

Lipid binding and the scaffolding function of the Kinase Suppressor of Ras

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# Lipid binding and the scaffolding function of the Kinase Suppressor of Ras

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The signal transduction field has recently seen a surge of interest in cascade scaffolding proteins. One of these, the Kinase Suppressor of Ras (KSR), has received a great deal of attention as a scaffold for the Ras/ERK signaling pathway. KSR interacts with both MEK and ERK, and possibly binds to Raf-1 as well. Very little is known about the regulation of KSR; however, it has been determined that membrane association is essential for its function in signal augmentation. KSR shares a high degree of sequence homology to Raf-1, including an almost identical phosphatidic acid binding region (PABR). Previous work in the Romero lab has determined the direct interaction of Raf-1 with phosphatidic acid is critical for its membrane recruitment. The PABR is a 35 amino acid sequence consisting of a poly-basic motif (PBM) flanked by two hydrophobic regions. Neutralization of the two arginine residues in the PBM abrogates the binding of Raf-1 to phosphatidic acid (PA), and consequently disrupts its membrane association. This thesis examines lipid-binding properties of the PABR and their potential role in the traffic and function of KSR. Using peptides corresponding to the PABR and tryptophan fluorescence spectroscopy, the data presented in the first section demonstrate that PA induces a blue-shift in the tryptophan emission spectra of WT KSR PABR, and this shift is specific for PA. The second section explores the cellular consequence of KSR PABR mutation.

A KSR protein lacking the arginine residues in the PBM expressed in HIRcB fibroblasts retains its membrane-binding ability, but inhibits MEK and ERK phosphorylation in a dominant negative fashion. The data presented here support the conclusion that, although an intact PABR may not be essential for the membrane localization of KSR, it is essential for proper coupling of the pathway.

## FOREWARD

Once upon a time, when imagination soared and possibilities were endless, I dreamed of what I would be “when I grew up.” I can remember intentions to become everything from a disc jockey to a commercial pilot to a concert pianist. Admittedly, I can also recall a great deal of indulgent smiling and nodding from my family during this time.

The years passed, and I settled on a career in science. I freely confess I initially pursued biology courses as a means to an end – that end being medical school. Then one day, during my AP Biology class, I had an epiphany: I found the phrase “by an unknown mechanism” for the first time in a textbook. I was stunned, for in my naiveté, I had assumed scientists already had all the answers. At the same time, however, I was profoundly confused – how could modern science and medicine hope to treat what was not yet completely understood? And suddenly, that simple phrase in my textbook became a call to arms.

There have been many who have questioned this decision, wondering why I did not become a musician. Indeed, I have devoted a tremendous portion of my life to my musical pursuits, and can therefore understand the perception that music is my greatest passion. Friedrich Nietzsche said, “Without music life would be a mistake,” and I could not agree more; however, I would like to take this opportunity to correct a misconception. My greatest passion is, in fact, *learning*. And where better to indulge my love of learning than the scientific field? A place where each day brings a new paper, a new piece of data, or a new outlook into the mechanics of what Douglas Adams called “Life, the universe, and everything.” I truly could not imagine myself doing anything else, and it is my fervent hope that someday, the girl who was riveted by the unknown once upon a time might learn just enough to make a difference.

My gratitude knows no bounds for the people who have helped me throughout my graduate studies. To Guillermo Romero, my thesis committee, and members of the lab: thank you for your guidance and for making this work possible. To my sister, cousin, and friends – you are my support network, and no one could ask for better.

I wish to dedicate the work presented here to my parents for their unwavering love and support...  
even though I'm now "all grown up."

Whoso neglects learning in his youth,  
Loses the past and is dead for the future.

- Euripides, *Phrixus*

When we remember we are all mad, the mysteries  
disappear and life stands explained.

- Mark Twain

...To strive, to seek, to find, and not to yield.

- Alfred Lloyd Tennyson, "Ulysses"

When you get to the end of your rope,  
tie a knot and hang on.

- Franklin D. Roosevelt

Nothing shocks me. I'm a scientist.

- Indiana Jones



*It is a tale told by an idiot,  
Full of sound and fury,  
Signifying nothing.*

*William Shakespeare, MacBeth*



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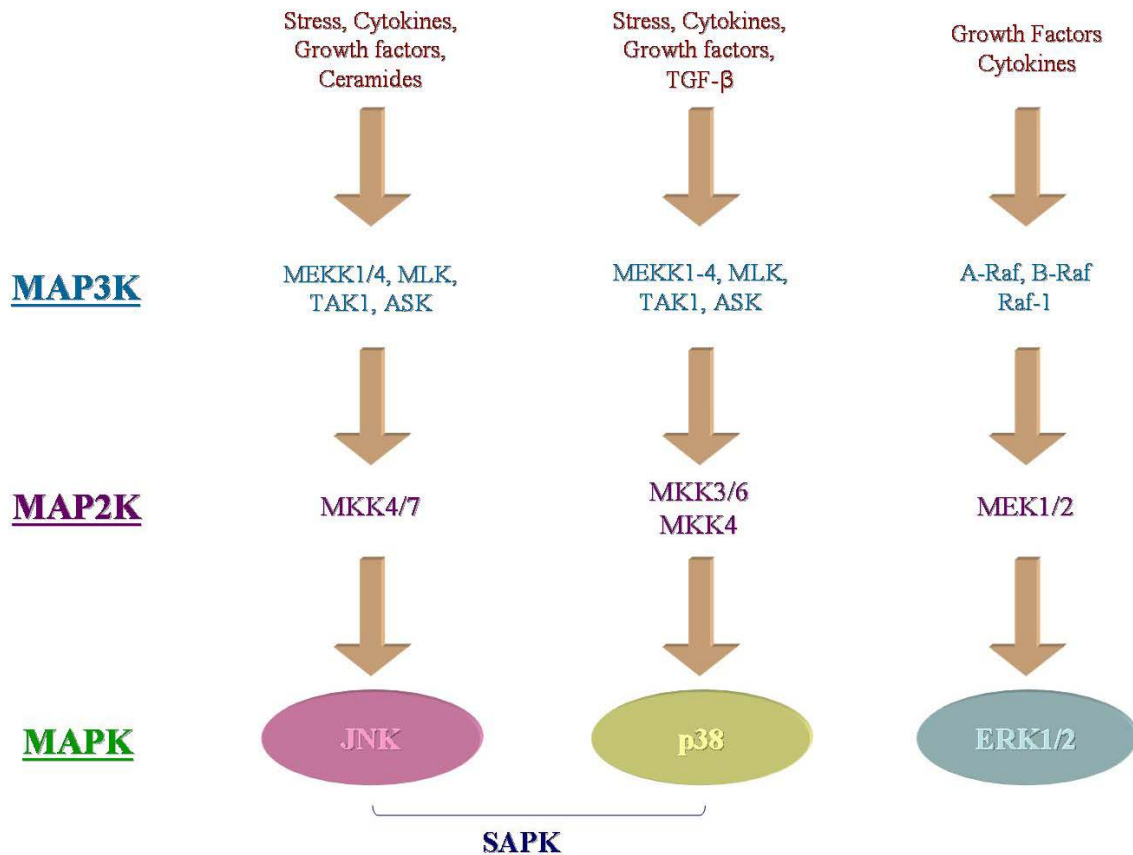
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## **1. Introduction**

### **1.1. MAPK Signaling**

Mitogen-activated protein kinases, or MAPK's, are a ubiquitous class of signaling proteins implicated in a number of cellular functions, such as proliferation, differentiation, and apoptosis. Three MAPK cascades have been identified to date: the extracellular-signal regulated kinases (ERK's), and the two stress-activated protein kinases (SAPK's) c-Jun N-terminal kinase (JNK) and p38. These three pathways, although they differ in activation and function, share a common structural thread. Each signaling cascade is comprised of three sequentially activated kinases: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and the ultimate MAPK. When the MAP3K is activated, it phosphorylates the MAP2K, which in turn phosphorylates and activates the MAPK [1].

The SAPK JNK is activated in response to various cellular stresses, cytokines, growth factors, and ceramides, and is functionally implicated in proliferation, differentiation, and apoptosis [2]. The JNK's get their name from their phosphorylation of c-Jun, a DNA-binding protein component of the AP-1 transcription complex. AP-1 regulates the transcription of many genes involved in the cell stress response [1]. The p38 MAPK is activated by many of the same stimuli as JNK, including stress, cytokines, growth factors, and TGF- $\beta$ . In addition to proliferation, differentiation, and apoptosis, p38 has also been implicated in development and inflammation [1]. p38 plays a critical role in the cell response to various stresses such as radiation, high osmolarity, and heat shock [2]. As there is a great deal of functional overlap between p38 and JNK, they share a number of the same upstream activators. These are summarized in Figure 1. Shared kinases include the class of MAP3K's known as mixed lineage kinases (MLK), apoptosis



**Figure 1 - JNK, p38, and ERK1/2 MAPK Cascades**

There are three main families of MAPK proteins, the SAPK's JNK and p38, and the ERK's. All MAPK cascades consist of three kinase modules: a MAP3K, which phosphorylates and activates a MAP2K, which in turn phosphorylates the MAPK. JNK's and p38 are implicated in the cellular response to various stresses, while the ERK's regulate growth and differentiation.

signaling kinase (ASK), and TGF-activated kinase (TAK). MAP2K's for these pathways include MKK3, 4, and 6 for p38, and MKK4 and 7 for JNK [2].

ERK1 and ERK2 are widely expressed signaling molecules which are involved in cell growth, differentiation, and development. Targets for ERK's include transcription factors such as ELK1 and STAT proteins [2]. In addition to basic phosphorylation of targets, duration of ERK activation has been implicated in pathway consequence. Nerve growth factor (NGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) all stimulate the rapid activation of ERK in PC-12 cells. The EGF response is transient, while FGF- and NGF-mediated MAPK activation is sustained. This protracted phosphorylation of ERK is essential for NGF- and FGF-stimulated differentiation [3]. ERK1/2 are activated by the dual-specificity kinases MEK1 and MEK2. MEK1/2, in turn, are phosphorylated by the MAP3K family of Raf proteins, which can be activated by the proto-oncogene Ras [1].

## **1.2. Phosphatidic acid in MAPK signaling**

The first evidence that phosphatidic acid (PA) played a role in ERK signaling came in 1996, when the PA-binding region (PABR) of Raf-1 was first described [4]. Previous work with the MAP3K Raf-1 had demonstrated its interaction with phosphatidylserine (PS) through its N-terminal cysteine-rich region [5]. Further lipid binding analysis of Raf-1 determined that the full-length protein also bound to PA. Deletion experiments determined the minimum region for PA binding is amino acid 389 through 423, located in the C-terminal kinase region. The PABR is characterized by hydrophobic residues flanking a poly-basic motif (PBM), which consists of two arginine residues (R398, R401). Substitution of these basic residues for neutral alanines results in a mutant unable to interact with PA [6, 7], indicating that there is an electrostatic component to this association. Raf-1 PA binding is not mediated exclusively by electrostatic



interactions, however. Increasing concentrations of salt do not disrupt the protein lipid interaction, as would be expected if association was regulated by charge alone. Lipid binding of the PABR is specific for PA; binding to other acidic lipids was negligible, as was binding to phosphatidyl alcohols [4]. In addition, using surface plasmon resonance (SPR), Rapp and colleagues have reported Raf-1 to bind to mixed composition liposomes containing phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol with an apparent  $K_D$  of 0.5nM [8]. Hence the PA-PABR interaction is specific, and requires not only electrostatic associations with the PBM but hydrophobic interaction with the lipid acyl chains as well.

Further characterization of this protein-lipid interaction revealed that PA is required for the recruitment of Raf-1 to the plasma membrane, a critical step in its activation by Ras [4, 7, 9, 10]. There are three mechanisms of PA synthesis in cells: 1) phosphorylation of diacylglycerol (DAG) by DAG kinase, 2) acylation of lyso-PA by acyl transferases, and 3) hydrolysis of phosphatidylcholine by phospholipase D (PLD) [9]. The initial work characterizing Raf-1 translocation implicated PLD as the source of PA. Primary short chain alcohols compete with water in the transphosphatidylation reaction catalyzed by PLD, creating a phosphatidyl alcohol instead of an acid. Incubation of cells with ethanol resulted in a decrease in Raf-1 present in the membrane fraction [4]. Further studies corroborated these data using Brefeldin A (BFA), which inhibits PLD activity through interference with its activator, ARF [10].

Two mammalian isoforms of PLD have been identified, PLD1 and PLD2 [9]. Transfection of cells with a PLD1 dominant negative construct had no effect, while PLD2 abrogated insulin-induced ERK phosphorylation, as well as Raf-1 membrane association [10]. Mutations of the arginine residues in the PBM interfere with Raf-1 membrane binding, as well as generate a

dominant negative mutant. Raf-1 R398A has a reduced affinity for PA *in vitro*, and inhibits insulin-induced activation of ERK. Furthermore, expression of the Raf-1 PABR fused to green fluorescent protein (GFP) decreases ERK phosphorylation in response to agonist, while a GFP-PABR PBM mutant had no effect [7]. Taken together, these data indicate that agonist-induced Raf-1 membrane translocation is mediated by a direct interaction with PLD2-generated PA.

Recent work by Ghosh and colleagues has also determined a physiological significance for the PABR. Mutation of the tetrapeptide Raf-1 PBM (residues 398-401) to alanines resulted in a Raf-1 incapable of PA interaction. Zebrafish embryos injected with RNA encoding this PBM mutant exhibit bent trunk and tail structures, a developmental defect in posterior axis formation. *In situ* hybridization of the *no tail* gene, which functions downstream of Raf-1, reveals this anatomical deficiency is due to aberrant signaling through Raf-1 [6]. Consequently, binding of PA is critical for Raf-1 function in embryonic development.

### **1.3. Endocytosis in MAPK signaling**

Endocytosis of cell surface receptors has long been considered a means by which signaling can be downregulated or terminated. Recent work has demonstrated, however, that internalization of receptors does not lead to signal cessation. Rather, receptor endocytosis is emerging as a critical step in the signaling process. Interference with endocytosis results in a decrease in ERK activation, whether accomplished by cholesterol depletion with cyclodextrin, cytochalasin D treatment [11], or dynamin negative mutants [12]. Cells defective in clathrin-mediated endocytosis exhibit a reduction in ERK phosphorylation in response to EGF [13]. Additional studies have demonstrated that ERK phosphorylation does not necessarily require internalization of the activated receptor. In Cos cells, G-protein coupled  $\beta$ 2A and  $\alpha$ 2A adrenergic receptors (AR) both activate ERK, and this activation is sensitive to inhibitors of clathrin-mediated

endocytosis. Of these two AR's, only the  $\beta$ 2A receptor is internalized, while the  $\alpha$ 2A receptor remains at the plasma membrane. Activation of both of these receptors, however, results in the transactivation of the EGF receptor, and its subsequent internalization [14]. Hence, although endocytosis of receptors appears to be required for activation of the ERK cascade, the internalization event is not necessarily specific for the activated receptor.

Insight into this endocytic requirement for signaling came with the discovery of Ras/ERK signaling components on the surface of early endosomes following insulin stimulation. These “signaling endosomes” contain activated Ras, Raf-1, MEK, and ERK, as well as the insulin receptor [7, 15]. These endocytic vesicles also contain PLD2 on their surface, and are enriched in phosphatidic acid (PA) [15] and cholesterol [11]. The lipid composition of the endosome also changes as a function of time. Maximum levels of PA are reached between five and fifteen minutes after stimulation, coincident with peak association of Raf-1 [15]. Mutation of the Raf-1 PBM (R398A) renders it incapable of associating with the endosomal compartment. Of particular interest, overexpression of a Raf-1 PABR GFP fusion abrogated the endosomal association of Raf-1 [7], presumably by binding to PA and preventing Raf-1 access. This data suggests that endosomal lipid composition plays an important role in signal coordination.

## **1.4. Scaffolds in MAPK signaling**

### **1.4.1. Scaffolding in yeast**

The *Saccharomyces cerevisiae ste5* gene product represents the first recognized MAPK scaffold. Ste5p was identified by a genetic screen for perturbations in hormone-regulated mating [16]. The yeast reproductive process is initiated by peptide mating hormones, which in turn activate a G-protein coupled MAPK cascade. This particular MAPK pathway consists of the MAP3K kinase, Ste20p, MAP3K Ste11p, MAP2K Ste7p, and MAPK's Fus3p and Kss1p [17].

Overexpression of Ste5p enhanced activation of Fus3p, while deletion experiments confirmed its positive regulation of the mating pathway [18, 19].

Insight into Ste5p's scaffolding function came about through the demonstration of its selective interaction with all four of the mating pathway kinases through separable binding sites [18, 20, 21]. Additionally, binding to Ste5p was determined to be the rate limiting step for activation of Fus3p [18]. The most compelling data for Ste5p function as a scaffold, however, was obtained through sedimentation experiments. The highest specific activity of Fus3p is observed when it is associated with a large multi-protein complex consisting of all the MAPK pathway kinases and Ste5p. Deletion of Ste5p abrogates the association of Fus3p with this complex, and consequently reduces its activity [22].

A role for Ste5p in mediating MAPK specificity has also been suggested. The MAP3K Ste11p has been implicated in multiple cellular responses, including filamentous growth, mating, and the high-osmolarity glycerol (HOG) response [23]. Each of these three pathways employs a distinct MAPK: Kss1p, Fus3p, and Hog1p, respectively [24]. Specificity was explored using protein fusions of Ste11p with different pathway components. Ste11p fused to Ste5p retained the ability to complement  $\Delta ste11$  yeast, while linking to Pbs2, a putative scaffold in the HOG response, cannot complement these deletion animals [25]. Thus, tethering function of Ste5p in yeast is not only essential for coupling of mating MAPK signaling, but may mediate pathway specificity and cellular response as well.

#### **1.4.2. SAPK scaffolding**

Scaffolds identified for mammalian SAPK scaffolding include  $\beta$ -arrestin-2, CrkII, Filamin, IKAP, JIP1-4, MKPX, POSH, and SKRP1 [26]. The best characterized of these is the family of proteins known as JNK interacting proteins, or JIP's. There have been four JIP's identified, all

encoded by distinct genes. Based on their sequence similarities, JIP's can be divided into two classes: JIP1/JIP2, which contain a C-terminal SH3 and PTB domain, and JIP3/JIP4, which consist of an extended coiled-coil domain [26]. Although mammalian JIP's share no sequence homology with yeast Ste5p, these two proteins are the most functionally equivalent scaffolds identified to date. As discussed above, Ste5p scaffolds all of the kinases of the mating MAPK pathway. JIP's also interact with complete three-kinase JNK modules, and in doing so not only augment signaling, but protect from aberrant JNK activation [27]. JIP1 and JIP2, for example, have been shown to bind to MAPK JNK, MAP2K MKK7, and various MAP3K mixed lineage kinases (MLK) [26]. It is also intriguing that the MAPK phosphatase MKP7 can also be found associated with both JIP1 and JIP2. Association of MKP7 to JIP1 scaffolds has been shown to inhibit JNK activation [28]. Hence, in addition to facilitating signaling through complex assembly, JIP scaffolding complexes also contain a mechanism by which signaling can be inhibited.

The physiological relevance of JIP proteins remains unclear. JIP3 null mice, for example, die soon after birth due to severe defects in brain development [29]. There have been three separate reports of JIP1-deficient mice. One group reported deletion of JIP1 to be embryonic lethal [30], while the most recent description of JIP1 knockouts reports the mice to be healthy and viable, but defective in neuron recovery following ischemic insult [31]. Yet another study confirms JIP null mice to be viable but defective in neuronal JNK regulation [32]. Additional studies will be required to more fully discern the physiological role of JIP scaffolds.

### **1.4.3. ERK1/2 scaffolds**

Determination of a bona fide scaffolding protein for the ERK1/2 signal cascade has proven more complex than for the other MAPK pathways. Scaffolding function has been attributed to a number of proteins, however, and these include MP1, MORG-1,  $\beta$ -arrestin, CNK, MEKK-1, RKIP, and KSR [26] (Figure 2).

#### **1.4.3.1. MP1 and MORG-1**

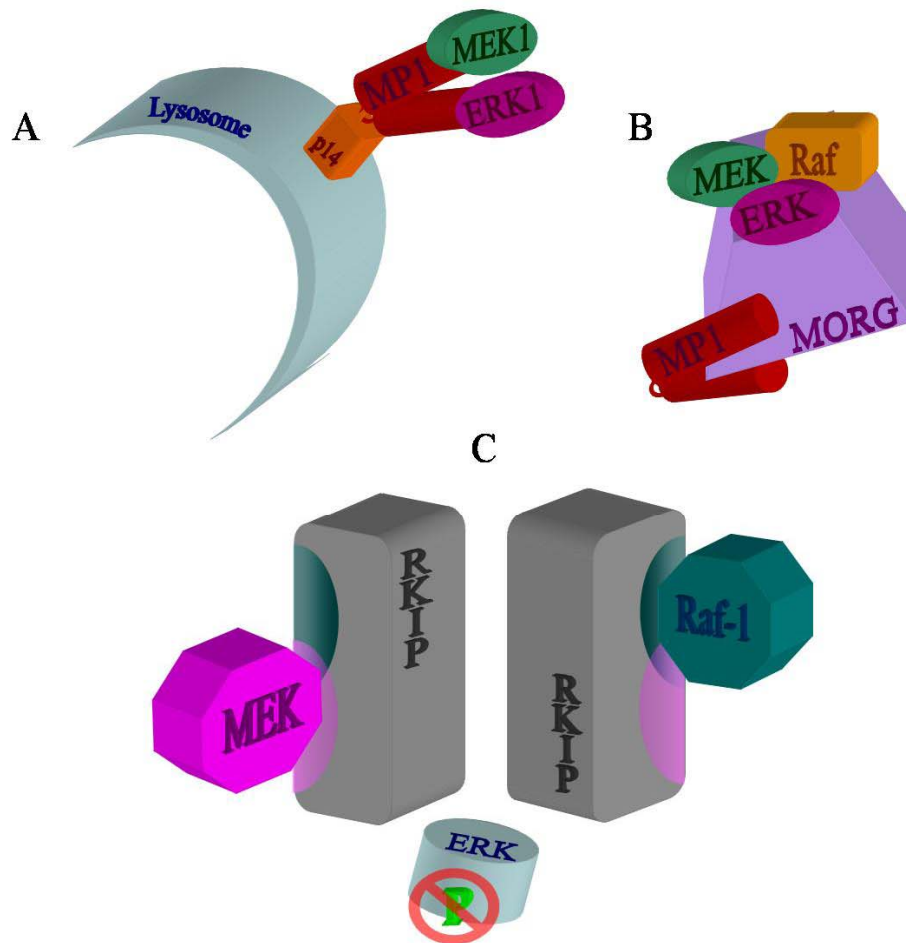
The first of these, MEK partner 1 (MP1), confers a level of specificity in ERK signaling, in that it binds only ERK1 and MEK1 [33]. MP1 has been demonstrated to localize MEK1 and ERK1 selectively to the surface of late endosomes and lysosomes. This membrane interaction is mediated by p14, a small protein on the lysosomal surface [34, 35]. By mistargeting p14 to the plasma membrane, MP1 localization is likewise misdirected [35], and siRNA knockdown of p14 results in MP1 being relocated to the cytosol. Furthermore, cells lacking p14 or MP1 are defective in ERK1/2 phosphorylation, with ERK1 being more severely inhibited than ERK2 [34]. MP1 association with ERK1 decreases in response to agonist or activated Ras, while levels of bound MEK1 do not change [36]. Recent work has also demonstrated that MP1 may mediate agonist specificity in MAPK phosphorylation. Pak-1-dependent phosphorylation of ERK requires MP1 and p14, while PDGF stimulation is independent of the scaffold [37]. Furthermore, the latest report concerning MP1 has shown it to dimerize in cells [36], but the physiological role of this dimerization has not been explored. Hence, as a scaffold, MP1 is not only required for proper activation of the pathway, but for localizing it to the proper intracellular compartment as well.

MP1 is a very small protein (14.5 kiloDaltons), which has raised speculation that it is not large enough to accommodate MEK1 and ERK1 as a scaffold. The identification of mitogen-activated

protein kinase organizer 1 (MORG-1) may serve to allay some of these concerns [38]. MORG-1 and MP1 have similar tissue expression patterns; both are ubiquitously expressed. mRNA transcripts for both have been isolated in heart, brain, lung, liver, muscle, kidney, spleen and testis, only differing slightly in relative abundance in several tissues [33, 38]. Although the only binding partners identified for MP1 to date are p14, MEK1 and ERK1, MORG-1 has been shown to interact with MP1, Raf-1, B-Raf, MEK1/2, and ERK1/2. Moreover, siRNA knockdown of MORG-1 reveals it to be an essential component of serum stimulated ERK phosphorylation, but not necessary for EGF-dependent ERK activation [38]. Intracellular localization patterns of MORG-1 have not been determined. If MORG-1 and MP1 subcellular distributions coincide, it is conceivable that these two proteins may work together to couple ERK signaling. MP1 does not bind to Raf-1, but MORG-1 could overcome this deficiency by its interaction with both Raf-1 and MP1.

#### **1.4.3.2. RKIP**

The scaffolds discussed to this point have all augmented MAPK signaling. The discovery of the Raf kinase inhibitor protein (RKIP) represents the first inhibitory ERK scaffolding molecule [39]. RKIP interacts with Raf-1, MEK, and ERK, but does not bind to Ras. Although MEK and ERK can associate simultaneously with RKIP, the binding sites for MEK and Raf-1 overlap. Concurrent occupation of RKIP by MEK and Raf-1 is therefore impossible, and the scaffold sequesters the proteins from each other – thereby ultimately inhibiting the activation of ERK. Moreover, in order to reverse RKIP-mediated inhibition of ERK phosphorylation, both the MEK and Raf-1 binding sites on the scaffold must be eliminated, implying that binding of one is sufficient for inhibition [40].



**Figure 2 - ERK1/2 Scaffolding Proteins**

ERK scaffolding function has been attributed to a number of proteins. MP1 (A) is a small molecule which selectively binds MEK1 and ERK1, and is tethered to the lysosomal surface by p14. Another putative scaffold, MORG (B), also interacts with MP1, in addition to Raf, MEK, and ERK. It has been suggested that these two proteins synergize to augment ERK phosphorylation. Conversely, RKIP (C) is an inhibitory scaffold. RKIP binds to MEK and Raf, but these binding sites overlap, making concurrent binding impossible. Thus, RKIP sequesters signaling components from activation.



Recently, RKIP has garnered a great deal of attention in oncology, in that it has been identified as a metastasis suppressor gene. Examination of prostate cancer cells determined that degree of metastasis corresponded to a progressive loss of RKIP expression [41]. Additionally, inhibition of ERK phosphorylation by rituximab, a drug shown to sensitize non-Hodgkin's lymphoma cells to chemotherapeutic drugs and apoptosis, has been shown to be mediated by upregulation of RKIP [42].

## 1.5. KSR

The Kinase Suppressor of Ras (KSR) represents one of the more enigmatic scaffolds identified for Ras/ERK signaling to date. Although KSR is widely accepted as an ERK scaffold, its exact role in facilitating this signaling pathway remains elusive.

### 1.5.1. Discovery

KSR was originally isolated in *Drosophila melanogaster* and *C. elegans* as a novel suppressor of phenotypes caused by overexpression of activated Ras [43-46]. In *Drosophila*, development of R7 photoreceptor cells requires activation of the RAS pathway. Overexpression of G12V RAS leads to the “rough-eye” phenotype, caused by an overproduction of R7 cells [46]. Flies examined expressing both activated RAS and KSR, however, maintain a normal phenotype. This suggested that KSR antagonized activated RAS, giving rise to its name as a suppressor of Ras [43, 46].

In the case of *C. elegans*, the let-60 Ras pathway is involved in vulval formation. Gain of function mutations in the pathway lead to the formation of multiple vulvas (MUV), whereas a loss of function results in the “vulvaless” phenotype [46]. Overexpression of activated Ras in this system leads to the MUV phenotype, but addition of *ksr-1* abrogates this effect. It is

interesting to note that although overexpression of the activated MAP3K in both of these systems also results in the “activated phenotype,” addition of *ksr-1* has no effect. Moreover, genetic epistasis experiments revealed that *ksr-1* appeared to be functioning downstream of Ras, but either upstream or in parallel with Raf [44-46].

### **1.5.2. Domain architecture**

KSR is a member of the “Tyrosine-kinase like” class of proteins (TKL), which is a broad class of proteins grouped together based on sequence similarity in their kinase domains which all members contain. The TKL group is diverse and contains both serine/threonine and tyrosine kinases, but shares high sequence identity to tyrosine kinase domains. The RAF protein family is a small family of highly related members of the TKL group and consists of all Raf isoforms, as well as KSR-1 and KSR-2. To date, KSR-1 homologs have been isolated in *C. elegans*, *Drosophila melanogaster* and *virilis*, *Mus musculus*, and *Homo sapiens* [43-45]. Quite recently, a second *ksr* has been cloned in *C. elegans*, and *ksr-2a* is 35% identical to *ksr-1* [47]. KSR-2 has also been described in humans, and is predicted to exist in a mouse model as well [48].

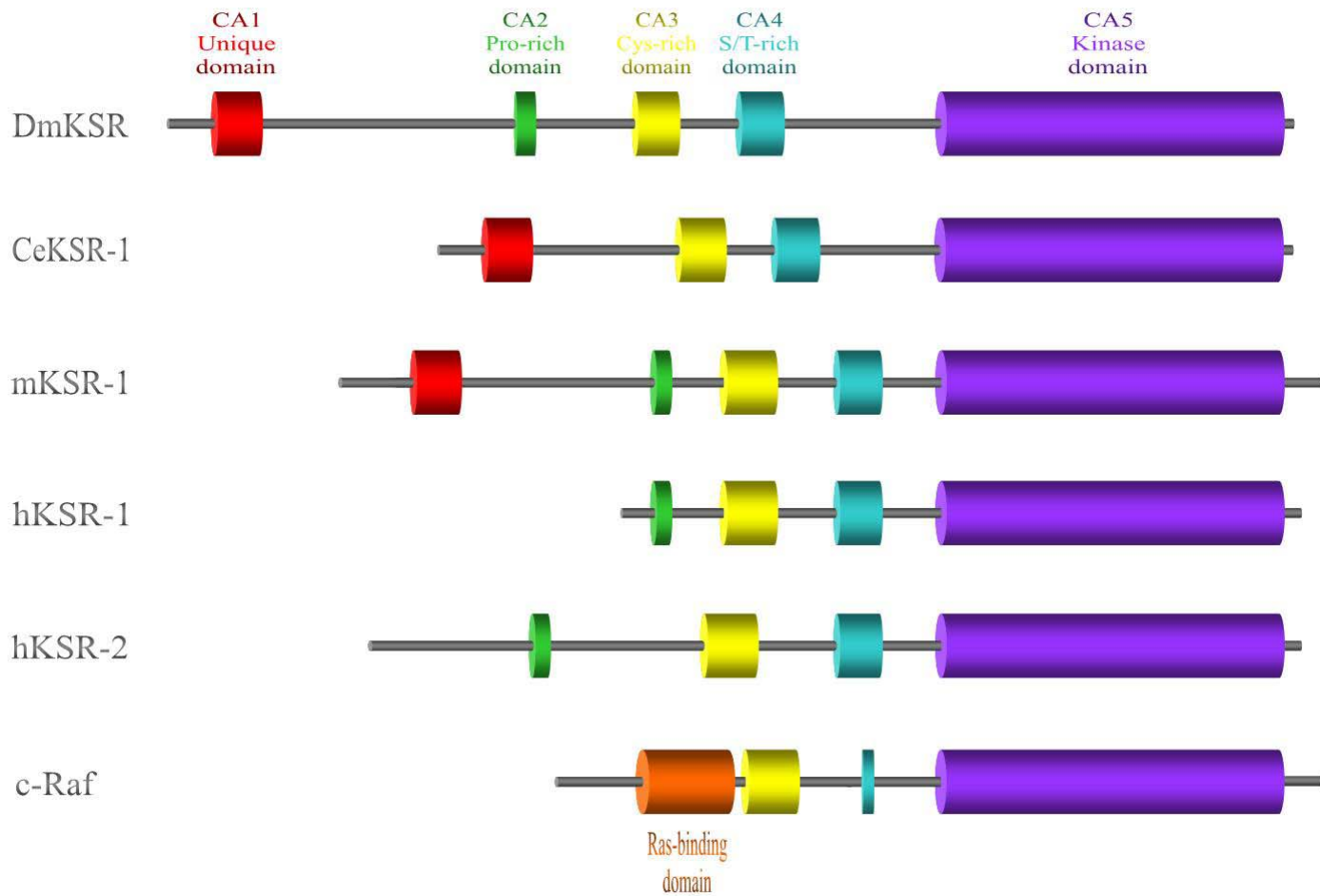
The domain architecture of the Raf protein family is shown in Figure 3. N-terminally, KSR contains a 40 residue segment not found in any other protein family (CA1, unique domain). The CA1 region is absent in hKSR-1 [43] and hKSR-2 [48]. Proceeding towards the C-terminus, KSR contains a proline rich region (CA2), a cysteine rich C1 domain (CA3), and a serine/threonine enriched region (CA4). The most C-terminal portion of the protein is the putative kinase domain (CA5), although the functionality of this region is controversial. All known protein kinases have a lysine residue downstream of a glycine-rich region, located in subdomain II, one of eleven subdomains conserved in all known kinases. This lysine

participates in the binding of the  $\alpha$ -phosphate of ATP. KSR contains an arginine at this position [43-45]. This region is discussed in detail in a later section (Section 1.5.4.2 – Kinase).

KSR and Raf are quite similar in sequence. While Raf is lacking the CA1 and CA2 domains and KSR lacks the Ras-binding domain, the remainder of the sequence starting with the CA3 domain is related. In mice, for example, this stretch shared by Raf-1 and KSR-1 is 28% identical and 46% similar. It is in the CA5 kinase domain that we find the most sequence similarity between KSR and Raf. *Drosophila* RAF and KSR kinase domains are 41% identical and 61% similar [43], while in *C. elegans*, KSR shares 30% identity with lin-45 Raf within the kinase domain, and 23% identity overall [44]. The most interesting aspect of this homology is KSR's possession of an almost identical PA- binding region. As mentioned above, the PABR consists of a PBM surrounded by hydrophobic sequences. This sequence is located between amino acid position 604-641 in mKSR1, with the essential arginine residues being at position 612 and 615 (Figure 4A). It is interesting to note that the PABR sequence is well conserved across species, including the recently isolated KSR-2 in *C. elegans* [47] and human KSR-2 [48], see Table 1. The role of this sequence in KSR function has not been explored.

### **1.5.3. Physiological role of KSR**

When examined in an invertebrate physiological context, KSR appears to be absolutely essential for Ras-mediated signaling in several species. In *C. elegans*, elimination of *ksr-1* alone is non-lethal; animals exhibit minor abnormalities in sex myoblast migration [44, 45, 49]. siRNA knockout of *ksr-2* also caused no gross anatomical defects, but these animals were sterile. This sterility was caused by arrest of developing oocytes in the pachytene stage of prophase I. These phenotypes are similar to those observed in animals having a loss-of-function mutation in let-60 Ras. Intriguingly, ablation of both *ksr-1* and *ksr-2* results in a severe Ras-like phenotype and



**Figure 3 - Domain Architecture of KSR**

KSR consists of five conserved regions. The N-terminal CA1 domain is a region unique to the KSR family of proteins. This region is absent in both human KSR homologues. Other domains include the CA2 proline-rich, CA3 cysteine-rich, and the CA4 serine/threonine-rich regions. The largest domain in KSR is the C-terminal putative kinase domain. (DmKSR: *D. melanogaster* KSR, CeKSR: *C. elegans* KSR, mKSR: *M. musculus* KSR, hKSR: *H. sapiens* KSR)

subsequent lethality due to a total lack of development of the excretory system. Individually, *ksr-1* and *ksr-2* are required for separate Ras-mediated processes: sex myoblast migration, and excretory and reproductive development, respectively [47]. Together, although there is a degree of redundancy in their function, *ksr-1* and *ksr-2* are apparently essential for Ras-dependent signaling in *C. elegans*. Likewise, KSR is a requisite component of insulin-induced activation of MEK and ERK in *Drosophila* S2 cells. siRNA knockout of KSR results in a loss of activity in this Ras-mediated pathway [50]. It is not surprising to note that loss of KSR expression in *Drosophila* is embryonic lethal [43], as this is the only species in which a second KSR homolog has not been identified.

In mammals, KSR-1 knockout mice have been generated. These animals appear grossly normal; however, ERK phosphorylation is sufficiently inhibited to hamper tumor cell development and block T-cell activation [51]. Targeted disruption of KSR-2 has not yet been examined, but the data from the invertebrate knockout experiments suggests that although single knockouts may have no overtly deleterious phenotype, ablation of both KSR homologs would be lethal.

#### **1.5.4. Function**

##### **1.5.4.1. Original theories**

The genetic data, combined with an analysis of the primary structure of KSR as a putative kinase, initially led to three possible models for its function. The first of these suggested that KSR represented a new MAPKKK, possibly leading to the discovery of a novel corresponding MAPKK and MAPK. In this model, KSR would be directly activated by Ras, and function in parallel to Raf. The second model suggested that KSR was a co-activator of Raf. Upon activation of a receptor tyrosine kinase, a pathway would be activated in parallel to Ras, leading ultimately to the activation of KSR, and consequent amplification of Raf activation. Finally, it

was postulated that KSR was an activator of Raf-1, serving as an intermediate step between Ras and Raf-1 activation [43]. These models have since been abandoned in the literature in favor of the role of KSR as a scaffolding molecule, as discussed in Section 1.5.4.3 – Scaffold.

#### **1.5.4.2. Kinase**

The most significant feature of the kinase domain of KSR is located in subdomain II. Mammalian kinases contain an invariant lysine residue in this region which is essential for the binding of ATP [52]. Mammalian KSR-1 isoforms have an arginine in place of this lysine, as does *C. elegans* KSR-2 [43, 47]. Although this may seem to be a conservative mutation, previous work with other kinases has demonstrated that mutation of this conserved lysine residue to any other amino acid, even arginine, can abolish activity [53]. The majority of known kinases contain a consensus sequence in subdomain VIb HRDL(K/R/A)XXN, where D and N are invariant residues [52]. KSR, however, has a lysine substitution in place of the arginine in VIb, distinguishing it from the majority of other kinases. Further, subdomain VIII has been implicated in substrate recognition specificity [52]. Although this region is highly conserved between KSR's of different species, it differs from Raf-1 significantly enough to suggest that, if KSR was a kinase, its spectrum of substrates would be quite different from that of Raf [43].

In an effort to determine if KSR kinase activity is essential for function, complementation studies were performed using two kinase dead mutants of *C. elegans*. Neither mutation of the magnesium-ATP binding motif (ceKSR K503M), nor the catalytic nucleophilic aspartate (ceKSR D618A) affected complementation [54], suggesting that the kinase function of the protein is immaterial to its role in signaling. Although the catalytic function of this region is questionable, expression of the isolated kinase domain of KSR dominantly inhibits Ras signal transduction [55-57]. The mechanism of this inhibition has not yet been determined.

Work by Kolesnick and colleagues has suggested that KSR is a ceramide-activated protein (CAP) kinase [58]. Additionally, using a special *in vitro* assay, they report that KSR phosphorylates Raf-1 at position 269, thus contributing to its activation by EGF [59]. Attempts have been made by other labs to corroborate these results, but with no success [60].

#### **1.5.4.3. Scaffold**

KSR was originally suspected of having scaffolding functions when it was discovered to bind to a number of signaling proteins. Demonstrable binding partners of KSR include Raf-1, MEK1/2, ERK1/2, G protein  $\beta\gamma$ , 14-3-3, Hsp70 and 90, cdc37, PP2A, and C-Tak1 [54, 57, 61-65].

As KSR has been put forth as an ERK scaffold, a great deal of effort in the literature has been dedicated to characterizing its interaction with pathway components. MEK is constitutively bound to KSR through the CA5 kinase domain, while association with ERK is Ras-dependent [66, 67]. The ERK1/2 binding site within KSR is an FxFP motif located in the serine/threonine-rich CA4 region of the protein [68]. Its binding to MEK which appears to be essential for KSR function. Mutations in mKSR-1 corresponding to loss-of-function alleles identified in *C. elegans* *ksr-1* [44, 45] prevent MEK association. Additionally, expression of exogenous KSR shifts the apparent molecular weight of MEK from 44 to more than 700 kDa in cells, moving it into a complex which also contains ERK1/2 [54].

The interaction of KSR with Raf-1, however, is not quite so straightforward, and appears to be species specific. This complex is most easily detected in *Drosophila* S2 cells, where Raf and KSR appear to be constitutively associated [50]. One of the loss-of-function mutations of KSR previously isolated in *Drosophila* (KSR<sup>L50S-R51G</sup>) corresponds to a mutation in the CA1 region of the protein [63]. Further examination of this mutant reveals that it has a decreased ability to bind Raf, indicating that KSR/Raf binding is mediated by the CA1 domain of DmKSR [50], and that

this constitutive binding is essential for function. In contrast, the detection of mKSR and Raf complexes is only possible in the membrane fraction of stimulated cells [64]. This agonist and membrane dependence of murine Raf/KSR interaction is consistent with the data reporting that KSR translocates from the cytosol in response to cell stimulation [62, 64, 65, 69, 70]. Furthermore, Raf activation requires its relocation to the plasma membrane as well, a necessary event for its interaction with Ras. Ergo, only when KSR and Raf are both localized to the plasma membrane can Raf be activated, subsequently phosphorylate KSR-bound MEK, and successfully propagate the signal.

The most compelling evidence for KSR's role as a scaffolding molecule came about through overexpression experiments. Loss-of-function studies in invertebrates demonstrate the unequivocal necessity of KSR for Ras-mediated signaling. Therefore, it was initially perplexing that, at high levels of KSR expression, Ras signaling was inhibited. Further analysis of this phenomenon revealed that, at low levels, KSR did indeed enhance signaling. Increasing KSR expression, however, inhibited ERK-dependent processes in a number of systems, such as R7 photoreceptor development in *Drosophila* and germinal vesicle breakdown in *Xenopus* oocytes [56, 57, 61, 67].

It is not surprising that KSR affects Ras pathway activation in a biphasic manner. MAPK signaling components exist in limited quantities in cells, which would suggest a corresponding optimum concentration of scaffold to facilitate signaling. Exceeding this optimum scaffold to signal protein ratio would therefore be predicted to segregate signal components from one another instead of enabling their activation [71]. In other words, excessive levels of KSR would increase the incidence of non-functional signal complexes, namely those lacking a complete repertoire of proteins required for signal coupling. Accordingly, increased expression of Raf and



MEK has been demonstrated to overcome this inhibition, and restore pathway activation in *Drosophila* [50], ostensibly by increasing the probability of a complete scaffold signal complex. Thus, based on the protein interactions of KSR and the overexpression data, this protein has been firmly established as an ERK scaffold.

While endogenous levels of KSR-1 have, to date, been shown to augment ERK phosphorylation, hKSR-2 is purportedly a signaling inhibitor. Recent studies with hKSR-2 have demonstrated that it is a negative regulator of Cot, a MAP3K family member involved in regulation of inflammatory and oncogenic signaling. Cot controls interleukin-8 production, which is thought to be mediated by ERK and NF- $\kappa$ B signaling. Overexpression of hKSR-2 selectively inhibits Cot-mediated ERK activation, while Ras-induced ERK signaling is unaffected. This is intriguing, as hKSR-2 also interacts with Raf, MEK, and ERK, but appears to selectively inhibit the Cot pathway [48]. The most recent work with this isoform suggests that hKSR-2 also blocks MEKK3 activation, an additional MAP3K implicated in the inflammation response. Activation of MEKK3 leads to ERK and JNK phosphorylation, as well as activation of the NF- $\kappa$ B pathway. hKSR-2-mediated inhibition of this signaling results in an inhibition of interleukin-8 production. Of interest, however, is the selectivity of this negative regulation: no effects were seen on other MAP3K's, such as MEKK4, TAK1, or Raf [72]. Consequently, hKSR-1 represents not only an additional scaffold for MAPK signaling, but a potential mediator of pathway selectivity as well.

#### **1.5.5. KSR and the plasma membrane**

A number of studies have demonstrated that KSR shuttles between the cytosol and the plasma membrane. In quiescent cells, its localization is mainly cytosolic. Upon addition of various agonists, including serum, EGF, and PDGF, there is a rapid translocation of KSR to the membrane compartment [62, 64, 65, 69, 70]. Membrane association of KSR is essential but not

sufficient for its function; artificial targeting by myristoylation does not activate the protein [60]. Although this traffic has been reported in a number of cell types in response to various agonists, the actual mechanism by which KSR is recruited to the membrane has not yet been elucidated.

#### **1.5.5.1. The C1 domain**

One of the initial studies characterizing KSR showed its murine isoform to cooperate with oncogenic Ras and enhance *Xenopus* oocyte meiotic maturation. This augmentation occurred at the level of MEK1 and MAPK, in that mKSR enhanced the kinetics of their activation [63]. Further studies demonstrated the cysteine-rich C1 domain was necessary and sufficient for this signal augmentation. In order to examine the role of this region, cysteines 359 and 362 in the CRD were mutated to serines (KSR CRM mutant). Whereas wild type mKSR accelerated *Xenopus* oocyte maturation induced by activated Ras, the CRM mutant had no such effect. Furthermore, expression of the CRD of KSR alone enhanced maturation, while the same construct containing the CRM mutation did not. Interestingly, the researchers report (in data not shown) that the rate of maturation is equivalent in oocytes injected with Ras<sup>V12</sup> alone and those with both Ras<sup>V12</sup> and KSR CRM, prompting them to conclude that mutation of the CRD does not generate a dominant negative KSR [60].

The mechanism by which the CRD mediates the cooperation between KSR and oncogenic Ras is still unclear. Additional studies have suggested that this region is required for KSR membrane localization. In 293 cells, the distribution of ectopically expressed mKSR1 was exclusively cytosolic. Co-transfection of KSR and Ras<sup>V12</sup> caused a pool of wild type KSR to redistribute to the membrane compartment, while CRM KSR remained in the cytosol. Work by another group has shown the CRD to be essential for the interaction of KSR with G-protein  $\beta\gamma$  subunits [69], although the significance of this interaction has not been extensively studied.

Despite the similarity between the CRD of KSR and Raf-1, these sequences are not interchangeable [70]. The C1 domains in these two proteins have the most homology with those classed as atypical, such as PKC $\gamma$ . Unlike typical C1 domains, the atypical class do not interact with diacylglycerol (DAG) or phorbol esters [73]. Swapping the CRD of Raf-1 with either the CA3 region of KSR or the C1b domain of PKC $\gamma$  abrogates its ability to activate MEK. Further, substitution of the Raf-1 CRD into KSR renders it unable to enhance oocyte maturation [70]. Further studies by this group demonstrated that membrane association of KSR is necessary but not sufficient for its functional contribution to Ras signaling. Artificial targeting of KSR to the membrane by myristoylation did not affect its ability to cooperate with oncogenic Ras, but was also not sufficient to promote oocyte germinal vesicle breakdown [60].

Taken together, the data pertaining to the KSR C1 domain indicate that this region of the protein is required for folding or function, but it not yet understood how this is accomplished. Moreover, although it has been reported that the CRD is essential for membrane recruitment, the mechanism by which this region may be involved in KSR traffic has not yet been determined.

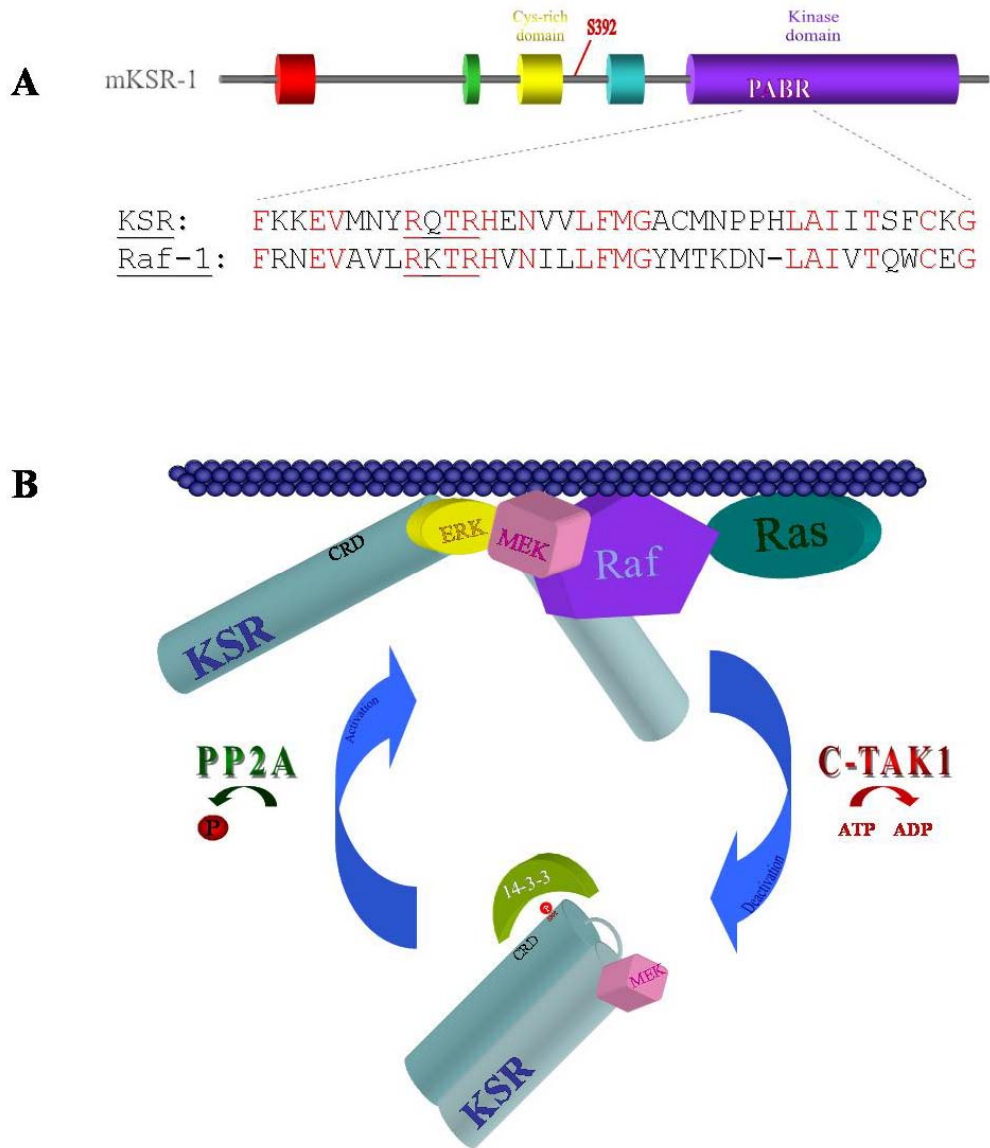
#### **1.5.5.2. Phosphorylation-regulated trafficking**

The first studies to explore the role of phosphorylation in KSR function revealed there to be a number of target sites in the N-terminal non-catalytic region. Phosphorylation of T260, T274, and S443 is induced by activated Ras, while S297 and S392 are phosphorylated in quiescent cells [67]. Phospho-serines at position 297 and 392 form consensus sites for 14-3-3 protein binding [62, 67]. These sites are essential for this interaction; alanine substitution at either S297 or S392 reduces the amount of 14-3-3 bound to KSR, while mutation of both residues eliminates it completely [67]. S392 is dephosphorylated in response to growth factor treatment, while there is no detectable change in phosphorylation at position 297 in response to EGF or PDGF.

Fractionation and immunostaining has also shown KSR S392A is constitutively associated with the membrane [62, 65], which results in enhanced ERK phosphorylation [62]. Together, these data suggest a model in which KSR is sequestered in the cytoplasm in quiescent cells by 14-3-3 proteins binding to phosphorylated S392.

Since neither kinase domain deletion nor kinase-dead mutants of KSR demonstrate decreased phosphorylation, these sites are not the result of autophosphorylation [74]. As mentioned above, KSR resides in cells as part of a large multi-protein complex, which has been shown to include Cdc25-associated kinase (C-TAK1). C-TAK1 phosphorylates KSR *in vitro*, and mutations which eliminate this interaction inhibit phosphorylation of KSR *in vivo* [62]. Hence C-TAK1 functions to retain KSR in the cytoplasm by phosphorylation of the 14-3-3 interaction site. Furthermore, since membrane association is required for KSR function, C-TAK1 constitutive association with KSR also represents a mechanism by which signaling could be terminated very quickly by redistribution of the scaffold back into the cytosol.

Recent studies have identified protein phosphatase 2A (PP2A) as the phosphatase required for KSR membrane translocation. PP2A structural and catalytic subunits (A and C, respectively) are constitutively bound to KSR within the N-terminal region, while association of the regulatory B subunit is agonist-dependent. Specific inhibition of PP2A with okadaic acid results in a dramatic increase in phosphorylation at position 392, and sequesters KSR in the cytoplasm. It also appears that bound 14-3-3 obscures the ERK binding site on KSR, as treatment with okadaic acid also reduces scaffolded ERK [65]. Interestingly, it has been shown previously that PP2A-mediated dephosphorylation of S259 and subsequent dissociation of 14-3-3 of Raf-1 is a critical step in its activation [75]. Thus, for both KSR and Raf-1, membrane association and therefore activation is gated by PP2A.



**Figure 4 – KSR trafficking between the cytosol and the plasma membrane**

A) S392 is located very close to the CRD in KSR. Additionally, the PABR of KSR is depicted aligned with that of Raf-1. Color coding of domains is as in Figure 3, see legend. Identical residues are shown in red, and the PBM is underlined. B) See text for additional details. Briefly, KSR traffic has been reported to be dependent on the phosphorylation state of S392. When S392 is phosphorylated, 14-3-3 proteins associate with KSR and sequester it in the cytosol. Dephosphorylation of this residue leads to the release of 14-3-3, and allows KSR to move to the plasma membrane.

### **1.5.5.3. A theoretical model for KSR traffic**

A potential model to combine all KSR trafficking data is as follows (Figure 4). Upon growth factor stimulation of cells, KSR S392 is rapidly dephosphorylated by associated PP2A, allowing for dissociation of 14-3-3 proteins. The proximity of the CRD to the S392 site where 14-3-3 binds suggests that association of 14-3-3 may mask this region. Dissociation of 14-3-3 would expose the C1 domain, thereby allowing it to facilitate KSR membrane recruitment. Furthermore, although MEK binding is unchanged, S392A KSR shows increased levels of ERK association, indicating the ERK binding site on KSR may be obscured by 14-3-3 binding [62]. Thus, dephosphorylation of KSR also allows for access of ERK to the scaffold. Once Raf-1, KSR, MEK and ERK are all colocalized in the membrane compartment, signaling can take place. Upon termination of the stimulus, bound C-TAK1 phosphorylates KSR on S392, prompting the reassociation of 14-3-3 proteins, movement of the scaffold into the cytosol, and prevention of any additional ERK binding.

It is important to note that, although this model and all data reported address the importance of KSR membrane recruitment, how this event occurs has not been determined. The CA3 CRD has been reported to be critical for KSR traffic, but neither a protein nor a lipid target for this region has been identified. Thus, while membrane association of KSR appears to be essential for its function, the mechanism underlying this membrane recruitment is unknown.

## **1.6. Statement of the problem**

The purpose of this thesis work was to investigate the role of lipid-binding in the scaffolding function of KSR. Membrane recruitment is essential for the ability of KSR to augment ERK activation; however, the mechanism of this translocation remains elusive. Previous work in the laboratory with Raf-1 has demonstrated that its membrane recruitment is mediated by direct interaction with PA in the membrane. Furthermore, mutation of the PBM renders Raf-1 incapable of membrane association. As KSR possesses an almost identical PABR, the following hypotheses were proposed:

1. The PABR of KSR interacts with PA, and this interaction can be affected by neutralization of the PBM.
2. Mutation of the KSR PABR will be detrimental to its ability to bind membranes and facilitate ERK activation.

The testing of these two hypotheses are described below in Chapters 2 and 3, respectively.

## 2. Lipid binding analysis of the KSR PABR

### 2.1. Introduction

Lipid second messengers are emerging as essential regulators of many signaling systems. One of these, phosphatidic acid (PA), has been implicated in multiple cellular processes including endocytosis [76-78], activation of PI<sub>4</sub>P 5-kinase [79], apoptosis [80], and activation of the MAP3K Raf-1 [7, 10]. PA synthesis in cells can occur by several different processes: 1) diacylglycerol (DAG) phosphorylation by DAG kinase, 2) lyso-PA acylation by acyl transferases, and 3) phospholipase D (PLD) mediated hydrolysis of phosphatidylcholine [9]. PA also serves as a substrate for lipid modifying enzymes. PA phosphohydrolases (PAP's) convert PA into diacylglycerol, while phospholipase A<sub>2</sub> removes an acyl chain to generate lyso-PA [80]. A number of PA-binding proteins have been described (Table 1). Phospholipase C $\gamma$  is activated by PA [81], while Ras-GAP is inhibited [82]. The function of mammalian Target of Rapamycin (mTOR) is also regulated by PA, the production of which recent studies have attributed to DAG kinase  $\zeta$  [83]. Additional targets of PA include the protein tyrosine phosphatase SHP-1 [84], and phosphodiesterase-4 [85].

Of particular interest, work with Raf-1 has established PA association as a critical step in its membrane recruitment and therefore activation. Mutations that disable PA-binding prevent Raf-1 membrane association and generate a dominant negative for ERK phosphorylation [7, 10]. The Raf-1 PABR is characterized by hydrophobic residues flanking a poly-basic motif (PBM) comprised of two arginine residues [4]. Alanine substitution within the PBM eliminates PA binding [6, 7]. Increasing concentrations of salt do not disrupt the protein lipid interaction, however; thus, binding is not exclusively mediated by electrostatic interactions. Furthermore,



lipid binding of the Raf-1 PABR is specific for PA and highly cooperative, while binding to other basic lipids and phosphatidyl alcohols was negligible [4].

The Kinase Suppressor of Ras (KSR) is a scaffolding protein for the ERK pathway originally identified in genetic screens for modifiers of an activated Ras phenotype [43-45]. Membrane recruitment of KSR has been reported in response to various agonists, and has been determined to be essential for its function in ERK cascade coupling [62, 64, 65, 69, 70]. Although it has been reported that the N-terminal cysteine rich domain is essential for KSR membrane association [60, 70], the mechanism underlying this recruitment has not been determined.

KSR shares a great deal of sequence similarity with Raf-1, especially within its putative C-terminal kinase domain. This region contains an almost identical PABR, a sequence which is conserved in all identified KSR isoforms. Using tryptophan-containing peptides corresponding to the PABR of KSR, the work in this chapter explores the lipid binding properties of this region. PA induces a blue-shift in the emission spectra of the WT peptide. This fluorescence change is specific for PA; no change is observed in the presence of phosphoinositides, phosphatidylcholine, or phosphatidylserine. This change in maxima is specifically induced by long chain PA's, and maximum fluorescence changes vary with the saturation of the hydrophobic chain. Furthermore, circular dichroism analysis of the PABR peptides reveals there to be a significant spectral change of the WT in the presence of lipid. This change is not observed with a PBM mutant peptide (R612A, R615A). Taken together, these data suggest a direct KSR-lipid interaction.

Raf-1	FRNEVAVL <u>RKTRH</u> VNILLFMGYMTKDN-LAIVTQWCEG
A-Raf	FKNEMQVL <u>RKTRH</u> VNILLFMGFMTKPGF-AIITQWCEG
B-Raf	FKNEVGVL <u>RKTRH</u> VNILLFMGYSTKPQ-LAIVTQWCEG
DmKSR	FRSEVANF <u>KNTRH</u> ENLVLFMGACMNPPYLAIIVTSLCKG
CeKSR-1	FKLEVSAY <u>ENTRH</u> DNIALFLGYFMSDGQYGMVMSLSKG
CeKSR-2a	ATEIVSAY <u>KNSRHDH</u> IALFYGYVSDPVTNTYAIIVTNFYQH
mKSR-1	FKKEVMNY <u>RQTRH</u> ENVVLFMGACMNPPHLAIITSFCKG
hKSR-1	FKKEVMNY <u>RQTRH</u> ENVVLFMGACMNPPHLAIITSFCKG
hKSR-2	FKREVMAY <u>RQTRH</u> ENVVLFMGACMSPPHLAIITSLCKG

**Table 1 – PABR sequence alignment of Raf-1 and KSR**

The PABR is highly conserved among Raf and KSR isoforms. The basic structural features of this domain in Raf and KSR include the poly-basic domain (underlined) and hydrophobic sequences on either side. Color coding of amino acids is as follows: green=non-polar, red=positively charged, purple=negatively charged, black=uncharged, polar.

## **2.2. Methods**

### **2.2.1. Materials**

DOPA, POPA, DPPA, C6-PA, Egg PC, and DOPS were obtained from Avanti Polar Lipids (Alabaster, AL). PI, PI<sub>3</sub>P, PI<sub>3,4</sub>P, PI<sub>3,5</sub>P, PI<sub>4,5</sub>P, and PI<sub>3,4,5</sub>P were purchased from Echelon Biosciences (Salt Lake City, UT). Peptides were synthesized and biotin-conjugated by the Molecular Medicine Institute Peptide Synthesis Facility (Pittsburgh, PA). Peptides were purified and determined by HPLC to be greater than 95% pure.

### **2.2.2. Peptides**

Tryptophan-containing peptides corresponding to the PABR of WT KSR (FKKEVMN YRQTRHENVVLWMGACMNPPHLA) and MT KSR (FKKEVMNYAQTAHENVVLWMGACMNPPHLA) were resuspended in 50mM Tris HCl, 100mM NaCl, and 100nM EGTA. Lipids were prepared initially as micelles in the same buffer (pH 7.4) + 1% CHAPS. Vesicles were prepared by dilution of detergent lipid solutions to well below the critical micelle concentration (CMC). The CMC for CHAPS is 6-10mM, and the concentration of CHAPS in the samples was at most 300μM. The pH of the experimental samples was maintained throughout at 7.4.

### **2.2.3. Fluorescence measurements**

Tryptophan fluorescence values were acquired using a FluoroMax-3 spectrofluorometer equipped with single-grating excitation and emission monochrometers. Excitation and emission slits were maintained at 5 nm, and measurements were made using a 1 x 1 cm quartz cuvette. Samples were excited at 280 nm, and emission was measured from 300-400 nm for full spectra, or at a constant 325 nm for titration experiments. Each measurement reflects the average fluorescence over five seconds of acquisition. Changes in fluorescence at 325 nm were plotted against log lipid concentration, and analyzed by curve fitting with GraphPad Prism software.

#### **2.2.4. Circular dichroism measurements**

CD measurements were taken of peptides ranging in concentration from 0.2-0.5mg/mL, in 50mM Tris, pH 7.4 at 25°C, in the presence or absence of 50uM lipid and 18mM CHAPS. All samples were horn-sonicated for 5 minutes just prior to analysis. Measurements were taken using an AVIV instrument built on a Varian Cary 60 platform using AVIV data acquisition and data processing software. Data reported reflects an average of ten spectra per sample. CD data was analyzed using CDSSTR, from DICHROWEB ([www.cryst.bbk.ac.uk/cdweb](http://www.cryst.bbk.ac.uk/cdweb)) using the built in databases available on the server (reference set 4 for the range 190-240 nm; data truncated at 240 nm). Analysis was confirmed using CDFIT ([www.structure.llnl.gov/cd](http://www.structure.llnl.gov/cd)).

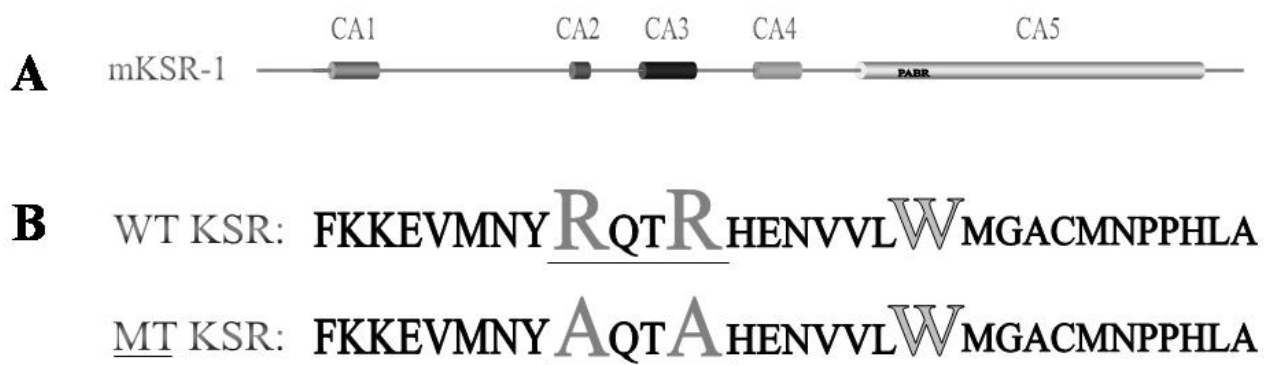
## 2.3. Results and Discussion

### 2.3.1. KSR PABR peptides interact with PA *in vitro*

The fluorescence of tryptophan is determined by its environment. Interactions between this amino acid and a hydrophobic milieu stabilize the dipole formed upon excitation, thus increasing the quantum yield and therefore fluorescence. This makes tryptophan fluorescence a powerful tool for determining protein structure as well as exploring protein-lipid interactions [86]. Tryptophan spectroscopy and fluorescence quenchers have been used extensively to determine the kinetics of protein-protein and protein-lipid interactions [87] and insertion depth of membrane proteins [88, 89], as well as peptide-membrane topology [90].

Accordingly, peptides corresponding to the KSR PABR (amino acids 603-633) were synthesized containing a tryptophan substitution at the position analogous to F622. These peptides are depicted in Figure 4. Wild type KSR PABR (WT KSR) possessed an intact PBM, while the two arginine residues in the MT KSR peptide PBM (R612/615) were mutated to alanines. Previous studies have shown that mutation of one or both of these arginine residues in the Raf-1 PABR results in an ablation of PA-binding [6, 7].

Sample emission traces of the two peptides are shown in Figure 5. Emission scans were made of the peptides between 310 and 400 nm at a constant excitation wavelength of 280 nm. In the absence of lipid, the emission maximum was 350 nm. Addition of PA to the WT PABR produced an increase in fluorescence as well as a more than 10nm blue-shifting of the curve. The amplitude of tryptophan fluorescence blue shift has been correlated with depth of membrane insertion. Emission maxima can shift from 337 nm for a tryptophan located near the membrane surface to 318 nm upon full insertion [88]. Structure of the PABR peptide supports this



**Figure 5 – KSR PABR peptide design**

(A) Domain structure of mKSR-1, depicting the location of the putative PABR. (B) PABR peptide sequences correspond to amino acids 607-636 of mKSR-1. Tryptophan was substituted for the phenylalanine at position 622. The underlined portion represents the PBM. Arginines 612 and 615 were mutated to alanines to produce the MT KSR peptide.

possibility, in that the PBM is surrounded by hydrophobic sequences ideal for membrane intercalation. Changes in fluorescence for the WT KSR peptide were detectable at nanomolar concentrations of lipid, while no change was observed in the MT KSR spectrum, even at lipid concentrations up to 10 $\mu$ M PA.

Closer examination of the MT KSR PABR curve reveals the tryptophan emission maxima to be notably blue-shifted relative to the WT peptide in the absence of lipid. This blue-shift indicates that in the case of the MT peptide, the milieu of the tryptophan residue is more hydrophobic than in the WT PABR. This could either indicate a structural change in the MT peptide, or the MT PABR may be aggregating. There is also no change in the emission peak of the MT peptide in the presence of lipid. There are two possible explanations for this. It is conceivable that the environment surrounding the tryptophan residue is so hydrophobic, binding of lipid does not change its fluorescent properties. Alternatively, there may be no peptide-lipid interaction with the MT KSR PABR.

In order to further explore the effects of lipids on the peptides, we conducted preliminary CD measurements to examine structural features of the PABR in the presence and absence of lipids in the WT and mutant peptides. The CD spectra of the WT KSR are shown in Figure 7A. The spectrum appeared largely devoid of defined secondary structure. Spectral deconvolution estimated WT KSR PABR to be composed of 70% random coil, with a very small beta-sheet and negligible alpha-helical component. Upon addition of detergent micelles containing PA, there were significant changes in the spectrum that may be consistent with a large proportion of beta-sheet content. Control experiments with detergent alone, or PC, which do not cause changes in fluorescence, did not cause these effects (data not shown).

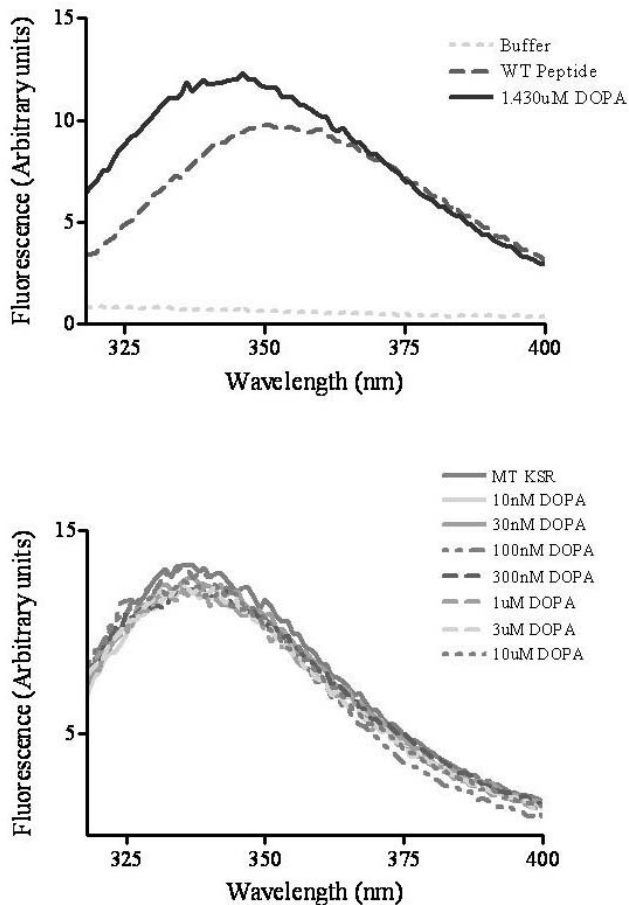
The CD spectra of MT PABR are shown in Figure 7B. Overall, the spectra are qualitatively different from those observed for the WT, and deconvolution of the spectrum in the absence of lipid suggested an increase in random coil contribution to 90% as compared to the WT spectrum. Furthermore, we could not detect similarly drastic changes in the spectrum in response to lipid addition. It appears that there may be a small increase in helix content. - While these experiments need to be considered preliminary, they suggest that WT KSR PABR may undergo conformational changes upon the addition of PA, while the mutation of the PBM may cause a change in peptide structure as compared to the WT that directly or indirectly could be the cause for preventing lipid-induced conformational changes in the mutant.

### **2.3.2. Changes in PABR fluorescence are specific for PA**

PA blue shifts the emission peak of the PABR tryptophan; thus, maximum fluorescence changes were easily detectable at a fixed emission wavelength of 325nm. Table 2 summarizes data for lipids tested. The shifting of the emission peak of KSR WT PABR was specific for phosphatidic acid; no change was detectable with phosphatidylserine, phosphatidylcholine or any of the phosphoinositides. Four different PA's were tested, dioleoyl PA (DOPA, C18:1), dipalmitoyl PA (DPPA, C16:0), palmitoyl oleoyl PA (POPA, C16:0-18:1), and dicaproyl PA (C-6 PA, C6:0). The PA's with longer acyl chains, DOPA, DPPA, and POPA, affected the emission peak of WT KSR PABR with half-maximal values of 1266nM, 1242nM, and 841nM, respectively. This effect was determined to be non-cooperative; Hill coefficients obtained through curve-fitting were approximately 1. The short-chain C-6 PA, however, had no effect on the fluorescence of the peptide (Figure 8A).

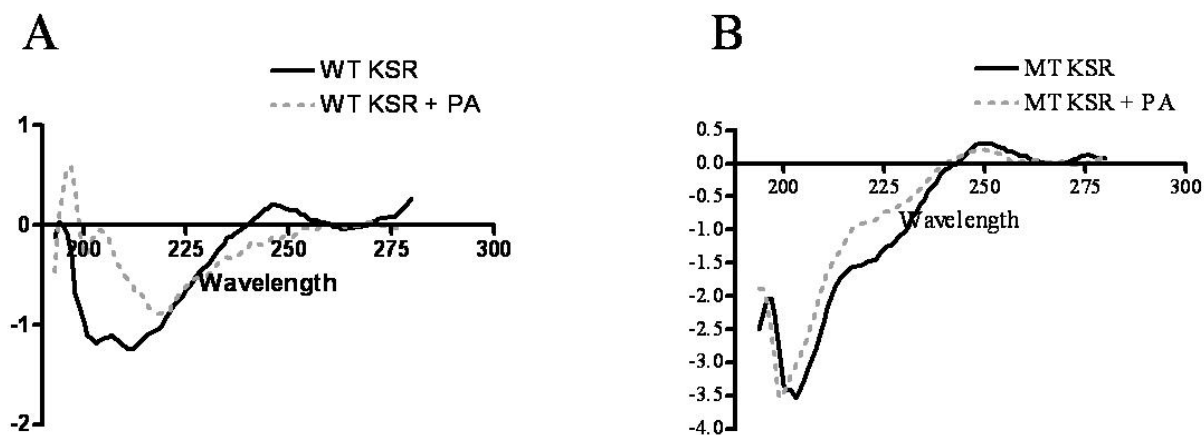
Additionally, acyl chain composition affects observed fluorescence values. Although the calculated  $EC_{50}$  values for DOPA, DPPA, and POPA are not statistically distinct, addition of





**Figure 6 – DOPA induces a blue-shift in the emission peak for WT KSR PABR but not MT KSR PABR**

Samples containing 200nM peptide were excited at 280nm, and emission scans were collected between 310nm and 400nm. A blue-shift in WT KSR fluorescence is detectable at submicromolar concentrations of lipid, but no change is observed in the MT even at concentrations of 10 $\mu$ M. The ideal control for this experiment would be to demonstrate binding of the Raf-1 PABR to PA as well. Unfortunately, no fluorescence change was detectable in the Raf-1 peptide upon addition of lipid. This may be due to a problem with the peptide, however, as synthesis of the Raf-1 PABR was reported to be problematic by the Peptide Facility. They, and other synthesis facilities contacted, reported that synthesis of a Raf-1 MT PABR was virtually impossible, due to the extremely hydrophobic nature of the mutant peptide. The substitution of negatively charged residues instead of alanine within the PBM may prove a means by which to avoid this problem. Additional experiments will also be required to verify that the tryptophan residue does not affect function in the full-length protein.



**Figure 7 – CD spectra of WT and MT KSR**

CD spectra for WT (A) and MT (B) KSR PABR peptides were analyzed using NCI's CD-Fit software. For analysis details, see Materials and Methods. WT KSR contains  $70\% \pm 15\%$  random coil in the absence of lipid, and  $70\% \pm 15\%$   $\beta$ -sheet in the presence of 100 $\mu$ M PA detergent micelles. MT KSR contains  $90\% \pm 15\%$  random coil, and this composition does not significantly change upon addition of PA micelles. Furthermore, no conformational change was observed with either peptide in the presence of detergent alone or PC micelles (data not shown). (Data contained in this figure and the corresponding methods were designed, generated, and presented as reported by G. Romero).

<b>Lipid</b>	<b>EC50 ± SEM</b>
DOPA	1266 ± 262 nM
POPA	841 ± 144 nM
DPPA	1242 ± 131 nM
C-6 PA	>> 10μM
DOPS	> 10μM
Egg PC	>> 10μM
PI	>> 3μM
PI <sub>3</sub> P	>> 3μM
PI <sub>3,4</sub> P	>> 3μM
PI <sub>3,5</sub> P	>> 3μM
PI <sub>4,5</sub> P	>> 3μM
PI <sub>3,4,5</sub> P	>> 3μM

**Table 2 – Calculated affinities of KSR PABR for lipids**

lipid caused significantly different changes in the fluorescence (Figure 8B). From the sigmoidal curve fit, theoretical maximum values were calculated for each lipid. The top of the DOPA curve ( $37122 \pm 1594$ ) was 2.9 times that of the POPA ( $12732 \pm 2814$ ), and 3.5 times the DPPA value ( $10527 \pm 1957$ ). The structure of these three lipids is shown in Figure 9, and their transition temperatures, defined as the temperature at which a lipid will transition from a rigid gel phase into the more fluid liquid crystalline state, are as follows: DOPA  $-8^{\circ}\text{C}$ , POPA  $28^{\circ}\text{C}$ , DPPA  $67^{\circ}\text{C}$ . All binding experiments were performed at room temperature ( $22^{\circ}\text{C}$ ), meaning that only DOPA is in the liquid crystalline phase.

Although more work is needed to establish a link between tryptophan levels in the KSR peptides and the fluidity of the lipid phase, it is interesting to speculate on the observed differences between DOPA and the other lipids with lower transition temperatures. We propose that the maximum levels of tryptophan fluorescence change are observed when the PABR is in the presence of vesicles composed of more fluid membrane, increased membrane fluidity possibly facilitating peptide insertion. Moreover, the apparent preference of the PABR for the less saturated lipids is supported by *in vivo* studies with PLD. Work in the Romero lab has determined PLD2 as the source of the PA required for Raf-1 membrane requirement through the PABR [10]. The two most abundant PA species generated in stimulated cells by PLD2 are POPA and DOPA, representing 20.5% and 12.8% total PA, respectively [91].

It is also of interest that the peptide is selective for PA compared to the similarly negatively charged PS. PA is not a particularly abundant membrane lipid. In erythrocyte plasma membrane, for example, PA comprises less than 1.5% of the membrane, compared to the 8% determined to be PS<sup>1</sup>. To date there have been no reports determining ratio of PA to PS specifically for the endosomal compartment, where the Ras/ERK

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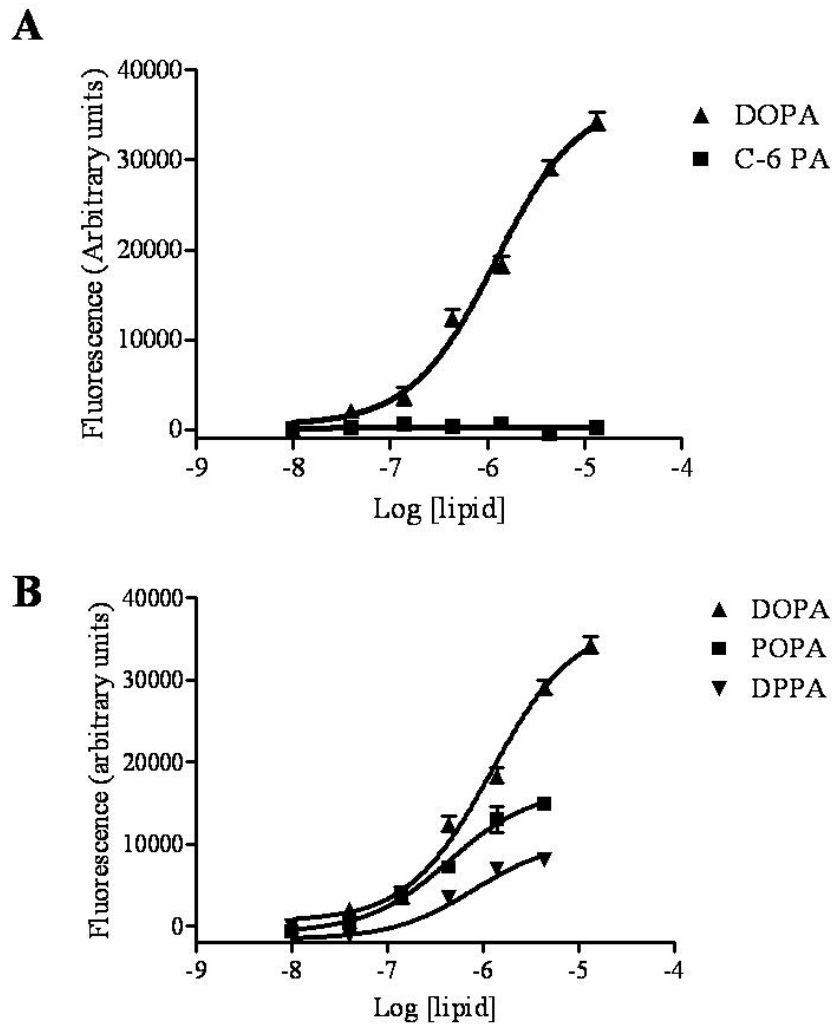
<sup>1</sup> Sanderson JM. *Org Biomol Chem*. 2005. 3(2):201-12.

signaling components assemble. Previous work in our lab has shown that treatment of HIRcB cells with insulin results in a greater than five-fold increase in endosomal PA. Assuming a constant level of PS between the plasma membrane and the endosome, stimulation with insulin results in a five-fold increase in the PA:PS ratio. Moreover, concentration of PA into microdomains may result in local exclusion of other negatively charged phospholipids. The observed selectivity of the PABR for PA may facilitate specific localization of Raf and KSR to lipid microenvironments, such as the PA-enriched endosome. Moreover, the CD studies discussed above did not include exploration of the effects of PS on PABR structure. It is possible that the energy of interaction associated with any peptide:PS interaction is insufficient to induce required specific conformational changes. Thus, although an association between the PABR and PS may be possible, it could be an ineffectual one. Finally, it is important to note that the peptide studies presented are reductionist, and that binding of the whole protein to lipids may differ from the isolated PABR, a point which remains to be explored in future studies.

Binding properties of the PABR remain largely uncharacterized. Early work with Raf-1 reported that PABR-PA binding was highly cooperative, with Hill coefficients ranging from 3.3 to 6.2. The same study also reported the apparent affinity of the PABR for PA to be 4.9-7.8% mol% PA [4]. It is important to note, however, that these values were determined using a solid phase binding assay, and do not provide an accurate representation of binding affinity. Furthermore, the use of immobilized lipid renders their values less physiologically relevant. Additional studies by Rapp and colleagues used surface plasmon resonance (SPR) and recombinant full-length Raf-1 to explore this lipid interaction. Using mixed composition liposomes, consisting of PC, PE, and sphingomyelin, they calculate the affinity of Raf-1 for PA to be 0.5nM [8]. This value bears further scrutiny, however. The highest affinity reported to date for a lipid binding

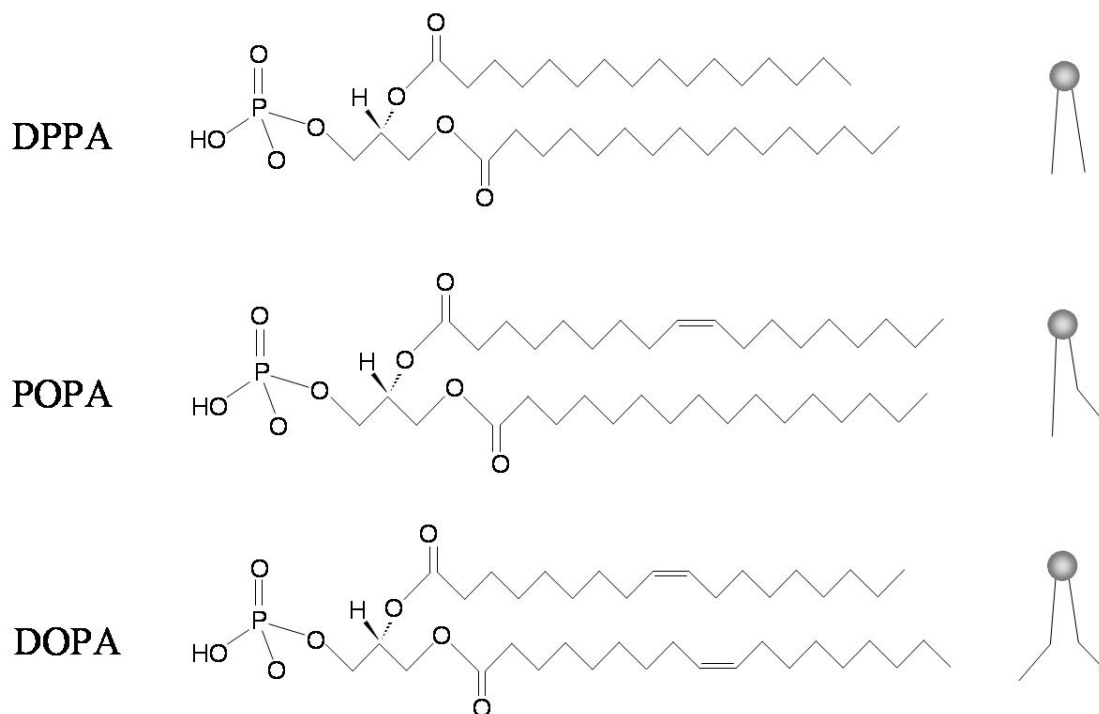
region is the PX domain of p47phox for PI3P, that being 50-100nM [92]. Moreover, the affinity of Ras for Raf is only 150nM [93]. Taking into account the possibility that the affinity of the full-length protein for lipid may be greater than for the binding region alone, it is still difficult to accept that the two values would be quite so disparate. Thus, further analysis of lipid-PABR interactions is warranted to definitively determine lipid-binding affinity of this domain, both as an isolated peptide and in the context of the full-length protein.

The data presented here strongly suggest a direct KSR lipid interaction. Furthermore, a KSR mutation that changes the structure of the PABR and may eliminate lipid binding has been defined. The cellular consequences of this mutation will be explored in the subsequent chapter.



**Figure 8 – Maximal fluorescence changes are correlated with long-chain mono- or di-unsaturated PA's**

(A) WT KSR peptides do not interact with short chain PA's. (B) Maximal fluorescence change is observed in the presence of DOPA (18:1), followed by POPA (16:0, 18:1) and DPPA (16:0). Theoretical tops of the curves (see text for details) were calculated with GraphPad Prism.



**Figure 9 – Structures of various PA's**

The defining characteristics of a PA molecule are the negatively charged headgroup, and the degree of saturation and length of the fatty acid chains. Simple depictions of lipid structures are shown at right. Membranes composed exclusively of DOPA, having two unsaturated acyl groups, are going to be more fluid than those made up of DPPA. Transition temperatures of DOPA are much lower than the other lipids tested ( $<0^{\circ}\text{C}$ ), thus making it the only lipid in the liquid crystalline phase at room temperature.



### **3. Binding of phosphatidic acid is essential for KSR function as a facilitator of mitogen-activated protein kinase activation, but not its membrane association**

#### **3.1. Introduction**

Recent work in the signaling field has shown that targeted membrane association of the components of the Ras/Raf/MEK/ERK signaling pathway is essential for their activation. Ras is targeted to cholesterol rich microdomains in the plasma membrane [11], while Raf-1 has been shown to interact directly with membrane lipids, namely phosphatidic acid (PA) [4, 10] and phosphatidyl serine [8]. Following cell stimulation, activated components of the ERK1/2 signaling pathway are localized to the surface of intracellular vesicles identified to be early endosomes [10, 15]. Neither ERK nor MEK possess any identified lipid binding regions, which suggests the involvement of additional scaffolding proteins in their membrane recruitment.

To date, several scaffolding proteins have been identified for the ERK1/2 pathway. One of these, MEK1 partner (MP1), has been shown to specifically interact with and localize MEK1 and ERK1 to the surface of late endosomes and lysosomes. This subcellular targeting is accomplished by the interaction of MP1 with p14, a small protein on the membrane surface. siRNA knockdown of MP1 results in cells defective in ERK signaling [34]. Furthermore, in the absence of p14, MP1 is unable to associate with lysosomes, and the efficiency of ERK signaling is compromised [34, 35].

A second scaffold which has proven much more enigmatic is the Kinase Suppressor of Ras (KSR). KSR has been shown to interact with MEK1/2 [51, 54, 57, 61, 94], ERK1/2 [51, 57, 67], and Raf-1 [64] *in vivo*. Furthermore, KSR shuttles between the cytoplasm and the plasma membrane in response to agonists. In quiescent cells, its localization is mainly cytosolic;

however, upon stimulation with agonist, there is a rapid translocation of KSR to the membrane compartment [62, 64, 65, 69, 70].

Sequence analysis of KSR reveals two well-defined lipid-binding motifs: an N-terminal C1 domain and a C-terminal PA-binding region (PABR). It has been suggested that the cysteine-rich C1 domain (CRD) may be involved in membrane binding [60, 70]. The CRD is located within the CA3 region of KSR, and is homologous to the atypical C1 domain of Raf-1. A bona fide lipid target for the CRD has not yet been found, although unpublished data by Morrison *et al* has ruled out ceramide and phorbol esters [70]. The PA-binding region (PABR) is located in the kinase domain of KSR. Like that of Raf-1, the KSR PABR consists of a positively charged polybasic motif (PBM) flanked by hydrophobic segments. Neutralization of the PBM has been shown to disable membrane binding in Raf-1 [7].

Data presented in this section demonstrate that mutation of the KSR PABR (MT KSR) generates a dominant negative for ERK phosphorylation. Furthermore, MT KSR is an agonist specific dominant negative, in that it inhibits ERK phosphorylation in response to insulin, but not EGF. Surprisingly, the subcellular localization of KSR is not changed with the PABR mutation. Both WT and MT KSR traffic constitutively between the cytoplasm and the membrane fraction independent of stimulation. Finally, both WT and MT KSR are found in the endosomal compartment, independent of PA-binding. Taken together, these data suggest that an intact PABR may not be necessary for recruitment of KSR to membranes, but it is essential for proper coupling of the signaling pathway.

## **3.2. Methods**

### **3.2.1. Cell culture**

HIRcB cells, rat-1 fibroblasts overexpressing human insulin receptor, were cultured at 37°C and 5% CO<sub>2</sub> in DMEM-F12 media supplemented with 10% fetal bovine serum and 100nM methotrexate. Cells destined for imaging experiments were transfected using Superfect (Qiagen), while Lipofectamine 2000 (Gibco) was used for biochemical samples, as per the manufacturers instructions. NIH-3T3 cells were treated as above, but grown in DMEM + 10% fetal calf serum.

### **3.2.2. Plamids**

Plasmids encoding mKSR-1 were kindly supplied by Dr. Richard Kolesnick and Dr. Kun-Liang Guan. KSR was amplified by PCR and subcloned into eGFP C1 vector (Clontech) using the EcoRI and Sall restriction sites. HA WT KSR was generated by PCR amplication and subsequent cloning into pCMV-HA (BD Biosciences) using the NotI and BglII sites. R612/615A KSR (MT KSR) was made by the four primer PCR method.

### **3.2.3. Antibodies**

Primary antibodies used were as follows: mouse anti-EEA-1 (Transduction Laboratories); rabbit anti-MEK, rabbit anti-ERK1/2, rabbit anti phospho-MEK, mouse anti-phospho ERK1/2 E10 clone (Cell Signaling Technology); mouse anti-HA ascites fluid (Covance). All primary antibodies were used at a concentration of 1:1000. Horseradish peroxidase conjugated secondary antibodies (Pierce) were used at 1:5000.

### **3.2.4. Cell fractionation**

HIRcB cells were transfected with pCMV HA-KSR WT or MT, and serum starved overnight. After stimulation with 200nM insulin, cells were washed with PBS on ice, and scraped in cell

cracking buffer (10mM HEPES pH 7.4, 2mM EDTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 50mM NaF, 1ug/mL leupeptin). Cells were then broken by 20 passes through a 22.5 gauge needle, and spun at 3000 RPM at 4°C for ten minutes to remove nuclei and unbroken cells. Soluble and particulate fractions were separated by a 90 minute 100,000 x g spin at 4°C. Pellets were resuspended in Laemmli buffer, and equal amounts of cytosol and membrane fractions were loaded on 10% acrylamide gels for Western blotting. Resultant blots were probed with anti-HA antibody.

### **3.2.5. Endosome isolation**

Endosomes were isolated as previously described [15]. Briefly, HIRcB cells were transfected with WT or MT KSR, and serum starved overnight. Cells were incubated with ferro-insulin conjugates for 20 minutes at 37°C, and scraped in homogenization buffer (1 mM Hepes, pH 7.2, 1 mM EDTA, 25 mM sucrose, 1mM NaVO<sub>4</sub>, 50mM NaF), broken by 20 passes through a 22.5 gauge needle, and loaded onto columns in a magnetic field. Endosomes were eluted from the columns using homogenization buffer + 0.5% TX-100. Samples were boiled in Laemmli buffer, run on 10% acrylamide gels, and Western blotted with anti-HA antibodies.

### **3.2.6. Live cell imaging**

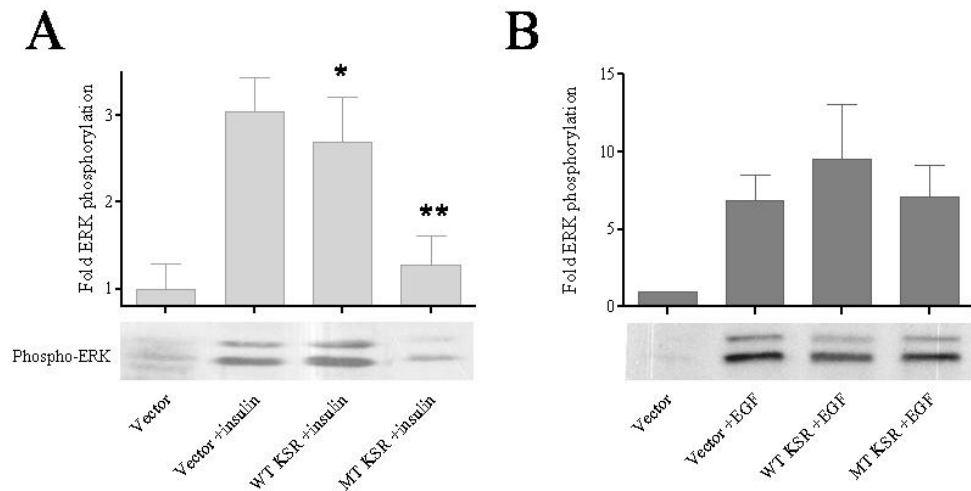
Cells were plated on 35mm poly-L-lysine coated coverslip dishes (MatTek), and transfected with eGFP-KSR WT or KSR MT constructs using Superfect Transfection Reagent (Qiagen). Cells were imaged at 37°C by placing the coverslip dish in a microperfusion incubator (Medical Systems Corp.). Images were acquired using a Zeiss Pascal confocal microscope, equipped with a 63x oil immersion objective. Samples were excited at 488nm, and emitted light was filtered through a 545nm long pass filter before photomultiplier detection.

### 3.3. Results and Discussion

#### 3.3.1. MT KSR inhibits ERK and MEK phosphorylation in response to insulin

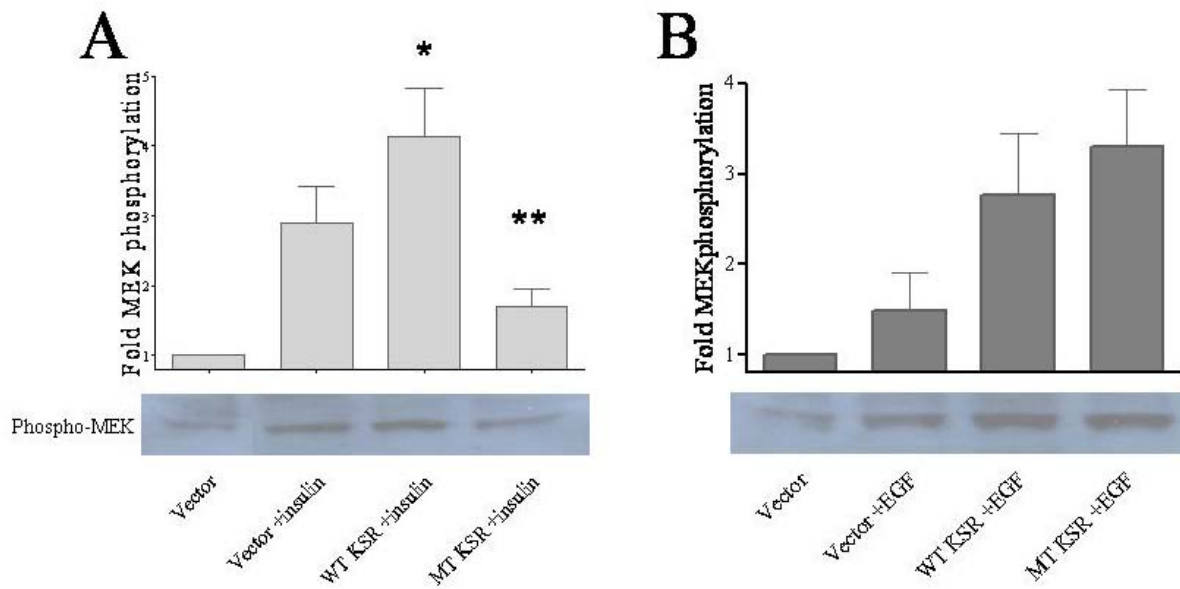
Neutralization of the PBM of Raf-1 has been demonstrated to eliminate PA binding, and thus membrane interaction and activation [6, 7]. Data presented in Chapter 2 of this thesis likewise demonstrate the same mutation in the KSR PABR eliminates any detectable PA-induced changes *in vitro*. In order to explore the cellular consequences of a KSR PABR mutant (MT KSR), the PBM arginine residues (R612/615) were mutated to alanines in the full-length protein. These constructs were then expressed in rat-1 fibroblasts overexpressing the human insulin receptor (HIRcB cells). Insulin-induced Ras/ERK signaling and the PA-dependent traffic of Raf-1 have been extensively characterized in this system [7, 10, 11, 15].

Mutation of the Raf-1 PBM generates a mutant which dominantly inhibits ERK phosphorylation in response to insulin, ostensibly by its inability to associate with the membrane compartment [7]. We therefore initially sought to determine if an intact KSR PABR is required for ERK activation. HIRcB cells were transfected with either empty pCMV-HA vector, HA-WT KSR, or HA-MT KSR, and serum starved overnight. Cell lysates were analyzed by SDS-PAGE, and Western blotted with antibodies specific for phosphorylated ERK. As shown in Figure 9A, MT KSR is a potent dominant negative for ERK phosphorylation, while WT KSR had no effect. A similar inhibition is seen for the ERK kinase, MEK (Figure 11A). Furthermore, this inhibition is dose dependent. Low levels of MT KSR expression have no effect; however, inhibition at higher doses is profound. These data demonstrate that an intact KSR PABR is required for ERK activation, and mutation of this domain generates a dominant negative for the pathway.



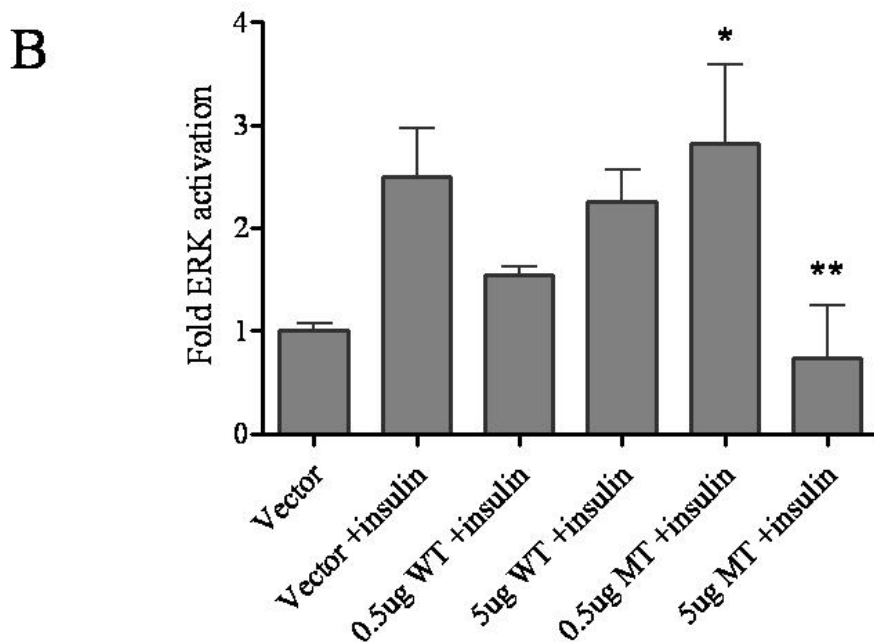
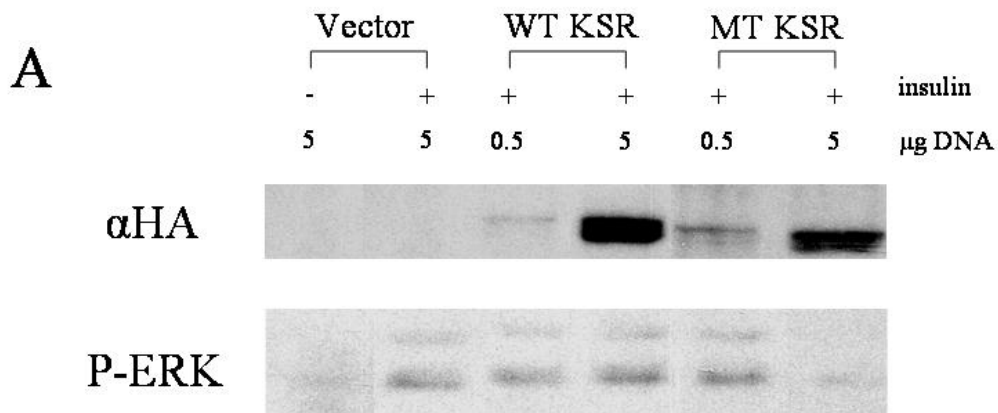
**Figure 10 – MT KSR inhibits ERK phosphorylation in response to insulin, but not EGF**

HIRcB cells were transfected with empty pCMV-HA vector, HA-WT KSR, or HA-MT KSR using Lipofectamine 2000 in the absence of serum. On the day following transfection, cells were stimulated with 200nM insulin (A) or 100ng/mL EGF (B) for 10 minutes at 37°C, washed with PBS, scraped and lysed in hot Laemmli buffer. Samples were sonicated and boiled prior to separation on a 10% acrylamide gel. Western blots were probed with antibodies specific for phospho-ERK. Samples represent equal numbers of cells plated the day prior to transfection, and no significant cell loss was observed following transfection with any construct used. Transfection efficiency was greater than 75%, as determined by a visual assay using GFP-labeled KSR (data not shown). One-way ANOVA and Dunnet's post test were used to determine that MT KSR (\*\*) is significantly different from WT KSR (\*) in the insulin treatment, but not the EGF. (n=3) Preliminary experiments conducted expressing comparable levels of KSR in these cells showed no significant change in total ERK expression upon transfection with the MT, but further studies will be required to confirm this result (data not shown).



**Figure 11 – MT KSR inhibits MEK phosphorylation in response to insulin, but not EGF**

Samples were treated exactly as described in Figure 9 for ERK phosphorylation, except Western blots were probed with antibodies against phosphorylated MEK. (n=3)



**Figure 12 – MT KSR inhibition of ERK phosphorylation is dose-dependent**

HIRcB cells were handled as has already been described. One-way ANOVA and Dunnet's post-test determined that the difference between the two doses of MT KSR are significant, while there is no significant change with higher doses of WT KSR.



### **3.3.2. Dominant negative effects of MT KSR are specific for insulin signaling**

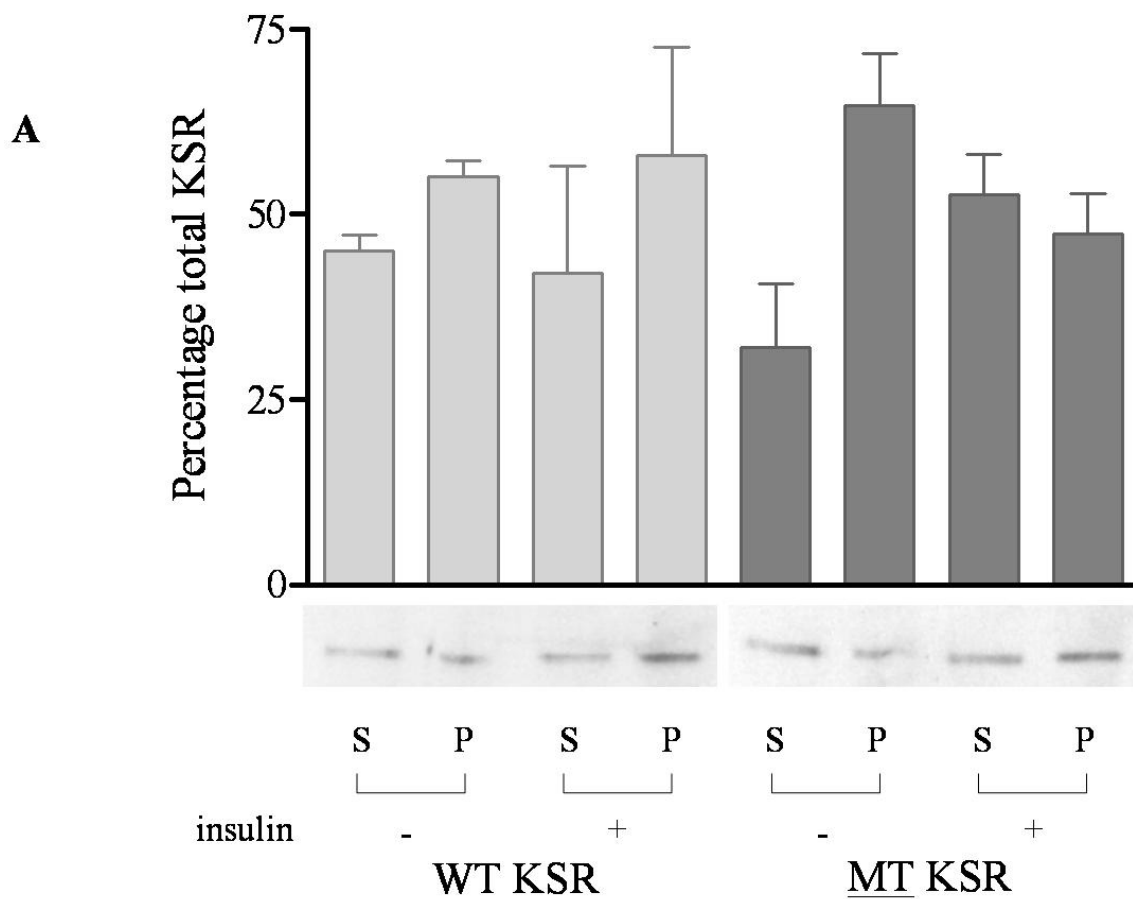
The Ras/ERK cascade is activated by a variety of agonists, and scaffolding proteins have been suggested as possible mediators of pathway specificity. We therefore investigated the necessity of the PABR in epidermal growth factor (EGF)-mediated activation of ERK. Intriguingly, MT KSR had no effect on the phosphorylation of MEK and ERK in response to EGF (Figure 10B, 11B). This data can be interpreted one of two ways: either KSR is not required for EGF signaling in HIRcB cells, or activation of ERK in this pathway does not require the KSR PABR. The idea of a scaffold having an agonist-specific function is not without precedent. MEK-partner 1, for example, has been reported to be essential for Pak-mediated activation of MEK in response to fibronectin. siRNA knockdown of MP1 had no effect on MEK phosphorylation stimulated by PDGF [37]. Yeast scaffolds have also been implicated in specificity of pathway response. The high osmolarity response and mating pathways share the same MAP3K, but differ in the ultimate MAPK activated. Using different MAP3K-scaffold fusion, studies have shown that MAPK specificity is conferred by the scaffolding protein [25].

### **3.3.3. MT KSR interacts with membranes independently of agonist**

Raf-1 interaction with membranes absolutely requires an intact PABR. Mutation of even one of the PBM arginines results in a mutant that acts as a dominant negative for insulin-induced ERK phosphorylation, correlating with its inability to bind membranes [7]. Data presented in Chapter 2 of this thesis demonstrate that a similar mutation in the KSR PABR affects PA binding. Thus, we sought to determine if the dominant negative effect of MT KSR was associated with a loss of membrane association. HIR cells were transfected with either HA-WT KSR or HA-MT KSR, disrupted in detergent-free buffer, and separated by a 90 minute centrifugation at 100,000x g. The isolated soluble (S) and particulate (P) fractions were boiled in Laemmli buffer, and

analyzed by SDS-PAGE. Western blots were probed with an antibody recognizing the HA epitope tag, and results are shown in Figure 13A. The amount of KSR in the S and P fractions was quantified by densitometry, and expressed as a fraction of the total amount of KSR detected. Surprisingly, there was no apparent difference in the membrane association of WT and MT KSR. Furthermore, there was also no change in KSR distribution in response to insulin. One-way ANOVA confirms this observation; there is no significant difference between quiescent and stimulated cells, nor is there a difference between WT and MT KSR.

These data are inconsistent with the reported model of KSR traffic in cells (for example, see [62]). KSR is reported to be a cytosolic protein in quiescent cells, and only translocates to the membrane fraction in response to agonist. Thus, it is conceivable that there may be a problem with our KSR constructs. To rule out this possibility, HA-WT KSR was expressed in NIH-3T3 cells, a cell type in which KSR traffic has been reported to be agonist dependent. Studies using 3T3 cells reported that approximately 25% of the total KSR associates with the membrane in quiescent cells, and increases to 70% in response to stimulation [64]. As shown in figure 13B, expression of our HA-WT KSR construct corroborates these results. Thus, despite their homology, KSR and Raf-1 traffic is regulated by different mechanisms. Furthermore, the equal distribution of KSR between the membrane and the cytosol appears to be unique to HIRcB cells. KSR membrane recruitment has been reported to hinge upon two factors. The first of these is the N-terminal cysteine-rich domain (CRD). Mutation of the cysteine residues in this region prevent association of the protein with the membrane [60, 70]. The CRD of KSR is similar to the atypical C1 domains of Raf-1 and PKC; however, the mechanism by which this region may



**Figure 13 – WT and MT KSR associate with membrane independently of agonist**

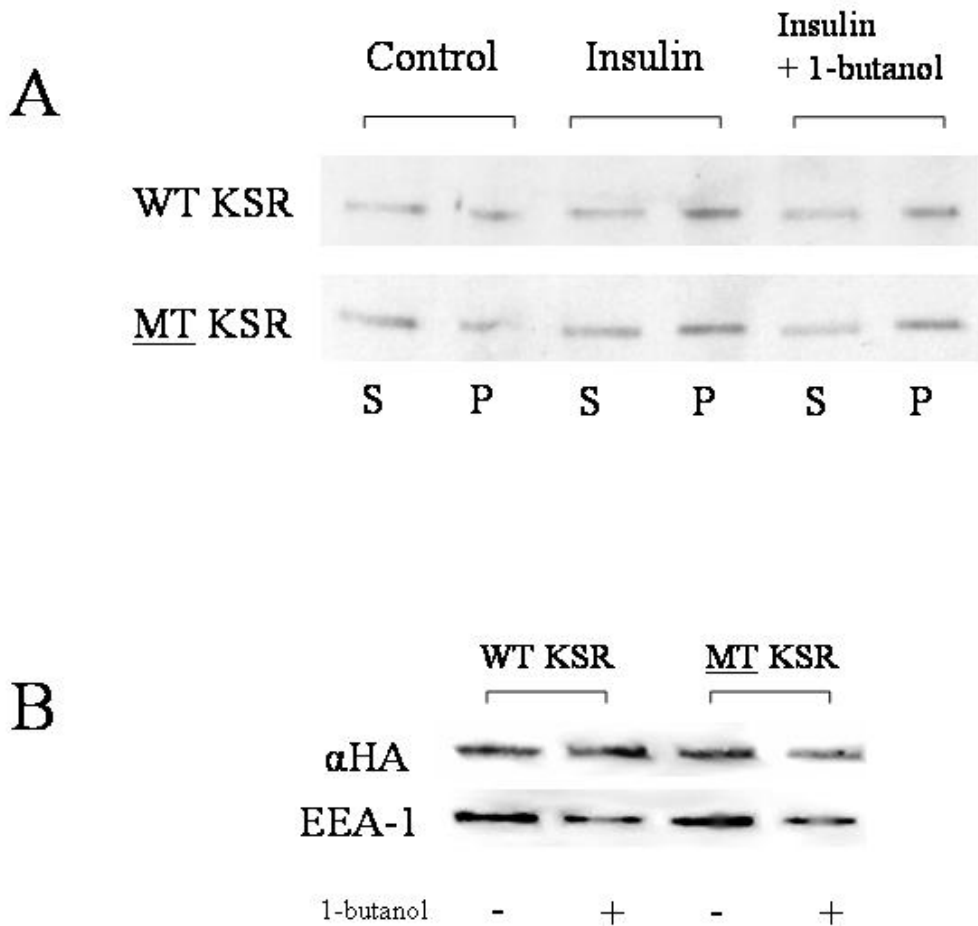
(A) HIRcB cells were fractionated by centrifugation at 100,000g, as described in the text. One-way ANOVA detected no statistical difference between groups (n=3). (B) Fractionation of NIH-3T3 cells expressing HA-WT KSR. Approximately 50% of KSR redistributes to the membrane in response to agonist in 3T3 cells.

mediate membrane association is yet to be determined. The second reported regulator of KSR traffic is 14-3-3 association, mediated by phosphorylation of serine 392. Mutation of this residue to an alanine results in a KSR constitutively associated with the membrane [25]. These two models are not mutually exclusive, however. Binding of 14-3-3 is very close to the CRD, implying that association to KSR may mask the CRD in quiescent cells.

#### **3.3.4. WT and MT KSR bind the surface of early endosomes**

Previous studies in the Romero lab have demonstrated that the components of the Ras/ERK signaling cascade assemble on the surface of early endosomes. Endosomal localization of Ras, Raf-1, MEK, and ERK is agonist dependent, and blockade of endocytosis inhibits ERK phosphorylation [7, 15]. The localization of Ras and Raf to these “signaling endosomes” is not surprising, as both of these proteins are known to interact with membranes. Neither MEK nor ERK possess any identified lipid binding domain, which suggests possible role for a scaffolding protein in their endosomal recruitment.

We therefore next sought to determine if KSR could be found in the endosomal fraction of HIRcB cells. Recent work in our lab has developed a novel method of isolating endosomes. Briefly, iron beads are conjugated to insulin, and internalized by the cells. The cells are then mechanically disrupted by multiple passes through a small-gauge needle, and loaded onto columns in a magnetic field. This technique allows for the specific isolation of endosomes containing activated insulin receptors [15]. Using this technique, we demonstrate that KSR associates with endosomes, and mutation of the PABR has no effect on this recruitment (Figure 14B).



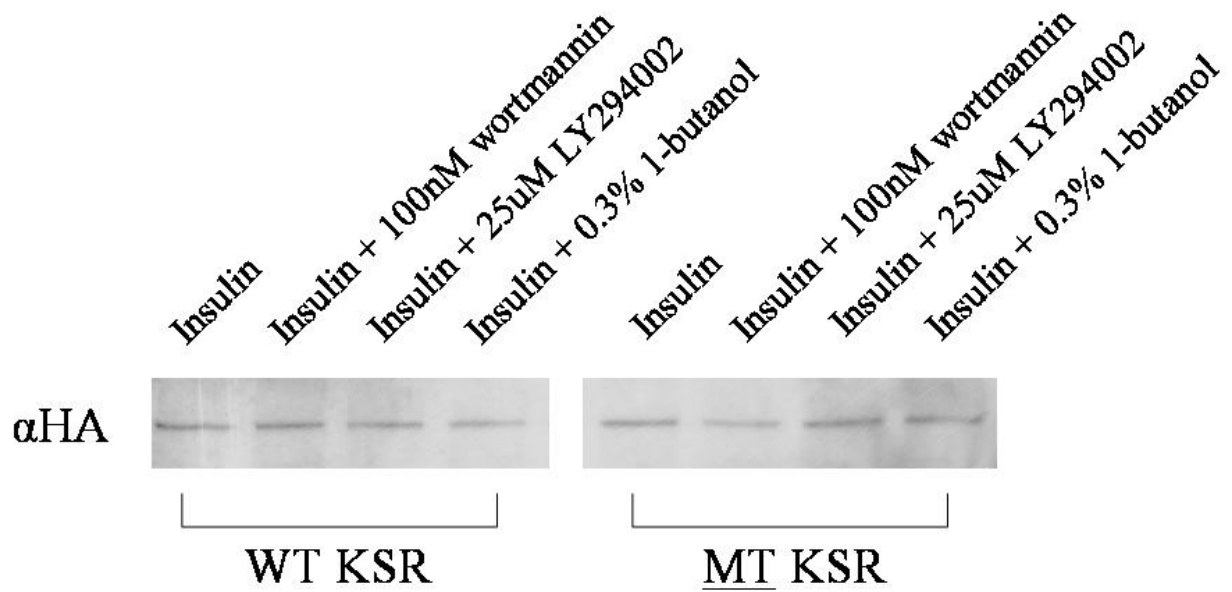
**Figure 14 – KSR associates with the membrane and endosomal fractions independent of PA binding**

HIRcB cells were transfected with WT or MT KSR, as described previously. Prior to stimulation with 200nM insulin, cells were incubated with 0.3% 1-butanol for 20 minutes at 37°C. For both the S100/P100 fractionation (A) and the magnetic endosome isolation (B), cells were collected and lysed after 20 minutes of agonist stimulation. Western blots were probed with an antibody against the HA epitope tag.

Localization of signaling molecules to specific subcellular localization is a common thread among scaffolding proteins. The A-kinase anchoring proteins, for instance, target PKA to organelles in close proximity to its substrates [95]. An example of this is dual-specificity AKAP-1. This scaffold tethers PKA to the surface of the mitochondria, where it phosphorylates and inactivates the pro-apoptotic BAD [96]. ERK scaffolds, too, have been reported to localize to specific subcellular locations. MP1 has been reported to localize to the surface of late endosomes and lysosomes. This lysosomal recruitment is mediated by MP1 interaction with p14 [35]. A physiological role for MP1 targeting of MEK1 and ERK1 to the lysosomal surface has not been elucidated; however, mistargeting the complex to the plasma membrane inhibits ERK phosphorylation [34], indicating proper subcellular localization is essential for pathway coupling.

### **3.3.5. WT and MT KSR traffic does not require PA or 3-phosphoinositides**

The data reported above seem to indicate that, although a functional KSR PABR is required for ERK phosphorylation, this domain is not implicated in membrane association. In an effort to more fully explore the role of lipid binding in KSR traffic, we used inhibitors of PI<sub>3</sub> kinase activity and PA-synthesis. There are multiple pathways by which PA can be generated in cells; however, the activity of PLD2 has been determined to be essential for Raf-1 membrane translocation. PA production by PLD-mediated hydrolysis of phosphatidylcholine can be inhibited by application of primary short-chain alcohols, which compete for water in the transphosphatidylation reaction. This competition results in the synthesis of a phosphatidylalcohol instead of PA [97]. Since the PABR of Raf-1 does not bind phosphatidylalcohols [4], 1-butanol and 1-propanol treatments were ideal for PA synthesis inhibition in our model. Only 0.25% of the total phosphoinositide in cells is phosphorylated at the 3 position [98], supporting the idea that these lipids serve a regulatory role in lieu of a



**Figure 15 – KSR association with endosomes does not require binding of PA or a 3-phosphoinositide**

HIRcB cells were transfected with WT or MT KSR, stimulated with ferro-insulin complexes for 20 minutes, and lysed. Samples were analyzed by Western blotting against the HA epitope tag.

structural one. Of these 3-phosphoinositides, PI<sub>3</sub>P is enriched in the endosomal compartment, where it becomes the target of FYVE and PX domains [99]. PI<sub>3</sub>K inhibitors wortmannin and LY294002 were therefore used to rule out 3-PI contribution to KSR traffic.

Cell fractionation studies depicted in Figure 14A reveal that inhibition of PA production by butanol treatment does not affect KSR membrane association. Likewise, KSR endosomal localization is unaffected by this treatment (Figure 14B). It is not surprising that treatment with butanol reduces the amount of EEA-1 in the sample slightly, as PA has been implicated in endocytosis and membrane fission [78]. It is clear, however, that there is no significant decrease in the amount of KSR associated with the endosome fraction in the presence of a PA-synthesis inhibitor. Additionally, neither wortmannin nor LY294002 treatment affected the presence of KSR in the endosome fraction (Figure 15.) These data imply that KSR membrane association may be independent of lipid association, either with PA or 3-PI's. To strengthen this conclusion, a useful control would be to validate the lipid-altering drugs used in these studies. For example, the efficacy of the LY294002 or wortmannin in inhibiting PI<sub>3</sub>K might be confirmed by incubation of cells with P<sup>32</sup>-orthophosphate, and measurement of PI levels relative to PI<sub>3</sub>P by HPLC. Alternatively, levels of phosphorylated Akt could be measured, as a downstream kinase in PI<sub>3</sub>K-mediated signaling. Additionally, the effectiveness of the butanol in inhibiting PA production could be assayed by confirming that this drug treatment alters Raf traffic, which has been previously determined to require this lipid.

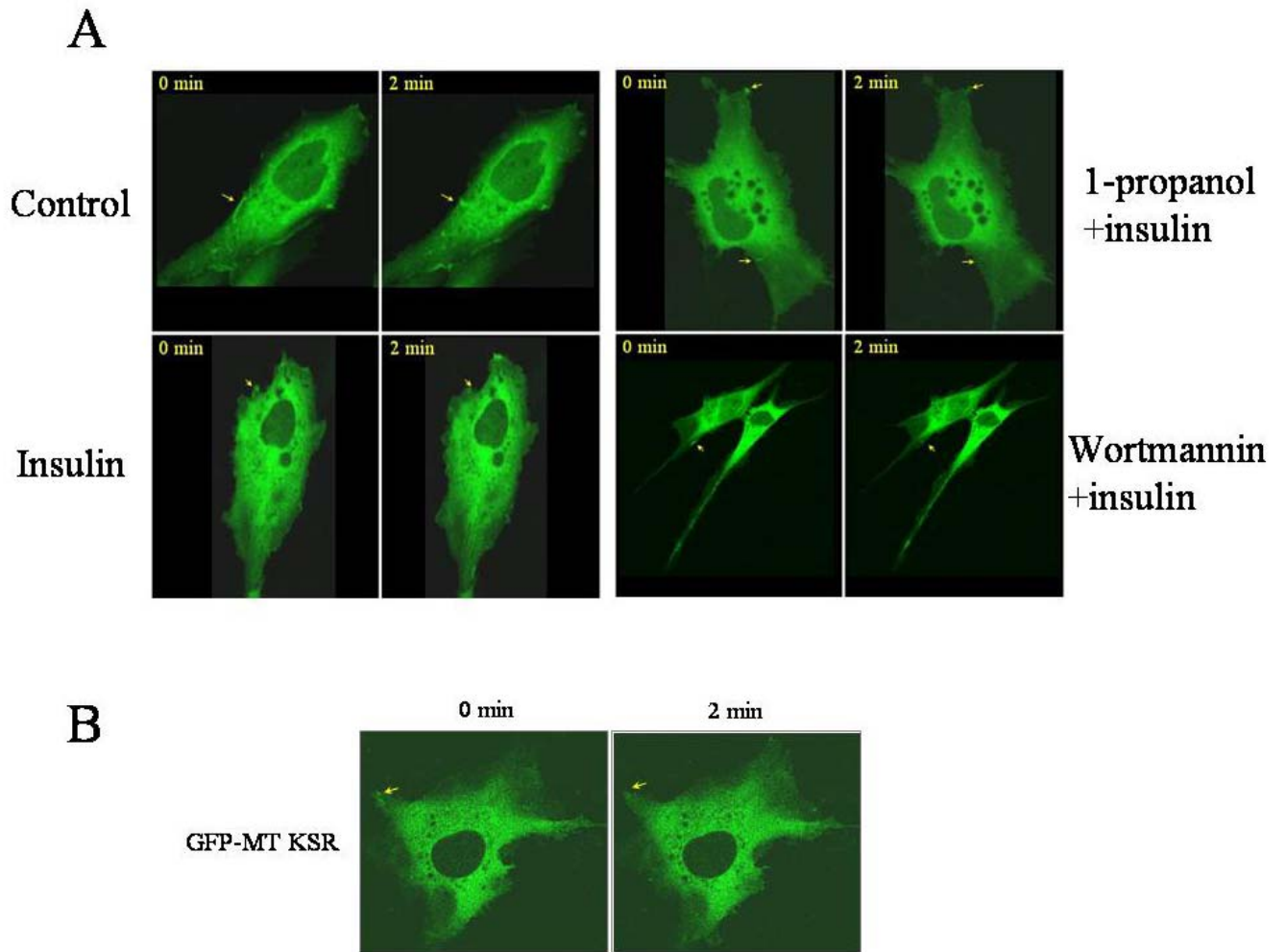
### **3.3.6. Live cell dynamics of KSR**

In order to visualize KSR traffic, green fluorescent protein (GFP) fusions of both the WT (Figure 16A) and MT (Figure 16B) were transfected into HIRcB cells, and imaged by confocal microscopy. Examination of the subcellular distribution of KSR reveals it to have a diffuse



cytosolic distribution, although a significant portion is found localized to membrane ruffle structures, both in unstimulated and stimulated cells. These ruffles assemble and are internalized rapidly, as seen by comparing the images where indicated with arrows. Pre-incubating the cells with the PA-synthesis inhibitor 1-propanol or the PI3K inhibitors wortmannin and LY294002 prior to stimulation had no effect on traffic of either the WT or the MT KSR protein. Thus, the live cell imaging supports the biochemical data indicating KSR traffic is both lipid- and agonist-independent.

Live cell dynamics of Ras/ERK family members in these cells have been well characterized by our lab. Ras, for example, can be easily visualized at the plasma membrane in quiescent cells. Upon stimulation of the cells with insulin, there is a rapid internalization of Ras in punctuate structures. This traffic is strictly agonist dependent [11]. Raf-1 traffic has been explored using confocal microscopy as well. By focusing on the region of the cell just above the level of the coverslip, it is possible to visualize the surge in Raf-1-GFP membrane association following cell stimulation [10]. Characterization of the endosomes isolated in response to insulin stimulation also show a definitive time course for association of Ras, Raf, MEK, and ERK. Both Ras and Raf association peak between 5 and 15 minutes following stimulation. MEK and ERK association is also transient; maximum levels of ERK and MEK are detectable at approximately 15 minutes of stimulation [15]. Thus, the purpose KSR constitutive association with both the plasma membrane and the endosomal compartment in these cells remains elusive.



**Figure 16 – Live cell dynamics of GFP-WT and GFP-MT KSR**

HIRcB cells were transfected with GFP fusions of the WT and MT KSR using the non-fluorescent transfection reagent Superfect. Each paired set of images reflects the same cell, two minutes apart. Points of interest are highlighted with arrows.

#### 4. Concluding Remarks and Future Work

In recent years, the field of signal transduction has seen a surge of interest in scaffolding proteins. Indeed, scaffolding provides an elegant mechanism by which to enhance the specificity and efficiency of signaling. Facilitation of signal protein interaction, concentration of kinases in a specific subcellular location, and sequestration of signaling components to prevent aberrant activation are all functions that have attributed to this class of proteins.

The work presented in this thesis has focused on the Kinase Suppressor of Ras, a scaffolding molecule for the Ras/ERK signaling cascade. Although it is widely accepted that KSR scaffolds MEK and ERK and enhances their activation, very little is actually known about the regulation of this protein. The purpose of my thesis was to investigate the role of the PABR in KSR traffic and function.

The data presented here suggest a direct KSR-lipid interaction. Membrane association of KSR is essential for its function in facilitating ERK phosphorylation [62], but the underlying mechanism of this recruitment has not been determined. Based on sequence identity (28% identity, 46% similarity) and *in vivo* functional studies, KSR is proposed to be similar in structure to Raf, and membrane binding of Raf depends upon interaction with PA. Mutation of the Raf PBM generates a dominant negative protein, and ablates its ability to interact with the membrane [7]. *In vitro* analysis of the same PBM mutation in the KSR PABR suggests possible reduced binding to PA. Moreover, expression of the full-length KSR PBM mutant in HIRcB cells dominantly inhibited ERK phosphorylation. Thus, it was quite surprising that neither the PABR mutation nor PA-synthesis inhibition altered KSR association with the membrane.

What, then, is the mechanism underlying the dominant inhibition conferred by the PBM mutation? In the case of Raf, the answer to this question is more straightforward. Mutation of the PABR disables membrane association, preventing Raf interaction with Ras, and uncoupling the signal. In the case of KSR, however, the PBM mutation does not seem to affect membrane association. It has been previously reported by our lab that the components of the Ras/ERK signaling cascade assemble on the surface of early endosomes, and that these endocytic vesicles are enriched in PA [7, 15]. Neither MEK nor ERK possess a defined lipid binding domain, suggesting the involvement of a scaffolding protein in their endosomal recruitment. KSR has been previously reported to interact with both MEK and ERK. The data presented here support a model in which the PABR of KSR binds PA *in vitro*. Further, KSR localizes to endosomes *in vivo*. Thus, the simplest model to incorporate all data states that KSR is involved in the endosomal recruitment of MEK and ERK, and its scaffolding function allows it to properly present MEK to Raf-1 for activation. Although the initial association of KSR with the membrane may not be mediated by PA, KSR's maneuvering of MEK and ERK into close enough proximity with Raf-1 may require direct interaction with the lipid. MT KSR therefore uncouples ERK and MEK phosphorylation by sequestering them away from PA- and cholesterol-rich subdomains where the upstream signaling components are located (Figure 17). Although the work presented here demonstrates the association of KSR with the membrane compartment, the model described above is predicated on the temporal and spatial colocalization of KSR and Raf. The association of Raf with membranes is agonist dependent, but data presented in this thesis demonstrate that KSR traffic is constitutive. Application of agonist may induce the redistribution of KSR among membrane compartments. The membrane to which KSR associates in quiescent cells may lack Ras and Raf, resulting in an incomplete signaling

unit. Agonist dependent processes would then redistribute KSR to a compartment which does contain the remaining components of the cascade. The binding of PA to the PABR of KSR could play a central role in this redistribution. Immunoelectron microscopy would prove useful in addressing this question. This technique would allow us to demonstrate concurrent association of KSR and Raf with the same vesicle compartment, and verify that they are in close proximity.

There is another possible explanation for these effects. *Ku68*, a loss of function mutation in *C. elegans* KSR [44], corresponds to an R615H mutation in mKSR-1. This point mutation is located within the PBM. The R615H mutant is reported to have a decreased affinity for MEK [54]. This arginine residue is located within the PBM, and is mutated to an alanine in MT KSR. Thus, the possibility that MT KSR simply has a decreased affinity for MEK cannot be ruled out<sup>2</sup>. However, it is important to note that the authors do not test the affinity of the R615H for MEK *in vitro*; they simply demonstrate that there is less MEK associated with this mutant in the context of an immunoprecipitation. MEK affinity for MT KSR versus WT KSR could be determined using recombinant proteins *in vitro*, and subsequent pull-down of KSR and Western blotting for scaffolded components. If MT KSR affinity for MEK is reduced, this does not necessarily preclude ERK binding. There has been no determination of cooperativity in MEK and ERK association with KSR, although a C809Y mutation in the kinase domain abolishes both MEK and ERK association with the scaffold [54]. This, too, could be determined by *in vitro* binding analysis. If MT KSR demonstrates reduced affinity for MEK, but retains binding to ERK, its dominant negative effects could be caused by sequestration of ERK from its upstream activators.

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<sup>2</sup> Unfortunately, attempts to determine if MT KSR retained the ability to bind MEK were unsuccessful; KSR-MEK complexes could not be detected in HIRcB cells. The reason for this failure to co-immunoprecipitate KSR with MEK or ERK is unclear.

Additionally, crosslinking experiments would be useful in determining the effects of inhibiting PA synthesis on the association of KSR with its scaffolded components.

It is feasible that PA binding may induce a conformational change of the protein. Circular dichroism experiments conducted by our lab for both KSR and Raf-1 PABR have provided preliminary evidence suggesting that there may be a structural rearrangement upon addition of lipid. The WT peptide alone appears to be predominantly (~70%) in a random coil conformation; however, addition of lipid induces a spectral change consistent with  $\beta$ -sheet formation. The CD spectrum of the MT PABR peptide was consistent with an even larger unstructured component (90% random coil), and addition of lipid induced very small spectral changes. From these data, we tentatively draw the following conclusions: mutations within the PBM induce a conformational change in the isolated PABR, and an intact PBM is required to observe a lipid-induced reordering of the peptide. A lipid-induced conformational change may either modulate binding of KSR for MEK and ERK, or change their relative spatial orientation. It would be particularly interesting to extend the studies of lipid effects on KSR alone to the investigation of the effects of lipids on KSR association with MEK and ERK. This will again require *in vitro* experiments with recombinant proteins.

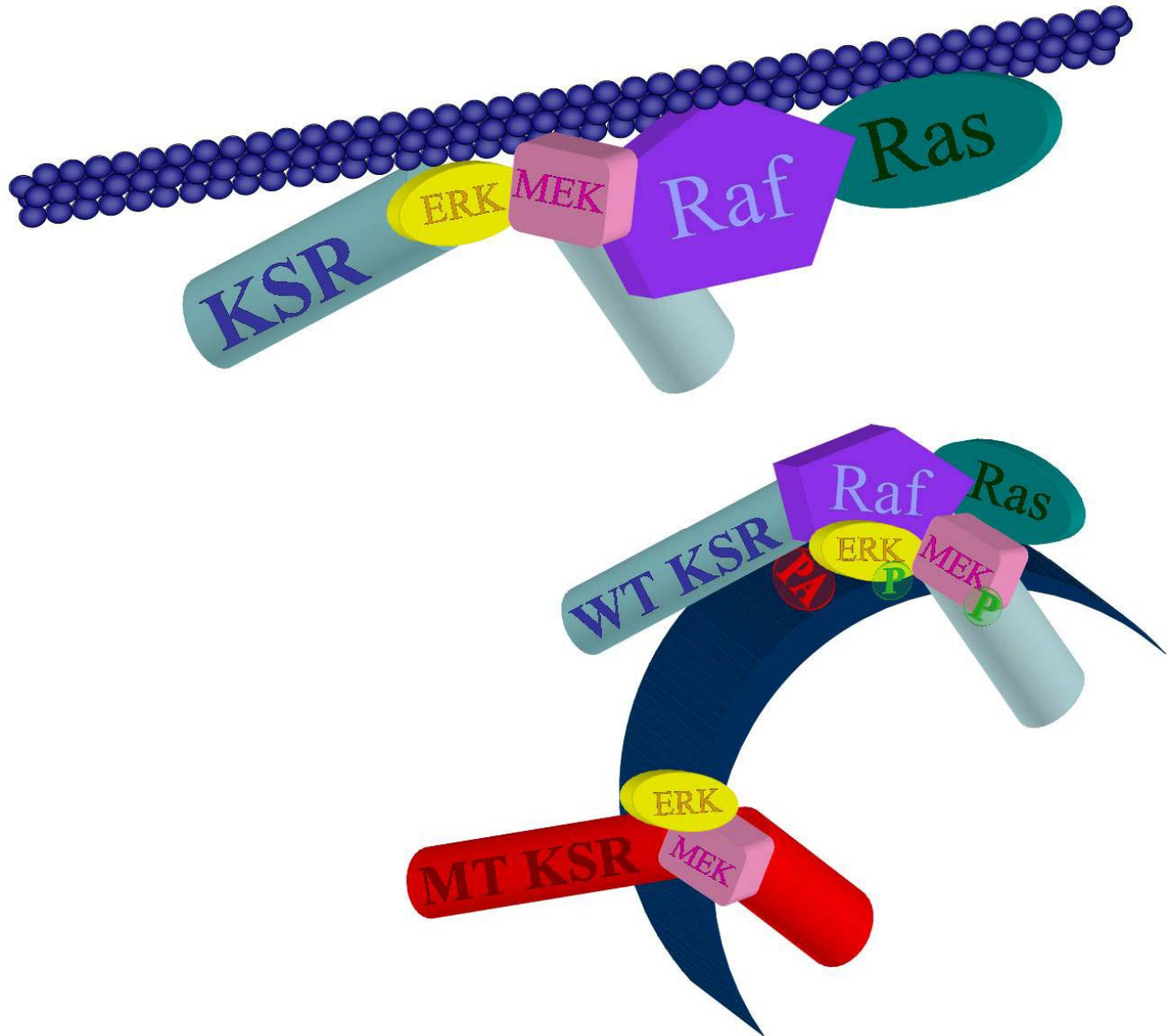
Here we also report that MT KSR is an agonist-specific dominant negative, although the import of this finding has not been determined. Inhibition of PLD2 in HIRcB cells results in a decrease in ERK activation and impedes Raf membrane association [10]. EGF does not activate PLD in these cells [100], indicating there may be an alternate mechanism for either PA synthesis (i.e. DAG kinase activity) or membrane recruitment of Ras/ERK proteins. The concept of agonist specificity of scaffolds is not unprecedented in the literature, although very little is understood about how this may occur. The recently identified MORG-1, for instance, is implicated in ERK

phosphorylation in response to serum, but not EGF [38]. MP1 mediates MEK phosphorylation during cell adhesion and spreading, but has no role in the PDGF response [37]. MP1 is particularly interesting, in that it serves to tether MEK1 and ERK1 specifically to lysosomes. Changing MP1 localization by artificially targeting it to the plasma membrane inhibits ERK activation [34]. This data indicates that impedance of scaffold interaction with the proper subcellular compartment can uncouple signaling, consistent with the model described above for KSR.

The KSR PABR is essential for its proper function in the Ras/ERK pathway. Mutation of this region may eliminate lipid binding *in vitro*, and produces a dominant negative scaffold *in vivo*. Mutation of the PABR is not sufficient to prevent the association of the full-length protein with the plasma membrane, nor the endocytic compartment. Taken together, these data indicate that, despite their sequence similarity, the traffic of KSR and Raf-1 is mediated by distinct mechanisms. As mentioned above, immunoelectron microscopy would be useful in determining the colocalization of Raf and KSR, to verify that they are in fact localized to the same vesicular compartment. It has been shown that the PABR of Raf-1 is sufficient to target GFP to the membrane and endosomal compartment. Mutation of the PBM results in a cytosolic distribution of this fusion protein [7]. This experiment could be expanded to include a KSR PABR-GFP fusion protein, to explore any differences in traffic of the two different domains. Another imaging technique that may prove useful is fluorescence resonance energy transfer (FRET). FRET between fluorophore fusion KSR and Raf-1 proteins could be used to determine not only that the proteins are in the same compartment, but that they are in close proximity to each other. Furthermore, live cell microscopy of cells expressing labeled KSR and Raf-1 would allow us to resolve any temporal differences in traffic of these two proteins. It would be expected, if the

model proposed in this thesis is correct, that WT KSR and Raf-1 would demonstrate energy transfer, but no FRET would be detectable with the PABR mutant.





**Figure 17 – A model for WT and MT KSR in HIRcB cells**

See text for a detailed explanation. Briefly, MT KSR (red) acts as a dominant negative by sequestering ERK and MEK away from PA-rich regions of the endosome. Only KSR possessing an intact PABR PBM (WT KSR, blue) can successfully couple the signaling pathway by associating with PA-enriched microenvironments.

## APPENDIX A

### Abbreviations

CRD	cysteine rich domain
DAG	diacylglycerol
DHPA	dihexanoyl phosphatidic acid
DOPA	dioleoyl phosphatidic acid
DOPS	dioleoyl phosphatidyl serine
DPPA	dipalmitoyl phosphatidic acid
EEA-1	early endosome antigen 1
EGF	epidermal growth factor
GFP	green fluorescent protein
KSR	kinase suppressor of Ras
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MP1	MEK partner 1
MT KSR	KSR R615/618A
MUV	multiple vulval phenotype
PA	phosphatidic acid
PABR	phosphatidic acid binding region
PBM	poly-basic motif of the PABR
PBS	phosphate buffered saline

PC	phosphatidyl choline
PDGF	platelet derived growth factor
PI	phosphatidyl inositide
PI <sub>3</sub> P	phosphatidyl inositide-3-phosphate
PI <sub>3,4</sub> P	phosphatidyl inositide-3,4-phosphate
PI <sub>3,5</sub> P	phosphatidyl inositide-3,5-phosphate
PI <sub>4,5</sub> P	phosphatidyl inositide-4,5-phosphate
PI <sub>3,4,5</sub> P	phosphatidyl inositide-3,4,5-phosphate
POPA	palmitoyl oleoyl phosphatidic acid
PS	phosphatidyl serine

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