MECHANISTIC BASIS OF SIGNAL AMPLITUDE

MODULATION BY THE RAS EFFECTOR IMP

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DEDICATION

Dedicated to my wife, mother, and sister

MECHANISTIC BASIS OF SIGNAL AMPLITUDE MODULATION BY THE RAS EFFECTOR IMP

by

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DISSERTATION

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MECHANISTIC BASIS OF SIGNAL AMPLITUDE MODULATION BY THE RAS EFFECTOR IMP

Chiyuan Chen, Ph.D.

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The RAF/MEK/MAP Kinase signal transduction cascade is the most extensively studied MAPK pathway that mediates diverse cellular responses to environmental cues, and makes a major contribution to Ras-dependent oncogenic transformation. The Ras effector and E3 ligase family member IMP (Impedes Mitogenic signal Propagation) acts as a steady-state resistor within the RAF-MEK-ERK kinase module. IMP concentrations are regulated by Ras, through induction of autodegradation, and can modulate signal/response thresholds by

directly limiting the assembly of functional KSR1-dependent RAF/MEK complexes. Here, we examine the mechanistic basis of signal amplitude modulation by the Ras effector IMP. We show that the capacity of IMP to inhibit signal propagation through RAF to MEK is a consequence of disrupting assembly of multivalent mitogenic complexes that are required for C-RAF kinase activation and functional coupling of active kinases to downstream substrates. We also study how Ras regulates IMP functions by isolating IMP mutants compromised for Ras interaction and how post-translational modifications such as sumoylation control IMP activities. Finally, we identify some candidate IMP binding proteins to further investigate how IMP impacts cell behaviors through protein-protein interactions and how IMP is modulated by other proteins.

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PRIOR PUBLICATIONS

Chiyuan Chen, Robert E. Lewis, and Michael A. White (2007). IMP modulates KSR1-dependent multivalent complex formation to specify ERK1/2 pathway activation and response thresholds. In Review.

Sharon A. Matheny, **Chiyuan Chen**, Robert L. Kortum, Gina L. Razidlo, Robert E. Lewis, and Michael A. White (2004). Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. *Nature*, **427**, 256-260.

Chiyuan Chen, and Michael A. White (March 2007). Molecular Mechanisms of IMP Function in Ras Signaling. Abstract, Proteomics and Cell Signaling (X6), Keystone Symposia.

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LIST OF ABBREVIATIONS

ATP	adenosine 5’-triphosphate
CNK	Connector Enhancer of KSR
CMV	Cytomegalovirus
CRD	cystein-rich domain
C-TAK1	Cdc25C-associated kinase
EGF	epidermal growth factor
ERK	Extracellular Regulated Kinase
FBS	Fetal bovine serum
GAP	GTPase-activating protein
GDP	guanosine 5’-diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine 5’-triphosphate
HA	hemagglutinin
HEK293	Human embryonic kidney cell line
Hog	high osmolarity glycerol
Hsp	heat shock protein
IMP	Impedes Mitogenic signal Propagation
KSR	Kinase Suppressor of Ras

MAP3K	MAPK kinase kinase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MS	mass spectrometry
PCR	polymerase chain reaction
PI3K	Phosphatidyl inositol 3’ kinase
RalGDS	Ral guanine nucleotide dissociation stimulator
Ras	rat sarcoma
RBD	Ras-binding domain
RING	Really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
siRNA	small interference RNA
Ste	Sterile
SUMO	Small ubiquitin-like modifier
Sur-8	Suppressor of Ras-8
ZnF_UBP	Ubiquitin carboxy-terminal hydrolase-like zinc finger

CHAPTER ONE

Introduction

Molecular Architecture in Signal Transduction

Eukaryotic cells receive diverse stimuli from their environment, such as growth factors, cytokines and hormones, which influence cellular processes at the level of metabolism, gene expression, cell division, morphology, and cell fate. In the past, numerous studies have been done and many signal transduction networks have been described to clarify how cells respond to different stimuli. However, little is known about how cells coordinate different responses to display different behaviors specifically and efficiently under complex environments since *in vivo* cells are always exposed to a diverse pool of extracellular signals.

To answer the above question, research could be narrowed down by taking the advantage that cells often utilize some important proteins or pathways to control multiple different activities. Studying how the same protein or pathway produces discrete outcomes could greatly increase our information about achieving specificity and efficiency in signal transduction. For example, in yeast, mating pathway and osmolarity pathway share the same core MAPKKK Ste11p to induce two non-related cell activities: mating and surviving osmotic stress. To gain the specificity and efficiency of two different signaling through Ste11p, two scaffold proteins are utilized to control the activation of those two discrete Ste11p

involved kinase cascades respectively. In mating pathway, scaffold protein Ste5p associates with Ste11p, MAPKK Ste7p and MAPK Fus3p, and regulates the formation and activation of Ste11p/Ste7p/Fus3p kinase complex in response to mating pheromone. Similarly, the MAPKK Pbs2p functions as a scaffold with interacting with Ste11p and the MAPK Hog1p, and controls Ste11p/Pbs2p/Hog1p module engagement in response to osmotic stress (Elion, 2001). Therefore, by inducing discrete complexes formation and keeping components of a cascade in close proximity to each other, scaffold proteins enhance Ste11p activating different downstream signaling both specifically and efficiently in response to appropriate physiological stimulation.

Another example is the small G protein Ras, activation of which induces activation of several effector proteins and results in many different even reverse fundamental cellular processes such as proliferation and differentiation, survival and apoptosis. Although structural studies have shown that the switch regions in Ras are very mobile and maybe account for its ability to bind different effectors, it is not known yet that how these effectors are specifically activated in response to distinct ligands. A couple of reasons make early free diffusion hypothesis unlikely. First, it is insufficient to achieve the optimal concentration of specific effectors at the action sites to result in a rapid response following stimulation regarding random diffusion rates for the effectors to be recruited to the action sites. Second, free diffused effectors could potentially cause “leaky” signal in cells and affect

other spatial and temporal regulation processes such as positive or negative feedback signaling. To overcome these two disadvantages, recent models suggested that some effectors and their downstream signal transducers may exist as complexes in cells and could be recruited to the action sites in response to stimulation. Considering the fact that some scaffold proteins have been found to associate with both certain effectors and their downsteam molecules, these recent models look more and more attractive.

The above two examples indicate that not only the scaffold proteins provide an important regulation process for signaling transduction but also there is a fine molecular architecture in cells. The temporal and spatial regulation of different complexes formation may be a key mechanism to control the specificity and efficiency of signaling flowing through this architecture.

Ras GTPases and Oncoproteins

Ras proteins are monomeric guanine nucleotide-binding proteins that are involved in signal transduction. In mammals, there are three major Ras isoforms: Ha-Ras, Ki-Ras, and N-Ras. All Ras proteins belong to the Ras small GTPases superfamily, which includes small G proteins with molecular masses of 20-25 kD and serves as molecular switches to regulate cell survival, growth, morphogenesis, migration, cytokinesis and trafficking. Ras is the first small GTPase to be discovered and there are now approximately 150 different small GTPases that

have been identified in mammalian cells. Like other GTPases, Ras proteins cycle between active (GTP-bound) and inactive (GDP-bound) state. Under basal conditions, Ras proteins are GDP-bound and can not interact with their effector molecules. Upon stimulation, guanine-nucleotide exchange factors (GEFs) catalyze the replacement of GDP with GTP. These GTP-bound Ras proteins become active, and able to bind multiple downstream effectors including RAF family serine/threonine kinases, phophatidylinositol 3-kinase (PI3K), and RalGDS, a GEF for the small G protein Ral (Shields et al., 2000). The active state Ras is transient because GTPase activating proteins (GAPs) can stimulate its intrinsic GTPase activity and cause GTP hydrolysis.

In normal cells, Ras activity is controlled by upstream cell-surface receptor stimulation. For example, when the receptors are stimulated by a hormone, Ras is activated and transduces signal to result in cell growth. If the receptors are not stimulated, Ras is not activated and so the downstream cell growth will not be initiated. However, in about 30 percent of cancers including 50 percent of colon cancers and 90 percent of pancreatic cancers (Bos, 1989), *Ras* gene is mutated so that it is permanently switched on. As a correlated result, these cancer cells keep growing regardless of whether receptors on the cell surface are activated or not. It has been suggested that Ras activating mutations significantly contribute to cell transformation processes presumably by providing continuous

mitogenic signals that release tumor cells from reliance on external growth stimulation (Hahn and Weinberg, 2002).

RAF Proteins Functions

RAF family proteins are the first and also most extensively studied Ras effector molecules. In vertebrates there are three isoforms: A-RAF, B-RAF, and C-RAF (Marais and Marshall, 1996). All RAFs are composed of an N-terminal regulatory domain, which includes two conserved regions, CR1 and CR2, and a C-terminal kinase domain, CR3 (Figure 1.1) (Wellbrock et al., 2004).

Genetic studies in mice have revealed non-redundant developmental functions of RAF isoforms. A-Raf-deficient mice display neurological and intestinal abnormalities, depending on the genetic background (Pritchard et al., 1996). Mice lacking B-RAF have defects in both neural and endothelial cell lineages and die around embryonic day 12 (E12) (Wojnowski et al., 1997). *C-RAF*$^{-/-}$ embryos show general growth retardadation and die at midgestation with anomalies in the placenta, lungs, skin and liver (Mikula et al., 2001; Wojnowski et al., 1998). Disruption of both *BRAF* and *CRAF* abrogates the differentiation of all embryonic lineages, but it has no arresting effect on the cell proliferation and implantation of the embryo (Wojnowski et al., 2000). It was also shown that loss of one additional Raf allele (*C-RAF*$^{-/-}$/*B-RAF*$^{+/-}$ or *C-RAF*$^{+/-}$/*B-RAF*$^{-/-}$) increases dramatically the extent of abnormalities and leads to the death of 90% of the

embryos before E10.5. *A-RAF*$^{-/-}$*/C-RAF*$^{-/-}$ double knockout mice show a generalized reduction in proliferation without change in apoptosis, and die at E10.5 (Mercer et al., 2005). Although it is difficult to interpret these studies in terms of functional differences among RAF proteins at the molecular level, they clearly suggested unique physiological roles for A-RAF, B-RAF, and C-RAF and indicated that there might be early developmental cooperation between all three RAF isoforms.

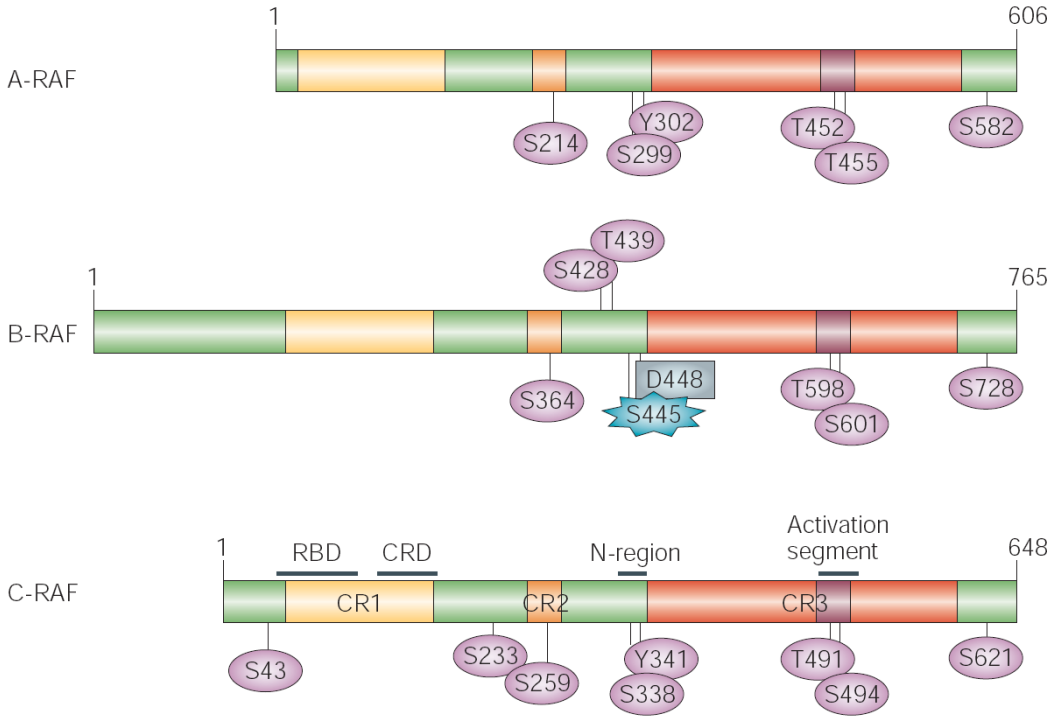


Figure 1.1 Structure of the RAF proteins (Wellbrock et al., 2004).

Recently it was found that B-RAF is highly mutated in human cancers including 30-60 percent of melanoma, 30-50 percent of thyroid cancer, 5-20 percent of colorectal cancer and 30 percent of ovarian cancer. The most common mutations elevate B-RAF kinase activity, indicating that *B-RAF* is an important human oncogene (Davies et al., 2002). This discovery combined with another fact that both *B-RAF* and *RAS* mutations are restricted to the same tumor types and usually mutually exclusive (Gustafsson et al., 2005) suggests that these two genes are in the same oncogenic signaling pathway and that RAS functions to activate B-RAF in these tumors.

RAF Proteins Regulation

Although multiple regulation mechanisms have been proposed, the whole activation process of RAF proteins has not been clearly elucidated. In the case of C-RAF, three main mechanisms of activation have been proposed: membrane localization, phosphorylation, and oligomerization.

It has been shown that C-RAF activation requires recruitment to the inner leaflet of the plasma membrane and may involve binding to Ras. The interaction with Ras is mediated by two short regions in C-RAF CR1 domain: Ras-binding domain (RBD) and cystein-rich domain (CRD) (Figure 1.1) (Dhillon and Kolch, 2002; Wellbrock et al., 2004). At the membrane, several important activation sites in C-RAF are phosphorylated by membrane-bound kinases including S338,

possibly by p21 activated kinases PAK1 and PAK3; Y341, possibly by Src and Src-family kinases. These two residues are located in a short region at the N-terminus of CR3 domain, which is called N-region. It has been proposed that the negative charge in this region could release the inhibition of N-terminus on the kinase domain. T491 and S494 are the other two essential activation sites and located in the activation region in the CR3 domain. The corresponding kinases of these two sites have not been identified. Except the activation sites, some inactivation phosphorylation sites are also important for regulation of C-RAF activation. For example, S259 on C-RAF is a target for inhibitory phosphorylation by Akt and PKA, which provides a 14-3-3 binding site, and must be dephosphorylated by phosphatases PP1 or PP2A to allow mitogenic activation of C-RAF (Chong and Guan, 2003; Dhillon et al., 2002; O'Neill and Kolch, 2004; Ory et al., 2003). Besides membrane localization and phosphorylation, recent studies suggested that oligomerization is also an essential step for optimal C-RAF activation (Farrar et al., 1996; Goetz et al., 2003; Luo et al., 1996; Weber et al., 2001). All these complicated processes with involvement of other chaperone/adaptor proteins including 14-3-3, heat shock protein 90 and 50 (Hsp90 and Hsp50), lead to a conformational change in C-RAF, whereby the regulatory N-terminus dissociates from the C-terminal catalytic domain, resulting in an activated kinase.

All important activating phosphorylation sites of C-RAF are conserved in A-RAF (Figure 1.1) (Wellbrock et al., 2004) and it is believed that A-RAF is activated by the similar mechanisms to C-RAF (Marais et al., 1997). However, regulation of B-RAF is different. The biggest difference is the regulation through the N region. In B-Raf, the corresponding site of Y341 in C-RAF is an aspartic acid (D448, Figure 1.1) (Wellbrock et al., 2004). Although the equivalent site of S338 in C-RAF is conserved in B-RAF (S445), it is constitutively phosphorylated (Mason et al., 1999). Therefore, whereas the N regions in C-RAF and A-RAF must be phosphorylated for those kinases to get activation, B-RAF bypasses these regulatory phosphorylation events, which also likely explains why B-RAF has a higher basal activity than the other two RAF proteins and is frequently mutated in human cancers and carcinomas.

RAF/MEK/ERK Kinase Module

RAF/MEK/ERK is the most extensively studied Ras effector pathway, and has been shown to be involved in proliferation, differentiation, survival, apoptosis, transformation, and other cellular processes (Pearson et al., 2001).

So far MEK is the best characterized and also dominant substrate for all Raf proteins. There are two isoforms of MEK: MEK1 (44 kD) and MEK2 (45 kD). Although MEK1 and MEK2 are 85% identical overall and greater than 90% identical in their catalytic cores, in many cell lines MEK1 was described as the

main RAF-activated MAPKK (Jelinek et al., 1994; Wu et al., 1996; Xu et al., 1997). MEK1 and 2 are activated by phosphorylation of two serine residues in the activation loop (Alessi et al., 1994; Yan and Templeton, 1994; Zheng and Guan, 1994) and contain a proline-rich sequence that is required for recognition and activation by RAF proteins (Catling et al., 1995). All three RAF isoforms can activate MEK proteins. However, some results from genetic model systems and transfection experiments suggested RAF family members can preferentially interact with and activate MEK1 or MEK2 under normal condition. A-RAF, the least well characterized member of RAF family, seems only activates MEK1 whereas C-RAF can activate both MEK1 and MEK2 equally well (Wu et al., 1996). B-RAF binds to both MEK1 and MEK2 but activates MEK1 better (Reuter et al., 1995). It is also reported that B-RAF might phosphorylate MEK1 approximately 10 times more efficiently than C-RAF and at least 500 times more efficiently than A-RAF (Pritchard et al., 1995).

As the only characterized substrates of MEK1 and MEK2 to date, ERK1 and ERK2 have been identified as ubiquitous Ser/Thr kinases that participate in many signaling processes. They are proteins of 44 kD (ERK1) and 42 kD (ERK2) with nearly 85% identity overall. ERK proteins can be activated by a variety of growth factors and mitogens through Ras-RAF-MEK pathway and relay the signaling information to multiple cytoplasmic, membrane and nuclear substrates (Pouyssegur et al., 2002). Both MEK proteins have been shown to fully activate

ERK1/2 in vitro (Robinson et al., 1996; Zheng and Guan, 1993). Activation of these kinases occurs as a result of phosphorylation of the Thr and Tyr residues in a Thr-X-Tyr (TXY) signature motif. The unique interaction between MEK and ERK proteins might provide a mechanism to regulate ERK cellular localization and keep specificity of signal transduction from MEKs to ERKs (Fukuda et al., 1997).

Scaffolding Proteins

The diversity of physiologic functions regulated by the Ras-RAF-MEK-ERK signaling cascade raises the question: What actually determines the specificity of the signals through this pathway? Although the answer of this question looks still far away from our knowledge territory, it is no doubt that a fine regulation system exists to control the activity of this pathway both spatially and temporally. So far a lot of regulation processes about RAF activity and RAF/MEK/ERK signaling have been characterized. Besides the protein-protein interaction between the key players in the pathway, an additional level of regulation appears to be provided by accessory scaffolding proteins, which are essential for signal transduction through this pathway. Certain observations suggested that these proteins are able to physically interact with both upstream and downstream molecules such as kinases and substrates, and recruit them into specific signaling complexes (Garrington and Johnson, 1999). Formation of these

signaling complexes may not only provide a spatial and temporal regulation mechanism for pathway activity, but also insulate particular pathways from nonproductive crosstalk with homologous signaling constructs.

Several scaffolding proteins have been identified and characterized to various extents within the past decade. The initial discoveries were made by genetic screens in *Caenorhabditis elegans* and *Drosophila melanogaster*. In *C. elegans*, activation of Ras/ERK pathway is essential for multiple developmental events including vulval induction, which is the best characterized phenotype of *C. elegans* development. Hyperactivation of this pathway during development causes extra vulval tissue (Multivulva or Muv phenotype) whereas its hypoactivation results in no vulva (Vulvaless or Vul phenotype) (Sternberg and Han, 1998). Similarly, Ras/ERK pathway plays an important role in the development process of *Drosophila* eye. Over-stimulation of this pathway results in a rough eye phenotype due to the presence of extra R7 cells while its insufficient stimulation results in the same phenotype but due to missing R7 cells (Kornfeld et al., 1995; Raabe, 2000; Sieburth et al., 1998; Sundaram and Han, 1995; Therrien et al., 1995; Therrien et al., 1998).

By using the phenotypes described above as readouts, one especially productive forward genetic screen has been used to identify components and regulators in the MAPK cascade in worms and flies, which is to look for suppressors of the Ras gain-of-function Muv or rough eye phenotype. Except key

components of Ras/ERK pathway, those screens identified numerous positive regulators of this cascade including some important scaffolding proteins such as Kinase Suppressor of Ras-1 (KSR1) in both *C.elegans* and *Drosophila* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995), Suppressor of Ras-8 (SUR-8) in *C.elegans* (Sieburth et al., 1998), and Connector-Enhancer of KSR (CNK) in *Drosophila* (Therrien et al., 1998).

Kinase Suppressor of Ras

Kinase Suppressor of Ras-1 (KSR1) was first identified in Ras-dependent genetic screens in *Caenorhabditis elegans* and *Drosophila melanogaster* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995), and homologous proteins were also found in mammals (Therrien et al., 1995). Genetic epistasis analysis indicated KSR is a positive regulator in Ras/ERK pathway, which acts between Ras and RAF or in a pathway parallel to RAF. KSR shares highly structural similarities with RAF proteins including a cysteine-rich motif (CA3) which is present in the CR1 domain of RAF proteins, a serine/threonine-rich region (CA4) that resembles the CR2 of RAFs, and a putative kinase domain (CA5) which is around 35% identical to those of RAF proteins (Figure 1.2) (Kolch, 2005). However, there are also several important structural differences between KSR and RAF proteins: 1) KSR has a unique CA1 domain at its N-terminus; 2) KSR does not contain the Ras-binding domain as RAFs; 3) In the

ATP binding pocket of KSR putative kinase domain (CA5), an arginine takes place of a lysine, which is involved in the phosphotransfer reaction and is usually required for enzymatic activity (Therrien et al., 1995).

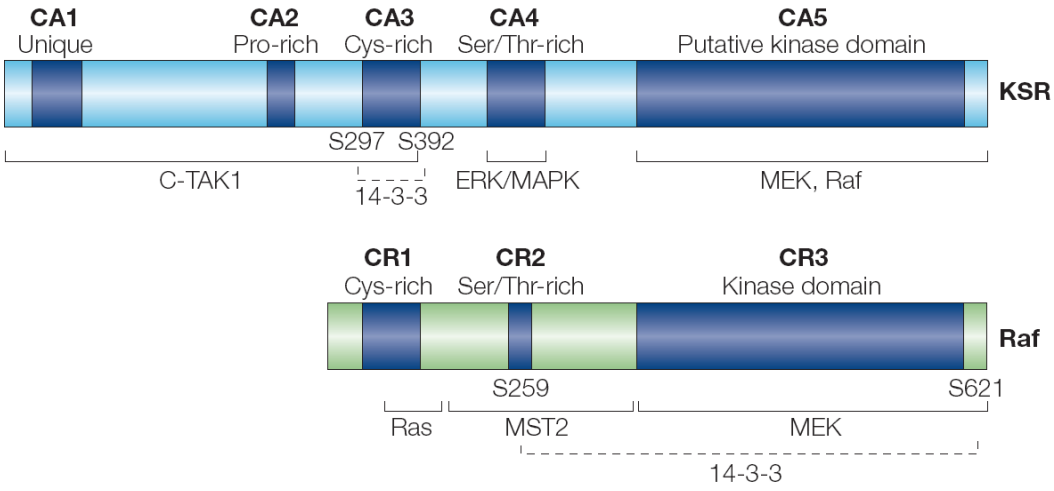


Figure 1.2 Domain structure of KSR and RAF proteins (Kolch, 2005).

The scaffolding activity of KSR is suggested by its ability to directly interact with multiple components and regulators of Ras/ERK pathway. Biochemistry studies have shown that KSR is able to interact with C-RAF (Therrien et al., 1996; Xing et al., 1997) and MEK (Denouel-Galy et al., 1998; Yu et al., 1998) through CA5 domain, ERK through FXFP motif in CA4 domain (Cacace et al., 1999; Jacobs et al., 1999; Muller et al., 2000), the γ subunits of heterotrimeric G proteins through CA3 domain (Bell et al., 1999), and 14-3-3

proteins through two phophorylation sites in CA3 domain (Cacace et al., 1999; Xing et al., 1997). More importantly, recent studies showed that KSR is likely required to couple RAF to upstream kinases, indicating that its scaffolding function may be essential for RAF activation (Anselmo et al., 2002; Douziech et al., 2006).

Consistent with the scaffolding function, the biological effects of KSR vary dramatically, depending on the level of KSR protein expressed (Cacace et al., 1999). Studies showed that KSR functions as a positive regulator of Ras signaling when expressed at low or near physiological levels (Cacace et al., 1999; Muller et al., 2000; Therrien et al., 1996), whereas it negatively regulates Ras signaling at high levels of expression (Cacace et al., 1999; Denouel-Galy et al., 1998; Joneson et al., 1998; Sugimoto et al., 1998; Yu et al., 1998). This property of scaffolding proteins has been suggested by studies about yeast protein Ste5p (Figure 1.3) (Ferrell, 2000): A scaffolding protein can only increase the amount of signaling output produced by a cascade when its concentration is within a limited range; If the scaffold concentration underachieves or exceeds that of the kinases that it coordinates, the output of the cascade will decrease. A prediction of this model is that coproduction of KSR and its binding kinases should reverse high-level-expression caused KSR inhibitory function into a stimulatory effect. This has been verified in *Drosophila* S2 cells (Nguyen et al., 2002; Roy et al., 2002; Stewart et al., 1999). The results showed that overexpressed KSR protein strongly

stimulates RAF mediated MEK activation in a Ras-dependent manner when RAF and MEK are coexpressed. Furthermore, KSR stimulatory effect depends on its interaction with both RAF and MEK. Colocalization of KSR and RAF is sufficient to induce MEK activation. Results of gel filtration assays also showed KSR and the kinases in the ERK pathway coexist in very high molecular weight complexes (250~500 kD in mouse brain lysate or >700 kD in 293T cells) that can not be detected in the absence of KSR (Nguyen et al., 2002; Stewart et al., 1999).

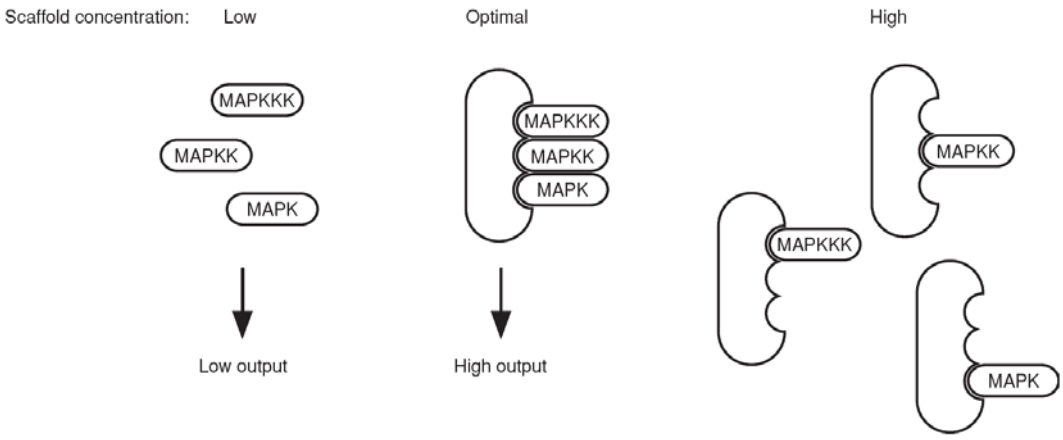


Figure 1.3 Signaling down a scaffolded protein kinase cascade (Ferrell, 2000).

Similarly as RAF proteins, KSR has been shown to translocate from the cytoplasm to the plasma membrane in response to Ras activation (Michaud et al., 1997; Xing et al., 1997) or ligands that activate G protein-coupled receptors (Bell

et al., 1999). In both cases, the CA3 domain, a cysteine-rich region homologous to the CR1 domain in RAF proteins (Figure 1.2) (Kolch, 2005), is required for KSR translocation (Bell et al., 1999; Michaud et al., 1997). These results suggested that mitogenic signaling could trigger the membrane localization of KSR, which might serve to bring MEK in close proximity to RAF, thus allowing RAF to phosphorylate MEK (Figure 1.4) (Morrison, 2001; Roy and Therrien, 2002). Further study showed that C-TAK1, a Cdc25C-associated kinase, regulates KSR localization by phosphorylating Ser297 and Ser392 on KSR, which in turn enable binding by 14-3-3 proteins (Muller et al., 2001). Strikingly, mutation of Ser392 constitutively targets KSR to the plasma membrane, accompanied by membrane colocalization of KSR, MEK and ERK proteins. Moreover, the KSR S392A mutant has enhanced biological activity to augment Ras-dependent *Xenopus* oocytes maturation. These findings indicated C-TAK1 can negatively regulate KSR scaffolding function by phosphorylating Ser392, which might result in 14-3-3 mediated sequestration of the KSR–MEK complex in the cytoplasm. Consistent with these results, Ory et al. showed that the serine/threonine protein phosphatase PP2A interacts with KSR and may regulate KSR localization by dephosphorylating Ser392 (Ory et al., 2003). Treating NIH3T3 cells with a PP2A inhibitor reverses PDGF induced wild-type KSR, but not KSRS392A, translocation to the plasma membrane. Overall, the above data provide insight into how scaffolding proteins may be regulated and underscore the importance of

controlling the formation of multiple protein complexes at specific intracellular locations (Figure 1.4) (Roy and Therrien, 2002).

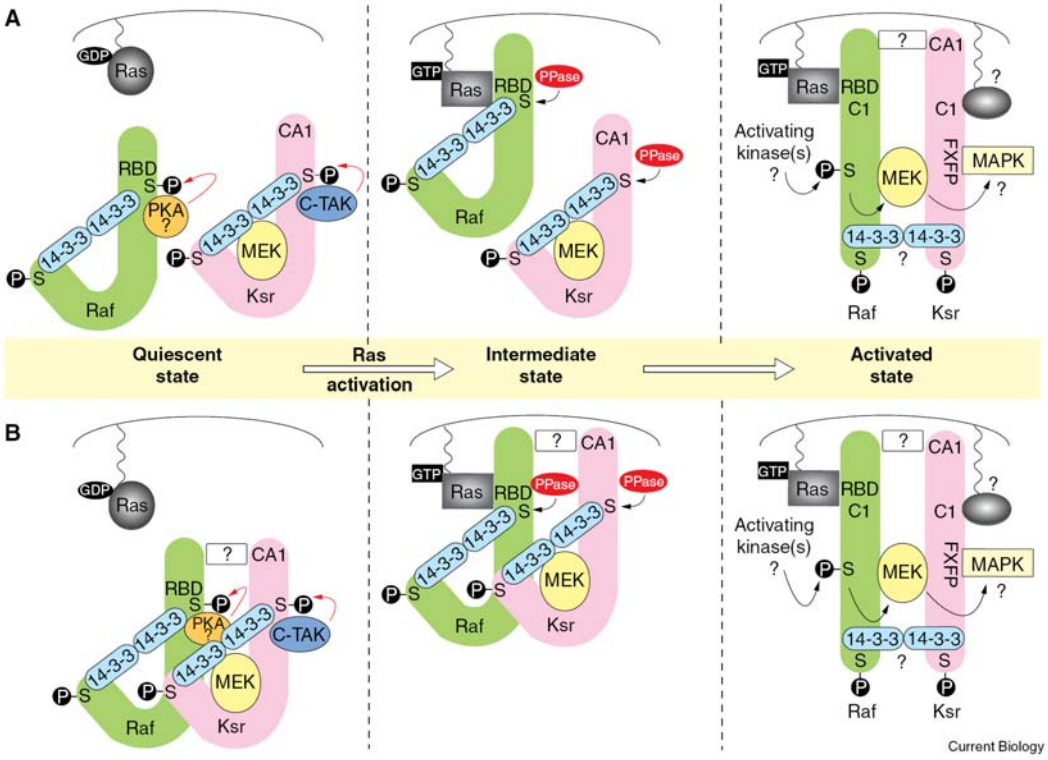


Figure 1.4 Models depicting the scaffolding function of KSR (Roy and Therrien, 2002).

Small Ubiquitin-related Modifier

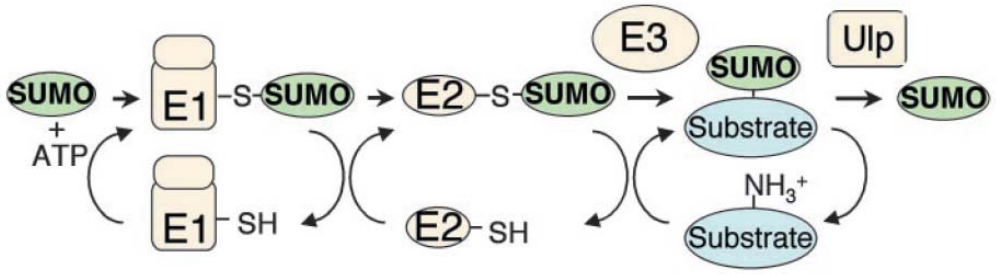
Small ubiquitin-related modifier (SUMO) is a family of proteins which are covalently attached to lysine residues in target proteins as post-translational

modifications. There are three isoforms of SUMO proteins in mammals: SUMO1 (also known as sentrin, Smt3c, PIC1, GMP1, and Ubl1), SUMO2 (also known as sentrin3 and Smt3a), and SUMO3 (also known as sentrin2 and Smt3b) (Hay, 2005).

Like ubiquitin and other ubiquitin-like proteins (Ubls), SUMO conjugation process (sumoylation) involves the activating enzyme (E1), the conjugating enzyme (E2), and the ligase (E3) while the reversible attachment of SUMO is controlled by ubiquitin-like proteases (Ulps) (Figure 1.5) (Johnson, 2004). In a sumoylation process, an E1 first activates SUMO in an ATP-dependent manner and forms a convalent intermediate with SUMO. Then the active SUMO is transferred to the SUMO specific E2 called Ubc9. Finally SUMO is transferred from Ubc9 to a substrate with the assistance of an E3. SUMO is usually conjugated to lysine residues in a short consensus sequence ΨKXE, where Ψ is a hydrophobic amino acid, generally valine, leucine, or isoleucine; K is the lysine residue where SUMO is covalently attached; X is any residue; and E is a glutamic acid. The attached SUMO can also be cleaved by enzymes of the Ulp family, which helps to maintain the balance of sumoylation process in cells.

To date, although multiple proteins have been reported to get sumoylated in cells, in many cases the function of SUMO modification still remains unclear. It has been shown that sumoylation is involved in diverse cellular processes, including nuclear transport, transcriptional regulation, chromosome organization

and function, DNA repair, and signal transduction (Johnson, 2004). Studies about sumoylation in ERK pathway revealed that two components of the pathway could be sumoylated: ERK upstream kinase – MEK (Sobko et al., 2002) and ERK downstream transcription factor – Elk-1 (Yang et al., 2003). However, function studies suggested that sumoylations of MEK and Elk-1 have different contribution to the signaling transduction through ERK pathway. In *Dictyostelium*, sumoylation of MEK1 is required for its localization in cytosol and cortex in reponse to chemoattractant stimulation, which indicated that SUMO may play a positive role in chemoattractant-mediated ERK stimulation to control chemotaxis (Sobko et al., 2002). On the contrary, in mammalian cells, SUMO conjugation and phosphorylation by ERK counteracts with each other to regulate Elk-1 transcriptional activity, which suggested that SUMO may negatively regulate the downstream signaling of ERK (Yang et al., 2003).



	E1	E2	E3	SUMO−cleaving protease
S. c.	Aos1·Uba2	Ubc9	Siz1 Siz2 (Nfi1)	Ulp1 Ulp2 (Smt4)
H. s., *M. m.,* *R. n.*	Aos1·Uba2 (SAE1,Sua1·SAE2)	Ubc9	PIAS1 (GuBP) PIAS3 (KChaP) PIASxαβ (ARIP3,Miz1) PIASy RanBP2 (Nup358) Pc2	SENP1 SENP2 (Axam,SuPr-1,SMT3IP2) SENP3 (SMT3IP1) SENP6 (SUSP1)

Figure 1.5 The SUMO conjugation pathway (Johnson, 2004). Enzymes present in *S. cerevisiae* (*S.c.*) and in human (*H.s.*), mouse (*M.m.*), and rat (*R.n.*) are listed.

CHAPTER TWO

IMP Modulates Multiple Mitogenic Complexes Formation to Specify ERK1/2 Pathway Activation and Response Thresholds

Abstract

The RAF/MEK/MAP Kinase signal transduction cascade is a key Ras effector pathway that mediates diverse cellular responses to environmental cues, and makes a major contribution to Ras-dependent oncogenic transformation. Recently we identified a novel Ras GTPase effector protein IMP (Impedes Mitogenic signal Propagation) that limits formation of RAF/MEK complexes by inactivation of KSR1, a scaffold/adaptor protein that couples activated RAF to its substrate MEK (Matheny et al., 2004). We further examine the mechanism underlying IMP inhibitory function in mitogenic signaling. We show that IMP inhibits KSR1 homooligomerization to block KSR1-dependent B-RAF coupling to MEK. IMP also suppresses B-RAF/C-RAF heterooligomerization, which may be essential for both C-RAF activation and C-RAF coupling to MEK. Importantly, we show that IMP affects C-RAF-dependent MEK activation, and C-RAF interactions with KSR1, 14-3-3, and heat shock protein 90 (Hsp90). Our observations indicate that IMP regulates both C-RAF kinase activation and functional coupling of active kinases to downstream substrates to modulate signal

propagation by targeting assembly of multiple mitogenic complexes involving RAF family proteins, KSR1, 14-3-3, and Hsp90.

Introduction

Ras/RAF/MEK/ERK pathway has been shown to be involved in proliferation, differentiation, survival, apoptosis, transformation, and other cellular processes (Pearson et al., 2001). Regarding the impact of this pathway on various human cancers and diseases, considerable efforts have been devoted to address how the signaling through this pathway is finely regulated. Among all the proposed and examined mechanisms, the essential role of multiple mitogenic complexes formation through protein-protein interactions involving key players, scaffolds and other regulators in the pathway has been explored (Kolch, 2005). Although most of these complexes have been extensively studied in the past years, some important aspects of their formation still remain unknown including the mysterious and complicated RAF activation process.

To date, the whole activation process of RAF proteins has not been clearly elucidated although three main mechanisms of activation have been proposed including membrane localization, phosphorylation, and oligomerization. Recent studies in mammalian cells and *Drosophila melanogaster* suggested that formation of some complexes, such as B-RAF/C-RAF, KSR/RAF and CNK/RAF, may be directly involved in RAF activation process (Douziech et al., 2006;

Garnett et al., 2005; Rushworth et al., 2006; Wan et al., 2004). Besides, a couple of proteins have been reported to regulate B-RAF/C-RAF complex formation to affect RAF activation including Rheb (Karbowniczek et al., 2004; Karbowniczek et al., 2006; Yee and Worley, 1997), a Ras-homologous GTPase, and mixed-lineage kinase 3 (MLK3) (Chadee and Kyriakis, 2004a, b; Chadee et al., 2006), a MAPK kinase kinase (MAP3K).

Recently we identified a novel Ras GTPase effector protein IMP that negatively regulates MAPK signaling pathway by inhibiting signal propagation through RAF to its downstream substrates MEK1/2 (Matheny et al., 2004). Preliminary data also suggested IMP limits formation of RAF/MEK complexes and functions by inactivating KSR1, a scaffold/adaptor protein that couples activated RAF to MEK. Here, we investigate the mechanistic basis of IMP function. We show that KSR1 oligomerizes to couple KSR1/B-RAF and KSR1/MEK complexes. KSR1 promotes B-RAF/C-RAF heterooligomerization. IMP inhibits these oligomerization processes to block both C-RAF activation and functional coupling of active RAF proteins to their substrates MEK. Importantly, we found that it is C-RAF, not B-RAF, that is essential for EGF stimulated MEK activation in HEK293 and HeLa cells. IMP suppresses C-RAF-dependent, not B-RAF-dependent, mitogenic signaling in those cells. Finally, we show that IMP inhibits C-RAF interactions with KSR1, 14-3-3, and Hsp90. Our data suggest that formation of multiple mitogenic complexes involving RAF family proteins, KSR1,

14-3-3, and Hsp90, may be essential for both C-RAF activation and functional coupling of active kinases to downstream substrates. This property is engaged by IMP for modulation of signal amplitude.

Results and Discussion

To further investigate how IMP inactivates KSR1 to limit MEK activation and function, we examined the impact of IMP on KSR1/RAF/MEK complex formation and function. By immunoprecipitating ectopic KSR1 in HEK293 cells, we found that IMP does not inhibit endogenous B-RAF or MEK binding to KSR1 but inhibits KSR1-bound MEK phosphorylation (Figure 2.1A). Same phenomenon was found when KSR1 C-terminus (CA5, putative kinase domain) was immunoprecipitated (Figure 2.1A). Results of IMP immunoprecipitation assay show that B-RAF can not be detected in the IMP-immunoprecipitated complex while both KSR1 and MEK are detected in the same complex (Figure 2.1B). These observations suggest that KSR1/B-RAF and KSR1/MEK may exist as two different KSR1 complexes in cells and IMP could affect those two complexes interacting with each other to block signal propagation from RAF to MEK. One possible mechanism of the interaction between two KSR1 complexes is KSR1 oligomerization. By coexpressing two different tagged KSR1 proteins, we found FLAG-tagged KSR1 is pulled down with immunoprecipitated HA-tagged KSR1 (Figure 2.1C), indicating KSR1 protein oligomerizes in HEK293

cells. Oligomerization is also detected between KSR1 C-terminus and coexpressed IMP inhibits KSR1 C-terminus oligomerization (Figure 2.1D). The above observations suggest that instead of affecting RAF or MEK binding to KSR1, IMP could impair KSR1-dependent B-RAF coupling to MEK, which maybe result in the inhibition of signal propagation through RAF to MEK.

Considering KSR1 is structurally homologous to RAF family proteins, KSR1 homooligomerization is reminiscent to homo- or hetero- oligomerization of RAF family proteins (Farrar et al., 1996; Garnett et al., 2005; Luo et al., 1996; Rushworth et al., 2006; Wan et al., 2004; Weber et al., 2001). We examined whether IMP could affect B-RAF/C-RAF heterooligomerization. As shown in Figure 2.2, expression of IMP blocks B-RAF/C-RAF complex formation as detected by immunoprecipitation of epitope-tagged B-RAF (Figure 2.2A) or by immunoprecipitation of endogenous C-RAF from cells expressing either wild-type B-RAF or the low-activity oncogenic B-RAF mutant, G596R (Wan et al., 2004) (Figure 2.2B). We also examined native B-RAF/C-RAF complex formation when IMP was knocked down. As shown in Figure 2.2C, inhibition of IMP expression (Figure 2.2D) results in elevated levels of native B-RAF/C-RAF complex formation as well as MEK activation induced by EGF stimulation. Thus, our accumulative observations suggest that KSR1 and RAF family proteins could form different kinds of complexes through homo- or heterooligomerizations in

response to mitogen stimulation. IMP targets the assembly of these complexes to regulate mitogenic signaling through RAF to MEK.

In regard to the previous result that IMP inhibits C-RAF-BXB, a constitutively active mutant of C-RAF, -induced MEK activation (Matheny et al., 2004) and the current data that IMP inhibits B-RAF/C-RAF interaction, we examined whether B-RAF is also involved in C-RAF-BXB-induced MEK activation. As shown in Figure 2.3A, both wild-type and kinase-dead B-RAF are pulled down by immmunoprecipitating C-RAF-BXB. More importantly, coexpression of kinase-dead B-RAF could elevate C-RAF-BXB-induced MEK activation as well as wild-type B-RAF, indicating that the kinase activity of B-RAF is dispensable for fortifying C-RAF-BXB-induced signaling. The interaction between B-RAF and C-RAF-BXB was further confirmed by reciprocal immunoprecipitation (Figure 2.3B). To gain better insight into the contribution of kinase-dead B-RAF to C-RAF-BXB-induced MEK activation, we performed in vitro kinase assay to evaluate C-RAF-BXB kinase activity. As shown in Figure 2.3C, kinase-dead B-RAF strongly elevates C-RAF-BXB kinase activity, indicating that B-RAF can induce C-RAF-BXB activation by either coupling other kinases to C-RAF-BXB or leading an activity-related structural change of C-RAF-BXB. Coexpressed IMP partially inhibits kinase-dead-B-RAF-induced C-RAF-BXB activation (Figure 2.3C). Moreover, IMP inhibits C-RAF-BXB binding to wild-type or kinase-dead B-RAF (Figure 2.3D, 2.3E, and data not

shown), as well as wild-type- or kinase-dead-B-RAF-enhanced C-RAF-BXB-induced MEK activation (Figure 2.3C and data not shown), suggesting that IMP targets B-RAF/C-RAF-BXB complex formation to negatively regulate B-RAF-dependent, but not B-RAF-kinase-activity-dependent, C-RAF-BXB activation. Thus, our results not only provide a mechanism for IMP to regulate C-RAF-BXB-induced MEK activation, but also indicate a novel aspect of B-RAF function in C-RAF-dependent signaling events. The observations that B-RAF, especially the kinase dead form, could bind both C-RAF-BXB and MEK (Figure 2.3B) and fortify C-RAF-BXB-induced downstream signaling (Figure 2.3D) by enhancing C-RAF-BXB kinase activity (Figure 2.3C) are reminiscent to KSR functions in Ras/ERK pathway (Claperon and Therrien, 2007), suggesting that except its kinase-activity-related function, B-RAF may play a role as a scaffold protein like KSR to couple C-RAF to MEK as well as regulate C-RAF activation.

When we examined IMP effects on B-RAF/C-RAF interaction, we noticed that IMP inhibits two B-RAF oncogenic mutants, G596R and G466V, induced MEK activation but not wild-type B-RAF induced MEK activation (Figure 2.2B and data not shown). Recent discoveries from Marais group showed G596R and G466V are two low-kinase-activity mutants of B-RAF and can only activate MEK through C-RAF (Wan et al., 2004) whereas wild-type B-RAF are able to activate MEK through C-RAF or by itself (Garnett et al., 2005). Therefore, our results suggest that IMP inhibits low-kinase-activity-B-RAF-mutants-induced

MEK activation by interfering B-RAF/C-RAF complex formation. Moreover, the fact that IMP does not affect wild-type-B-RAF-induced signaling indicates that the inhibitory effects of IMP on mitogenic signaling may be C-RAF dependent.

To further characterize IMP effects on different RAF isoforms dependent signaling events, we first examined IMP effects on MEK activation induced by expression of B-RAF or C-RAF in HEK293 cells. We found that IMP suppresses both EGF-induced and overexpressed-C-RAF-enhanced endogenous MEK activation (Figure 2.4A) while IMP has no effect on B-RAF-induced MEK activation (Figure 2.4B). We further knocked down B-RAF or C-RAF in HEK293 cells and examined IMP effects on EGF induced MEK activation. As shown in Figure 2.4C, B-RAF knock-down does not affect EGF induced MEK activation (compare lane 3 and 7) and IMP still inhibits the signaling in those cells (compare lane 7 and 8). However, knocking down C-RAF causes decreased MEK activation induced by EGF (compare lane 3 and 11) and IMP does not further block the decreased signaling (compare lane 11 and 12). Consistent with these results, knocking down C-RAF in HeLa cells also results in decreased MEK activation induced by EGF stimulation. More importantly, inhibition of both IMP and C-RAF expression reverses the increased level of MEK activation caused by knocking down IMP protein alone (Figure 2.4D). These observations suggest that two novel aspects of EGF signaling: 1. It is C-RAF, not B-RAF, that is essential for EGF-induced MEK activation at least in 293 and HeLa cells; 2. IMP

preferentially inhibits C-RAF-dependent, not B-RAF-dependent, mitogenic signaling.

We show that IMP specifically blocks C-RAF-dependent mitogenic signaling. Considering IMP inhibits MEK phosphorylation in KSR1 complex but not B-RAF binding to KSR1 (Figure 2.1A), we also examined whether IMP could affect C-RAF binding to KSR1. As shown in Figure 2.5A, IMP blocks C-RAF interacting with KSR1. Moreover, coexpressed KSR1 also blocks C-RAF binding to IMP (Figure 2.5B), suggesting that C-RAF, KSR1, or IMP competitively interacts with each other. Regarding the fact that IMP function depends on KSR1 expression (Matheny et al., 2004), our results suggest that KSR1 may be involved in C-RAF-dependent, but not B-RAF-dependent, mitogenic signaling. To further examine KSR1 and IMP effects on B-RAF/C-RAF heterooligomerization, we coexpressed C-RAF, KSR1 and IMP proteins in 293 cells and immunoprecipitated C-RAF. Clearly, ectopic KSR1 protein is sufficient to induce B-RAF/C-RAF complex formation (Figure 2.5C, compare lane 2 and 4) and co-expressed IMP suppresses KSR1-induced B-RAF/C-RAF heterooligomerization (Figure 2.5C, compare lane 4 and 5). Same phenomenon was found when C-RAF-N' (1-330) is immunoprecipitated (Figure 2.5C). Since 14-3-3 protein and phosphorylation at serine 621 (S621) of C-RAF, which provides a binding site for 14-3-3, have been shown to be involved in both C-RAF activation (Jaumot and Hancock, 2001; Light et al., 2002; Tzivion et al., 1998; Yip-Schneider et al., 2000)

and B-RAF/C-RAF oligomerization process (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001), we examined whether IMP impacts 14-3-3 binding to B-RAF or C-RAF. Results show that IMP blocks 14-3-3 binding to C-RAF (Figure 2.5C, compare lane 2 and 3), but not B-RAF (Figure 2.2B). However, IMP does not affect 14-3-3 binding to C-RAF-N' (Figure 2.5C). These results indicate that IMP may regulate C-RAF activation by affecting both 14-3-3 binding to C-RAF C-terminus and B-RAF or KSR1 binding to both N- and C-terminus of C-RAF. Moreover, the fact that we did not detect an obvious change of phosphorylation at S621 of C-RAF (Figure 2.5C) by using a phospho-specific antibody (6B4) (Hekman et al., 2004) suggests that IMP may act downstream of phosphorylation at S621 to regulate C-RAF/14-3-3 complex formation. Noticeably, western blotting reveals IMP blocks a heat shock protein, Hsp90, binding to C-RAF (Figure 2.5C). Regarding that Hsp90 has been reported to be essential for RAF kinase activity (Grammatikakis et al., 1999; Jaiswal et al., 1996; van der Straten et al., 1997), these observations indicate that IMP may target formation of multiple C-RAF-involved complexes including B-RAF/C-RAF, C-RAF/14-3-3, and C-RAF/Hsp90 to regulate C-RAF activation process.

Overall, we have examined the mechanism underlying IMP inhibitory function in mitogenic signaling. We show that IMP targets KSR1 homooligomerization to regulate KSR1-dependent B-RAF coupling to MEK. IMP inhibits B-RAF/C-RAF heterooligomerization, which can be induced by KSR1

expression. We find that the kinase-dead form of B-RAF activates C-RAF-BXB by forming a complex with it, which is suppressed by IMP. Importantly, we show that IMP specifically affects C-RAF-dependent MEK activation and formation of multiple C-RAF-involved complexes including B-RAF/C-RAF complex, KSR1/C-RAF complex, C-RAF/14-3-3 complex, and C-RAF/Hsp90 complex. As a conclusion, our results reveal that there are multiple mitogenic complexes in cells including RAF family proteins, KSR1, 14-3-3 and Hsp90, formation of which may be essential for both C-RAF activation and signaling propagation from RAF to MEK. The capacity of IMP to limit the formation of such complexes highlights this regulatory step as a key axis of control for signal modulation.

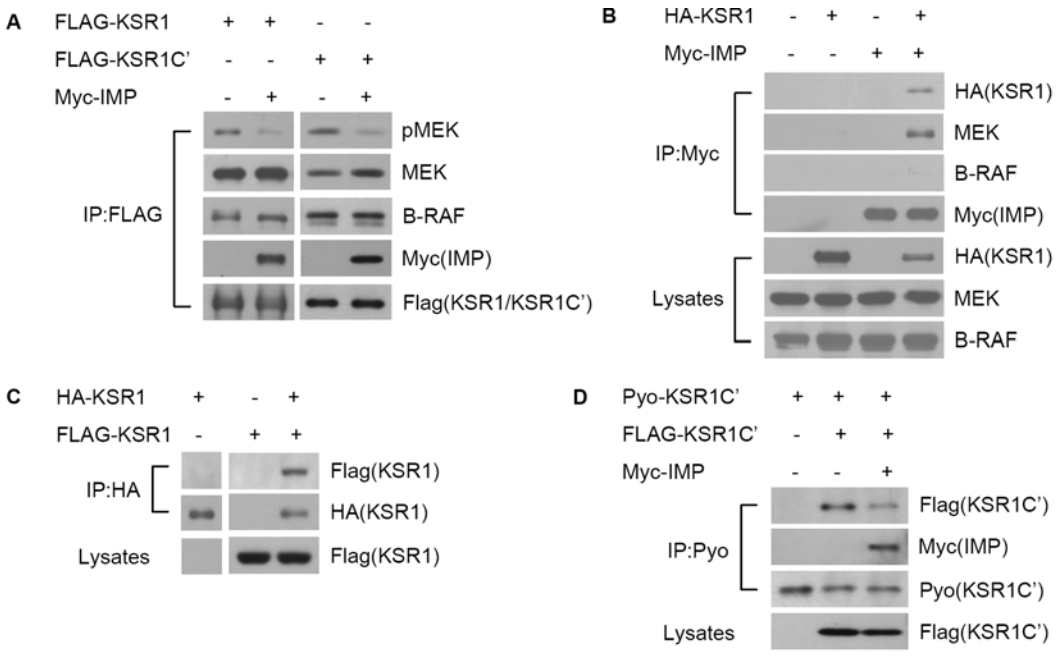


Figure 2.1 IMP blocks KSR1 homooligomerization to separate KSR1/B-RAF and KSR1/MEK complexes. A, IMP does not inhibit endogenous B-RAF or MEK binding to KSR1 but inhibits KSR1-bound MEK phosphorylation. FLAG-tagged KSR1 or KSR1C' was immunoprecipitated from HEK293 cells. Western blots for phospho-MEK (pMEK), MEK, B-RAF, Myc-IMP, and FLAG-KSR1/KSR1C' in FLAG-KSR1/KSR1C' immunoprecipitates are shown. B, Both KSR1 and MEK are detected in IMP immunocomplexes while B-RAF is not. Myc-IMP was immunoprecipitated from HEK293 cells. Upper: Western blots for HA-KSR1, MEK, B-RAF, and Myc-IMP in Myc-IMP immunoprecipitates. Lower: Western blots for HA-KSR1, MEK, and B-RAF in triton-soluble cell lysates. C, KSR1 oligomerizes in HEK293 cells. HA-KSR1 was immunoprecipitated from cells coexpressing FLAG-KSR1, HA-KSR1, or both. Upper: Western blots for FLAG-KSR1 and HA-KSR1 in HA-KSR1 immunoprecipitates. Lower: Western blot for FLAG-KSR1 in triton-soluble cell lysates. D, IMP inhibits KSR1 C-terminus homooligomerization in HEK293 cells. Pyo-KSR1C' was immunoprecipitated from cells coexpressing Pyo-KSR1C', FLAG-KSR1C', or Myc-IMP. Upper: Western blots for Pyo-KSR1C', FLAG-KSR1C', and Myc-IMP in Pyo-KSR1C' immunopricipitates. Lower: Western blot for FLAG-KSR1C' in triton-soluble cell lysates.

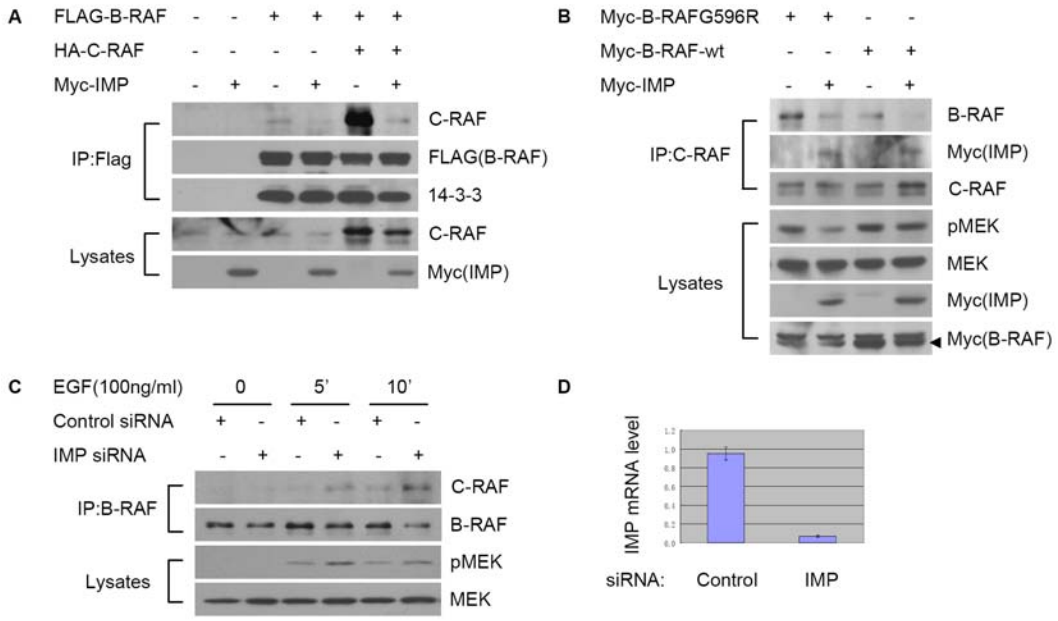


Figure 2.2 IMP inhibits B-RAF/C-RAF complex formation. A and B, Overexpressed IMP suppresses B-RAF/C-RAF complex formation. A, Immunoprecipitation of ectopic B-RAF protein from HEK293 cells coexpressing FLAG-B-RAF, HA-C-RAF, or Myc-IMP. Upper: Western blots for C-RAF, FLAG-B-RAF, and 14-3-3 in FLAG-B-RAF immunoprecipitates. Lower: Western blots for C-RAF and Myc-IMP in triton-soluble cell lysates. B, Immunoprecipitation of native C-RAF protein from HEK293 cells coexpressing B-RAFG596R, wild-type B-RAF (B-RAF-wt), or Myc-IMP. Upper: Western blots for B-RAF, Myc-IMP, and C-RAF in C-RAF immunoprecipitates. Lower: Western blots for phospho-MEK (pMEK), MEK, Myc-IMP, and Myc-B-RAF in

triton-soluble cell lysates. C and D, Inhibiting native IMP enhances EGF-induced B-RAF/C-RAF complex formation and MEK activation. C, Immunoprecipitation of endogenous B-RAF from HeLa cells transfected with control or IMP siRNA and stimulated with EGF (100ng/ml) for 5 min, 10 min, or left untreated as indicated. To allow accumulation of B-RAF/C-RAF complex, cells were pretreated with MEK inhibitor U0126 before EGF stimulation (Rushworth et al., 2006). Upper: Western blots for C-RAF and B-RAF in B-RAF immunoprecipitates. Lower: Western blots for phospho-MEK (pMEK) and MEK in triton-soluble cell lysates. D, Quantification of IMP mRNA level in cell samples shown in C. mRNA was extracted from duplicated cell samples shown in C and RT-PCR assay was performed as described in Materials and Methods. Values are representative of two independent experiments performed in duplicate and error bars represent standard deviation among replicates.

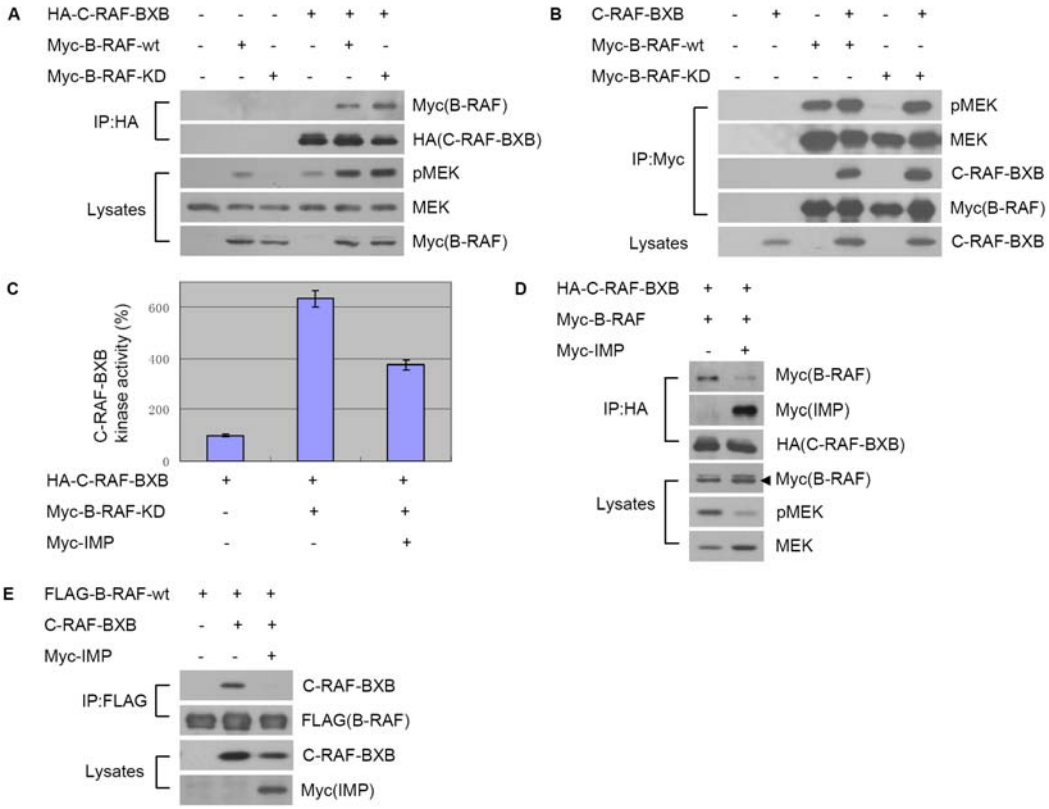


Figure 2.3 IMP blocks B-RAF/C-RAF-BXB complex formation to inhibit B-RAF-dependent C-RAF-BXB activation and C-RAF-BXB-induced MEK activation. A and B, Formation of B-RAF/C-RAF-BXB complex in HEK293 cells coexpressing wild-type B-RAF (B-RAF-wt), kinase-dead B-RAF (B-RAF-KD), or C-RAF-BXB. A, Immunoprecipitation of C-RAF-BXB. Upper: Western blots for Myc-B-RAF and HA-C-RAF-BXB in HA-C-RAF-BXB immunoprecipitates. Lower: Western blots for phospho-MEK (pMEK), MEK, and Myc-B-RAF in triton-soluble cell lysates. B, Immunoprecipitation of B-RAF. Upper: Western

blots for phospho-MEK (pMEK), MEK, C-RAF-BXB, and Myc-B-RAF in Myc-B-RAF immunoprecipitates. Lower: Western blot for C-RAF-BXB in triton-soluble cell lysates. C, IMP inhibits B-RAF-dependent C-RAF-BXB activation. C-RAF-BXB kinase activity, from protein immunoprecipitated from HEK293 cells expressing C-RAF-BXB alone or together with kinase-dead B-RAF (B-RAF-KD) or Myc-IMP, was measured in vitro using kinase-dead recombinant MEK as the substrate. Values are specific activity normalized to the activity of C-RAF-BXB expressed alone (arbitrarily set at 100%). Error bars represent standard deviation from the mean from three independent experiments. D and E, IMP inhibits B-RAF/C-RAF-BXB complex formation and C-RAF-BXB-induced MEK activation in HEK293 cells. D, Immunoprecipitation of C-RAF-BXB. Upper: Western blots for Myc-B-RAF, Myc-IMP, and HA-C-RAF-BXB in HA-C-RAF-BXB immunoprecipitates. Lower: Western blots for Myc-B-RAF, phospho-MEK (pMEK), and MEK in triton-soluble cell lysates. E, Immunoprecipitation of B-RAF. Upper: Western blots for C-RAF-BXB and FLAG-B-RAF in FLAG-B-RAF immunoprecipitates. Lower: Western blots for C-RAF-BXB and Myc-IMP in triton-soluble cell lysates.

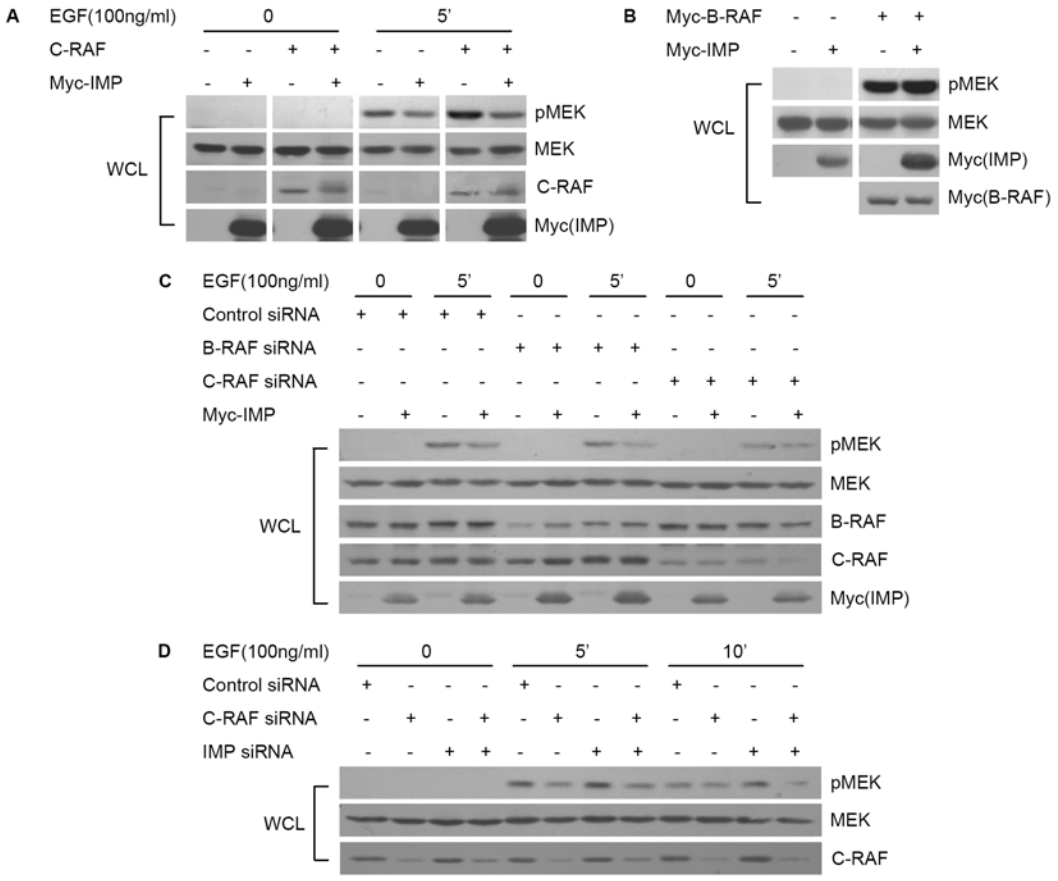


Figure 2.4 C-RAF function in mitogenic signaling is sensitive to IMP expression. A, IMP suppresses both EGF-induced and overexpressed-C-RAF-enhanced MEK activation. Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with C-RAF or Myc-IMP and stimulated with EGF (100ng/ml) as indicated. Western blots for phospho-MEK (pMEK), MEK, C-RAF, and Myc-IMP are shown. B, IMP does not affect B-RAF-induced MEK activation. Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with Myc-B-

RAF or Myc-IMP. Western blots for phospho-MEK (pMEK), MEK, Myc-B-RAF, and Myc-IMP are shown. C, Overexpressed IMP inhibits MEK activation in B-RAF-knock-down cells, but not in C-RAF-knock-down cells. Whole cell lysates (WCL) were collected from HEK293 cells transfected with Myc-IMP, control, B-RAF, or C-RAF siRNA, and stimulated with EGF (100ng/ml) for 5 min or left untreated as indicated. Western blots for phospho-MEK (pMEK), MEK, B-RAF, C-RAF, and Myc-IMP are shown. D, Inhibition of both IMP and C-RAF expression reverses the increased level of MEK activation caused by depleting IMP protein alone. Whole cell lysates (WCL) were collected from HeLa cells transfected with control, C-RAF, or IMP siRNA and stimulated with EGF (100ng/ml) for 5 min, 10 min or left untreated as indicated. Western blots for phospho-MEK (pMEK), MEK, and C-RAF are shown.

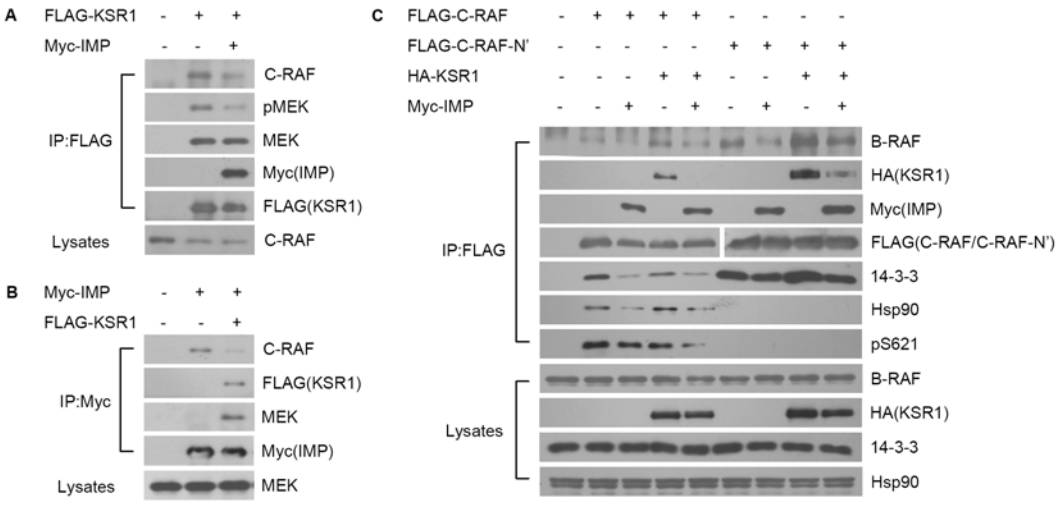


Figure 2.5 IMP inhibits C-RAF-involved multiple complexes formation. A and B, IMP blocks KSR1/C-RAF complex formation in HEK293 cells. A, Immunoprecipitation of KSR1. Upper: Western blots for C-RAF, phospho-MEK (pMEK), MEK, Myc-IMP, and FLAG-KSR1 in FLAG-KSR1 immunoprecipitates. Lower: Western blot for C-RAF in triton-soluble cell lysates. B, Immunoprecipitation of IMP. Upper: Western blots for C-RAF, FLAG-KSR1, MEK and Myc-IMP in Myc-IMP immunoprecipitates. Lower: Western blot for MEK in triton-soluble cell lysates. C, IMP suppresses formation of multiple C-RAF-involved complexes including KSR1-involved B-RAF/C-RAF complex, KSR1/C-RAF complex, C-RAF/14-3-3 complex, and C-RAF/Hsp90 complex. FLAG-tagged C-RAF or C-RAF-N' (1-330) was immunoprecipitated from HEK293 cells coexpressing FLAG-C-RAF/C-RAF-N', HA-KSR1, or Myc-IMP. Upper: Western blots for B-RAF, HA-KSR1, Myc-IMP, FLAG-C-RAF/C-RAF-N', 14-3-3, Hsp90 and phospho-C-RAFSer621 (pS621) in FLAG-C-RAF/C-RAF-N' immunoprecipitates. Lower: Western blots for B-RAF, HA-KSR1, 14-3-3, and Hsp90 in triton-soluble cell lysates.

Materials and Methods

Cell culture and Transfection. HEK293 cells were cultured in DMEM without sodium pyruvate (Gibco) with 10% FBS, and transfected with Lipofectamine and Plus Reagent (Invitrogen). HeLa cells were cultured in DMEM supplemented with 10% FBS, and RNAi was performed with Dharma*FECT* 1 (Dharmacon).

siRNAs. The following siRNA sequences were used. IMP-FW, GGACACAGCAGAGGAAAUUUU; IMP-RV, AAUUUCCUCUGCUGUGUCCUU; B-RAF-FW, AAGAGAUGAGAGACCACUCUU; B-RAF-RV, GAGUGGUCUCUCAUCUCUUUU; C-RAF-FW, GACGUUCCUGAAGCUUGCCUU; C-RAF-RV, GGCAAGCUUCAGGAACGUCUU; and Control-FW, AUGAACGUGAAUUGCUCAAUU; Control-RV, UUGAGCAAUUCACGUUCAUUU.

Plasmids. pCMV5-Myc-IMP, pcDNA3-HA-KSR1, pCMV5-FLAG-KSR1, pDCR-HA-RasG12V, pLNCX-FLAG-C-RAF, and pSRα-C-RAF-BXB have been previously described (Matheny et al., 2004). pCMV5-FLAG-KSR1C' (amino acids 540-873) (Joneson et al., 1998) and pcDNA3-Pyo-KSR1C' (amino acids 542-873) (Therrien et al., 1996) were gifts from Robert Lewis. pEFm-B-RAF,

pEFm-B-RAFG596R (Wan et al., 2004), and pEFHA-C-RAF-BXB (Garnett et al., 2005) were gifts from Richard Marais. pLNCX-FLAG-B-RAF and pLNCX-FLAG-C-RAF-N' (amino acids 1-330) (Tran et al., 2005) were gifts from Jeffrey Frost.

Antibodies. Antibodies against c-Myc (A-14, 9E10), HA (Y-11, F-7), B-RAF (H-145, F-7), C-RAF (C-12), and 14-3-3 β (FL-246) were from Santa Cruz. FLAG antibody was from Sigma. Pyo (Glu-Glu) antibody was from Delta Biolabs. Phospho-MEK and MEK antibodies were from Cell Signaling. Monoclonal antibody against C-RAF was from Transduction Laboratories. Monoclonal antibody against MEK was from BD Biosciences. Monoclonal antibody against Hsp90 (SPA-840) was from Stressgen. Phospho-C-RAFS621 antibody (6B4) (Hekman et al., 2004) was a gift from Ulf Rapp.

Immunoprecipitation. For coimmunoprecipitation, HEK293 cells were lysed 48 hr after transfection in modified RIPA buffer (20mM Tris, [pH 8.0], 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 20mM NaF, and protease inhibitors). Lysates were rotated for 20 min at 4°C, cleared by centrifugation at 17,000 × g for 30 min, and immunoprecipitated overnight with antibody-conjugated beads. Immunoprecipitates were washed four times in lysis buffer.

Kinase assay. C-RAF-BXB was immunoprecipitated and washed 3X in modified RIPA buffer, 2X in the same buffer plus 500mM NaCl, and 2X in 25mM HEPES 7.5, 10mM $MgCl_2$. The kinase reaction was performed as previously described (Matheny et al., 2004).

RT-PCR. Total RNA was prepared with the High Pure RNA Isolation kit (Roche). Reverse transcriptase reaction was done with the SuperScript First-Strand Synthesis System (Invitrogen). PCR amplification was carried out using Light Cycler (Roche). The following primers were used for PCR amplification: IMP-FW, TGCACGGTGTGTCTGGAG; IMP-RV, GCAAACAGGACACGTGGT. To quantify the transcripts in this study, parallel experiments were done by using the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) as the internal control. Values were normalized using GAPDH and analyzed using the relative quantification mathematical model (Pfaffl) as previously described (Whitehurst et al., 2007).

CHAPTER THREE

Isolating IMP Mutants Compromised for Ras Interaction

Abstract

IMP is a novel Ras effector which negatively regulates mitogenic signaling through Ras/ERK pathway. Preliminary results showed that Ras can inactivate IMP through induction of IMP auto-ubiquitination, facilitating KSR-dependent engagement of MEK by activated RAF (Matheny et al., 2004). Regarding Ras transduces signal to its downstream effectors through protein-protein interactions, one strategy to study the functions of Ras effectors is to isolate the mutants of these effectors which can not bind Ras and examine whether they still respond to upstream signaling. Here, we isolate some Ras-binding domain (RBD) mutants of IMP that are compromised for Ras interaction by error-prone PCR coupled with yeast two-hybrid screen. However, all examined IMP-RBD mutants compromised for Ras interaction in yeast still interact with Ras in HEK293 cells. Moreover, all examined full-length IMP mutants comprising the corresponding RBD mutations bind Ras in HEK293 cells and have the same effects on Ras12V-induced or C-RAF-BXB-induced MEK activation as wild-type IMP protein. These observations suggest that there may be other proteins mediating IMP interaction with Ras in mammalian cells but not in yeast. We show that a spontaneously appeared RBD mutant IMP-RBDS414G/K444E

does not bind Ras in HEK293 cells while the full-length mutant IMPS414G/K444E still interact with Ras and function as wild-type IMP. These results indicate that either there are other domains in IMP which can bind Ras besides RBD, or S414G/K44E destabilize the structure of RBD to prevent it from binding other proteins while full-length protein can bypass the effects caused by these two mutations and stabilize RBD.

Introduction

The small G protein Ras is a key regulator of multiple fundamental cellular processes including cell proliferation, differentiation, apoptosis, motility and metabolism (Shields et al., 2000). In pathology, oncogenic Ras mutants are also found to be involved in the growth and metastasis of various tumors and cancers (Webb et al., 1998). To further explore the biological role of Ras, much work has been done to discover and characterize Ras effector proteins. In quiescent cells, Ras is GDP-bound and can not interact with its effector proteins. Upon stimulation, Ras becomes GTP-loaded and able to bind multiple downstream effectors including RAF family serine/threonine kinases, phophatidylinositol 3-kinase (PI3K), and RalGDS, a GEF for the small G protein Ral (Shields et al., 2000). Since protein-protein interactions between Ras and its downstream effectors are essential for signal transduction through Ras, one strategy to study the functions of Ras effectors is to isolate the mutants of these

effectors which can not bind Ras and examine whether they still respond to upstream signaling.

Recently we identified a novel Ras GTPase effector protein IMP that limits formation of RAF/MEK complexes by inactivation of KSR, a scaffold/adaptor protein that couples activated RAF to its substrate MEK (Matheny et al., 2004). Ras can inactivate IMP through induction of IMP auto-ubiquitination, facilitating KSR-dependent engagement of MEK by activated RAF. Thus, Ras activation has dual effector inputs on the ERK cascade. First, Ras stimulates translocation of cytoplasmic RAF to the plasma membrane and initiates the RAF activation process. Second, by recruiting IMP and stimulating the autoubiquitination of IMP, Ras relieves the inhibition on KSR, allowing KSR to translocate to the plasma membrane and mediate complex formation between RAF, MEK and ERK.

To further investigate how Ras regulates IMP inhibitory function in mitogenic signaling, we isolate some IMP mutants compromised for Ras interaction by generating point mutantions in the Ras-binding domain (RBD; amino acids 304-456) (Matheny et al., 2004) of IMP and screening for interaction deficiency with Ras protein by yeast two-hybrid system. We show that all examined IMP-RBD mutants compromised for Ras interaction in yeast and full-length IMP mutants comprising the corresponding RBD mutations still bind Ras in HEK293 cells. We show that all examined full-length IMP mutants can inhibit

C-RAF-BXB-induced, but not Ras12V-induced, MEK activation, indicating they function as wild-type IMP protein. These observations suggest that in mammalian cells there may be other proteins mediating IMP interaction with Ras while they do not exist in yeast. Interestingly, a spontaneously appeared RBD mutant IMP-RBDS414G/K444E does not bind Ras in HEK293 cells. However, the full-length mutant IMPS414G/K444E still interacts with Ras and does not affect Ras12V-induced MEK activation, indicating that this mutant also functions as wild-type IMP. These results suggest two possibilities: 1. There are other domains in IMP which can bind Ras besides RBD; 2, S414G/K44E destabilize the structure of RBD to prevent it from binding other proteins while full-length protein can bypass the effects caused by those two mutations and stabilize RBD.

Results and Discussion

To identify mutations in IMP that would interfere with its interaction with Ras, we generated a library of mutants from the minimal RBD of IMP by error-prone PCR with Taq polymerase. The library was cloned into a yeast two-hybrid vector that expresses inserts as Gal4 activation domain fusions, and screened for interaction deficiency with Ras12V37G186S expressed as a LexA DNA-binding domain fusion. As a result, nine mutants of IMP-RBD were repeatedly isolated by both nutritional selection for histidine and an assay for β-galactosidase activity. These mutants are C337Y, K385E/V428A, C329F/Q388L, I421T, V365A,

L326H/R339W/W423L, Y373H/E398G/F445S, D367G/L377P and Y408F (Figure 3.1, 3.2). The interaction deficiency between some isolated variants and Ras was also characterized biochemically in HEK293 cells. Surprisingly, co-immunoprecipitation results show that all examined IMP-RBD mutants can still bind Ras12V in HEK293 cells (Figure 3.3) except I421T, which does not get expressed (Figure 3.6). Furthermore, full-length IMP proteins containing the corresponding mutations also interact with Ras12V (Figure 3.4). To further explore the functions of those mutants, we examined the effects of those mutants on Ras12V-induced or C-RAF-BXB-induced MEK activation. As shown in Figure 3.5, just as wild-type IMP protein, all mutants inhibit C-RAF-BXB-induced, but not Ras12V-induced, MEK activation, indicating these mutants have the same functions as wild-type IMP protein.

When we studied the interaction between Ras and IMP-RBD mutants, we found that a spontaneously appeared RBD mutant, S414G/K444E, can not interact with Ras12V in HEK293 cells (Figure 3.6). However, full-length IMPS414G/K444E still binds Ras12V (Figure 3.7) and does not inhibit Ras12V-induced MEK activation, suggesting it functions as wild-type IMP (Figure 3.8).

The above results suggest that all examined mutations identified in the screen do not affect Ras binding in mammalian cells although they do in yeast. One possible explanation is that there may be other proteins that can mediate IMP binding to Ras in mammalian cells but not in yeast. Identification of these

proteins in the future may not only reveal how Ras regulates IMP activities, but also lead to novel information about how signal is transduced from Ras to its effector proteins. Moreover, the fact that S414G/K444E suppresses IMP-RBD, but not full-length IMP, interacting with Ras indicates two possibilities: 1. There are other domains except RBD in IMP which can mediate the interaction between IMP and Ras. 2, S414G/K444E may destabilize the structure of RBD to prevent it from binding other proteins while full-length protein can bypass the effects caused by those two mutations and stabilize RBD.

Noticeably, IMPS414G/K444E displays stronger interactions with Ras12V and other two Ras effector mutants, Ras12V35S and Ras12V37G, than wild-type IMP (Figure 3.7). Since Ras12V35S and Ras12V37G preferentially activate RAF and Ral-GEFs respectively (White et al., 1995), these observations suggest that IMPS414G/K444E can not be preferentially activated by previously identified Ras effector mutants and may respond to Ras signaling better than wild-type IMP although it has the same effect on Ras12V-induced MEK activation as wild-type IMP.

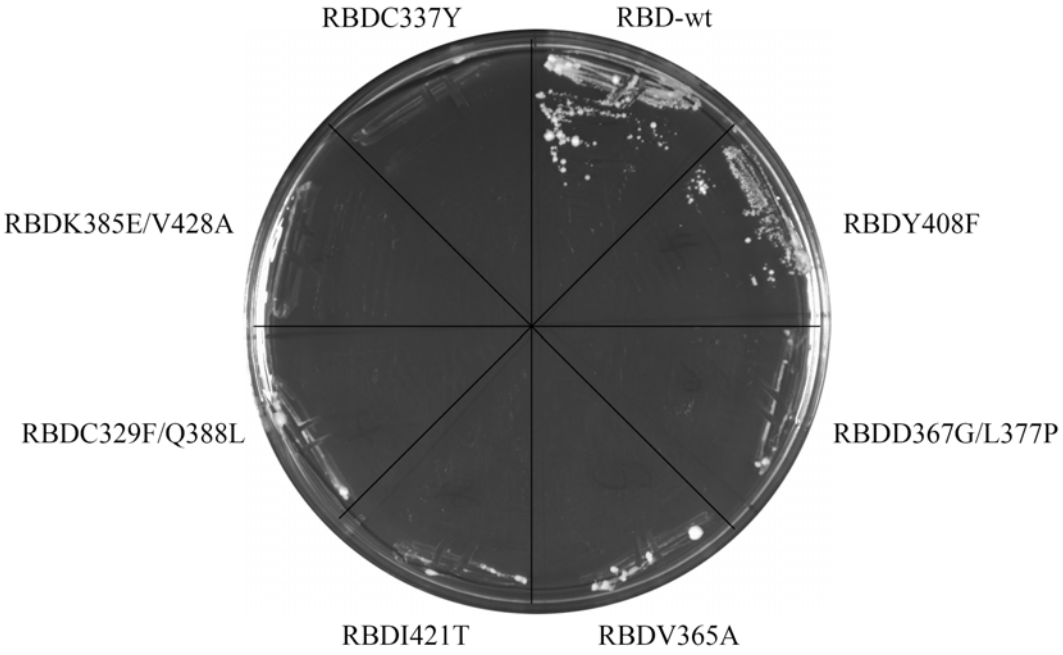
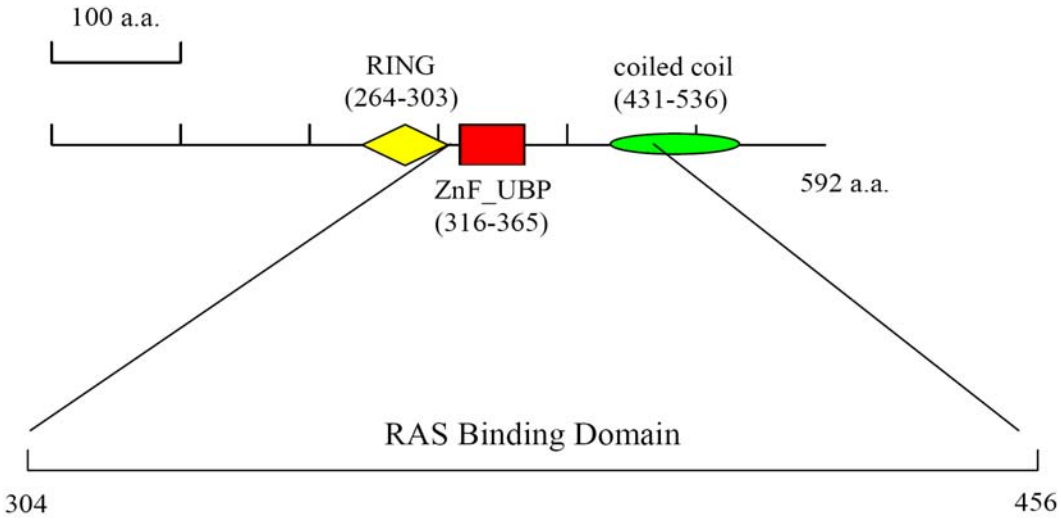


Figure 3.1 Interaction deficiency of IMP-RBD mutants with Ras in two-hybrid binding assay. The indicated IMP-RBD mutants were expressed as fusions to the Gal4 activation domain, together with Ras12V37G186S expressed as a fusion to the LexA DNA-binding domain, in the yeast reporter strain L40. Interaction deficiency is indicated by no growth on media lacking histidine (above) and no blue color in X-gal assay (not shown).



Mutants:

1. 337 Cys/Tyr
2. 385 Lys/Glu; 428 Val/Ala
7. 329 Cys/Phe; 388 Gln/Leu
8. 421 Ile/Thr
10. 365 Val/Ala
12. 326 Leu/His; 339 Arg/Trp; 423 Trp/Leu
13. 373 Tyr/His; 398 Glu/Gly; 445 Phe/Ser
16. 367 Asp/Gly; 377 Leu/Pro
17. 408 Tyr/Phe

Figure 3.2 Domain structure of IMP protein and list of isolated IMP-RBD mutants.

	1	2	3	4	5	6	7	8	9
HA-Ras12V	+					+	+	+	+
Myc-IMP-RBD-wt		+				+			
Myc-IMP-RBDC337Y			+				+		
Myc-IMP-RBDV365A				+				+	
Myc-IMP-RBDY408F					+				+

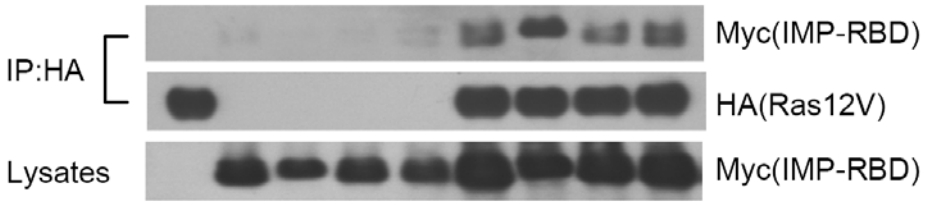


Figure 3.3 IMP-RBD mutants bind Ras in HEK293 cells. HA-Ras12V was immunoprecipitated from HEK293 cells coexpressing HA-Ras12V, Myc-IMP-RBD-wt, Myc-IMP-RBDC337Y, Myc-IMP-RBDV365A, or Myc-IMP-RBDY408F. Upper: Western blots for Myc-IMP-RBD and HA-Ras12V in HA-Ras12V immunoprecipitates. Lower: Western blot for Myc-IMP-RBD in triton-soluble cell lysates.

	1	2	3	4	5	6	7	8	9
HA-Ras12V	+					+	+	+	+
Myc-IMP-wt		+				+			
Myc-IMPC337Y			+				+		
Myc-IMPV365A				+				+	
Myc-IMPY408F					+				+

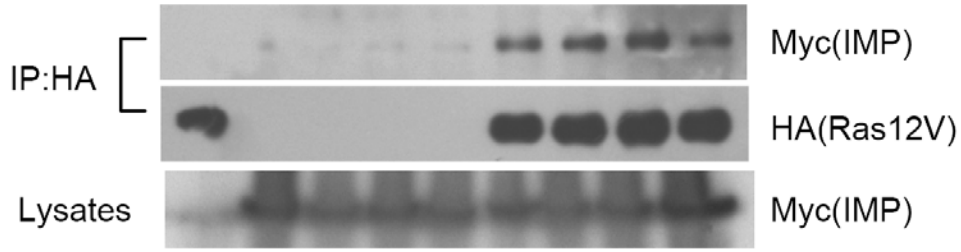
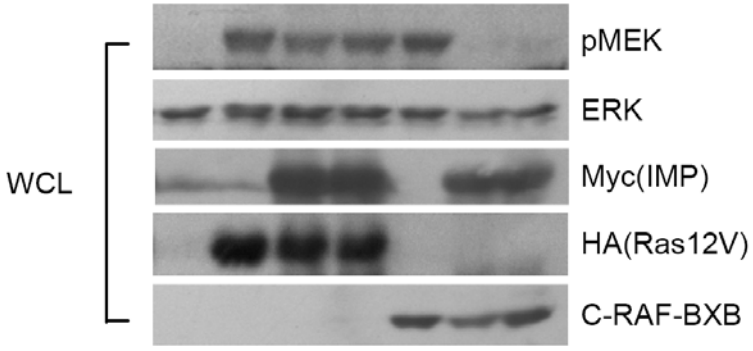


Figure 3.4 Full-length IMP mutants bind Ras in HEK293 cells. HA-Ras12V was immunoprecipitated from HEK293 cells coexpressing HA-Ras12V, Myc-IMP-wt, Myc-IMPC337Y, Myc-IMPV365A, or Myc-IMPY408F. Upper: Western blots for Myc-IMP and HA-Ras12V in HA-Ras12V immunoprecipitates. Lower: Western blot for Myc-IMP in triton-soluble cell lysates.

A

	1	2	3	4	5	6	7
HA-Ras12V		+	+	+			
C-RAF-BXB					+	+	+
Myc-IMP-wt			+			+	
Myc-IMPC337Y				+			+



B

	1	2	3	4	5	6	7	8	9
HA-Ras12V		+	+	+	+				
C-RAF-BXB						+	+	+	+
Myc-IMP-wt			+				+		
Myc-IMPV365A				+				+	
Myc-IMPY408F					+				+

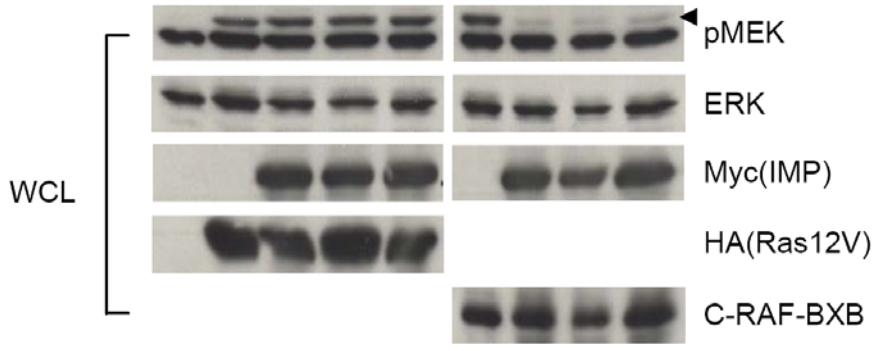


Figure 3.5 Full-length IMP mutants act as wild-type IMP to regulate mitogenic signaling. A, IMPC337Y inhibits C-RAF-BXB-induced, but not Ras12V-induced, MEK activation as wild-type IMP (IMP-wt). Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with HA-Ras12V, C-RAF-BXB, Myc-IMP-wt, or Myc-IMPC337Y. Western blots for phospho-MEK (pMEK), ERK, Myc-IMP, HA-Ras12V, and C-RAF-BXB are shown. B, IMPV365A and IMPY408F inhibit C-RAF-BXB-induced, but not Ras12V-induced, MEK activation as wild-type IMP (IMP-wt). Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with HA-Ras12V, C-RAF-BXB, Myc-IMP-wt, Myc-IMPV365A, or Myc-IMPY408F. Western blots for phospho-MEK (pMEK), ERK, Myc-IMP, HA-Ras12V, and C-RAF-BXB are shown.

	1	2	3	4	5
HA-Ras12V	+	+	+	+	+
Myc-IMP-RBDS414G/K444E	+				
Myc-IMP-RBDC337Y		+			
Myc-IMP-RBDI421T			+		
Myc-IMP-RBDV365A				+	
Myc-IMP-RBDY408F					+

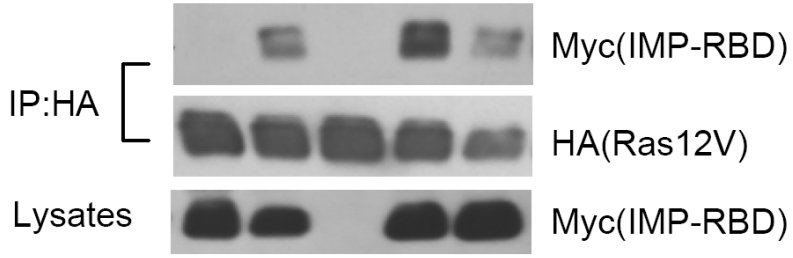


Figure 3.6 IMP-RBDS414G/K444E does not bind Ras and IMP-RBDI421T is not expressed in HEK293 cells. HA-Ras12V was immunoprecipitated from HEK293 cells coexpressing HA-Ras12V, Myc-IMP-RBDS414G/K444E, Myc-IMP-RBDC337Y, Myc-IMP-RBDI421T, Myc-IMP-RBDV365A or Myc-IMP-RBDY408F. Upper: Western blots for Myc-IMP-RBD and HA-Ras12V in HA-Ras12V immunoprecipitates. Lower: Western blot for Myc-IMP-RBD in triton-soluble cell lysates.

	1	2	3	4	5	6	7	8	9	10	11
HA-Ras12V	+					+	+				
HA-Ras12V35S		+						+	+		
HA-Ras12V37G			+							+	+
Myc-IMP-wt				+		+		+		+	
Myc-IMPS414G/K444E					+		+		+		+

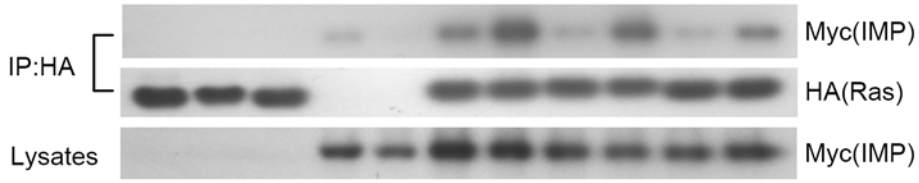


Figure 3.7 Full-length IMPS414G/K444E binds Ras in HEK293 cells. HA-Ras12V, HA-Ras12V35S, or HA-Ras12V37G was immunoprecipitated from HEK293 cells coexpressing HA-Ras12V, HA-Ras12V35S, HA-Ras12V37G, Myc-IMP-wt, or Myc-IMPS414G/K444E. Upper: Western blots for Myc-IMP and HA-Ras in HA-Ras immunoprecipitates. Lower: Western blot for Myc-IMP in triton-soluble cell lysates.

	1	2	3	4
HA-Ras12V		+	+	+
Myc-IMP-wt			+	
Myc-IMPS414G/K444E				+

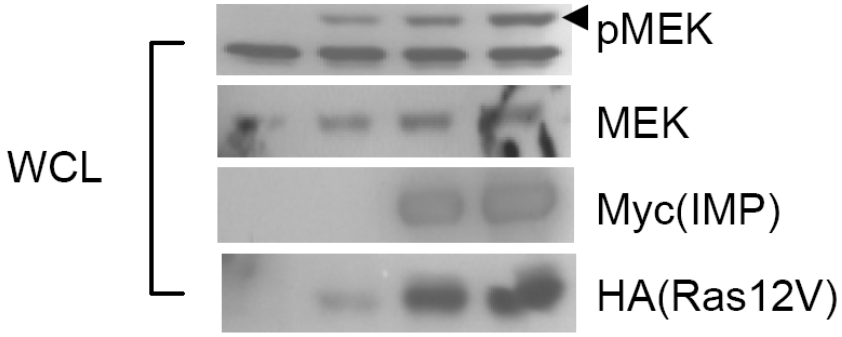


Figure 3.8 Full-length IMPS414G/K444E does not affect Ras12V-induced MEK activation as wild-type IMP. Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with HA-Ras12V, Myc-IMP-wt, or Myc-IMPS414G/K444E. Western blots for phospho-MEK (pMEK), MEK, Myc-IMP, and HA-Ras12V are shown.

Materials and Methods

Library construction and Screening. IMP-RBD (amino acids 304-456) was randomly mutagenized by PCR and ligated into the vector pGADGE to create in-frame fusions with the GAL4 transcription-activation domain as previously described (White et al., 1995). The S. cerevisiae strain L40 (*MATa HIS3 Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ gal4*) was transformed by lithium acetate precipitation with the pGADGE-IMP-RBD cDNA library (1ug) and pBTM116-RasG12V/E37G/C186S (2ug) as bait. Transformants were selected in media lacking leucine (selecting the library plasmid) and tryptophan (selecting the bait plasmid). The cells growing on –leu-trp were screened for interaction deficiency by replica-plating on -leu-trp-his, where histidine production is a reporter for interaction. The colonies growing on –leu-trp were also replica-plated onto filters to test for negativity in an assay for β-galactosidase acitivity, evidenced by no blue colonies in the presence of X-gal substrate. DNA were extracted from negative colonies and sequenced.

Plasmids. pBTM116-Ras12V/37G/186S, pDCR-HA-Ras12V, pSRα-C-RAF-BXB, and pCMV5myc-IMP have been previously described (Matheny et al., 2004). pRK5myc2-IMP-RBDC337Y, pRK5myc2-IMP-RBDI421T, pRK5myc2-IMP-RBDV365A, and pRK5myc2-IMP-RBDY408F were generated by subcloning IMP-RBD cDNAs from corresponding pGADGE vectors isolated

from the screen into EcoRI/SalI-digested pRK5myc2-RASSF1A construct (Shivakumar et al., 2002). pRK5myc2-IMP-RBD was generated by subcloning IMP-RBD cDNA from pCMV5myc-IMP into SalI/XbaI-digested pRK5myc2-IMP-RBDV365A construct. pCMV5myc-IMP-RBDS414G/K444E was a spontaneously appeared mutant identified by sequencing pCMV5myc-IMP-RBD construct, which was previously made by Sharon Matheny. pCMV5myc-IMPC337Y was generated by subcloning IMP-RBDC337Y cDNA from pRK5myc2-IMP-RBDC337Y into PmlI/XbaI-digested pCMV5myc-IMP construct. pCMV5myc-IMPV365A, pCMV5myc-IMPY408F, and pCMVmyc-IMPS414G/K444E were generated by subcloning IMP-RBD cDNAs from corresponding pRK5myc2-IMP-RBD mutants into SalI/XbaI-digested pCMV5myc-IMP construct.

Antibodies. Antibodies against c-Myc (A-14, 9E10), HA (Y-11, F-7), ERK1 (C-16), and C-RAF (C-12) were obtained from Santa Cruz. Phospho-MEK and MEK antibodies were purchased from Cell Signaling.

Cell culture reagents. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose w/o sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen).

Transfection. HEK293 cells were grown to 50-70% confluence on 35mm plates and medium was changed to Optimum (Invitrogen) before transfection. For each plate, 2ug DNA was mixed with 6ul Plus Reagent (Invitrogen) in 100ul Optimum for 15 minutes at room temperature while 4ul Lipofectamine (Invitrogen) was incubated with 100ul Optimum under the same condition. Both mixtures were then pooled together for another 15 minutes and delivered to cells. After 24 hours, medium were changed to normal growing medium with 10% FBS or starvation medium without serum (for assays examining MEK activation). Cell lysates were collected 72 hours after transfection.

Co-immunoprecipitation. Cells were lysed 72 hours following transfection in modified RIPA (RadioImmunoPrecipitation Assay) buffer (20mM Tris pH8.0, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 20mM NaF, and protease inhibitors) and homogenized by rotation at 4℃ for 20min. The lysates were clarified by centrifugation at 14,000 RPM for 30min at at 4℃. HA-conjugated agarose (Santa Cruz) was added to the supernatant for overnight incubation at 4℃. Beads were washed 4X in lysis buffer. After aspirating the last wash, the immunoprecipitates were boiled in 2X sample buffer.

CHAPTER FOUR

Sumoylation of IMP

Abstract

Post-translational modifications regulate protein activities and functions. Preliminary results suggested that IMP is regulated by ubiquitination (Matheny et al., 2004) and phosphorylation (Sharon Matheny, personal communication). Preliminary data also indicated that SUMO exerts negative effects on IMP activity (Sharon Matheny, personal communication), raising the possibilities that IMP could be modulated by SUMO and sumoylation of IMP may regulate its activity. Here, we show that SUMO1 expression rescues IMP-induced KSR1 inactivation. IMP has three potential sumoylation sites, $LK^{246}SE$, $LK^{488}EE$ and $LK^{509}EE$, which matches the sumoylation consensus sequence ΨKXE. We show that IMP binds SUMO1 and is directly modified by three SUMO1 molecules. Modulation at K246 may be a prerequisite for the other two sumoylation events. Importantly, we find that sumoylated IMP is specifically detected in a triton-insoluble cell fraction. Thus, our data reveal that IMP is directly sumoylated and sumoylation of IMP may regulate its cellular localization and function.

Introduction

Post-translational modifications play essential roles in protein activities and functions. In mammalian cells, there are multiple post-translational modifications and most of them are involved in the activation of signaling pathways such as phosphorylation, glycosylation, acetylation, methylation, hydroxylation, ubiquitination, and sumoylation. Compared with other extensively studied modifications, sumoylation is a recent discovered reversible post-modification of proteins by the small ubiquitin-related modifier (SUMO). SUMO is a family of proteins which are covalently attached to lysine residues in target proteins. Three isoforms of SUMO proteins have been found in mammals: SUMO1 (also known as sentrin, Smt3c, PIC1, GMP1, and Ubl1), SUMO2 (also known as sentrin3 and Smt3a), and SUMO3 (also known as sentrin2 and Smt3b) (Hay, 2005). Previous studies have shown that sumoylation is involved in diverse cellular processes including nuclear transport, transcriptional regulation, chromosome organization and function, DNA repair, and signal transduction (Johnson, 2004).

To date, two components in Ras/ERK pathway have been shown to be modified by SUMO: ERK upstream kinase – MEK (Sobko et al., 2002) and ERK downstream transcription factor – Elk-1 (Yang et al., 2003). It was suggested that sumoylations of MEK and Elk-1 function differently to regulate signaling transduction through Ras/ERK pathway. In *Dictyostelium*, sumoylation of MEK1 is required for its localization in cytosol and cortex in reponse to chemoattractant

stimulation, suggesting that sumoylation may play a positive role in chemoattractant-mediated ERK stimulation to control chemotaxis (Sobko et al., 2002). On the contrary, in mammalian cells, SUMO conjugation and ERK-induced phosphorylation counteract with each other to regulate Elk-1 transcriptional activity, indicating that SUMO may negatively regulate the downstream signaling of ERK (Yang et al., 2003).

IMP is a novel Ras effector that negatively regulates Ras/ERK pathway. Preliminary data suggested IMP is regulated by ubiquitination and phosphorylation (Matheny et al., 2004) (Sharon Matheny, personal communicatoion). Moreover, preliminary results indicated SUMO reverses IMP inhibitory effects on Ras/ERK pathway (Sharon Matheny, personal communication). We have examined the mechanism underlying SUMO negative regulation of IMP function. We show that SUMO1 expression reverses IMP-induced KSR1 inactivation and this effect is not affected by EGF stimulation. IMP protein sequence analysis reveals three potential sumoylation sites, $LK^{246}SE$, $LK^{488}EE$ and $LK^{509}EE$, matching the sumoylation consensus sequence ΨKXE, where Ψ is a hydrophobic amino acid, generally valine, leucine, or isoleucine; K is the lysine residue where SUMO is covalently attached; X is any residue; and E is a glutamic acid. We show that IMP interacts with SUMO and is directly modified by tri-sumoylation. Modification at K246 may be a prerequisite for the other two sumoylation events. Importantly, we find that sumoylated IMP can only

be detected in a triton-insoluble cell fraction. These observations suggest that IMP is sumoylated in certain cell compartment, which may reveal a mechanism to regulate IMP ability to affect mitogenic signaling.

Results and Discussion

To verify that SUMO counteracts IMP function, we examined SUMO1 effect on IMP-induced KSR1 inactivation. As shown in Figure 4.1, coexpression of IMP induces accumulation of a higher molecular weight species of KSR1, highlighted by the band shift. This phenotype has been described previously as hyperphosphorylation of KSR1, indicating its inactivation (Matheny et al., 2004). Coexpression of SUMO1 partially reverses IMP effect on KSR1 with no change in response to EGF stimulation (Figure 4.1). These results suggest that SUMO1 may function as a stable negative regulator of IMP to rescue IMP-induced KSR1 inactivation.

Analysis of the primary amino acid sequence of IMP reveals three potential sumoylation sites matching the consensus motif ΨKXE: $LK^{246}SE$ in the N terminus, $LK^{488}EE$ and $LK^{509}EE$ K246 in the C terminus (Figure 4.2). We first examined whether three different IMP truncation mutants, IMP-N' (amino acids 1-255), IMP-C' (amino acids 457-592) and IMP-RBD, could be modified by SUMO1. The results of co-immunoprecipitation assays show that all three truncation mutants can bind SUMO1 (Figure 4.3). However, there is no evidence

that any mutant could be directly sumoylated. The fact that all three mutants also interact with SUMO1 Δ GG, a variant which can not be conjugated, suggests that there are multiple domains in IMP which could bind either free SUMO1 protein or other sumoylated proteins. Moreover, an IMP-RBD mutant deficient for Ras binding, IMP-RBDS414G/K444E (see Chapter 3), can not bind SUMO1 either (Figure 4.4), indicating that this mutant may not have a stable structure for interacting with any other protein.

To rule out the possibility that structural defects may prevent all three IMP truncation mutants from getting sumoylated, we examined full-length IMP protein for direct modification by SUMO1. We also mutated one potential sumoylation site, K246, to alanine and examined this mutant for sumoylation. As shown in Figure 4.5, SUMO1 immunoprecipitates from triton-insoluble pellets display a strong band at approximately 120 kD higher than expected for unmodified wild-type IMP (68 kD). However, this band can not be detected in the lane of IMPK246A. There are lighter bands approximately 40 and 80 kD higher than unmodified IMP. These results are not apparent in cells expressing SUMO1 Δ GG (Figure 4.5). Regarding the molecular weight of coexpressed GFP-tagged SUMO1 is around 40 kD, those bands mentioned above may represent IMP protein with one, two, or three modifications, respectively. These observations suggest that IMP may be directly modified by up to three SUMO1 molecules and tri-sumoylated IMP is the dominant form of modified IMP protein. In the process

of IMP tri-sumoylation, modification at K246 may be a prerequisite for the other two modification events. We also noticed that sumoylated IMP can only be detected in SUMO1 immunoprecipitates from triton-insoluble pellets, but not triton-soluble supernatants (Figure 4.5). Regarding that SUMO expression reverses IMP-induced KSR1 inactivation (Figure 4.1) and IMP can drive KSR1 into a triton-insoluble cell fraction (Matheny et al., 2004), our results suggest that IMP sumoylation in certain cell compartments may provide a mechanism to relieve IMP inhibitory effect on KSR1 and facilitate mitogenic signaling propagation.

Overall, we present data suggesting that SUMO1 is a negative regulator of IMP activity. IMP has three potential sumoylation sites, $LK^{246}SE$, $LK^{488}EE$ and $LK^{509}EE$, which matches the sumoylation consensus sequence ΨKXE. IMP is directly modified by three SUMO1 molecules and sumoylation at K246 may be a prerequisite for the other two modification events. Moreover, both full-length IMP (Figure 4.5) and IMP truncation mutants (Figure 4.3) bind SUMO1, indicating that there are multiple domains in IMP which can interact with either free SUMO1 protein or other sumoylated proteins. Finally, the facts that IMP can drive KSR1 into the triton-insoluble cell fraction and sumoylated IMP can only be detected in the same fraction suggest that sumoylation of IMP in certain cell compartments may be essential to regulate IMP inhibitory effect on mitogenic signaling through KSR1.

	1	2	3	4	5	6	7	8	9
EGF (100ng/ml)	0			5'			10'		
FLAG-KSR1	+	+	+	+	+	+	+	+	+
Myc-IMP		+	+		+	+		+	+
HA-SUMO1			+			+			+

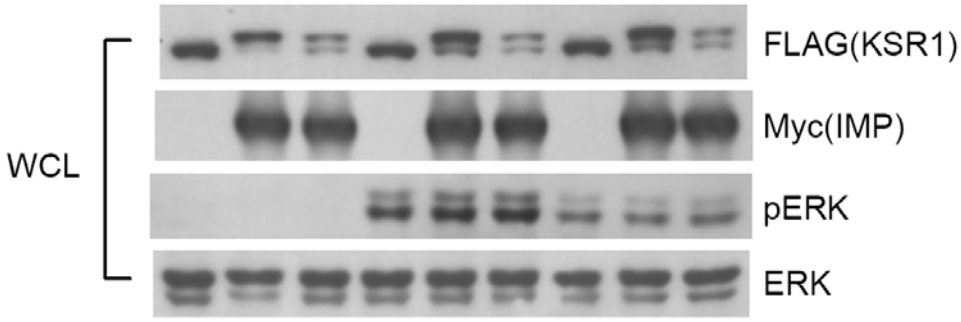


Figure 4.1 SUMO-1 partially blocks IMP-induced hyperphosphorylation on KSR1. Whole cell lysates (WCL) were collected from HEK293 cells cotransfected with FLAG-KSR1, Myc-IMP, or HA-SUMO1, and stimulated with EGF (100ng/ml) for 5 min, 10 min or left untreated as indicated. Western blots for FLAG-KSR1, Myc-IMP, phospho-ERK (pERK), and ERK are shown.

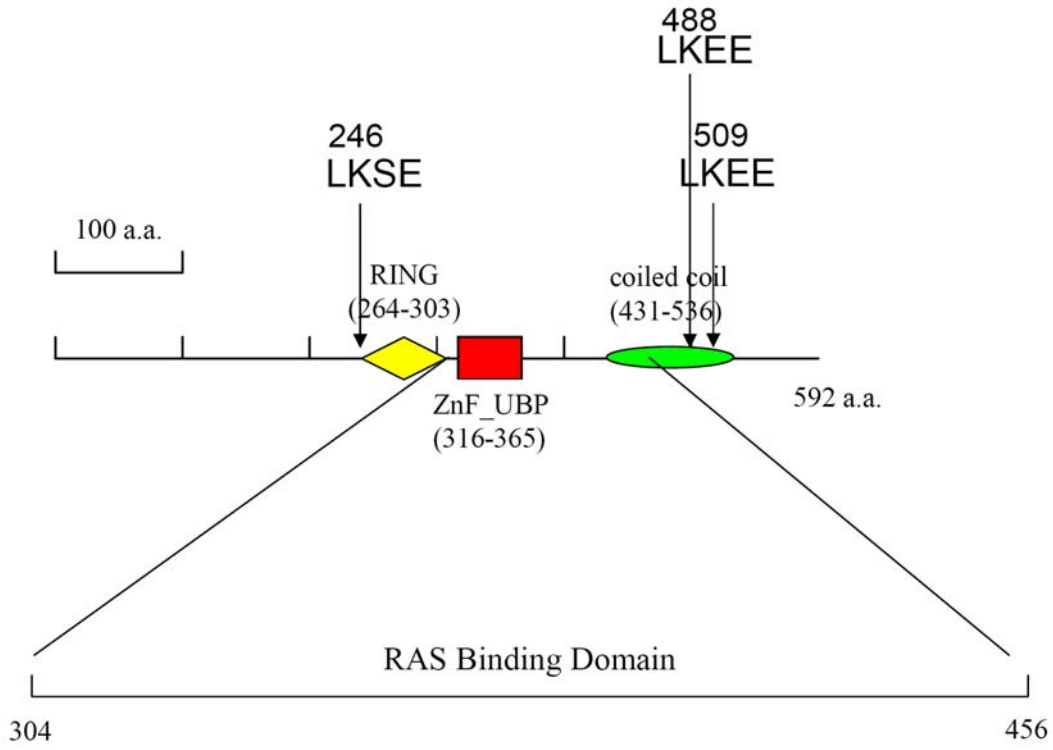


Figure 4.2 Potential sumoylation sites in IMP protein.

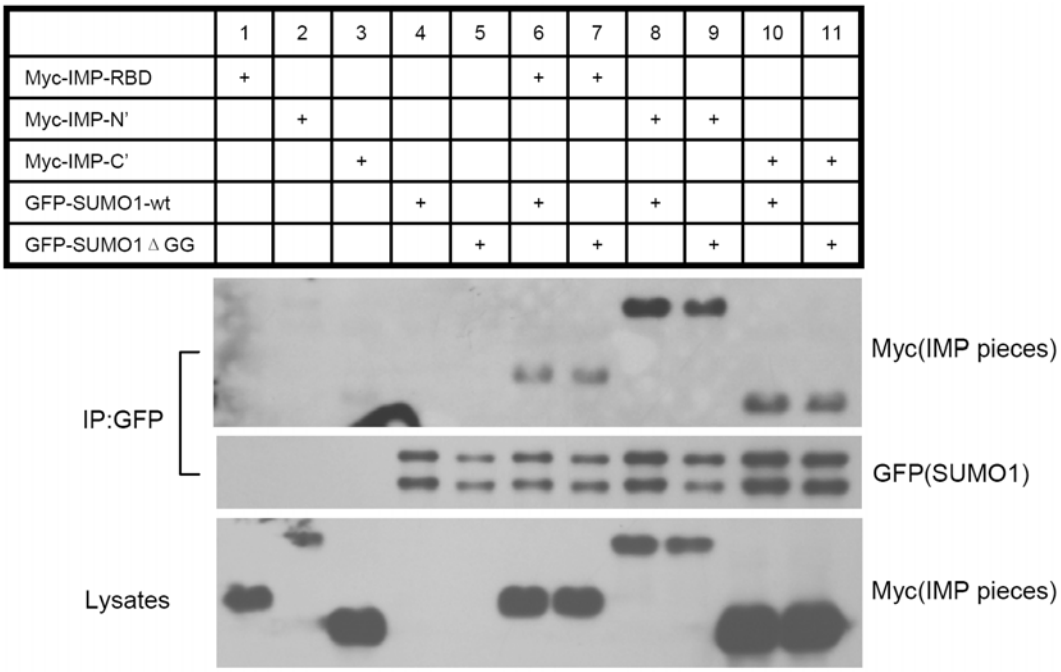


Figure 4.3 IMP truncation mutants bind SUMO1 in HEK293 cells. GFP-SUMO1 was immunoprecipitated from HEK293 cells coexpressing Myc-IMP-RBD, Myc-IMP-N', Myc-IMP-C', GFP-SUMO1-wt, or GFP-SUMO1 Δ GG. Upper: Western blots for Myc-IMP-RBD/N'/C' and GFP-SUMO1 in GFP-SUMO1 immunoprecipitates. Lower: Western blot for Myc-IMP-RBD/N'/C' in triton-soluble cell lysates.

	1	2	3	4	5	6	7	8
Myc-IMP-RBD-wt	+				+	+		
Myc-IMP-RBDS414G/K444E		+					+	+
GFP-SUMO1-wt			+		+		+	
GFP-SUMO1 Δ GG				+		+		+

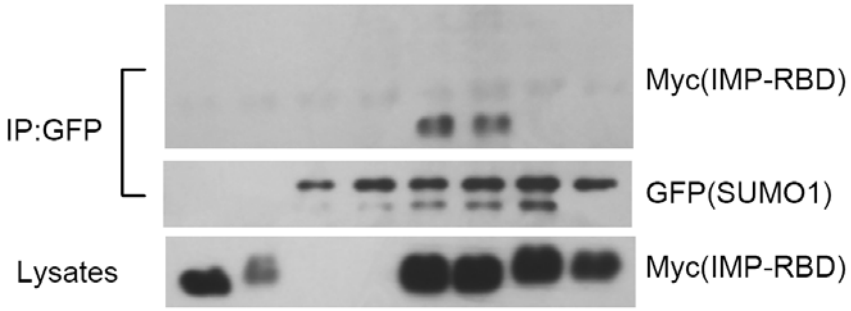


Figure 4.4 IMP-RBDS414G/K444E does not bind SUMO1. GFP-SUMO1 was immunoprecipitated from HEK293 cells coexpressing Myc-IMP-RBD-wt, Myc-IMP-RBDS414G/K444E, GFP-SUMO1-wt, or GFP-SUMO1 Δ GG. Upper: Western blots for Myc-IMP-RBD and GFP-SUMO1 in GFP-SUMO1 immunoprecipitates. Lower: Western blot for Myc-IMP-RBD in triton-soluble cell lysates.

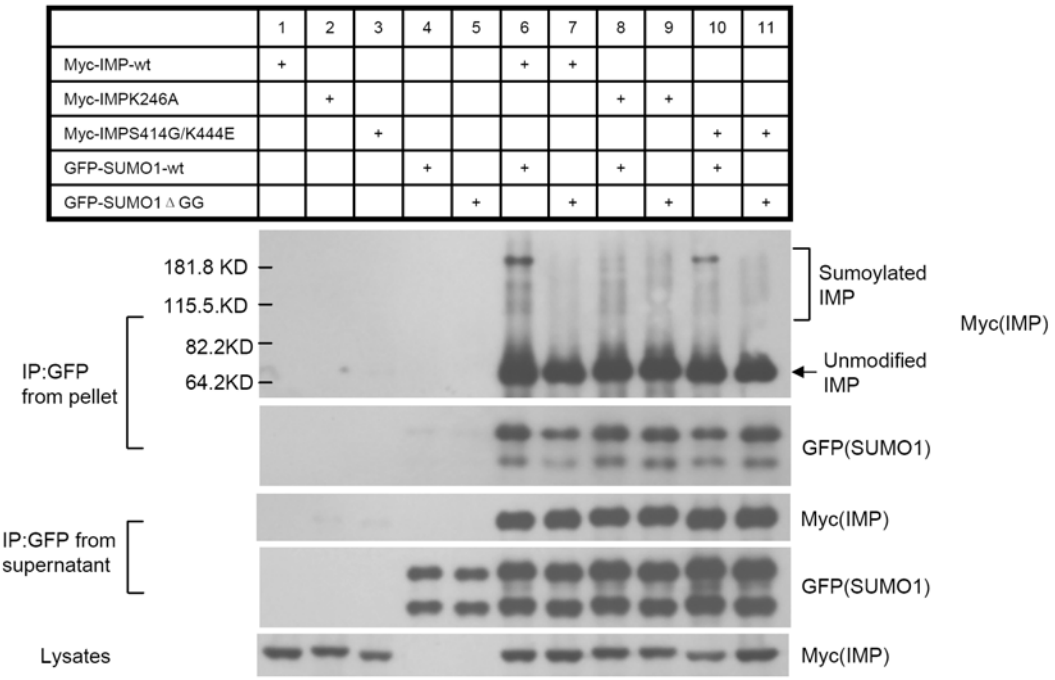


Figure 4.5 IMP is modified by SUMO1 in a triton-insoluble cell fraction. GFP-SUMO1 was immunoprecipitated from HEK293 cells coexpressing Myc-IMP-wt, Myc-IMPK246A, Myc-IMPS414G/K444E, GFP-SUMO1-wt, or GFP-SUMO1 Δ GG. Top: Western blots for Myc-IMP and GFP-SUMO1 in GFP-SUMO1 immunoprecipitates from triton-insoluble pellets. Sumoylated and unmodified IMP proteins are highlighted as indicated. Middle: Western blots for Myc-IMP and GFP-SUMO1 in GFP-SUMO1 immunoprecipitates from triton-soluble supernatants. Bottom: Western blot for Myc-IMP in triton-soluble cell lysates.

Materials and Methods

Plasmids. pCMV5-FLAG-KSR1, pCMV5myc-IMP, pCMV5myc-IMP-N' (amino acids 1-255) (Matheny et al., 2004), pCMVmyc-IMPS414G/K444E, pRK5myc2-IMP-RBD, pCMV5myc-IMP-RBDS414G/K444E (Chapter 3), and pcDNA3-HA-SUMO1 (Orth et al., 2000) have been previously described. pCMV5myc-IMP-C' (amino acids 457-592) and pCMV5myc-IMPK246A were previously made by Sharon Matheny. pCS2-GFP-SUMO1-wt/ Δ GG are gifts from Hongtao Yu (Gocke et al., 2005).

Antibodies. Antibodies against c-Myc (A-14, 9E10), GFP (FL, B-2), and ERK1 (C-16) were obtained from Santa Cruz. Phospho-ERK antibody was purchased from Cell Signaling. FLAG antibody was obtained from Sigma.

Co-immunoprecipitation. HEK293 cells were transfected by lipofectamine and plus reagent (Invitrogen) on 35mm dishes with the indicated plasmids. Cells were lysed 72 hours following transfection in modified RIPA buffer (20mM Tris pH8.0, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 20mM NaF, and protease inhibitors) and homogenized by rotation at 4℃ for 20min. The lysates were clarified by centrifugation at 14,000 RPM for 30min at 4℃. HA-conjugated agarose (Santa Cruz) was added to the supernatant for overnight incubation at 4℃.

Beads were washed 4X in lysis buffer. After aspirating the last wash, the IPs were boiled in 2X sample buffer. For immunoprecipitation from triton-insoluble pellets, the pellets were solved in 1% SDS and 10mM Tris pH7.5, sonicated 4 sec at 90% power, diluted 1:10 in modified RIPA buffer (see above), and centrifuged at 14,000 RPM for 30 min at 4℃. The supernatants were used for immunoprecipitation as described above.

CHAPTER FIVE

Identifying Candidate IMP Binding Proteins

Abstract

IMP is a novel Ras effector which negatively regulates mitogenic signaling through Ras/ERK pathway. Preliminary results showed that IMP is regulated by several post-translational modifications such as phosphorylation, ubiquitination and sumoylation. To further investigate how IMP regulates mitogenic signaling and how IMP function is regulated, we have identified a panel of candidate IMP binding proteins by yeast two-hybrid system and immunoprecipitation in mammalian cells coupled with mass spectrometry (MS).

Introduction

Protein-protein interactions play crucial roles in biological processes. For example, cell signaling cascades rely on direct protein-protein interactions to transduce messages from the extracellular environment to certain intracellular effector proteins and lead to various cell activities and behaviors. In order to elucidate how signaling proteins transfer information to affect cell activities, experimental methods have been developed to survey the proteome for interacting partners. Here, we have screened the proteome for IMP binding proteins by yeast

two-hybrid system and immunoprecipitation in mammalian cells coupled with MS. As a result, we have identified several candidate IMP binding proteins.

Results and Discussion

A Jurkat T-cell library was screened for interaction with IMP expressed as a LexA DNA-binding domain fusion, as determined by both nutritional selection for histidine and an assay for β-galactosidase activity. Three protein fragments were repeatedly isolated: a fragment of C2orf29 protein (178-372; GenBank accession # AAH18664), a fragment of uncharacterized protein C9orf78 (126-289; GenBank accession # Q9NZ63) and a fragment of uncharacterized protein C5orf37 (389-528; GenBank accession # Q8NA72). The interactions between two isolated protein fragments and IMP were further characterized biochemically in HEK293 cells. Co-immunoprecipitation results show that the fragment of uncharacterized protein C9orf78 (126-289; GenBank accession # Q9NZ63) interacts with IMP while the fragment of uncharacterized protein C5orf37 (389-528; GenBank accession # Q8NA72) can not get expressed in HEK293 cells (Figure 5.1).

We also examined the proteins in IMP complex by immunoprecipitation coupled with MS. As shown in Figure 5.2A, colloidal blue staining of SDS-PAGE reveals three specific bands in the lane of Myc-IMP immunoprecipitates from starved cells, which are not present in the control lane (compare lane 1 and 3),

suggesting that these three bands may present specific binding proteins of IMP. Analysis of immunoprecipitates from cells growing in 10%-FBS-adapted cells shows same results (compare lane 3 and 7), indicating that serum stimulation may not affect other proteins binding IMP or the effects caused by serum stimulation are insensitive for detection in this assay. By MS analysis of the three bands described above, more than 20 peptides of different proteins are found in isolated IMP complexes (Figure 5.2B). Most of these proteins are major component proteins of nucleus such as nucleolin (found in band 2 and 3), ribonucleoprotein (found in band 1, 2 and 3), and other nuclear enzymes and proteins. Since it has been shown that IMP can bind the nuclear localization signal (NLS) of BRCA1 (Li et al., 1998) and p21 (Asada et al., 2004), our results suggest that IMP may translocate into the nucleus with BRCA1 and p21, where it may bind other nuclear proteins, although it is a proposed cytoplasmic protein (Asada et al., 2004; Li et al., 1998; Matheny et al., 2004). The other possibility is that the interactions between IMP and these nuclear proteins are nonspecific. Future characterization of these interactions in mammalian cells will reveal which proteins specifically bind IMP and may provide more indications about how IMP impacts cell activities and how IMP itself is regulated. The facts that peptides of IMP (also named BRCA1 binding protein 2) (Li et al., 1998) are present in band 1 (around 110 kD) and 3 (around 90 kD) and the molecular weight of IMP is 68 kD suggest that IMP protein may get certain modifications as discussed in Chapter 4.

Overall, we have identified several candidate IMP binding proteins by yeast two-hybrid system and immunoprecipitation in mammalian cells coupled with MS. Although the interactions of these proteins with IMP and their effects on IMP functions need to be further characterized, our results may lead to some novel information about IMP-associated proteins. Regarding all three proteins identified in yeast two-hybrid system are unidentified and their functions remain unknown, our findings indicate that they might play roles in mitogenic signaling through interactions with IMP.

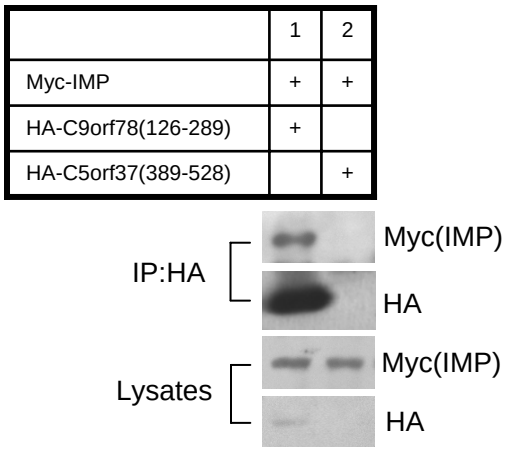
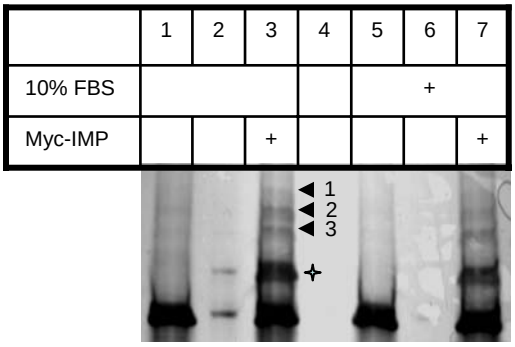


Figure 5.1 A peptide of C9orf78 (126-289) binds IMP protein in HEK293 cells while a peptide of C5orf37 (389-528) does not get expressed. HA-C9orf78(126-289) or HA-C5orf37(389-528) was immunoprecipitated from HEK293 cells coexpressing HA-C9orf78(126-289), HA-C5orf37(389-528), or Myc-IMP. Upper:

Western blots for Myc-IMP and HA-C9orf78(126-289)/C5orf37(389-528) in HA-C9orf78(126-289)/C5orf37(389-528) immunoprecipitates. Lower: Western blot for Myc-IMP and HA-C9orf78(126-289)/C5orf37(389-528) in triton-soluble cell lysates.

A



B

Gel Band ID	MW on Gel	Protein Name	Protein MW	Sequence	GI Number	Note
1	110 kDa	poly(ADP-ribose) polymerase	113.0 kDa	VVSEDFLQD VSASTK	190167	
		Similar to heterogeneous nuclear ribonucleoprotein U	78.6 kDa	DIDIHEVR	14044052	
		proliferating cell nuclear protein P120	95.4 kDa	IQDIVGILR	189422	?
		hypothetical protein FLJ10774; N-acetyltransferase-like	115.7 kDa	LDYLGVSYG LTPR	13399322	?
		BRCA1 associated protein	67.2 kDa	ANQVLLQNK	2665906	?

		MYB binding protein 1a; p53-activated protein-2	148.7 kDa	VYSTALSSFLTK	7657351	?
		ATP-binding cassette, sub-family F, member 1	91.6 kDa	TFFEELAVEDK	10947135	?
2	100 kDa	nucleolin	74.3 kDa	KFGYVDFESAEDLEK	21750187	major component
		RNA helicase Gu - human (fragment)	89.2 kDa	TFSFAIPLIEK	2135315	
		heterogeneous nuclear ribonucleoprotein U isoform b	88.9 kDa	NFILDQTNVSAAAQR	14141161	?
		poly(ADP-ribose) synthetase	113.1 kDa	VVSEDFLQDVSASTK	337424	?
		nuclear corepressor KAP-1	88.5 kDa	ADVQSIIGLQR	1699027	?
		nucleolar protein 1, 120kDa	94.0 kDa	IQDIVGILR	5453792	?
		GPI-anchored protein p137	72.7 kDa	TVLELQYVLDK	2498733	?
		Hypothetical protein FLJ10377	85.7 kDa	LINNNPEIFGPLK	15530220	?
		methionine-tRNA synthetase	100.8 k	ITQDIFQQLLK	15929104	?
		splicing factor proline/glutamine rich	76.1 kDa	FGQGGAGPVGGQGPR	4826998	?
		heat shock 90kDa protein 1, beta	83.2 kDa	NPDDITQEEYGEFYK	20149594	
3	90 kDa	nucleolin	76.3 kDa	GLSEDTTEETLK	4885511	
		DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	79.6 kDa	EAQELSQNSAIK	13787209	
		anti-colorectal carcinoma heavy chain	50.6 kDa	VNSAAFPAPIEK	425518	
		BRCA1 associated protein	67.2 kDa	DLQITEIQEQLR	10800417	
		poly(ADP-ribose) polymerase	113.0 kDa	VVSEDFLQDVSASTK	190167	?

		heterogeneous nuclear ribonucleoprotein R	64.9 kDa	NLATTVTEEILEK	13629286	?
		DEAH (Asp-Glu-Ala-His) box polypeptide 15	92.8 kDa	EVDDLGPEVGDIK	4557517	?
		DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	82.4 kDa	FGFGFGGTGK	4826686	?
		mitogen-activated protein kinase-activated protein kinase 2 isoform 2	45.5 kDa	ARALEAAALAH	32481209	?
		Probable ATP-dependent RNA helicase DDX27	89.8 kDa	SADFNPDFVFTEK	29427946	?

Figure 5.2 Identification of candidate IMP-associated proteins by co-immunoprecipitation in HEK293 cells coupled with mass spectrometry. (A) Wild-type IMP complexes were isolated from cells growing in starvation medium or 10%-FBS-adapted medium, resolved by SDS-PAGE, and stained with colloidal blue. Myc-IMP immunoprecipitates from cells growing in starvation medium or serum-adapted medium are loaded in lane 3 and 7 respectively as indicated while Myc-antibody immunoprecipitates from cells growing under same conditions are loaded in lane 1 and 5 as negative controls. There is no protein loaded in lane 2, 4 and 6. Star indicates IMP protein and arrows indicate three gel bands which were cut and analyzed by mass spectrometry (MS). (B) Identified peptide sequences by MS analysis.

Materials and Methods

Plasmids. pCMV5myc-IMP has been previously described (Matheny et al., 2004). pBTM116-IMP was generated by subcloning IMP cDNA from pCMV5myc-IMP plasmid into the EcoRI/BamHI-digested pBTM116 vector. pcDNA3.1-HA-C9orf78(126-289) and pcDNA3.1-HA-C5orf37(389-528) were generated by subcloning C9orf78(126-289) and C5orf37(389-528) cDNAs respectively from corresponding pGADGE vectors isolated from the screen into EcoRI/XhoI-digested pcDNA3.1-HA-Bim expression construct, a gift from Xiaodong Wang.

Yeast two-hybrid library screen. The S. cerevisiae strain L40 (*MATa HIS3 Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ gal4*) was transformed by lithium acetate precipitation with the pGADGE vector based Jurkat T-cell cDNA library and pBTM116-IMP as bait. Transformants were selected in media lacking leucine (selecting the library plasmid) and tryptophan (selecting the bait plasmid). The cells were initially screened for interaction deficiency by plating on -leu-trp-his, where histidine production is a reporter for interaction. For a second round of screening, the his+ colonies were replica-plated onto filters to test for positivity in an assay for β-galactosidase acitivity, evidenced by blue colonies in the presence of X-gal substrate. DNA were extracted from positive colonies and sequenced.

Isolation of IMP Complexes and Mass Spectrometry Analysis. Ectopically expressed IMP protein was immunopricipated from HEK293 cells growing in starvation medium or 10%-FBS-adapted medium. IPs were examined by SDS-PAGE and Colloidal blue staining (Invitrogen). Protein bands of interest were excised and in-gel digested. The resulting tryptic peptides were identified as previously described (Chien et al., 2006).

CHAPTER SIX

Discussion and Future Directions

Ras/RAF/MEK/ERK pathway is the most extensively studied MAPK pathway, and has been shown to be involved in multiple basic cellular processes and implicated in pathology of various cancers and diseases (Pearson et al., 2001). Although the essential biochemistry of the pathway has been established, it still remains unknown that how the pathway mediates specific inputs into diverse biological behaviors specifically and efficiently under complex environments. Recent studies of scaffolds and regulators have revealed that Ras/ERK pathway is organized as a complicated communication network (Kolch, 2005). Temporal and spatial regulation of multiple protein complexes formation controls the process of signal flux through this pathway and crosstalk with other pathways. Here, by examining the mechanistic basis of signal amplitude modulation by the Ras effector IMP, we explore how the signal flux through Ras/ERK pathway is regulated by protein complexes formation, especially in two important processes: RAF activation and signal propagation from RAF to MEK. We also study how Ras regulates IMP functions by isolating IMP mutants compromised for Ras interaction and how post-translational modifications such as sumoylation control IMP activities. Finally, we identify some candidate IMP binding proteins to

further investigate how IMP impacts cell activities by protein-protein interactions and how IMP is regulated by other proteins.

To date, RAF activation still remains as a complicated process and is only partially understood. In comparison with poor knowledge of activation processes of A-RAF and B-RAF, C-RAF activation has been extensively studied. Previous research has revealed that there is an intramolecular inhibition between C-RAF N-terminal regulatory domain and C-terminal kinase domain. It has also been proposed that three main mechanisms may lead to a conformational change in C-RAF, whereby the regulatory N-terminus dissociates from the C-terminal catalytic domain, resulting in an activated kinase. These mechanisms are membrane localization, phosphorylation, and oligomerization, which may be involved with each other. Among them, oligomerization has not been caught much attention until recently. Original research showed that ectopically forced dimerization induces C-RAF kinase activity (Farrar et al., 1996; Luo et al., 1996) and Ras could induce B-RAF/C-RAF complex formation in an overexpression system (Weber et al., 2001). Recent studies revealed that heterooligomerization of B-RAF/C-RAF occurs as a physiological process in mammalian cells (Rushworth et al., 2006), and it is an essential step for C-RAF activation in both normal cells and melanoma cells (Garnett et al., 2005; Wan et al., 2004). However, it still remains unclear that how B-RAF/C-RAF complex formation triggers C-RAF activation and whether it requires B-RAF kinase activity. Data from Marais group (Garnett

et al., 2005) showed that B-RAF kinase activity is essential for C-RAF activation, suggesting that a trans-phosphorylation event is involved. On the contrary, results from Kolch group (Rushworth et al., 2006) showed that the catalytic function of B-RAF is dispensable for stimulating C-RAF, indicating that B-RAF may activate C-RAF by other mechanisms besides trans-phosphorylation such as recruiting other C-RAF activators, sequestering C-RAF inhibitors, or inducing a conformational transition of C-RAF to its active state. Regarding the inhibitory effect of C-RAF N-terminus on its C-terminal kinase domain, it is possible that binding B-RAF may induce dissociation of C-RAF C-terminus from its N-terminus and lead to its activation. Here, we present data to show that the kinase-dead form of B-RAF could form a complex with C-RAF-BXB, a constitutively active variant of C-RAF which only includes C-RAF kinase domain, and induces its activation. These results are consistent with previous data that B-RAF kinase activity is not required for C-RAF activation (Rushworth et al., 2006). Moreover, our data suggest that kinase-dead-B-RAF may induce C-RAF activation by directly regulating its C-terminal catalytic domain instead of relieving the inhibitory N-terminus from the C-terminus. Importantly, IMP suppresses B-RAF/C-RAF heterooligomerization and B-RAF-induced C-RAF-BXB activation, suggesting that IMP regulates B-RAF/C-RAF complex formation to control C-RAF activation.

To further explore how RAF proteins are activated, numerous studies have been done to identify and characterize other regulators of RAF activation. Recent studies in *Drosophila* suggested that KSR, a scaffolding protein supposedly to couple RAF and MEK, is also involved in RAF activation process (Anselmo et al., 2002; Douziech et al., 2006) and functions at a step before the phosphorylation of the activation segment of RAF (Douziech et al., 2006). Considering the fact that KSR is a proposed pseudokinase and structurally homologous to RAF proteins, it is highly possible that KSR activates drosophila RAF (dRAF) in a same way as kinase-dead-B-RAF activates C-RAF. Noticeably, previous studies never ruled out the possibility that KSR or B-RAF may activate dRAF or C-RAF by recruiting a dRAF/C-RAF activator or sequestering a dRAF/C-RAF inhibitor. We show that KSR1 enhances B-RAF/C-RAF heterooligomerization and IMP inhibits C-RAF interaction with KSR1 through competitive binding, indicating that KSR1 may activate C-RAF by enhancing B-RAF/C-RAF complex formation and sequestering the negative regulator IMP.

Besides KSR, other proteins have been shown to be involved in RAF activation process including 14-3-3 and Hsp90. Previous studies showed that binding of 14-3-3 to the C-terminus of C-RAF is essential for its activation and this is mediated by phosphorylation of S621, which provides a binding site for 14-3-3 (Jaumot and Hancock, 2001; Light et al., 2002; Tzivion et al., 1998; Yip-Schneider et al., 2000). Recent results also showed that 14-3-3 binding and

phosphorylation at S621 are involved in B-RAF/C-RAF oligomerization process (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001). We show that IMP inhibits 14-3-3 binding to full-length C-RAF, but not C-RAF N-terminus. In comparison, IMP inhibits B-RAF and KSR1 interactions with both full-length C-RAF and its N-terminus. These observations suggest IMP blocks 14-3-3 binding to the C-terminus of C-RAF, which may interfere with the 14-3-3-dependent active conformation of C-RAF as indicated by previous study (Tzivion et al., 1998). Moreover, IMP may bind to both N- and C-terminus of C-RAF to block its interactions with B-RAF and KSR1. The fact that IMP does not suppress phosphorylation at S621 indicates that IMP regulates 14-3-3 binding to C-RAF C-terminus without affecting phosphorylation at S621 of C-RAF. Similar as 14-3-3, Hsp90 has also been reported to be essential for RAF kinase activity (Grammatikakis et al., 1999; Jaiswal et al., 1996; van der Straten et al., 1997). Although some studies suggested that Hsp90 regulates the stabilities of RAF proteins to affect mitogenic signaling (Grbovic et al., 2006; Schulte et al., 1995; Schulte et al., 1996), we did not detect that IMP affects C-RAF stability although IMP inhibits Hsp90 binding to C-RAF, indicating that the interaction between C-RAF and Hsp90 may regulate mitogenic signaling by another mechanism besides affecting C-RAF stability, perhaps through stabilizing the active conformation of C-RAF as 14-3-3 protein does. Therefore, our results suggest that IMP regulates formation of multiple C-RAF-involved complexes including B-RAF/C-RAF,

KSR1/C-RAF, KSR1/B-RAF/C-RAF, C-RAF/14-3-3 and C-RAF/Hsp90 to control C-RAF activation.

Besides affecting C-RAF activation, our preliminary results showed that IMP also interferes with EGF-induced C-RAF binding to MEK (Matheny et al., 2004). This raises the possibility that IMP may function at two levels to regulate signal propagation through RAF to MEK: RAF activation and RAF binding to MEK. We show that IMP inhibits KSR1 oligomerization to separate KSR1/B-RAF and KSR1/MEK complexes, indicating IMP may block KSR1-dependent B-RAF binding to MEK. Previous studies showed that C-RAF binding to MEK is correlated to C-RAF activation process (Xiang et al., 2002). Considering our preliminary result that C-RAF does not bind MEK in serum-starved cells (Matheny et al., 2004) and current data that kinase-dead-B-RAF interacts with MEK in starvation state, we suspect that B-RAF may also function as a scaffold protein like KSR1 to couple C-RAF and MEK. IMP inhibits both B-RAF and KSR1 binding to C-RAF, indicating IMP may suppress C-RAF binding to MEK by two manners. Moreover, the facts that IMP only partially suppresses kinase-dead-B-RAF-induced C-RAF-BXB activation and IMP strongly inhibits C-RAF-BXB-induced MEK activation (Matheny et al., 2004), we suspect that IMP may block MEK binding to C-RAF-BXB as well as full-length C-RAF (Matheny et al., 2004).

In summary, we show that IMP inhibits B-RAF/C-RAF complex formation to suppress C-RAF activation. We show that IMP blocks KSR1/C-RAF, KSR1/B-RAF/C-RAF, C-RAF/14-3-3 and C-RAF/Hsp90 complexes formation, which are also essential for C-RAF activation. Furthermore, IMP inhibits KSR1-dependent B-RAF binding to MEK by separating KSR1/B-RAF and KSR1/MEK complexes. IMP may regulate C-RAF binding to MEK by affecting KSR1/C-RAF and B-RAF/C-RAF complexes formation. Thus, by examining the mechanistic basis of signal amplitude modulation by the Ras effector IMP, we find that formation of multiple complexes including RAF family proteins, KSR1, 14-3-3 and Hsp90 is involved in the processes of both RAF activation and RAF binding to MEK. IMP controls the response threshold for MEK activation by regulating the availability of these complexes.

Since IMP negatively regulates signal propagation through Ras/ERK pathway, the obvious question is that how this inhibitory effect is relieved when the pathway is activated by upstream signaling. Our preliminary results showed that IMP is a Ras effector protein and active Ras can inactivate IMP through induction of IMP auto-ubiquitination (Matheny et al., 2004). A general method to study the functions of Ras effectors is to isolate the mutants of these effectors which can not bind Ras and examine their activities. Here, we isolate some IMP mutants compromised for Ras interaction and examine their functions. We show that all examined IMP RBD mutants compromised for Ras interaction that we

isolated from yeast two-hybrid still interact with Ras in HEK293 cells, indicating that there are other proteins mediating IMP binding to Ras in mammalian cells but not in yeast. Identifying these proteins and characterizing their functions in the future may not only reveal how Ras regulates IMP functions but also provide more information about how the signal is transduced from Ras to its downstream effectors.

Post-translational modifications normally regulate protein activities and functions. Preliminary results suggested that IMP is regulated by ubiquitination (Matheny et al., 2004) and SUMO exerts negative effects on IMP activity (Sharon Matheny, personal communication). We verify that SUMO1 counteracts IMP functions and this phenomenon is not affected by EGF stimulation, suggesting that SUMO1 may act as a stable inhibitor of IMP. IMP protein sequence analysis reveals three potential sumoylation sites, $LK^{246}SE$, $LK^{488}EE$ and $LK^{509}EE$, which matches the sumoylation consensus sequence ΨKXE. We show that IMP may be directly sumoylated by three SUMO1 molecules and K246 sumoylation might be a prerequisite for the other two sumoylation events. Importantly, we find that IMP sumoylation can only be detected in a triton-insoluble cell fraction. Regarding our previous results that IMP can drive KSR1 into the same cell fraction, this phenomenon indicates that sumoylation of IMP in certain cell compartments may be essential for relieving IMP inhibitory effect on KSR1 and facilitating mitogenic signal propagation.

Finally, to further investigate how IMP impacts cell activities by protein-protein interactions and how IMP is regulated by other proteins, we screen the proteome and identify some candidate IMP binding proteins. We show that three uncharacterized proteins are isolated by yeast two-hybrid system and their interactions with IMP still remain to be verified in mammalian cells. Considering the functions of all these proteins are unknown, our results suggest that they may play roles in mitogenic signaling through interaction with IMP. We isolate more than 20 candidate IMP binding proteins by immunoprecipitation of IMP in HEK293 cells coupled with mass spectrometry (MS) analysis. Most of these proteins localize in the nucleus such as nucleolin and ribonucleoprotein. Regarding previous studies showed IMP can bind the nuclear localization signal (NLS) of BRCA1 (Li et al., 1998) and p21 (Asada et al., 2004), it may shuttle between nucleus and cytoplasm and interact with some nuclear proteins although it is a proposed cytoplasmic protein (Asada et al., 2004; Li et al., 1998; Matheny et al., 2004). The other possibility is those nuclear proteins may nonspecifically interact with IMP and were pulled down by immunoprecipitation. Future verification of the interactions between IMP and these candidate proteins may reveal specific binding proteins of IMP and lead to novel information about how IMP impacts cell activities and how IMP itself is regulated by other proteins.

Overall, we show that IMP regulates multiple mitogenic complexes formation to control both RAF activation and RAF binding to MEK. We isolate

some IMP mutants compromised for Ras interaction and find that there may be other proteins mediating IMP interaction with Ras in mammalian cells. We also show that IMP can be sumoylated in certain cell compartments, which may be essential for regulating mitogenic signaling. Finally, we identify some candidate IMP binding proteins by yeast two-hybrid and immunoprecipitation in mammalian cells coupled with MS study. All of these results and observations not only reveal the mechanistic basis of IMP functions in Ras/RAF/MEK/ERK pathway, but also provide information about directions of future research such as how IMP itself is regulated by post-translational modifications, how IMP impacts cell activites through protein-protein interactions, and how other proteins regulates IMP and Ras/ERK signaling through interactions with IMP.

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VITAE

Chiyuan Chen was born in Xuzhou, Jiangsu, China, on July 17, 1976, the son of Jinshan Chen and Changying Qiao. After completing his work at the First High School of Xuzhou, Xuzhou, Jiangsu, China, in 1994, he entered Fudan University at Shanghai, China. He received the degree of Bachelor of Science with a major in Biology in July, 1998. He then entered the Graduate School of Life Sciences in Fudan University and was awarded the degree of Master of Science in Zoology in July, 2001. In August, 2001, he joined the Graduate School of Biomedical Sciences in University of Texas Southwestern Medical Center at Dallas, where he did his Ph.D. study in the laboratory of Dr. Michael A. White in the Department of Cell Biology. In 2003, he met Ying Chen, a UT Southwestern graduate student from Weifang, Shandong, China, and married her in 2006.

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