC12 cells were transfected with control, Sur-8, CNK2, or TRKA oligos and stimulated with 100ng/ml NGF for forty-eight hours. GFP was cotransfected along with the oligos so that neurites could be easily visualized. In control transfected cells, NGF induced neurites. Neurites were not induced in the CNK2 or TRKA knockdown cells, as previously reported. However, in the Sur-8 knockdown cells, neurite induction was comparable to the control transfected cells (Figure 3.5C). These results were seen three times. A caveat to this experiment is that the cells were not lysed to show that Sur-8 expression was reduced. However, the fact that the controls, CNK2 and TRKA, results recapitulated published work argues that the oligo transfection was successful. These data would indicate that even though Sur-8 may be involved in NGF induced MEK activation; it appears, at least preliminarily, that Sur-8 is either not involved in NGF induced neurite induction or that the degree of inhibition of MEK activation was insufficient for a robust inhibition of “neurite” induction. However, without stronger data no conclusion can be made. The combination of data with PDGF, EGF, and NGF seems to suggest that Sur-8 is downstream of at least one RTK, though it is presently unclear whether Sur-8 is involved in various different RTK pathways.

Discussion

Although much is known about the RAS/RAF signal transduction pathway such as the extracellular stimuli that activate the pathway, the corresponding cellular behavior that is mediated by the activation, as well as the core catalytic components (RAS, RAF, MEK, and ERK), the regulation of the pathway is not well understood. Non-enzymatic components, or scaffolds, have recently been genetically implicated in the pathway and have been hypothesized to contribute to pathway regulation. However, it is not known whether the scaffolds are directly involved in pathway activation and if so what their function is. Because the scaffolds have been reported to bind multiple pathway components in a non-competitive manner, three possible functions include 1) increasing the speed (or kinetics) of pathway activation, 2) contributing to the efficiency of pathway activation, or 3) restricting kinase specificity.

Sur-8 and KSR, two scaffolds implicated in the RAS/RAF signal transduction pathway, were investigated to determine whether they are directly involved in pathway activation, and if so what their function may be. In this work, we show that both Sur-8 and KSR contribute to RAS/RAF signal transduction pathway activation. In addition, our observations suggest that a function of these scaffolds may be to restrict kinase specificity. One of the main questions about the regulation of the RAS/RAF signal transduction pathway is how the same four core catalytic components can accurately and consistently couple pathway activation by a particular stimulus to the appropriate cellular behavior. We show that Sur-8 is involved in EGF induced MEK activation, but not LPA induced MEK activation. In contrast, KSR contributes to LPA induced MEK activation but is not limiting for EGF induced MEK activation. These observations suggest that scaffolds can couple the pathway to particular stimuli. This concept is also seen with Connector Enhancer of KSR 2 (CNK2), a neuronal specific scaffold of the RAS/RAF signal

transduction pathway. Bumeister et al has shown that CNK2 is involved in NGF induced pathway activation but not EGF induced pathway activation (63). Mitogen-activated Protein Kinase Organizer 1 (MORG1) is a binding partner of the scaffold MEK Partner 1 (MP1) and has been implicated in the LPA, phorbol 12-myristate 13-acetate (PMA), and serum but not EGF induced pathway activation (64). Taken together this data suggest that scaffolds can help couple pathway activation from to particular stimulus to a particular response -- Sur-8 for EGF, KSR and MORG1 for LPA, and CNK2 for NGF induced MEK activation. It will be interesting to determine whether KSR and MORG1 act together to mediate LPA induced MEK activation and how their mechanism of action differs.

In this study we investigated the role of Sur-8 in NGF induced MEK activation. Although knocking down Sur-8 inhibited NGF induced MEK activation, there was no effect on neurite induction. It could be that the there are distinct pools of MEK and that the pool that is activated in the Sur-8 knockdown cells can still couple to neurite induction while the pool that is activated in the CNK2 and TRKA knockdown cells cannot couple to neurite induction. However, the MEK activation and neurite induction are from separate experiments with no direct data showing the extent of Sur-8 knockdown. The reduction of Sur-8 expression is inferred from the phenotype of CNK2 and TRKA, which is known. Therefore, no conclusion can be drawn concerning the role of Sur-8 in NGF signaling and further investigations need to be done.

We extended our studies to determine if Sur-8's function involved more than coupling pathway activation to the stimulus. However, knocking down Sur-8 suppressed the level of EGF induced MEK activation without influencing activation kinetics. If Sur-8 contributed to pathway efficiency, it would be expected that Sur-8 would be involved in MEK activation in response to low doses of stimulus, but would be dispensable for higher concentration of stimulus. A dose

curve of EGF in control and Sur-8 knockdown cells suggest that Sur-8 does not contribute to the efficiency of pathway activation. Our observations are not in agreement with Li et al. who report that overexpression of Sur-8 enhances ERK activation in response to low EGF concentrations, but not high levels of EGF. However, they only show data for the low EGF concentration (31). This discrepancy is probably the result of the differences in methods – overexpression versus RNAi.

In conclusion, this study suggests that Sur-8 and KSR contribute to selective coupling of MEK/ERK to distinct stimuli. Specifically, Sur-8 contributes to EGF induced pathway activation and KSR is involved in LPA induced pathway activation. Sur-8 contributes to EGF induced MEK activation in a variety of cell lines (HeLa, human primary pre-adipocytes, BJs, and PC12) and appears to be involved in the magnitude but not the duration of the EGF induced MEK activation. KSR is involved in LPA induced MEK activation.

These results raise the question of how the scaffolds are coupled to the specific receptor. KSR is reported to bind to the heterotrimeric protein Gγ which binds to the LPA receptor. This binding to Gγ may be the means by which KSR is coupled to the LPA receptor. However, further studies are needed to test this. It is unclear how Sur-8 might couple to the EGF receptor. Also, the mechanism of action of KSR and Sur-8 on MEK activation needs accessed.

Materials and Methods

Cell culture and transfection:

HeLa cells were obtained from the ATCC (CCL-2). They were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). RNAi was performed using Oligofectamine (Invitrogen). 100 nM oligo was transfected using manufactor's protocol. BJ cells were treated similar to HeLas. PC12 cells were cultured in RPMI1640 supplemented with 10% horse serum (heat inactivated for 25 minutes at 55C) and 5% FBS. Cells were transfected with 120 nM oligo using Dharmacon's reverse transfection protocol with Lipofectamine 2000 (Invitrogen). Human pre-adipocytes were obtained from Zen-Bio, Inc (Research Triangle Park, NC) and cultured according to their protocols. Cells were transfected using 400 nM oligo using oligofectamine according to the protocol with the exception that the transfection cocktail was added to 1 x 10^5 cells as they were being seeded into a 35mm dish.

siRNAs and antibodies

The following siRNAs sequences were used.

Control Forward: CACCUAAUCCGUGGUUCAATT

Control Reverse: UUGAACCACGGAUUAGGUGTT

Sur-8 (1) Forward: UCCAGCAAUGCAGAGGUGATT

Sur-8 (1) Reverse: UCACCUCUGCAUUGCUGGATT

Sur-8 (2) Forward: GAAGCUGCGGAUGCUUGAUTT

Sur-8 (2) Reverse: AUCAAGCAUCCGCAGCUUCTT

KSR Forward: AGCUGCUGAUGUCUAUGCATT

KSR Reverse: UGCAUAGACAUCAGCAGCUTT

CNK2 Forward: UCGAGACGCCACGACAAGTT

CNK2 Reverse: CUUGUCAGUGGCGUCUCGATT

TRKA Forward: AUGUGGACAGAGGAGCAAATT

TRKA Reverse: UUUGCUCCUCUGUCCACAUTT

Antibodies against P-MEK, P-ERK, ERK, and Actin were from Sigma. P-AKT and AKT were from Cell Signaling. Monoclonal MEK was from Santa Cruz while c-Fos was from Upstate. Antibody against Sur-8 was a generous gift from Michael Stern (30).

RT-PCR

HeLa cells were transfected with siRNAs. Three days post-transfection total RNA was prepared using High Pure RNA isolation kit (Roche). Reverse transciptase reaction was performed using SuperScript First-Strand Synthesis System (Invitrogen). PCR amplification was performed using the following primers.

B-Actin Forward: TGACGGGGTGCGCCACACTGTGCCCATCTA

B-Actin Reverse: CTAGAAGCATTTGCGGTGGACGATGGAGGG

KSR Forward: CAAGTCCCATGAGTCTCA

KSR Reverse: GAAGTTGAACCTGCTGTC

Neurite Formation Assay

PC12 cells were transfected with 120 nM siRNA and 0.1ug pCEP4-GFP in antibiotic free media according to the Invitrogen protocol. 24 hours after transfection neurite projections were induced with 100ng/ml mouse NGF 2.5S (Alamane 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.

ERK Activation Assay

HeLa cells were transfected with 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 overnight before being stimulated for five minutes with EGF (Calbiochem). For immunocytochemistry cells were fixed for 10 minutes with 4% formaldehyde diluted in PBS. Then cells were incubated in methanol for 10 minutes at -20C. Cells were blocked for one hour in blocking buffer (5% BSA and 1% Tween in PBS). P-ERK (Sigma) was diluted 1:100 in blocking buffer. Signal was detected with goat anti-mouse FITC-conjugated antibodies (Jackson) used at a 1:300 dilution in blocking buffer. The primary antibody was incubated for one hour at room temperature while the secondary antibody was incubated for 30 minutes at 37C. Images were collected using Zeiss Axioplan 2 epifluorescence microscope and Open Lab 2.2.5 imaging software. Phospho-ERK signal intensities were analyzed using Open Lab software. More specifically, total intensity of the phosph-ERK signal was determined by multiplying the area of the manually determined cell by the mean R.O.I. intensity. The background was

subtracted to get an arbitrary value for the individual cells. The values were then plotted on a histogram using Cricket Graph.

Figure 3.1

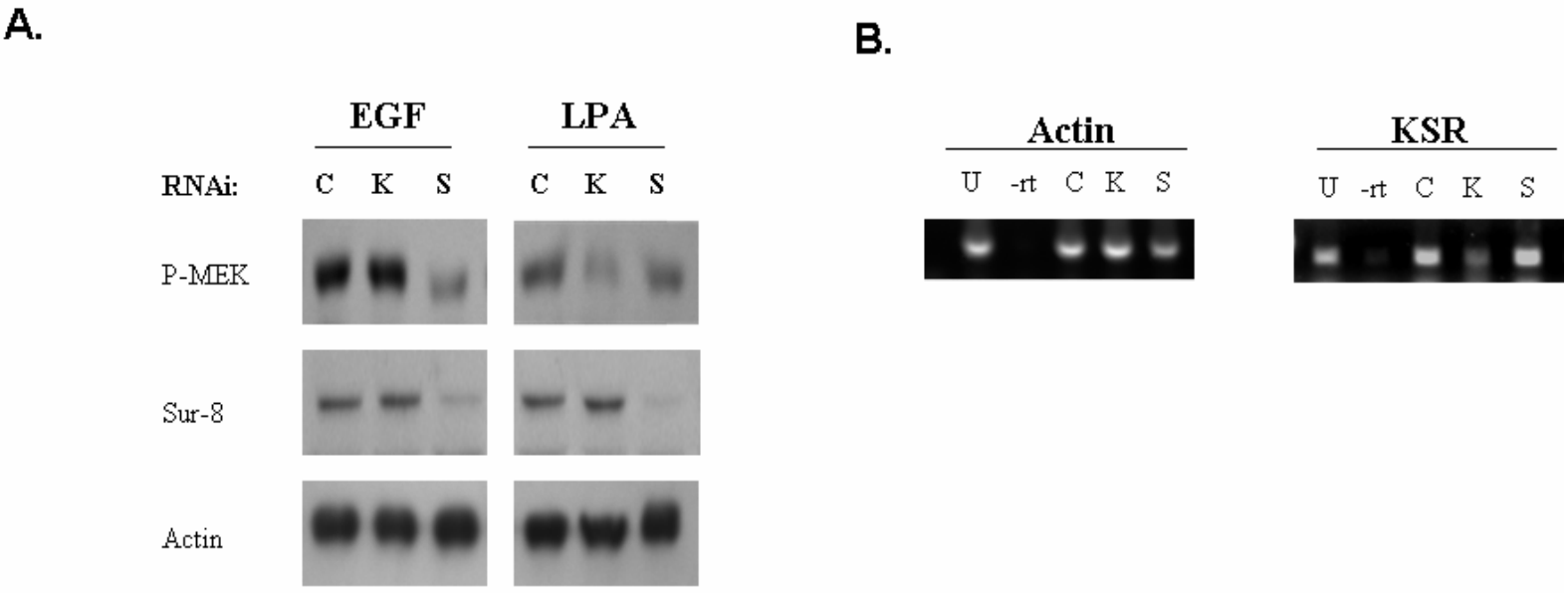


Figure 3.1 Sur-8 and KSR are involved in MEK activation. HeLa cells were transfected with Control (C), Sur-8 (S), or KSR targeted (K) oligos. A) After 48 hours, cells were serum starved overnight and stimulated with either 1ng/ml EGF or 10µM LPA for five minutes. Western blots were probed with indicated antibody. B) 72 hours post-transfection, RT-PCR was performed to determine KSR expression.

Figure 3.2

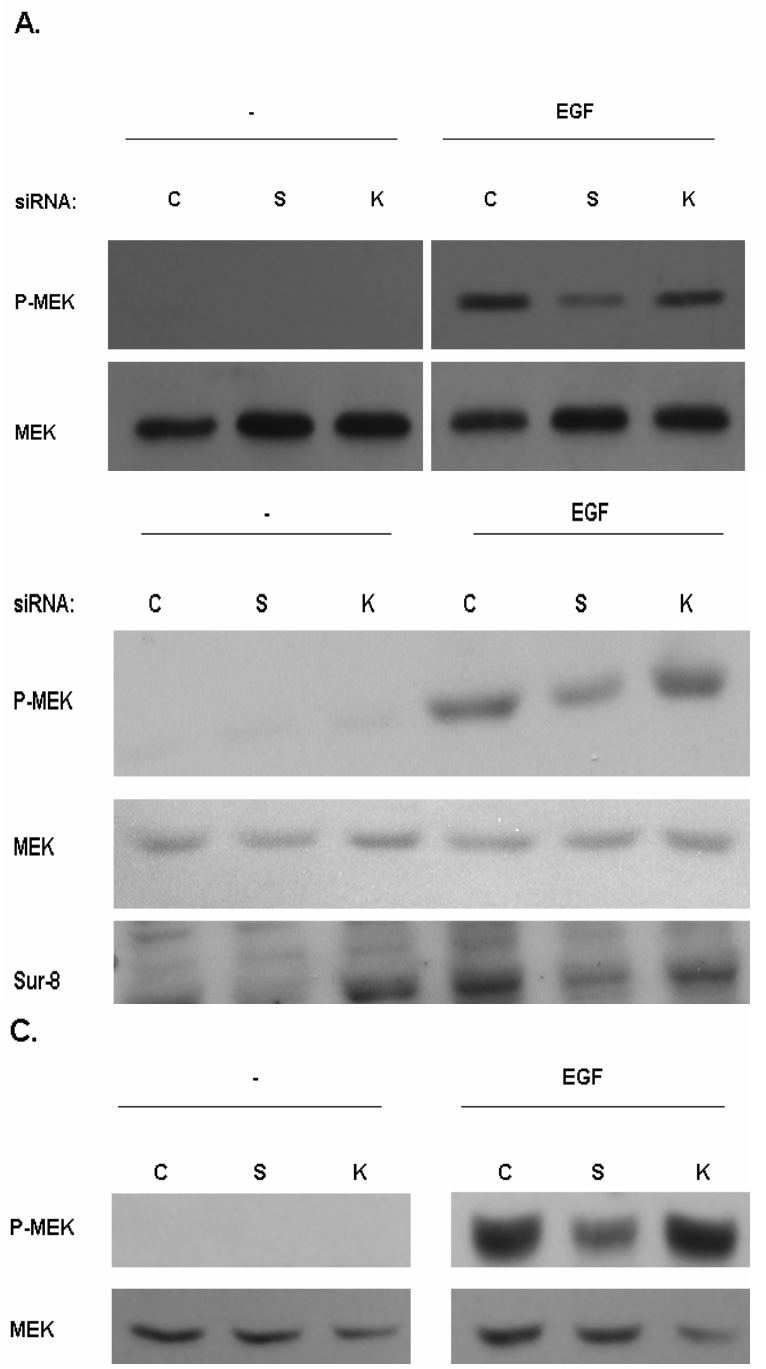


Figure 3.2 Sur-8 is involved in EGF induced MEK activation in various cells lines. Human Pre-adipocytes (A), BJ cells (B), or PC12 cells (C) were tranfected with control (C), Sur-8 (S), or KSR (K) oligos. After 48 hours cells were serum starved overnight and stimulated with EGF for five minutes. Western blot analysis was performed probing with indicated antibody.

Figure 3.3

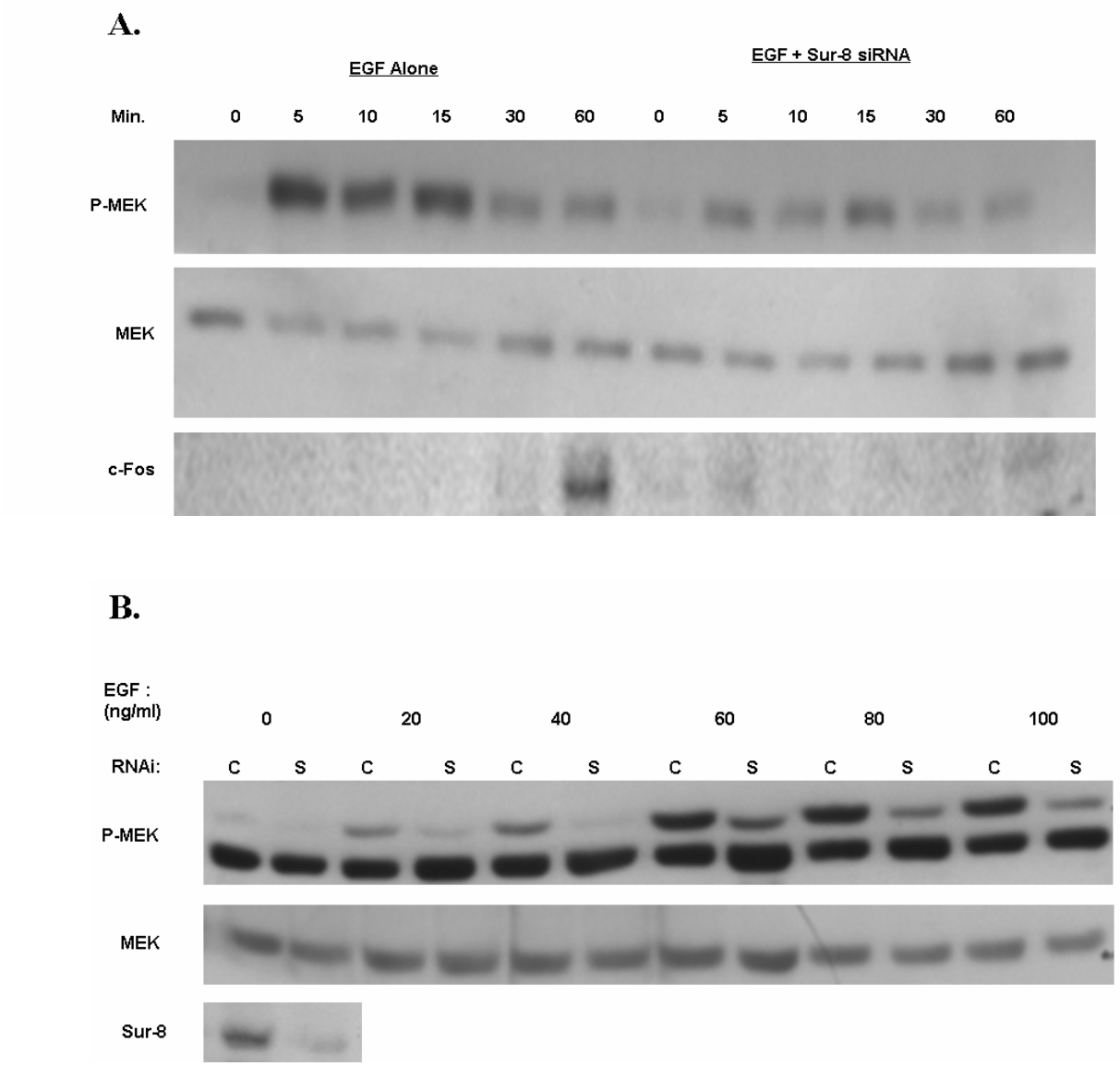


Figure 3.3 EGF time course and dose curve. HeLa cells were transfected as in Figure 3.1. A) Cells were stimulated with 1ng/ml for the indicated times. B) Cells were stimulated for five minutes with the indicated doses of EGF. Western blot analysis was performed using indicated antibodies.

Figure 3.4

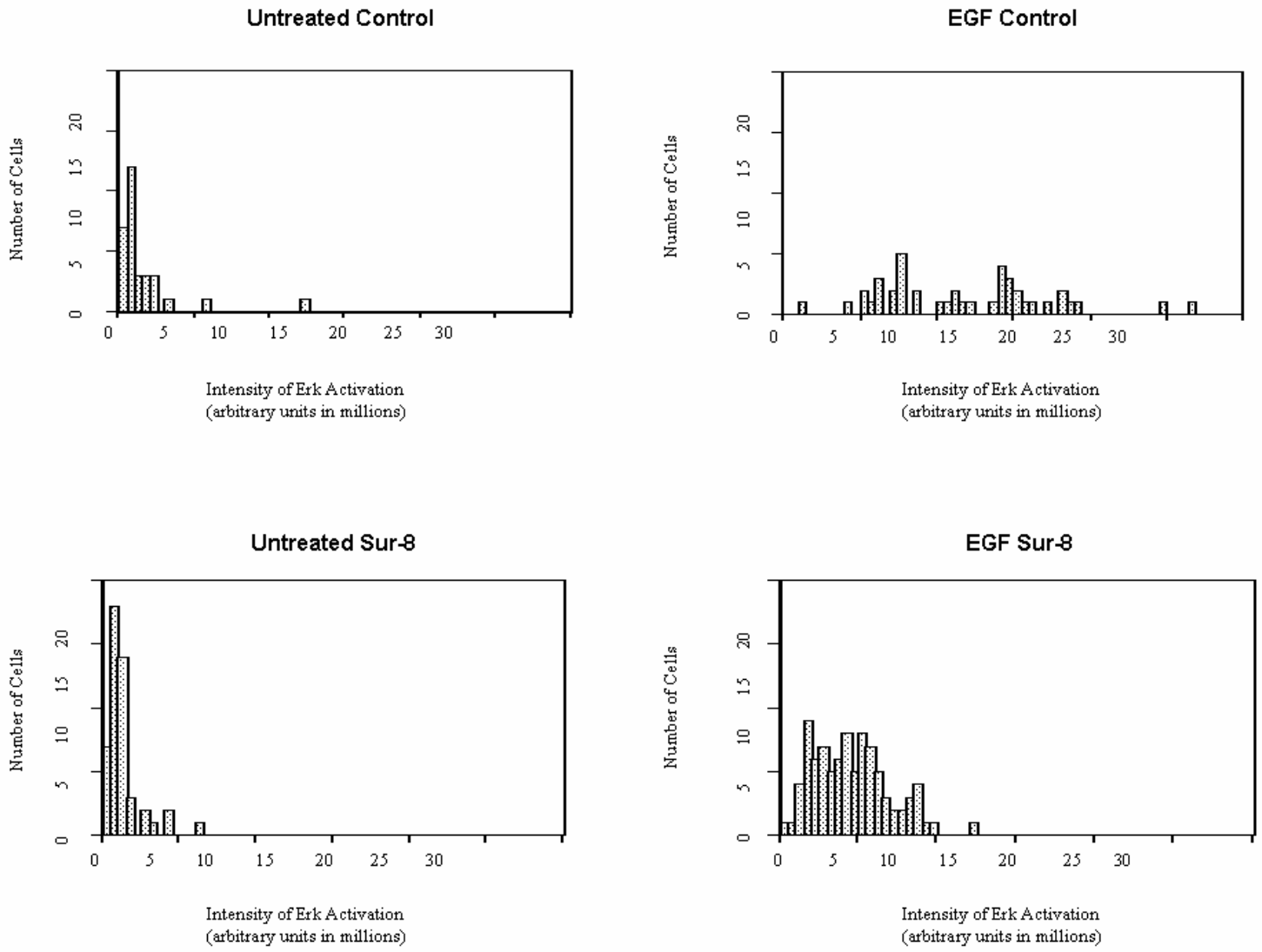


Figure 3.4 Residual MEK activation is not the result of untransfected cells. HeLa cells were transfected with control or Sur-8 targeted oligos. After 48 hours cells were serum starved overnight and stimulated with 1ng/ml of EGF for five minutes. Cells were immunolabeled with antibody against active ERK. The intensity of the immunofluorscence was measured using Open Lab software.

Figure 3.5

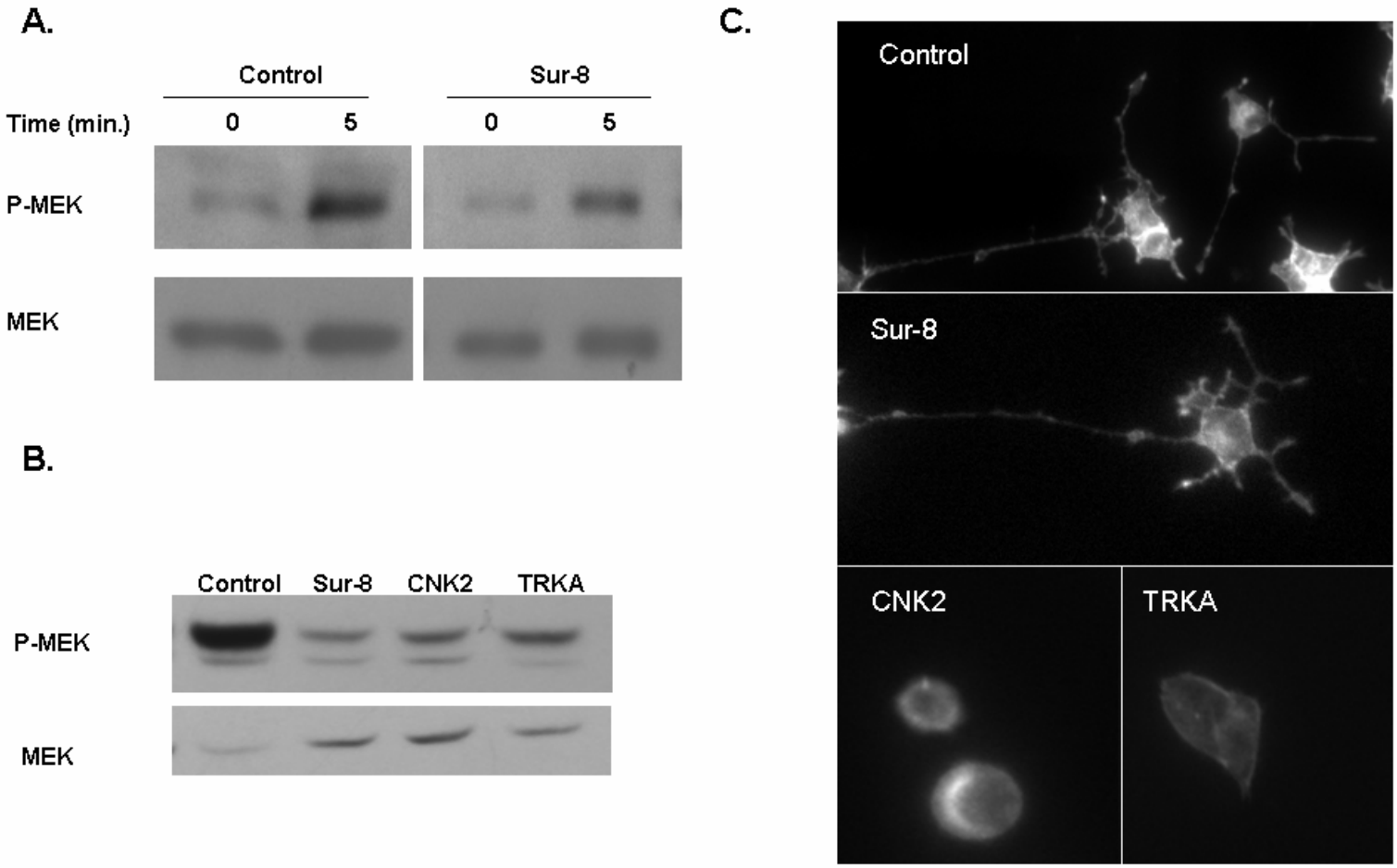


Figure 3.5 Sur-8's involvement in PDGF and NGF Signaling A) HeLa cells were transfected with control or Sur-8 oligos. After 48 hours cells were serum starved overnight and stimulated with 50ng/ml PDGF for five minutes. B) PC12 cells were transfected with control, Sur-8, CNK2, or TRKA oligos, after 48 hours cells were serum starved overnight and stimulated with 100ng/ml NGF for five minutes. C) PC12 cells were tranfected with control, Sur-8, CNK2, or TRKA targeted oligos along with p-CEP4-GFP. Cells were induced with 100ng/ml NGF for 48 hours and then pictures taken of GFP positive cells.

CHAPTER FOUR

SUR-8 AND KSR ARE INVOLVED IN RAF ACTIVATION

Abstract

We have shown that Sur-8 and KSR are integral components of the RAS/RAF signal transduction pathway. Moreover, we have shown that Sur-8 is involved in EGF mediated pathway activation while KSR is required for LPA induced pathway activation. The purpose of this study is to determine the mechanism of action of Sur-8 and KSR in RAS/RAF signal transduction pathway activation. We found that Sur-8 contributes to both RAF-1 and B-RAF activation by EGF. Sur-8 is also involved in bringing a kinase to RAF-1 that result in an activating phosphorylation. We also have preliminary evidence that Sur-8 contributes to RAF-1 localization. KSR does not contribute to LPA induced MEK activation by impacting RAF-1 or B-RAF activation. However, KSR does impact EGF induced RAF-1 activation even though it does not impact EGF induced MEK activation.

Introduction

In chapter three, we demonstrated that Sur-8 and KSR contribute to RAS/RAF signal transduction pathway activation in mammalian cells. Specifically, we determined that Sur-8 contributes to EGF induced RAS/RAF signal transduction pathway activation whereas KSR

is involved in LPA induced RAS/RAF signal transduction pathway activation. The purpose of this study is to determine the mechanism of action of Sur-8 and KSR on RAS/RAF signal transduction pathway activation. The major components of the RAS/RAF signal transduction pathway are RAS, RAF, MEK, and ERK. From the studies previously described in chapter 3, we placed both Sur-8 and KSR upstream of MEK. Mutations in both Sur-8 and KSR can genetically suppress an activated RAS phenotype but not an activated RAF phenotype, leading to the hypothesis that they both act downstream of RAS but either upstream or parallel to RAF (31,52-54). Our lab had shown that KSR is required for insulin induced RAF activation in *Drosophila* (65). Therefore, we began by examining the contribution of Sur-8 and KSR on RAF activation.

In mammalian cells there are three isoforms of RAF—A-RAF, B-RAF, and C-RAF (or RAF-1). RAF regulation is a complex process that even though well studied is not completely understood. Most work to date has been performed with RAF-1. The most well studied aspects of RAF-1 activation can be broken down into three groups – 1) phosphorylation and dephosphorylation, 2) RAS binding, and 3) localization. In the inactive state RAF-1 is cytoplasmic and phosphorylated on S259. Upon stimulation S259 is dephosphorylated, S338 and Y341 are phosphorylated, RAF-1 binds to RAS, and it translocates to the plasma membrane. Other less well characterized sites that are phosphorylated upon stimulation include S43, S621, T491, and S494 (18). Fringer et al has also shown that in unstimulated cells RAF-1 is localized at the cell edges while upon serum stimulation RAF-1 staining appears not only at the edges but throughout the cell (66).

B-RAF regulation is similar to RAF-1 in that upon stimulation it binds to RAS and translocates to the plasma membrane. However, regulation by phosphorylation differs between the two isoforms. S364, the analogous site to S259 of RAF-1, is not phosphorylated in the inactive state. Also, the analogous site for S338 (S445) is constitutively phosphorylated on B-RAF. Whereas, the analogous site of Y341 of RAF-1 (D448) in B-RAF acts as a phosphomimetic. However, T598 and S601 of B-RAF are known to be regulated by phosphorylation. The analogous sites of RAF-1 are T491 and S494 (18).
We found that KSR does not appear to regulate LPA induced MEK activation through either RAF-1 or B-RAF and the mechanism remains elusive. However, one possibility is that KSR may be involved in the RAF/MEK association. Sur-8 is required for EGF induced RAF-1 activation. Sur-8 was not limiting for EGF induced S259 dephosphorylation, S338 and Y341 phosphorylation of RAF-1, RAS/RAF-1 binding, or RAF-1 recruitment to the membrane. However, we show that Sur-8 is required for RAF-1 "clustering" as determined by immunofluorescence. We also observed that Sur-8 binds an unknown kinase that is capable of phosphorylating and increasing RAF-1 activation *in vitro.* Sur-8 also contributes to EGF induced B-RAF activation.

Results

To determine whether Sur-8 contributed to RAF-1 activation, HeLa cells were transfected with either control or Sur-8 targeted oligos, serum starved overnight and then left untreated or stimulated with 1ng/ml EGF for five minutes. RAF-1 was immunoprecipitated and placed

in an *in vitro* kinase assay using kinase dead MEK as a substrate. Western blot analysis was performed on the kinase assays probing for MEK phosphorylation on S217 and S221, the activating phosphorylations (60-62). Post-nuclear supernant was included for verification of Sur-8 knockdown. In control transfected unstimulated cells there was no MEK activation. In response to EGF, MEK is phosphorylated in the control transfected cells, but the phosphorylation was reduced in the Sur-8 knockdown cells. (Figure 4.1A) When RAF-1 activation was assessed, there was little RAF-1 activation in the control transfected, unstimulated cells. RAF-1 was activated in response to EGF in the control transfected cells. In the Sur-8 knockdown cells, EGF induced RAF-1 activation was reduced. Similar results were observed at least three times.

To test whether Sur-8 contributed to B-RAF activation as well as RAF-1, a B-RAF activation assay was performed. The experiment was set up similarly to the RAF-1 activation assay in that control or Sur-8 oligo transfected cells were serum starved and stimulated with 1ng/ml EGF for five minutes. B-RAF was immunoprecipitated and placed in an *in vitro* kinase assay using kinase dead MEK as a substrate. In unstimulated, control transfected cells there was little B-RAF activation, whereas in the Sur-8 knockdown unstimulated cells, there was an increase in B-RAF activation. However these results were only observed once. B-RAF was activated in EGF stimulated control transfected cells, and this activation was decreased in the Sur-8 knockdown cells stimulated with EGF. (Figure 4.1B) Similar results were observed three times. These results suggest that Sur-8 positively contributes to both RAF-1 and B-RAF activation in response to EGF and suppresses B-RAF activation in the absence of stimulation. In response to LPA, B-RAF activation was increased in the Sur-8 knockdown

cells. (Figure 4.1) These results, seen twice, would suggest that Sur-8 suppresses B-RAF activation in response to LPA.

RAF-1 phosphorylation, RAS/RAF-1 binding, and RAF-1 localization were investigated to determine how Sur-8 positively contributed to RAF-1 activation. The phosphorylation sites on RAF-1 that were examined were S259, a negative phosphorylation site, and S338 and Y341, both of which are positive phosphorylation sites. S259 phosphorylation was examined by Western blot analysis of whole cell lysates of control or Sur-8 knockdown HeLa cells. As seen in figure 4.2A, S259 phosphorylation was comparable in EGF stimulated control and Sur-8 knockdown cells. Similar results were observed at least three times. Post-nuclear supernants of control and Sur-8 knockdown cells were analyzed for S338 phosphorylation. In unstimulated, control transfected cells there were no S338 phosphorylation, but the phosphorylation was induced upon EGF stimulation. The phosphorylation in the Sur-8 knockdown cells was comparable to the controls. (Figure 4.2B) Similar results were seen three times. RAF-1 immunoprecipitates were used to investigate the contribution of Sur-8 to Y341 phosphorylation of RAF-1. There was no Y341 phosphorylation seen in either control or Sur-8 knockdown unstimulated cells, though this phosphorylation event was induced upon EGF stimulation. The level of phosphorylation was comparable between the control and Sur-8 knockdown cells. (Figure 4.2C) These results were observed twice. Together the data suggest that Sur-8 does not contribute to S259 dephosphorylation or S338 or Y341 phosphorylation. These data also suggest that EGF signaling was not completely ablated since enough signal was propagated to mediate these phosphorylation events even though RAF-1 activation was decreased.

To determine if Sur-8 contributed to RAS/RAF-1 binding, RAS was immunoprecipitated and the presence of RAF-1 was examined. In control transfected unstimulated cells there was some RAF-1 bound to RAS. RAF-1 binding to RAS increased upon EGF stimulation. There was a comparable amount of RAF-1 bound to RAS in the control and Sur-8 knockdown EGF stimulated cells. There was an increase in RAF-1 bound to RAS in the unstimulated Sur-8 knockdown cells. Therefore, reducing Sur-8 expression increased RAS/RAF-1 binding in unstimulated cells. (Figure 4.3) These results were observed at least three times. Sur-8 is not limiting for EGF induced RAS/RAF-1 binding, but may be inhibitory to RAS/RAF-1 interaction in the basal state.

Two separate approaches were used to address whether Sur-8 contributed to RAF-1 localization. The first approach was membrane isolation by centrifugation at 100,000 x g. In control transfected unstimulated HeLa cells there was a small amount of RAF-1 at the membrane which increased upon EGF stimulation. There was a comparable amount of RAF-1 in the membrane fraction in the EGF stimulated control and Sur-8 knockdown cells. However, as seen with the RAS/RAF-1 interaction, there was more RAF-1 at the membrane in the unstimulated Sur-8 knockdown cells as compared to the controls. The same amount of RAF-1 was seen in the membrane fraction in the unstimulated and EGF stimulated Sur-8 knockdown cells. (Figure 4.4A) These observations were seen at least three times.

The second approach to investigate RAF-1 localization was by immunofluorescence. Fringer et al. has shown in human foreskin fibroblasts that RAF-1 in unstimulated cells is localized towards the ends of the cells and upon serum stimulation the RAF-1 translocates throughout the cells (66). In HeLa control transfected unstimulated cells, RAF-1 was located towards

the edges of the cell and upon EGF stimulation there was more punctate staining and the puncta appeared larger, but remained around the cell edges. In the EGF stimulated Sur-8 knockdown cells, the RAF-1 puncta appeared smaller and spread throughout the cell. As with the P100 fractionation, the Sur-8 knockdown cells that were unstimulated looked remarkable similar to the control EGF stimulated cells. (Figure 4.4B) These observations were seen at least three times. These data suggest that even though Sur-8 does not contribute to the overall amount of RAF-1 at the membrane (as detected by membrane fractionation), Sur-8 is involved somehow in the localization, or "clustering" of RAF-1.

We next sought to determine if RAF-1 clustering was due to dimerization and if RAF-1 was autophosphorylated in the process. Immunoprecipitatied Sur-8 was added to immunoprecipitated RAF-1 *in vitro.* Endogenous RAF-1 was immunoprecipitated from HeLa cells treated in one of four conditions: 1) unstimulated control transfected, 2) unstimulated Sur-8 knockdown, 3) EGF stimulated control transfected, or 4) EGF stimulated Sur-8 knockdown. Myc-tagged Sur-8 was overexpressed in HEK293 cells that were unstimulated or EGF stimulated, and immunoprecipitated using anti-Myc agarose beads. These conditions were used because it was uncertain whether certain steps in RAF-1 regulation (such as RAS binding, membrane recruitment, dephosphorylation, or phosphorylation) were a prerequisite for Sur-8's function regarding RAF-1 activation. Both Sur-8 and RAF-1 immunoprecipitates were washed with 0.5M NaCl, 0.5% deoxycholate, and 1% Triton X-100. The immunoprecipitates were mixed in an *in vitro* kinase assay using ^{32}P-labeled ATP to detect phosphorylation. Very little phosphorylation was detected if immunoprecipitates from empty vector transfected cells were added to the RAF-1

immunoprecipitates. However, when immunoprecipitated Sur-8 was added to immunoprecipitated RAF-1, RAF-1 phosphorylation increased. This effect was the same whether the RAF-1 was immunoprecipitated from unstimulated, EGF stimulated, control transfected, or Sur-8 siRNA transfected cells. (Figure 4.5A)

To determine if the phosphorylation was the result of RAF-1 autophosphorylation, the assay was repeated using either wild-type or kinase-dead overexpressed RAF-1. As can be seen in figure 4.5B, again there was little to no phosphorylation with RAF-1 alone, but RAF-1 was phosphorylated when immunoprecipitated Sur-8 was added to immunoprecipitated wild-type RAF-1. However, RAF-1 was also phosphorylated when exogenous Sur-8 was added to immunoprecipitated kinase-dead RAF-1, suggesting that the RAF-1 is not autophosphorylated. Sur-8 does not contain a kinase domain and phosphorylation was seen with kinase dead RAF-1. Therefore, an unknown kinase must have been immunoprecipitated with Sur-8.

To determine whether this increase in phosphorylation resulted in an increase in RAF-1 activation, the above mentioned assay was repeated using kinase-dead MEK as a substrate. Again, there was little MEK phosphorylation when immunoprecipitates from empty plasmid transfected cells was added to RAF-1, but there was detectable phosphorylation when exogenous Sur-8 was added to immunoprecipitated RAF-1. This activation was not seen when immunoprecipitated Sur-8 alone was added to kinase-dead MEK. These results were obtained at least three times. Since Sur-8 does not contain a kinase domain, there must be a kinase bound to Sur-8 that was phosphorylating RAF-1. In an attempt to wash off the candidate associated kinase, the Sur-8 immunoprecipitates were washed with 3% empigen.

However, RAF-1 activation was still increased when Sur-8 immunoprecipitates were washed with empigen. (Figure 4.5C) (There was still RAF-1 phosphorylation as well (data not shown). Since empigen was unsuccessful in washing off the associated kinase activity, the Sur-8 immunoprecipitates were washed with 1.0 M NaCl, 1% SDS, or 3% BME. The only condition that reduced RAF-1 phosphorylation was 1% SDS. However, with that concentration of SDS, Sur-8 may be unfolded and therefore can no longer bind to either RAF-1 or the unknown kinase. (Figure 4.5D) Combined, these *in vitro* kinase assays suggest that Sur-8 binds a kinase that phosphorylates RAF-1 resulting in an increase in RAF-1 activation.

Next, we examined if KSR contributed to RAF-1 activation. An endogenous RAF-1 kinase assay was repeated in KSR knockdown HeLa cells. In the post-nuclear supernant there was no MEK phosphorylation in the unstimulated cells. There were comparable amounts of MEK phosphorylation in the control and KSR knockdown EGF stimulated cells. However as seen previously there was a reduction in MEK phosphorylation in the KSR knockdown LPA stimulated cells. In the corresponding *in vitro* RAF-1 kinase assay, there was no RAF-1 activation in either the control or KSR knockdown cells that were unstimulated. In response to EGF, RAF-1 was activated in the control transfected cells. Interestly, even though MEK activation was normal in KSR knockdown EGF stimulated cells, RAF-1 activation in these cells was dramatically reduced. (Figure 4.6A) These results were observed three times. These results suggest that KSR contributes to EGF induced RAF-1 activation even though it is not limiting for EGF induced MEK activation.

To investigate how KSR contributed to LPA induced MEK activation, a B-RAF activation assay was performed in control and KSR knockdown cells. (Endogenous RAF-1 was not activated in response to LPA; therefore KSR mediated LPA induced MEK activation does not act through RAF-1 (data not shown). As seen previously, in the post-nuclear supernants KSR knockdown cells displayed reduced MEK activation in response to LPA, but not in response to EGF. In the B-RAF activation assay, there was a slight amount of activation in the control transfected cells that were unstimulated. This activation was decreased in the KSR knockdown cells. In response to EGF, B-RAF activation was increased over baseline. This EGF induced B-RAF activation is slightly decreased in the KSR knockdown cells. In response to LPA there were comparable amounts of B-RAF activation in the control and KSR knockdown cells. (Figure 4.6B) These results were seen three times. These results suggest that KSR does not contribute to LPA induced MEK activation through B-RAF. It also suggests that KSR does contribute to B-RAF activity in the basal state and may have a slight influence in EGF induced B-RAF activation.

In the preceeding experiments we used RNAi to investigate the role of the scaffolds Sur-8 and KSR in the activation of RAF-1 and B-RAF following EGF or LPA stimulation. We used overexpression to address whether Sur-8 and KSR would be sufficient for EGF and LPA induced RAF-1 activation. RAF-1 was overexpressed along with the scaffolds in HEK293 cells. In unstimulated cells, there was a small amount of RAF-1 activation when either empty plasmid or KSR was overexpressed with RAF-1. However, this activity was reduced when Sur-8 was overexpressed along with RAF-1. There was an equal amount of RAF-1 activation when empty plasmid or Sur-8 was overexpressed with RAF-1 in EGF

stimulated cells. However, in EGF induced cells where RAF-1 and KSR was overexpressed, RAF-1 activation was increased. When the cells were stimulated with LPA, RAF-1 was activated and this activation was comparable between empty plasmid and Sur-8 overexpressed. However, there was a significant increase in RAF-1 activation when KSR was overexpressed. (Figure 4.7) Because of the slight increase in basal RAF-1 activation when KSR is overexpressed, no conclusion can be make regarding KSR sufficiency in EGF induced RAF-1 activation although with the large increase in LPA induced RAF-1 activation, it seems likely that KSR is sufficient for LPA induced RAF-1 activation. Sur-8 does not appear to be sufficient for EGF induced RAF-1 activation although it is necessary.

Discussion

In chapter 3, we determined that Sur-8 and KSR were integral components of the RAS/RAF signal transduction pathway in mammalian cells. Moreover, we observed that the scaffolds displayed ligand specificity in that Sur-8 was required for EGF induced RAS/RAF signal transduction pathway activation whereas KSR contributes to LPA induced RAS/RAF signal transduction pathway activation. At that time, we had placed both scaffolds upstream of MEK activation since reduction of protein expression of the scaffold resulted in reduction of MEK phosphorylation of S217 and S221 by the particular stimulus. In this study, we sought to determine the mechanism of action of Sur-8 and KSR. The investigation began with RAF since RAF is directly upstream of MEK in the RAS/RAF signal transduction pathway and our lab has previously shown that in *Drosophila* KSR is required for insulin induced RAF

activation (65). Moreover, mutations in both Sur-8 and KSR suppress an activated RAS phenotype but not an activated RAF phenotype suggesting, at least genetically, that Sur-8 and KSR function downstream of RAS but upstream of or parallel to RAF (53,67). By overexpression analysis Sur-8 was reported to facilitate RAS/RAF binding (31).

In the Sur-8 knockdown cells, we observed a reduction in both EGF induced RAF-1 and B-RAF, as well as MEK activation. Therefore, the decrease in EGF induced MEK phosphorylation observed in the Sur-8 knockdown cells correlates with a decrease in RAF-1 activation. RAS activation assays were performed in control and Sur-8 knockdown cells to determine whether Sur-8 would positively impact RAS activation in response to EGF. However, there was no difference in the amount of EGF induced RAS activation in the control or Sur-8 knockdown cells. (data not shown) Therefore, by transient loss-of-function analysis, Sur-8 acts at the level of RAF.

There are many components to RAF-1 regulation including RAS/RAF-1 binding, several RAF-1 phosphorylation and dephosphorylation events, and RAF-1 localization. By transient loss-of-function analysis we observe that Sur-8 is not limiting for EGF induced RAS/RAF-1 binding. This is in disagreement with Li et al. who observed that overexpression of Sur-8 increased RAS/RAF-1 complex (31). This discrepancy may be the result of an overexpression artifact.

We observe that Sur-8 is not limiting for EGF induced S259 dephosphorylation nor EGF induced phosphorylation of either S338 or Y341, but we do observe changes in EGF induced RAF-1 localization in the Sur-8 knockdown cells. RAF-1 localization visualized by immunofluorescence did display Sur-8 dependent changes in RAF-1 localization even though

EGF induced RAF-1 recruitment to the membrane was unchanged in the Sur-8 knockdown cells as detected by membrane fractionation. The RAF-1 immunofluorescence staining in control transfected EGF treated cells appears as large RAF-1 clusters around the edges of the cells. It is unclear what the immunoflurescence staining is depicting. Preliminary experiments were performed with rhodamine labeled EGF to determine whether this RAF-1 "clustering" was RAF-1 going into endosomes. However, the pattern shown in figure 4.4B occurred after five minutes EGF stimulation whereas endocytosis of rhodamine labeled EGF did not occur until fifteen minutes. Also, the RAF-1 punta and the endocytosed rhodamine-labeled EGF never overlapped (data not shown) suggesting that the RAF-1 clustering was not the result of RAF-1 being recruited to endosomes. We prepared to carry out electron microscopy (EM) studies to further investigate these RAF-1 clusters, however when cells were permeabilized with Triton X-100 the staining is nonspecific whereas for the immunofluorescence studies, cells were permeablized with methanol. Therefore, studies could not be carried out to further characterize the RAF-1 "clustering" or punta. However, one possibility was that the clustering is RAF-1 dimerization leading to autophosphorylation. *In vitro* RAF-1 kinase assays were carried out to determine whether Sur-8 added to RAF-1 *in vitro* could result in RAF-1 dimerization and consequently an increase in RAF-1 phosphorylation. We observed that mixing immunoprecipitated Sur-8 and immunoprecipitated RAF-1 results in an increase in RAF-1 phosphorylation, but it is not autophosphosphorylation. This increase in RAF-1 phosphorylation and RAF-1 activation, was seen only when immunoprecipitated Sur-8 was added to RAF-1, not with Sur-8 or RAF-1 alone. Because Sur-8 only contains leucine rich repeats (LRRs), there is a kinase that is

immunoprecipitating with Sur-8 that can phosphosphorylate RAF-1. Sur-8 immunoprecipitates were washed with 0.5M NaCl, 1.0M NaCl, 3% BME, 1% emigen, and 1% SDS. The only condition that reduced the phosphorylation was 1% SDS. However, with that high concentration of SDS, Sur-8 could be unfolded and cannot bind to RAF-1. Therefore, our conclusion is that there is a kinase that is tightly associated with Sur-8 that can phosphorylate RAF-1 resulting in an increase in RAF-1 activation.

We found that Sur-8 may have dual function in RAF-1 activation – it may act as both an activator and an insulator of RAF-1 activation. We observed that in unstimulated Sur-8 knockdown cells, there was more RAS/RAF-1 binding and more RAF-1 present at the membrane by membrane fractionation than in the control. Also, with the RAF-1 immunofluorescence, the punta in the Sur-8 knockdown cells closely resembled the pattern of the control EGF stimulated. Therefore, it would appear that Sur-8 acts as an insulator to keep RAF-1 in an "inactive" state until the presence of an activation signal. We were unable to consistently detect an increase in basal RAF-1 activation in unstimulated cells, and were never able to detect an increase in basal MEK or ERK activation. Recent data has suggested that RAF-1 may have kinase independent functions and it may be that the role of Sur-8 in unstimulated cells is to sequester RAF-1. Though it would be tempting to suggest that Sur-8 may sequester RAF-1 in the cytoplasm until EGF stimulation, preliminary data suggest that Sur-8 is at the membrane in unstimulated cells and the amount of Sur-8 at the membrane decreases upon EGF stimulation (data not shown).

KSR is required for EGF induced RAF-1 activation even though we do not detect any consequence of KSR on EGF induced MEK activation. These results are in contrast to the

knockout mice where the embryo fibroblast displays decrease ERK activation in response to EGF (68,69). The two observations may be in disagreement because of the methods used. By using RNAi, we are observing the consequence of a transient reduction in KSR expression whereas with knockout mice, there may be some genetic compensation so that the mice can survive. However, we observe, in the same experiment, that KSR knockdown cells have equal amounts of EGF induced MEK activation as compared to control, but drastically reduced EGF induced RAF-1 activation. This would suggest that EGF induced RAF-1 activation is coupled to something other than MEK. Although MEK is the most well-known RAF-1 substrate, increasing evidence suggests that RAF-1 has other substrates such as ASK1, MST2, and ROK-α (70). EGF induced B-RAF activation was slightly reduced in the KSR knockdown cells. LPA induced B-RAF activation was unaffected in the KSR knockdown cells. Therefore, the mechanism of how KSR contributes to LPA induced MEK activation is unclear. It could be that KSR is acting through the other RAF isoform, A-RAF, though this seems unlikely since A-RAF is the weakest MEK activator of the RAF isoforms (18). Another possibility is that KSR binds B-RAF to MEK. It is known that KSR constitutively binds to MEK and shuttles MEK to the plasma membrane (36,71,72). In *Drosophila* it has been shown that KSR couples RAF to MEK (73) (dRAF is most similar to B-RAF).

We have addressed the necessity of Sur-8 and KSR for RAF-1 activation by transient loss-of-function analysis; we addressed the concept of sufficiency by overexpressing both the scaffold and RAF-1. KSR and RAF-1 coexpression lead to elevated RAF-1 activation in a unstimulated state. Therefore, it was difficult to determine whether the increase in RAF-1

activation observed in EGF stimulated cells was just a consequence of KSR and RAF-1 coexpression. However, it did appear that KSR was sufficient to enhance LPA induced RAF-1 activation. Overexpression of Sur-8 was not sufficient to elevate RAF-1 activation in any of the context examined – unstimulated, EGF, or LPA stimulated. We did not find that Sur-8 could potentiate RAF-1 activation in response to EGF. This conflicts with reports where overexpressed Sur-8 potentiates RAF-1 activation in response to EGF (31). Our results may differ in the technical details of the RAF-1 immunoprecipitation and kinase assay. Li et al. used immunoprecipitation buffer containing 0.5% NP-40 and 100mM NaCl. Our immunoprecipitations were washed with 500mM NaCl, 0.5% deoxycholate, and 1% Triton X-100. Also, Li et al. used a coupled kinase assay where the final read-out was ELK-1 phosphorylation, three steps downstream of RAF-1. Our readout was MEK phosphorylation, the direct substrate of RAF-1. Therefore, Li et al may have gotten potentiation because of a contaminating kinase and the contaminating activity amplified as the result of a coupled assay, or they may simply be using a more sensitive assay to read out relatively small changes.

In conclusion, our model would be that Sur-8 and KSR are required for EGF induced RAF-1 activation; however the activation is uncoupled from MEK activation. Either MEK activation is mediated by B-RAF or there is a redundancy between B-RAF and RAF-1 for EGF coupling to MEK. In support of B-RAF being the major MEK activator, EGF fails to stimulate ERK in B-RAF null fibroblast while EGF signaling to ERK is intact in RAF-1 null cells (74,75). Sur-8 also contributes to RAF-1 localization in an unstimulated state, though this localization appears to be kinase-independent. We hypothesize that KSR contributes to

LPA induced MEK activation by coupling MEK to B-RAF. Further studies are needed to 1) test KSR's role in coupling B-RAF to MEK, 2) determine the kinase that is bound to Sur-8, and 3) what the phosphorylation site on RAF-1 is, and the physiological relevance. It would be tempting to speculate that the site might be TT491 or S494 of RAF-1 or T598 or S601 of B-RAF since these sites are regulated by phosphorylation in both B-RAF and RAF-1. However, it could be that this is a novel phosphorylation site since this site can be phosphorylated even after EGF stimulation.

Materials and Methods

Antibodies and plasmids

Anti-Ha was purchased from Babco. Polyclonal B-RAF, anti-myc 9E10 agarose conjugates, and RAF-1 C-12 (for immunofluorescence) was purchased from Santa Cruz. RAF-1 from Transduction Labs was used for Westerns and immunoprecipitations. Anti-FLAG M2 agarose conjugates was purchased from Sigma. Anti-phospho 340/341 RAF-1 was purchased from Biosource. Anti-phospho 259 RAF-1 and anti-phospho 338 RAF-1 was purchased from Cell Signaling. pCDNA3- Myc Sur-8 was a gift from Dr. Kiran Kaur. pCDNA3-Ha-KSR1 (58) and pLNX2-FLAG RAF1 (76) has previously been described. The kinase dead MEK protein was a kind gift from Dr. Melanie Cobb. Kinase dead RAF-1 was a kind gift from Dr. Jeffrey Frost.

Cell Culture and Transfection

HEK293 cells were cultured in DMEM without sodium pyruvate (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS). For transfection, cells were plated in a 35 cm dish to approximately 70% confluency, the next day cells were transfected using lipofectamine 2000 (Invitrogen) using 1ug of DNA.

Immunoprecipitation Kinase Assay

Forty-eight hours after transfection, cells were serum starved overnight and stimulated with 1ng/ml EGF or 10 µM LPA for five minutes. Cells were lysed in 200 ul of lysis buffer containing 30mM Hepes, 1% Triton X-100, 20mM B-glycerophosphate, 2mM sodium phosphate, 1 mM sodium vanadate, and 1mM sodium fluoride. Lysates were rotated at 4°C for twenty minutes to lyse. Lysates were spun at 13,000 x g for fifteen minutes. The supernant was pre-cleared with protein A/G beads for thirty minutes. Samples were spun at 13,000 x g for fifteen minutes. 20 µl of the supernant was saved as post-nuclear supernant. To the rest of the supernant antibody was added. For RAF-1 immunoprecipitation, 2 µl of monoclonal anti-RAF-1 (Transduction Labs) was used. For B-RAF immunoprecipitation, 4 µl goat polyclonal anti-B-RAF antibody from Santa Cruz was used. For immunoprecipitation of overexpressed protein, 15 µl of FLAG beads were used. For B-RAF

immunoprecipitations, only 0.1mg of supernant was used for the immunoprecipitation while all of the superanant from a 35mm dish was used for RAF-1 immunoprecipitation. For immunoprecipitation of endogenous proteins, the antibody was tumbled overnight and then 15 µl of A/G beads were added for two hours. While with the overexpressed immunoprecipitation, 15 µl of coupled beads were added and incubated for two hours. Beads were washed three times with lysis buffer, twice with lysis buffer containing 500mM NaCl, and twice with 10mM $MgCl_2$ and 25mM Hepes. For the kinase assay 30 µl of kinase buffer was added (10mM MgCl2, 200ng kinase-dead MEK, 12uM ATP, and 25 mM Hepes). The kinase assay was incubated for thirty minutes at 30°C for RAF-1 and fifteen minutes at 30°C for B-RAF. For radioactive assays 10µCi ^{32}P-ATP was used.

RAS Activation Assay

48 hours post-transfection cells were serum starved overnight. The next day, cells were either left unstimulated or stimulated with 1ng/ml EGF for 5 minutes. Cells were lysed in buffer containing 30mM Hepes pH 7.4, 1% Triton X-100, 20mM β-glycerophosphate, 2mM sodium pyrophosphate, 1mM sodium vanadate, 1mM sodium fluoride, 20mM potassium chloride, 3mM EDTA, 2mM EGTA, 3.75mM Magnesium chloride, and 14mM β-mercaptoethanol. Lysates were rotated at 4°C for twenty-five minutes and then spun for 15 minutes at 13,000 x g. Supernant was pre-cleared for forty-five minutes with protein A/G agarose beads and then spun for fifteen minutes at 13,000 x g. 10 µl of anti-H-RAS agarose

beads were added to the supernant and incubated for six hours. Beads were spun down for at 1,000 x g for two minutes and then washed three times in lysis buffer.

Figure 4.1

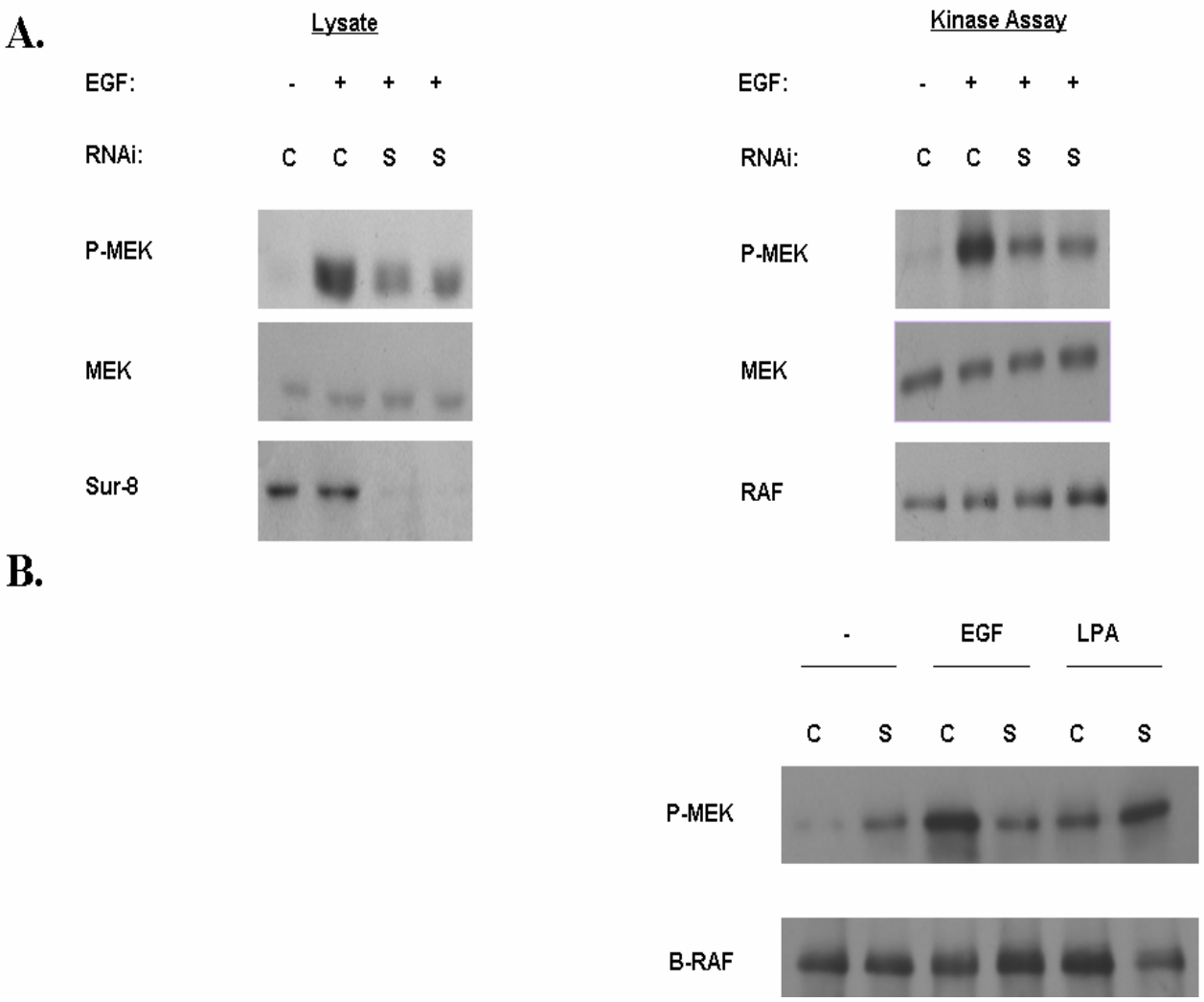


Figure 4.1 Sur-8 contributes to EGF induced RAF-1 and B-RAF activation. (A) HeLa cells were transfected with either control (C) or Sur-8 (S) oligos. After forty eight hours cells were serum starved overnight and then stimulated with 1 ng/ml EGF for five minutes. RAF-1 was immunoprecipitated from the cells and placed into an *in vitro* kinase assay using kinase-dead MEK as a substate. B) Cells treated as in A except B-RAF was immunoprecipitated.

Figure 4.2

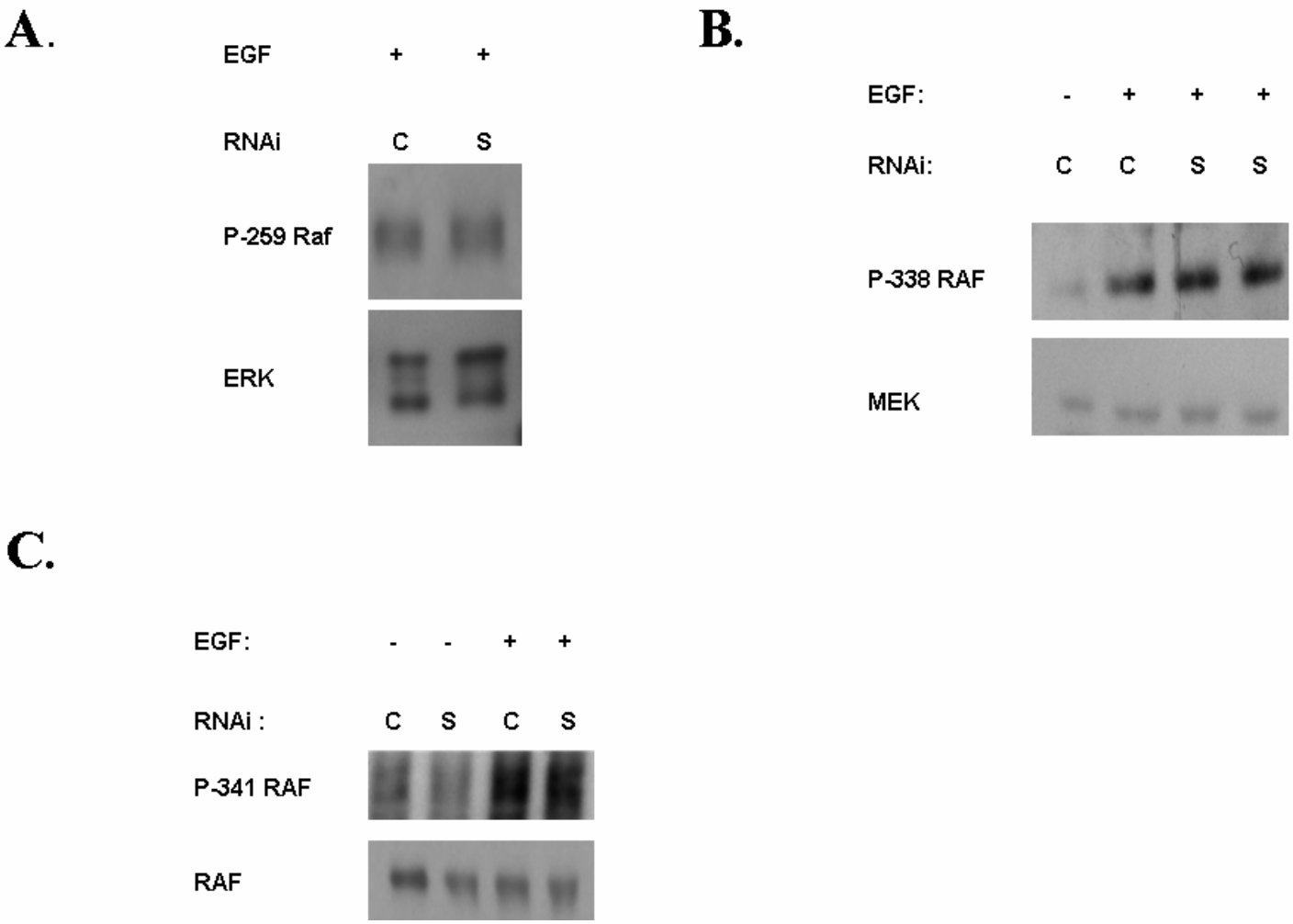


Figure 4.2 Sur-8 is not limiting for RAF-1 dephosphorylation of S259 or phosphorylation of S338 or Y341. A) Whole cell lysate from control (C) or Sur-8 (S) transfected cells were probed with phospho-specific S259 RAF-1 antibody. B) Post nuclear supernants from immunoprecipitations were probed with phospho-specific S338 RAF-1 antibody. C) RAF-1 immunoprecipitates were probed with phospho-specific Y341 RAF-1 antibody.

Figure 4.3

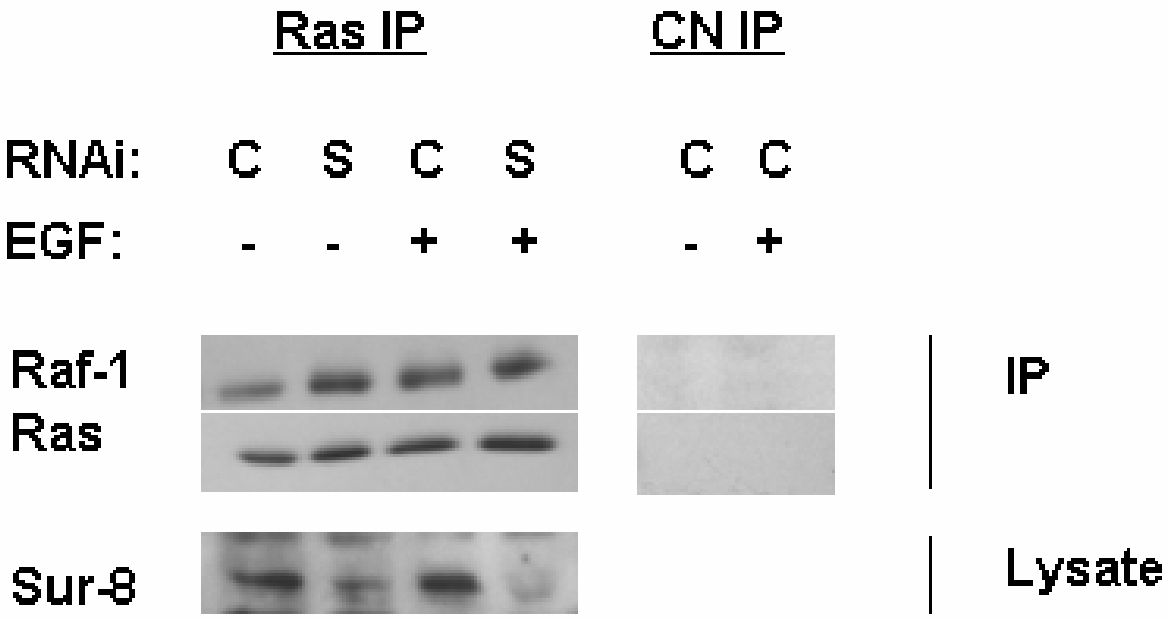


Figure 4.3 Sur-8 is not limiting for EGF induced RAS/RAF-1 binding. HeLa cells were transfected with either control (C) or Sur-8 (S) oligos, serum starved and stimulated with 1ng/ml EGF. Activated RAS was immunoprecipitated by binding to the RAS binding domain (RBD) of RAF-1. Western blot analysis was performed on immunoprecipitates probing for RAF-1.

Figure 4.4

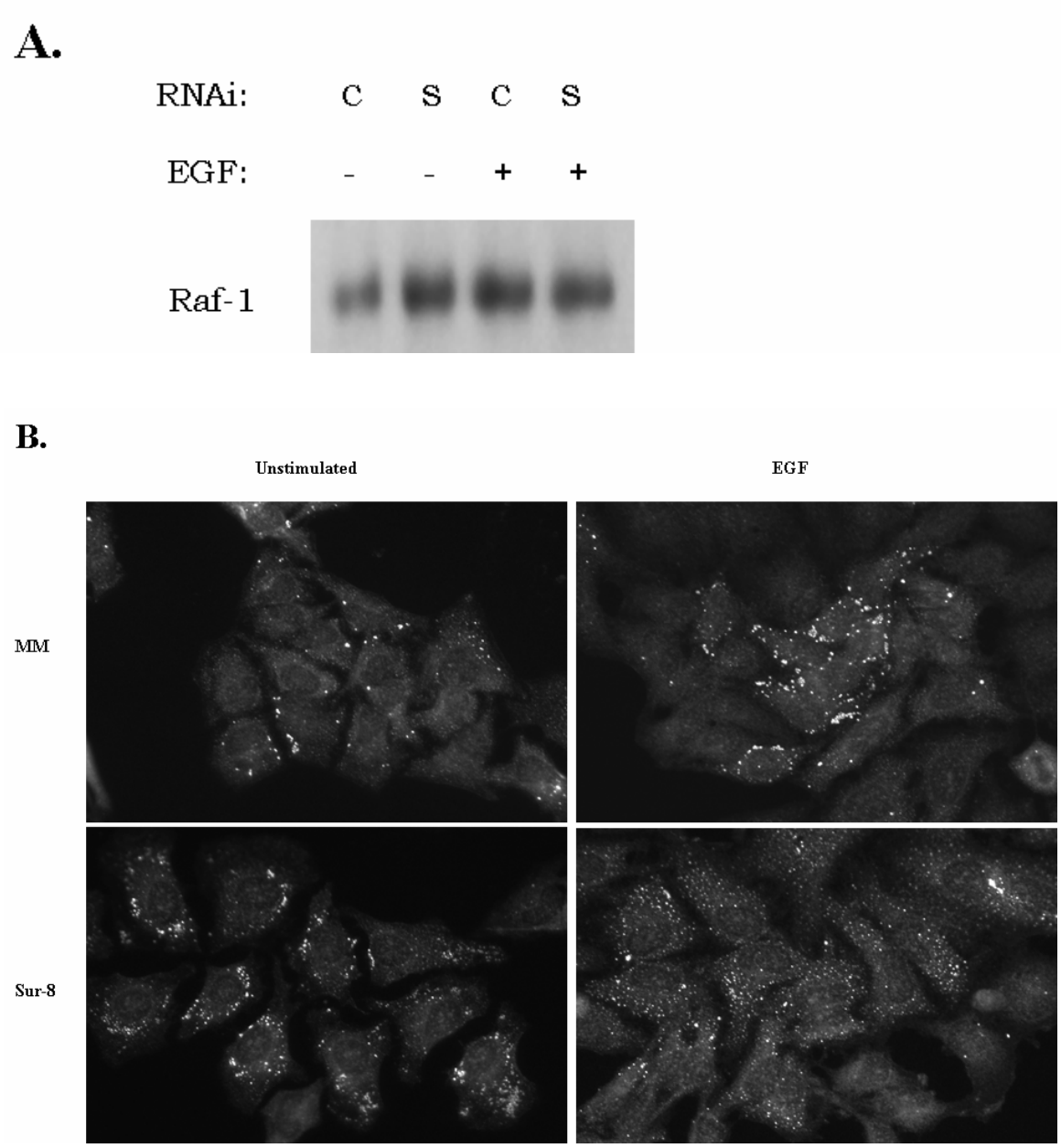


Figure 4.4 Sur-8's contribution to RAF-1 localization. A) HeLa cells were transfected with control (C) or Sur-8 (S) oligos, serum starved overnight and stimulated with 1ng/ml EGF for five minutes. A P100 fractionation was performed and the pellet analyzed by Western blot analysis. B) Cells were treated as in A except RAF-1 localization was detected by immunofluorescence with anti-RAF-1 antibody.

Figure 4.5

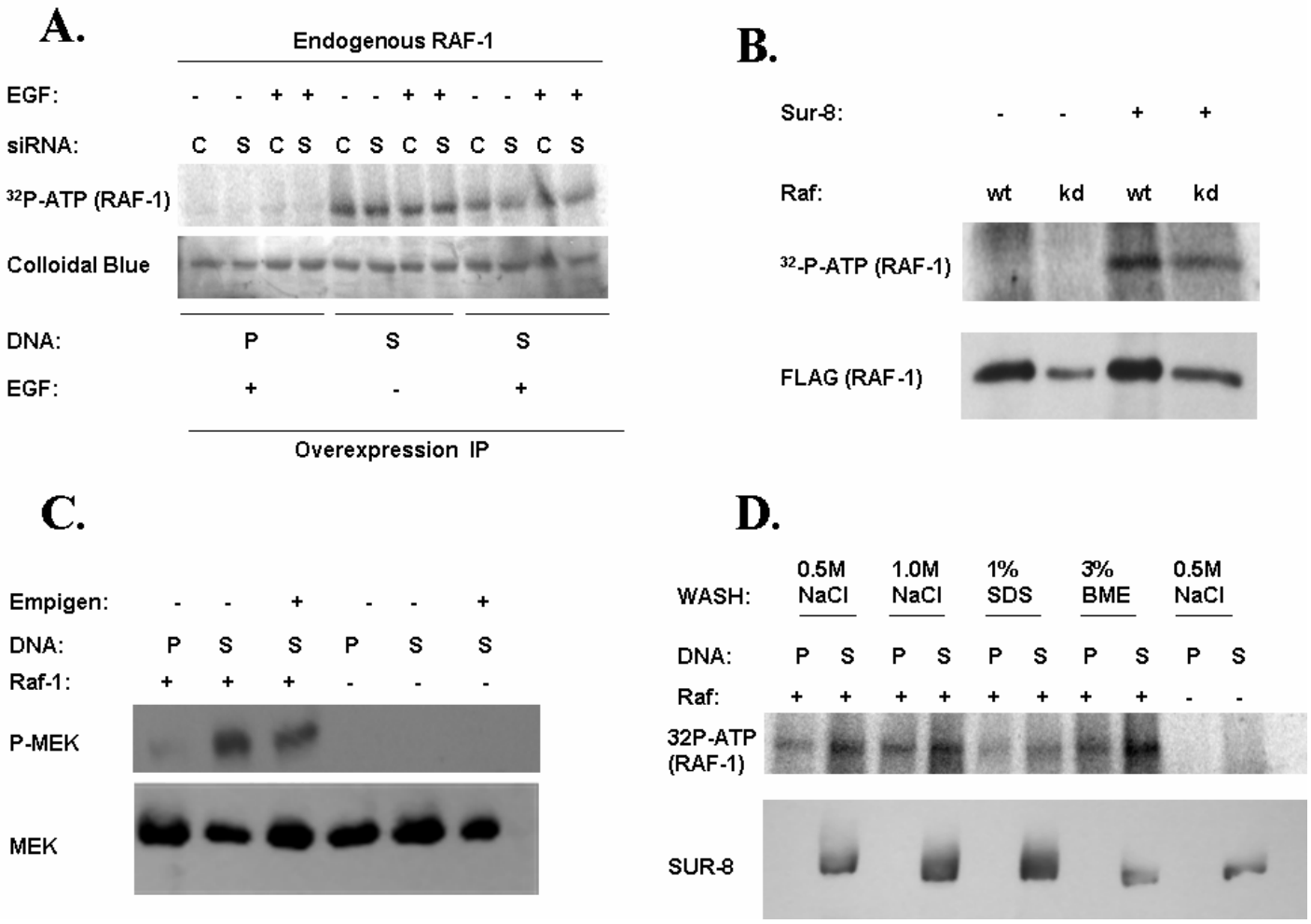


Figure 4.5 Sur-8 complex mediates phosphorylation of RAF-1. A) Endogenous RAF-1 was immunoprecipitated from cells transfected with control (C) or Sur-8 (S) oligos that were serum starved or stimulated with 1ng/ml EGF. Sur-8 was immunoprecipitated from cells ectopically expressing Sur-8. The RAF-1 and Sur-8 immunoprecipitates were mixed and then placed in an *in vitro* kinase assay using radiolabeled phosphate. B) The assay was repeated using RAF-1 immunoprecipitated from cells ectopically expressing either wild-type (wt) RAF-1 or kinase-dead (kd) RAF-1. C) Ectopically expressed RAF-1 and Sur-8 were immunoprecipitated. The Sur-8 immunoprecipitates were washed with 1% Empigen. The kinase assay was analyzed by Western blot analysis probing with antibody specific for active MEK. D) Sur-8 immunoprecipitates were washed as indicated and then added to RAF-1 immunoprecipitates in an *in vitro* kinase assay.

Figure 4.6

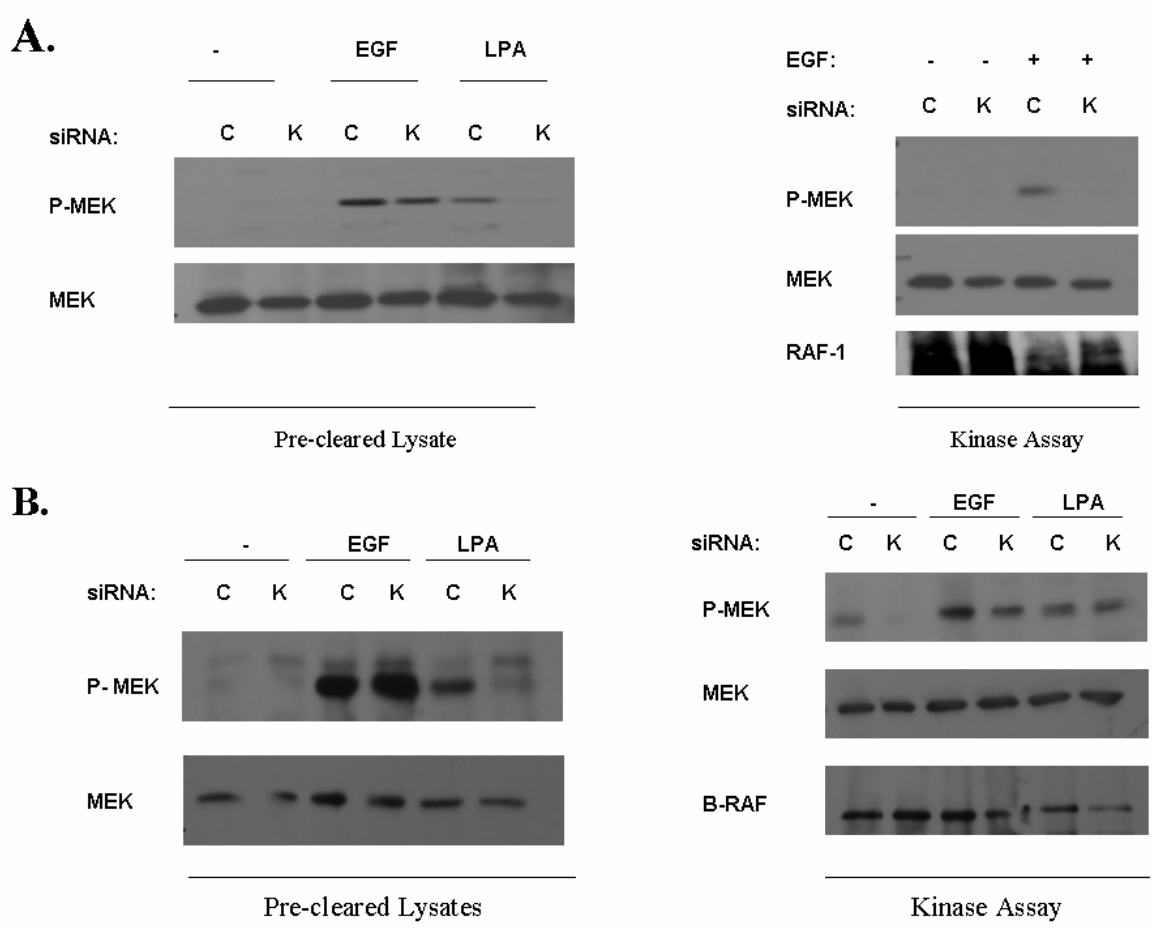


Figure 4.6 KSR's involvement in RAF-1 and B-RAF activation. A) HeLa cells were transfected with Control (C) or KSR (K) oligos. After forty eight hours, cells were serum starved and stimulated with either 1ng/ml EGF or 10µM LPA for five minutes. RAF-1 was immunoprecipitated and placed in a kinase assay using kinase dead MEK as a substrate. B) Cells were treated as in A except B-RAF was immunoprecipitated.

Figure 4.7

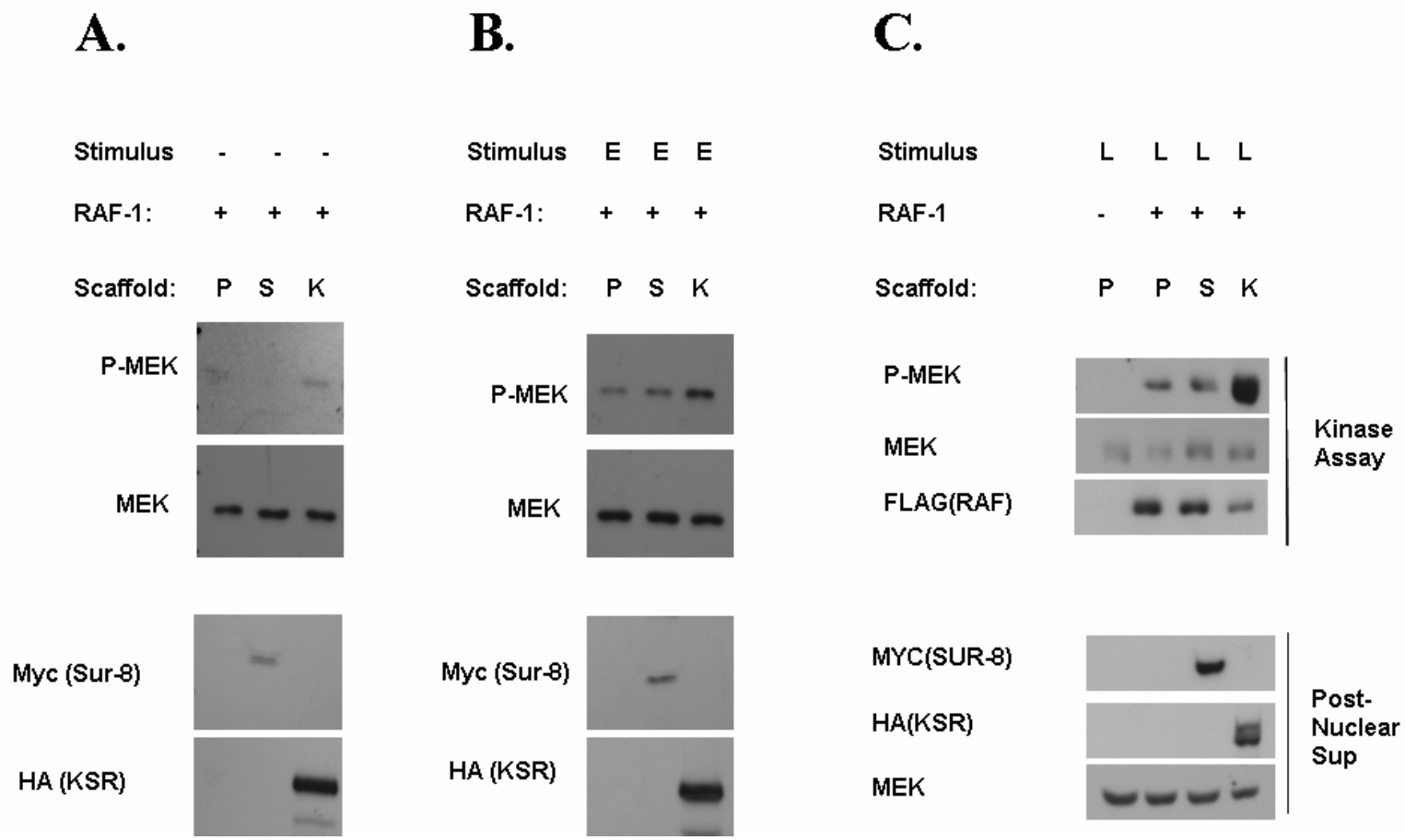


Figure 4.7 Overexpression of KSR is sufficient for RAF-1 activation. RAF-1 was ectopically expressed along with empty plasmid (P), myc-Sur-8 (S), or HA-KSR (K). Cells were serum starved (-), EGF (E), or LPA (L) stimulated. RAF-1 was immunoprecipitated and placed in an *in vitro* kinase assay using kinase dead MEK as a substrate.

CHAPTER FIVE

Investigating biological relevance of Sur-8 and KSR

Abstract

The RAS/RAF signal transduction pathway has been implicated in various cellular phenotypes such as migration, proliferation, and apoptosis. Not only have we shown that Sur-8 and KSR are required components of the RAS/RAF signal transduction pathway, but they are involved in providing ligand specific coupling, i.e. Sur-8 is required for EGF induced pathway activation whereas KSR is involved in LPA induced pathway activation. In this study, we wanted to determine whether that ligand specific coupling is manifested in cellular phenotypes. We examined the role of Sur-8 and KSR in cellular migration and adipocyte differentiation and the role of Sur-8 in melonocyte pigment production. We found that Sur-8 is required for basal pigment production in melanocytes and Sur-8 and KSR do display ligand specific coupling of the MEK/ERK signal transduction pathway in adipogenesis.

Introduction

The RAS/RAF-1 pathway has been implicated in numerous cellular phenotypes ranging from proliferation to migration and apoptosis to differentiation. In the previous studies we have

shown that Sur-8 and KSR display ligand specificity in that they are selectively involved in EGF and LPA induced MEK pathway activation, respectively. However, thus far the data has been biochemical; therefore the purpose of this study is to determine whether Sur-8 and KSR are involved in facilitating particular cellular phenotypes that are MEK/ERK mediated. Migration, melanocyte pigment production, and adipogenesis were the cellular phenotypes examined.

The cellular response of migration was chosen since both EGF and LPA can induce migration in normal rat kidney (NRK) cells. The rationale is that Sur-8 knockdown cells would migrate in response to LPA, but not in response to EGF while KSR knockdown cells would migration in response to EGF but not LPA.

The contribution of Sur-8 to melanocyte pigment production was also investigated. It is known that phorbal myristate acid (PMA) induced RAS/RAF-1 activation activates pigment production and in our initial observations we found that Sur-8 contributes to PMA induced MEK activation. To increase pigment production, PMA activates both RAS dependent and RAS independent pathways. The RAS independent pathways include Protein Kinase A (PKA), phosphoinositide 3 kinase (PI3K), and Protein Kinase C (PKC). The RAS pathway is inhibitory to pigment production although the cumulative effect of PMA is an increase in pigment production. The dogma of pigment production is that the increase in cAMP production leads to an increase in micropthalmia associated transcription factor (MITF) expression, which is an essential component in pigment production. MITF then activates the transcription of tyrosinase, the limiting enzyme in the pigment production pathway (77). The RAS/RAF-1 pathway negatively influences this pathway by phosphorylation of MITF on

S73 (78,79) leading to ubiquitination and degradation (80). Also, knockdown of B-RAF results in an increase in pigment production (81). Since Sur-8 contributes to PMA induced MEK activation, we hypothesized that limiting the amount of Sur-8 would decrease the inhibition mediated by the RAS/RAF-1 pathway such that the net result would be an increase in PMA induced pigment production.

The third cellular output examined was adipogenesis. Most of the work done on adipogenesis has used 3T3-L1 cells, a mouse pre-adipocyte cells line, or primary pre-adipocytes, cells that have committed to become adipocytes but have not matured. The maturation of a pre-adipocyte into an adipocyte involves growth arrest, clonal expression, early gene expression of adipocyte specific genes, and morphological changes. The first step in adipogenesis is growth arrest which can occur either through contact inhibition or by removal of serum. Clonal expansion is controversial. Studies using 3T3-L1 cells determined that clonal expansion is a required step, however studies using primary human adipocytes found that clonal expansion was a non-essential step. The next step of adipogenesis is early gene expression of specific adipoctyte genes which include CCAATT/ Enhancer Binding Protein (C/EBP) and Peroxisome Prolerator Activated Receptor (PPAR) γ (82). The last step is morphological changes. The cells lose their fibroblast-like shape and become spherical and filled with lipid droplets (83).

The RAS/RAF-1 pathway has been implicated in adipogenesis. Overexpression of H-Ras bypasses the need for insulin, a necessary component of differentiation. Activated RAF-1 induces pre-adipocyte differentiation while dominant-negative RAF-1 blocks differentiation (82). It is also known that the cocktail used to induce differentiation (insulin,

dexamethasone, isobutylmethylxanthine, and serum) is known to activate ERK, coinciding with induction of C/EBP expression (84). However, mitogenic signals such as EGF are inhibitory to adipogenesis (85). This inhibition has been associated with induction of cell proliferation (85).

We wanted to use this system to determine whether Sur-8 and KSR impact downstream cellular outputs. The rationale is that in control and KSR knockdown cells EGF would block differentiation whereas in Sur-8 knockdown cells the EGF induced RAS/RAF-1 activation signal would be diminished such that the cells would differentiate. Work from our lab has shown that KSR is necessary for insulin signaling in *Drosophila (44),* also the KSR knockout mouse has a decrease number of adipoctyes that could be restored by addition of KSR (86). Therefore, we hypothesize that KSR knockdown cells would not differentiate in response to insulin, but that the control and Sur-8 knockdown cells would differentiate.

Results

Both scratch and transwell assays were performed to determine if Sur-8 and KSR would contribute to EGF and LPA induced migration, respectively. Scratch assays were begun in HeLa cells. Untransfected cells were plated to confluency, serum starved overnight, scratched with a P200 tip, and stimulated with either 100ng/ml EGF or 10μM LPA overnight. Both the EGF and LPA treated scratches healed while the untreated scratch did

not. When the cells were transfected with control oligos, the cells did not display any stimulus dependent migration. Therefore, experiments could not be performed with HeLa cells. Next, Normal Rat Kidney (NRK) cells were used next to perform the scratch assays. Experiments were performed in untransfected cells to determine the optimal time of migration (4 hours). However, when cells were transfected, stimulus dependent migration was inconsistent in that even though there was some minor movement in the control transfected cells, the scratch was not filled and therefore it was difficult to determine whether Sur-8 and KSR knockdown cells migrated differently from the control transfected cells. Movies were taken over fourteen hours to try to quantitate the small amount of migration; however there was no substantial stimulus dependent migration (data not shown). Therefore, transwell migration assays were performed to determine whether Sur-8 and KSR would contribute to stimulus dependent migration. Initial experiments were performed with untransfected cells to determine optimal time of migration (8 hours). Again, transfected cells showed inconsistent stimulus dependent migration (data not shown). Thus the experiments could not be pursued and no conclusion could be made about the contribution of Sur-8 and KSR to EGF and LPA induced cell migration.

A melanoma cell line, MNT-1, from a darkly pigmented patient was used in experiments to determine whether Sur-8 contributed to PMA induced pigment production. We had observed that Sur-8 contributed to PMA induced MEK activation. HeLa cells were transfected with control, Sur-8 or KSR oligos, serum starved overnight, and stimulated with 1μM PMA. Control and KSR knockdown cells displayed similar amounts of phosphorylated MEK while the Sur-8 knockdown cells showed reduced MEK activation. (Figure 5.1A) Sur-8

knockdown was verified by protein expression while KSR knockdown was measured at the RNA level by RT-PCR. (Figure 5.1B)

Since Sur-8 contributed to PMA induced MEK activation in HeLa cells, we tested whether Sur-8 influenced PMA induced melanocyte pigment production. MNT-1 cells were transfected with control, tyronisase, or Sur-8 oligos and the absorbance read at 490 nm after 72 hours. The unstimulated control transfected cells had an absorbance of 0.06 while the tyronisase knockdown cells were lighter with an absorbance of 0.03 and the Sur-8 knockdown cells were darker with an absorbance of 0.15. (Figure 5.2) Similar results were observed at least three times. Because Sur-8 knockdown contributed to basal melanocyte pigment production experiments were not pursued to determine whether Sur-8 contributed to PMA induced pigment production.

Next, we investigated whether Sur-8 and KSR contributed to adipogenesis. Human primary pre-adipoctyes were obtained from Zen-Bio Labs. Control transfected, Sur-8 knockdown, or KSR knockdown cells were treated with either 60 ng/ml EGF or differentiation cocktail (insulin, dexamethasone, isobutylmethylxanthine, and serum) for five minutes and lysates were analyzed by Western blot analysis to determine whether MEK activation was changed in the knockdown cells. In unstimulated cells, there was no MEK activation. MEK was activated by both EGF and differentiation cocktail in the control transfected cells. The Sur-8 knockdown cells displayed reduced MEK activation in both the EGF and differentiation cocktail stimulated cells. (Figure 5.3) The KSR knockdown cells had a comparable amount of MEK activation to the controls in the EGF stimulated cells. The KSR knockdown cells stimulated with the differentiation cocktail showed a slight increase in MEK activation, but

there was also an increase in MEK protein levels. (Figure 5.3) This experiment was only performed once. These results suggest that Sur-8 positively contributed to both EGF induced and differentiation cocktail induced MEK activation while KSR does not influence EGF induced MEK activation. It is difficult to make a conclusion about KSR's role in differentiation cocktail induced MEK activation because of the unequal protein load. Experiments were carried out to determine if Sur-8 or KSR contribute to adipocyte differentiation. Pre-adipocytes were either control, Sur-8, or KSR oligio transfected and either left undifferentiated, differentiated, or EGF added from the sixth day of differentiation onward. After fourteen days, cells were stained with Oil Red O to detect lipid droplets as a measurement of mature adipoctyes. Pictures were taken with Axiovert 100M microscope and Open Lab software. The control and KSR knockdown pre-adipocytes that were left undifferentiated showed very little staining while the Sur-8 knockdown cells did display some Oil Red O staining although the lipid droplets were much smaller than in the mature adipocytes. In the control transfected pre-adipocytes that were treated with differentiation cocktail, Oil Red O staining showed mature lipid droplets correlating with differentiation into mature adipocytes. In the Sur-8 knockdown pre-adipocytes treated with differentiation cocktail there were more adipocytes while in the KSR knockdown cells there were less mature adipocytes (Figure 5.4). In the EGF plus differentiation cocktail treated pre-adipocytes there were no mature adipocytes in either the control transfected or the KSR knockdown cells. However there were some mature adipocytes in the Sur-8 knockdown cells that were treated with EGF plus differentiation cocktail. (Figure 5.4) There also appeared to be less cell proliferation as depicted by cell density though this was not formally tested.

These results suggest that Sur-8, but not KSR, is required for EGF to block adipoctye differentiation. KSR is required for adipocyte differentiation while Sur-8 may play an inhibitory role since there was more differentiation in the Sur-8 knockdown cells that were treated with differentiation cocktail. Also, lipid droplets were forming in the Sur-8 knockdown pre-adipocytes that were left undifferentiated. This experiment was performed only once.

Discussion

In previous studies we have shown that Sur-8 and KSR contribute to RAS/RAF-1 pathway activation. Moreover, Sur-8 is required for EGF induced pathway activation while KSR is involved in LPA induced pathway activation. In this study we wanted to investigate whether Sur-8 and KSR would contribute to a RAS/RAF-1 mediated cellular output. In particularly, if Sur-8 would contribute to an EGF induced cellular output, but not an LPA induced cellular output and visa versa for KSR. To this end, migration assays were performed since both EGF and LPA mediate migration. However with either the scratch or transwell migration assays we were unable to obtain consistent stimulus dependent migration in the control transfected cells. Therefore, we could not examine the role of Sur-8 and KSR in EGF and LPA induced migration. We may be able to obtain consistent stimulus dependent migration if we utilize a tetracycline inducible stable shRNA cell line.

We investigated whether Sur-8 would contribute to PMA induced melanocyte pigment production since in our initial studies we observed that Sur-8 was required for PMA induced MEK activation. Our studies were carried out using MNT-1 cells which is a melanoma cell line from a darkly pigmented patient. Pigment production was measured by reading the absorbance at 490 nm. Cells were transfected with either control, Sur-8, or tyrosinase oligos. Tyrosinase is the limiting enzyme in pigment production; therefore knockdown of the enzyme should result in less pigment production and was used as an internal control. We found that Sur-8 knockdown cells were darker in the unstimulated state. This observation is consistent with the fact that a decrease in either MEK activation (by the inhibitor PD90859) or MEK protein expression (proteolytic cleavage by anthrax lethal toxin) resulted in an increase in pigment production (80,87). Therefore experiments could not be carried out to determine if Sur-8 contributed to PMA induced pigment production because if a decrease in pigment production was seen with PMA induction. However, we discovered that Sur-8 was an inhibitory factor in pigment production. A caveat to using MNT-1 cells for these experiments is that melanomas have a high incidence of RAS/RAF mutations (15-30% for N-RAS and 26-70% for B-RAF) (4,88-90), therefore by using the MNT-1 cells, we may not be able to measure the contribution of PMA may have on the RAS/RAF signal transduction pathway because the oncogenic form of either N-RAS or B-RAF may have the pathway maximally activated.

Next, we investigated whether Sur-8 and KSR were involved in adipogenesis. The KSR null mice have a decreased number of adipoctyes (86) and our lab has shown previously that in *Drosophila* KSR is required for insulin induced RAF activation (65) and insulin is a

stimulator of adipoctye differentiation. It has been shown that EGF, when added to the differentiation cocktail, will block differentiation, presumably by stimulating proliferation (85). Therefore, Sur-8 knockdown cells should differentiation in the presence of EGF. We found that KSR knockdown cells do not differentiate when stimulated with the differentiation cocktail. Sur-8 knockdown pre-adipocytes accumulated small lipid droplets suggesting that Sur-8 may play an inhibitory role in helping to keep pre-adipocytes undifferentiated.

When MEK activation was assessed after five minutes stimulation with either EGF or differentiation cocktail, Sur-8 knockdown cells displayed decreased MEK activation in the EGF stimulated cells, but they also showed a decrease in the differentiation cocktail treated cells. In *Drosphila* reduction of protein expression of Sur-8 by RNAi did not alter MEK activation by insulin (65); therefore we are uncertain how Sur-8 is contributing to MEK activation in response to the differentiation cocktail. However, this decrease in MEK activation did not result in a decrease in adipocyte differentiation. We also found that Sur-8 knockdown cells differentiated in the presence of EGF. These results make it difficult to conclude changes in differentiation phenotypes with changes in MEK activation.

We were able to show that Sur-8 and KSR do display ligand specificity as measured by cellular output in that KSR, but not Sur-8, positively contributes to adipocyte differentiation while Sur-8, but not KSR, positively affects EGF mediated inhibition of adipocyte differentiation. These experiments need to be repeated for confirmation. Also, further studies to determine how Sur-8 contributes to basal pigment production would also be of interest. Studies to show a cellular phenotype with LPA and EGF would also help to tie together the biochemical data. The transfection efficiency in the pre-adipoctyes was assumed

to be low based on the low penetrance of the phenotype and the fact that it was a transient transfection in a human primary cell line. Experiments are underway to make an lentivirus that will knockdown Sur-8 and KSR and will increase transfection efficiency.

Materials and Methods

Materials and cell culture

MNT-1 cells were a kind gift from Michael Mark (University of Pennsylvia) and cultured in 15% serum with DMEM, 10% AIM V media, and 1x minimal essential amino acids. Primary human pre-adipocytes were obtained from Zen-Bio Labs (Research Triangle Park, NC) and cultured according to their protocols. MNT-1 cells were transfected using a reverse transfection protocol. 6.7 x 10^4 cells were transfected using 50 nM oligo and dharmafect 2 as the transfection reagent. NRK cells were transfected using a reverse transfection protocol where 1 x 10^4 cells were plated with 120 nM oligos using lipofectamine 2000 as the transfection reagent.

Scratch assays

Day one, cells were transfected. The next morning 3 - 35 cm dishes were combined and in the evening cells were serum starved. After 48 hours of serum starvation, cells were

scratched with a P200 tip, washed twice with DMEM and then DMEM containing the stimulant was added for four hours.

Transwell assays

Twenty-four hours after the cells were transfect 1 x 10^4 cells were placed on the membrane. The next day cells were serum starved overnight. 72 hours post-transfection the bottom of the membrane was scraped and the stimulant was added for 8 hours. Cells were stained with dapi.

Adipocyte differentiation

Preadipoctyes were transfected a day –1. At day 0 cells were changed to differention cocktail. At day 3 cocktail was changed to adipocyte cocktail. EGF was added at day 6 when indicated.

Oil Red O Staining

Cells were washed twice in PBS and then fixed in 2.5% paraformaldehyde in PBS for thirty minutes. Cells were then washed twice with PBS. 0.6% Oil Red O was added to the cells for ten minutes in the dark. Stain was diluted away using water. Cells were then washed six times in PBS.

Figure 5.1

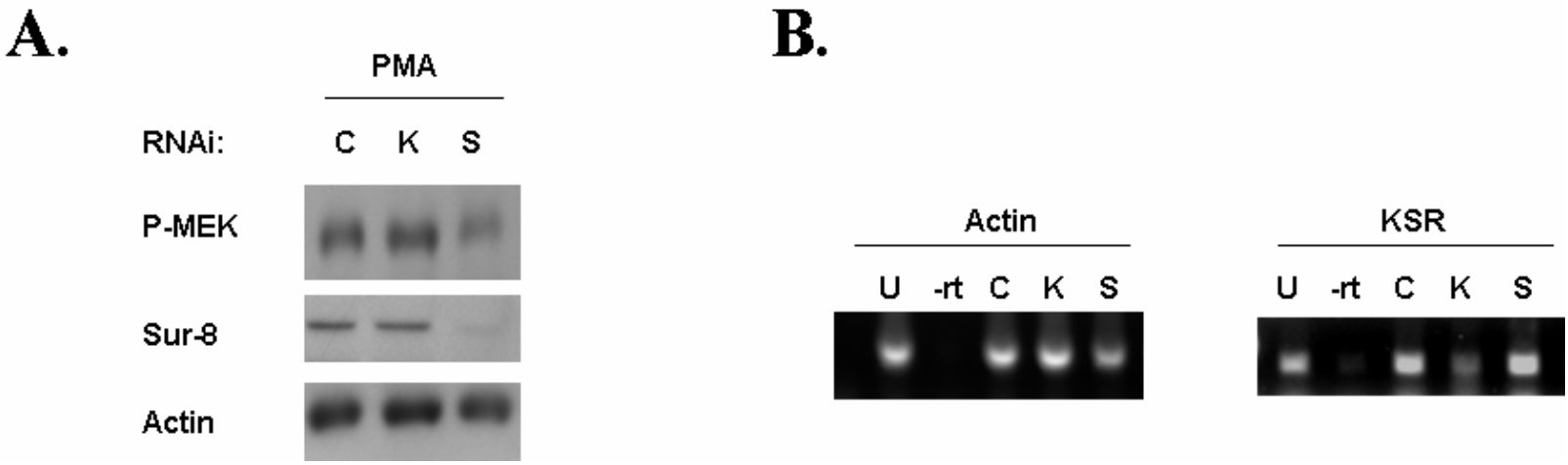


Figure 5.1 Sur-8 is involved in PMA induced MEK activation. (A) HeLa cells were transfected with control (C), KSR (K), or Sur-8 (S) oligos. After 48 hours cells were serum starved overnight and stimulated with 1 µM PMA for five minutes. Western blot analysis was performed with indicated antibodies. (B) Cells were treated as in (A) except RT-PCR was performed on either untreated (U), no reverse transcriptase (-rt), control knockdown (C), KSR knockdown (K), or Sur-8 knockdown (S) samples.

Figure 5.2

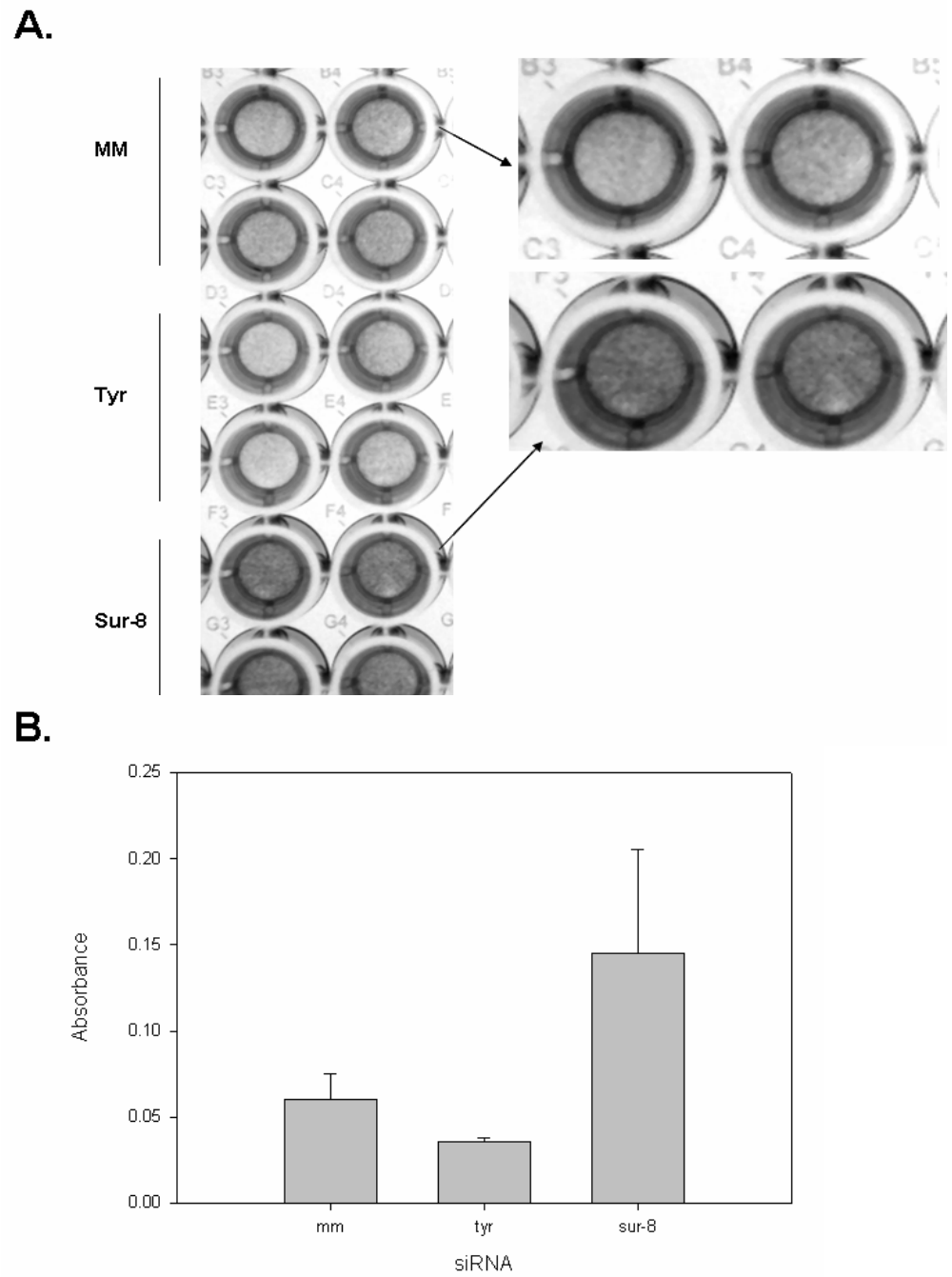


Figure 5.2 Sur-8 is an inhibitor of basal pigment production. MNT-1 cells were transfected with mismatch (mm), tyrosinase (tyr), or Sur-8 oligos. Absorbance was read at 490nm. A) Representative picture B) graph of absorbance

Figure 5.3

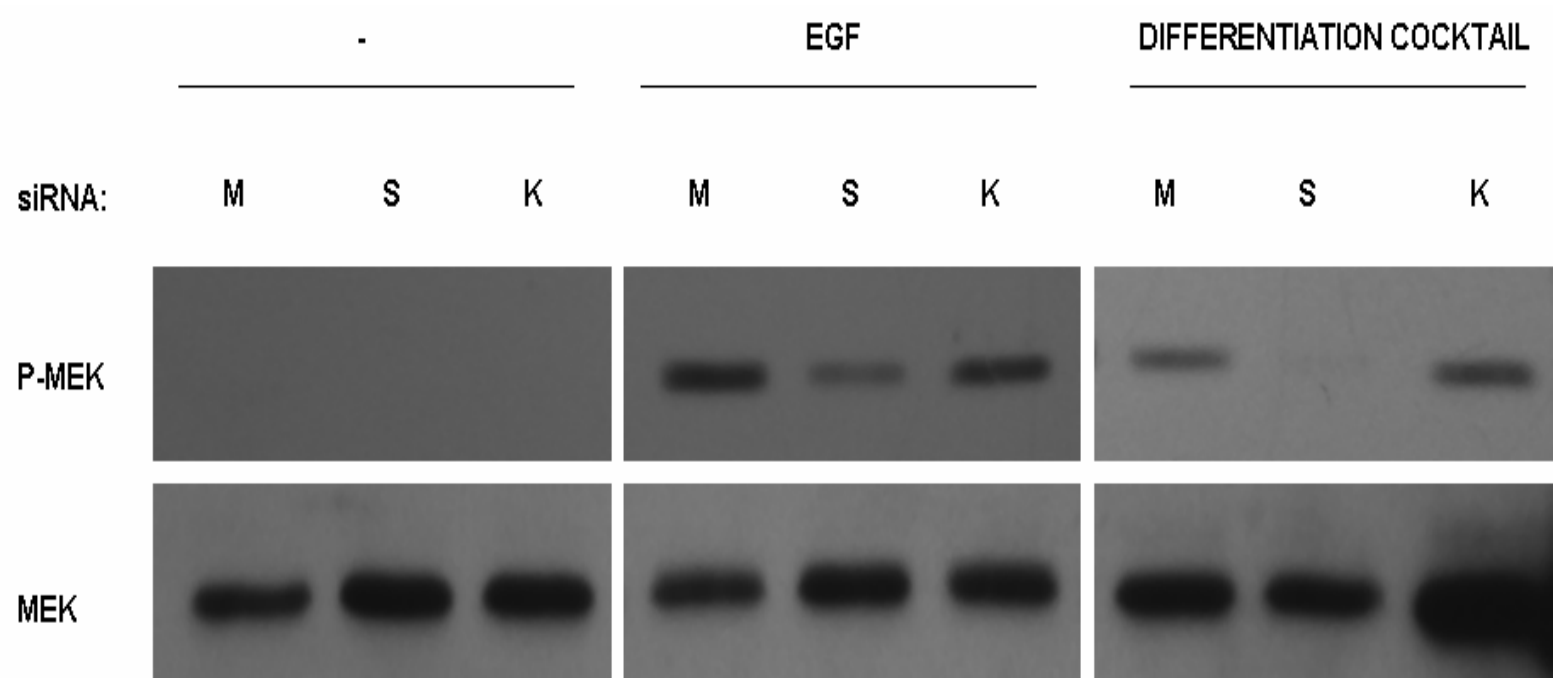


Figure 5.3 Sur-8 contributes to EGF and differentiation cocktail induced MEK activation. Primary human pre-adipocytes were transfected with mismatch (M), Sur-8 (S), or KSR (K) targeted oligos. Cells were stimulated with either 60ng/ml EGF or differentiation mix for five minutes. Western blot analysis was performed using antibodies indicated.

Figure 5.4

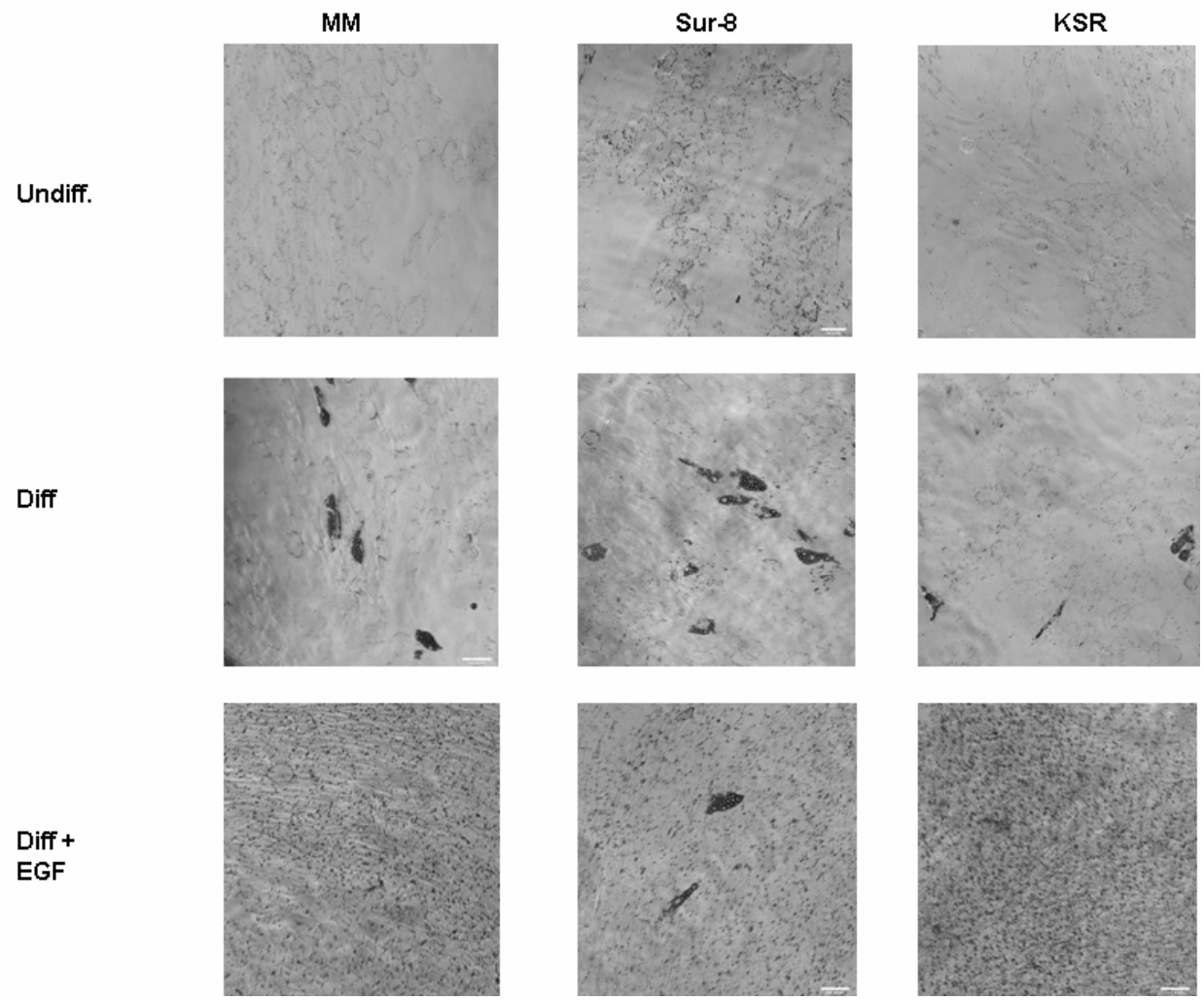


Figure 5.4 Sur-8 and KSR's contribution to adipogenesis. Primary human pre-adipocytes were transfected with mismatch (mm), Sur-8, or KSR oligos. The cells were either left undifferentiated (undiff), differentiated (diff), or EGF (diff + EGF) added along with differentiation mix. After 14 days cells were stained with Oil Red O and pictures taken.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

The RAS/RAF signal transduction pathway has been reported to be activated by numerous ligands including growth factors, chemokines, and apoptic agents. Pathway activation has been implicated in a variety of cellular outputs including proliferation, migration, apoptosis, and differentiation (91). Even though the core enzymatic components (RAS, RAF, MEK, and ERK) have been identified, pathway regulation is not completely understood. Studies aimed at understanding pathway regulation have identified non-enzymatic components, or scaffolds. These scaffolds include Sur-8 and KSR. In genetic experiments, both Sur-8 and KSR can rescue an activated RAS phenotype but not an activated RAF phenotype, suggesting that they both function downstream of RAS, but either upstream or parallel to RAF (54,92-94). However, it is not known whether they are integral pathway components and if so what their function may be. Proposed functions of scaffolds include altering pathway kinetics, enhancing pathway efficiency, and restricting kinase specificity.

In this study, we found that both Sur-8 and KSR contribute to RAS/RAF signal transduction pathway activation. Moreover, we found that Sur-8 is involved in EGF induced MEK activation while KSR contributes to LPA induced MEK activation. We determined that this ligand specificity is manifested not only at a biochemical level but also in cellular outputs. KSR positively contributes to adipocyte differentiation while Sur-8 positively contributes to EGF induced inhibition of adipogenesis. This suggests that scaffolds may aid in providing signal specificity.

We investigated the molecular mechanism of how Sur-8 and KSR impacted RAS/RAF signal transduction pathway activation. We found that both Sur-8 and KSR impact EGF induced RAF-1 activation. However, KSR is not required for EGF induced MEK activation. Therefore, EGF induced RAF-1 activation is coupled to something other than MEK. Although MEK is the most well known RAF-1 substrate, increasing evidence suggests that RAF-1 has other substrates such as ASK1, MST2, and ROK-α (70).

In the Sur-8 knockdown cells, we observed a reduction in both EGF induced RAF-1 and MEK activation. Although Sur-8 is not limiting for EGF induced S259 dephosphorylation nor EGF induced phosphoyrlation of either S338 or Y341, we did observe changes in EGF induced RAF-1 localization in the Sur-8 knockdown cells. RAF-1 localization, visualized by immunofluorescence, displayed Sur-8 dependent changes in RAF-1 localization even though EGF induced RAF-1 recruitment to the membrane was unchanged in the Sur-8 knockdown cells as detected by membrane fractionation.

In a RAF-1 *in vitro* kinase assay, addition of immunoprecipitated Sur-8 leads to RAF-1 phosphorylation correlating with an increase in RAF-1 activity. Therefore, we hypothesize there is a kinase that is tightly associated with Sur-8 that can phosphorylate RAF-1 resulting in an increase in RAF-1 activation.

We observed that Sur-8 may have dual functions in RAF-1 activation—it may act as both an activator and an insulator of RAF-1 activation. We found that in unstimulated Sur-8 knockdown cells, there was more RAS/RAF-1 binding and more RAF-1 present at the membrane by membrane fractionation than in the controls. Also, with the RAF-1 immunoflourescence, the punta in the Sur-8 knockdown cells closely resembled the pattern

of the control EGF stimulated. However, in a RAF-1 *in vitro* kinase assay, using RAF-1 immunoprecipitated from unstimulated Sur-8 knockdown cells, we were unable to detect an increase in RAF-1 kinase activity. Therefore, even though, RAF-1 may be "partially" activated – i.e. increase binding to RAS and increase in membrane recruitment, this is not translated into RAF-1 being able to activate its substrate, MEK. However, recent data suggest that RAF-1 may have kinase independent functions (95,96) and it may be that the role of Sur-8 in unstimulated cells is to sequester RAF-1.

Our model would be that Sur-8 and KSR are required for EGF induced RAF-1 activation; however the activation is uncoupled from MEK activation. Either MEK activation is mediated by B-RAF or there is a redundancy between B-RAF and RAF-1 for EGF coupling to MEK. Sur-8 also contributes to RAF-1 localization in an unstimulated state. We hypothesize that KSR contributes to LPA induced MEK activation by coupling MEK to B-RAF.

Further studies are needed to determine what the downstream target(s) of EGF induced RAF-1. It would also be interesting to determine what the RAF-1 activating kinase is that is bound to Sur-8. One possible kinase is FAS/FADD- interacting Serine/threonine kinase (FIST)/Homeodomain interacting protein kinase 3 (HIPK3) since Sur-8 binds to FIST/HIPK3 by yeast two hybrid and overexpressed co-immunoprecipitation (data not shown).

It is necessary to know how Sur-8 and KSR provide the signal specificity, i.e. what is the upstream and downstream of the scaffolds that aid in the signal specificity. It is known that KSR can bind Gγ which may be how KSR couples to LPA signaling (97). Preliminary experiments preformed suggest that Sur-8 does not bind directly to the EGF receptor.

Mechanistic studies of Sur-8 could be aided by knowledge of other binding partners. Yeast two hybrid studies along with co-immunoprecipitation experiments, found that Sur-8 binds to BAX (a pro-aptotic factor). Preliminary data suggest that Sur-8 knockdown HeLa cells are more sensitive to FAS induced apoptosis than TNF-α induced apoptosis. This is consistent with the fact that RAF-1 null fibroblasts are more sensitive to FAS than TNF-α induced apoptosis (98). Interestly, the downstream target of RAF-1in apoptosis may be MST2 instead of MEK. This requirement of RAF-1 in apoptosis is also kinase independent (99). I also observed that knockdown of KSR in HeLa cells caused some cell death in HeLa cells that was reversed if Sur-8 was knocked down simultaneously.

Sur-8's involvement in proliferation also needs to be addressed. Preliminary experiments suggest that Sur-8 is inhibitory to proliferation. Sur-8 knockdown cells grow 1.5-2 times faster than controls as detected by BrDu incorporation. When Sur-8 knockdown cells were serum starved and placed in suspension, there were approximately eight times more cells than the controls. One possibility for this may be a RAF-1 kinase independent function since Sur-8 impacts RAF-1 localization but not kinase activity in unstimulated cells.

We also began to examine Sur-8 and KSR's role in global signaling. Experiments suggest that Sur-8 is involved in JNK and AKT activation in response to EGF while KSR may contribute to JNK activation in response to LPA.

This work has helped to understand the complexity of scaffolding proteins and that much work is needed to understand their complex role in signal transduction networks.

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