Analysis of the Mechanism of Ras Activation: Mapping of Important Functional Domains of the Son of Sevenless Protein

Linda Sue McCollam-Guilani
University of Massachusetts Medical School

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A Dissertation Presented

By

Linda Sue McCollam-Guilani

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

(February 10, 1998)

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ANALYSIS OF THE MECHANISM OF RAS ACTIVATION: MAPPING OF IMPORTANT FUNCTIONAL DOMAINS OF THE SON OF SEVENLESS PROTEIN

A Dissertation Presented

By

Linda Sue McCollam-Guilani

Approved as to style and content by:

(Signature) Dr. Anthony Carruthers, Chair of Committee

(Signature) Dr. Reid Gilmore, Member of Committee

(Signature) Dr. Beth Luna, Member of Committee

(Signature) Dr. Steve Shoelson, Member of Committee

(Signature) Dr. Wayne Zhou, Member of Committee

(Signature) Dr. Michael P. Czech, Dissertation Mentor

(Signature) Dr. Thomas Miller, Dean of Graduate School of Biomedical Sciences

Department or Program Biochemistry

Month, Day, and Year February 10, 1998
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Last, I would like to thank the person who has contributed the most in the successful completion of my graduate studies, my loving and caring husband, Brad Guilani. Thank you Brad for helping me in so many different ways throughout my graduate studies.
The questions outlined in this thesis dissertation were proposed in order to provide insight regarding the mechanism by which the Drosophila Son of sevenless (dSOS) protein activates Ras. Ras proteins are GTP-binding proteins which bind guanine nucleotides very tightly and cycle between the inactive GDP-bound state and the active GTP-bound state. To address the mechanism by which the dSOS proteins activates Ras, a structure-function analysis of the dSOS protein was performed using truncation and deletion mutants of dSOS. In vivo Ras activation experiments using transiently transfected cells revealed that the NH₂-terminal domain of dSOS is required in order for the catalytic domain of dSOS to exhibit exchange activity in cultured mammalian cells. The COOH-terminal GRB2 (Growth Factor Receptor Binding Protein) binding domain on the otherhand was insufficient to confer Ras exchange activity to
the dSOS catalytic domain. Further analysis of the NH$_2$-terminal domain of the dSOS protein demonstrated that the function of promoting catalytic domain activity could be localized by mutational analysis to the pleckstrin (PH) and DBL (Diffuse B-cell Lymphoma) homology sequences. Fractionation studies of cells transiently transfected with various dSOS mutant proteins demonstrated that the NH$_2$-terminus of dSOS is also necessary for membrane association. These findings suggested that the model proposing that the recruitment of SOS via the adaptor protein GRB2 to the membrane is the main mechanism by which SOS activates Ras is unlikely to be the only mechanism by which SOS can activate Ras. From our data, a model can be proposed which postulates that SOS can activate Ras as a consequence of at least two steps. One step involves the SOS/GRB2 interaction and the second step involves the NH$_2$-terminal domain of SOS associating with unidentified cellular elements.
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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>βARK</td>
<td>Beta-adrenergic receptor kinase</td>
</tr>
<tr>
<td>BOSS</td>
<td>Bride of sevenless</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle protein</td>
</tr>
<tr>
<td>CSF-IR</td>
<td>Colony stimulating factor receptor</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4 diazabicyclo-(2,2,2)-octane</td>
</tr>
<tr>
<td>DBL</td>
<td>DBL homology domain</td>
</tr>
<tr>
<td>DER</td>
<td><em>Drosophila</em> epidermal growth factor receptor</td>
</tr>
<tr>
<td>DMEMK</td>
<td>Dulbecco’s minimum essential media</td>
</tr>
<tr>
<td>DOC</td>
<td>Sodium cholate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>F-Actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF-R</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
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<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GNEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor binding protein</td>
</tr>
<tr>
<td>GRP1</td>
<td>General receptor for phosphoinositides</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor receptor</td>
</tr>
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<td>Ins(1,4,5)P₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun nuclear kinase</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>OSBP</td>
<td>Oxysterol-binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor receptor</td>
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<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
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<tr>
<td>PI(3,4,5)P₃</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
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<td>PI(3,4)P₂</td>
<td>Phosphatidylinositol 3,4-bisphosphate</td>
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<td>PI(4)P</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3’ Kinase</td>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-P₂</td>
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<tr>
<td>PKCζ</td>
<td>Protein kinase C zeta</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C gamma</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PTP1C</td>
<td>Phosphotyrosine phosphatase</td>
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<tr>
<td>RAP1A</td>
<td>Ras related protein</td>
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<tr>
<td>Ras</td>
<td>GTP-binding protein</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
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<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecy sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>sev</td>
<td>sevenless</td>
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<tr>
<td>SH2</td>
<td>Src homology-2 domain</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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</tr>
<tr>
<td>SH3</td>
<td>Src homology-3 domain</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
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<tr>
<td>TBS</td>
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CHAPTER I

BACKGROUND

A. Ras superfamily

The Ras superfamily comprises over 60 small guanine nucleotide binding proteins that regulate a diverse array of biological functions (1,2). The molecular weight of these guanine nucleotide binding proteins ranges from 20-25 kDa. Based on primary amino acid sequence, the proteins are divided into subfamilies designated Ran, Rab, Rho, Arf, and Ras (1,2). Specifically, certain members of the Ras subfamily have been shown to be important in cell proliferation and differentiation (1-4). Ran has been implicated to play a role in nuclear functions such as nuclear transport (1-2,5). There is evidence that Rab and Arf proteins play an important role in intracellular vesicular transport (1-2,6,7). The Rho subfamily regulates cytoskeletal organization, for example, membrane ruffling (1-2,8,9) and generation of the superoxide free radical in NADPH oxidase catalyzed reactions (1-2,10). All members of the Ras superfamily have the ability to cycle between the active GTP-bound state and the inactive GDP-bound state (Figure 1). The proteins become biologically active when GDP is released.
and replaced with GTP. The intrinsic rate of this exchange is extremely slow. Ras superfamily proteins are regulated by the rate at which they hydrolyze bound GTP and the rate at which they exchange GDP for GTP. Negative regulatory proteins referred to as GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP to GDP, thus leading to inactivation of the Ras superfamily proteins (11,12). Positive regulatory proteins referred to as guanine nucleotide exchange factors (GNEFs) accelerate the exchange step by interacting with members of the Ras superfamily (12,13). In addition, Rho and Rab proteins interact with yet a third class of regulatory proteins referred to as guanine nucleotide dissociation inhibitors (GDIs). GDIs interact with GTPases to maintain the nucleotide bound state (14,15).

B. Ras subfamily

Ras proteins are present in all eukaryotic organisms from human to yeast. Ras was originally identified 15 years ago in animal tumor viruses containing the activated allele of the ras gene which is referred to as the oncogene. There are three human ras genes which encode four Ras
Figure 1. The GTPase Cycle. Ras superfamily proteins cycle between the active GTP-bound and inactive GDP-bound states as a consequence of the actions of GAPs (GTPase activating proteins) and GNEFs (Guanine Nucleotide Exchange Factors).
proteins: H-ras (Harvey), K-ras4a (Kirsten), K-ras4B, and N-ras. Specific point mutations in these three ras genes have been found in thirty percent of human tumors (3). The glycine at position 12 seems to be the most frequently mutated residue in Ras, and amino acids 13 and 61 have also been found to be mutated in mammalian Ras. Mutations at these three positions in Ras have been demonstrated to cause a ten-fold decrease in the intrinsic hydrolysis rate of GTP.

Ras proteins have a molecular weight of 21 kDa. Primary sequence elements which dictate guanine nucleotide binding and are conserved in all proteins that comprise the Ras superfamily are found in Ras at the following positions: amino acids 10-16, GXXXXGK; amino acids 57-162, DXXGXE; amino acids 116-119, NKXD; and amino acids 143-147, EXSAX (2). Additionally, primary sequence analysis reveals another conserved region in Ras proteins referred to as the effector domain at position amino acids 32-40. Mutations in this region (amino acids 32-40) caused no change in the ability of Ras proteins to bind and hydrolyze GTP. However, these mutations eliminated the ability of Ras proteins to transform (16). Therefore, this region seems to be important in the interaction of Ras with cellular targets. Further experiments demonstrated
that the effector domain does bind other proteins, for example GAP proteins, which will be discussed in a later section in more detail.

Ras proteins are posttranslationally modified. Ras proteins possess the conserved CAAX box at the C-terminus. The cysteine in this box is farnesylated via a thioether linkage by a farnesyl-protein transferase. The three C-terminal amino acids are then proteolytically cleaved, and the C-terminus is carboxymethylated. Finally, Ras proteins are palmitoylated on cysteine residues within the C-terminal hypervariable region. As a result Ras becomes associated with the inner side of plasma membranes where it can participate in signaling cascades. In addition, K-RAS4B requires a polybasic domain of six consecutive lysine residues located within the hypervariable region in order to have efficient plasma membrane targeting.

C. Receptor Tyrosine Kinases

The binding of polypeptide growth factors or hormones to receptor tyrosine kinases present on the surface of cells initiates a cascade of signaling events that are important in cellular regulation. Receptor
tyrosine kinases generally consists of five major structural domains: 1.) the extracellular domain is responsible for binding the growth factor or hormone and for subsequent transmission of the signal; 2.) the hydrophobic transmembrane domain is responsible for connecting the extracellular and intracellular domains; 3.) the juxtamembrane domain connects the transmembrane and kinase domains; 4.) the tyrosine kinase domain consist of two conserved structural motifs, an ATP-binding site (lysine residue and GXGXXG consensus sequence) and a tyrosine residue which can accept the γ phosphate from ATP; and 5.) the carboxyl-terminal domain consists of two possible regulatory domains (C-terminal tail or kinase inserts) both of which contain many autophosphorylation sites (17,18).

Mammalian cells contain several receptor tyrosine kinases all of which comprise the conserved catalytic core and diverse regulatory sequences. Analysis of the primary amino acid sequence of receptor tyrosine kinases suggest that they fall in two main categories, based on similar structural motifs (see Figure 2). Receptors in the first category contain cysteine-rich regions in the extracellular domain and an uninterrupted kinase domain. One group of receptors in this category is the EGF-R family which consist of two cysteine-rich domains (19). A second group of
receptors, in this category, consist of disulfide-linked heterotetramers (2α and 2β subunits) and a single cysteine-rich domain, for example, IR (20,21), IGF-1R (22), and c-met receptor (23). The second category of receptors encompasses those which have more regularly spaced cysteines and a varying number of immunoglobulin-like domains. In addition, a regulatory domain is inserted into the conserved kinase domain. Examples of receptor tyrosine kinases in this category include PDGF-R (24), CSF-1-R (25), c-kit (26), and FGF-R (27).

Although the above mentioned receptor tyrosine kinases are structurally distinct, the early events following their respective ligand binding appear mechanistically similar. The tyrosine kinase is activated, and the receptor undergoes autophosphorylation on specific tyrosine residues located in the kinase domain. This activation of the kinase results from a conformational change caused by receptor oligomerization or receptor phosphorylation. Once the kinase is activated the receptor can then phosphorylate a variety of intracellular substrates. These substrates are proteins containing elements that bind the phosphotyrosine residues present on the intracellular domains of the activated receptors. Protein regions that bind phosphorylated tyrosine residues are either SRC homology-2 domains (SH2 domains) or phosphotyrosine-binding domains referred to as PTB domains.
SH2 domains consist of approximately 100 amino acids that are conserved in a wide variety of signaling molecules (28-31). Cantley et al. have illustrated that high affinity binding of SH2 domains to phosphotyrosine residues requires that the phosphorylated tyrosine residue be within a specific amino acid sequence context (32-34). For example, the SH2 domains of GAP, PLCγ, and PI3K recognize distinct autophosphorylated phosphotyrosine residues on the β receptor for PDGF (35-41). SH2 domains are found in proteins with a known catalytic domain and also in proteins without a catalytic domain. Examples of SH2-containing proteins with a catalytic domain are SRC (tyrosine kinase), ABL (tyrosine kinase), SYK (tyrosine kinase), PTP1C (phosphotyrosine phosphatase), PLCγ (phospholipase C), and GAP (42). SH2-domain containing proteins without a catalytic domain are referred to as adapter proteins. These proteins which include p85, SHC, c-CRK, NCK, and GRB2, are responsible for coupling tyrosine kinase activity to downstream targets. SH2 domains rely on amino acids carboxy-terminal to the phosphotyrosine for specific recognition.

PTB domains also bind specifically to phosphotyrosine containing peptides. However, specificity is achieved in a different manner than with SH2 domains. The PTB domain was first identified in the SHC protein
(202,203) and subsequently found in several other signaling proteins, including IRS-1 (204). Unlike SH2 domains, PTB domains rely on amino acids amino-terminal to the phosphotyrosine for specificity (204). Structural studies revealed that PTB domains share a common fold. More specifically, the PTB domains of SHC and IRS-1 share a related structure, and the general orientation for peptide binding to the two PTB domains is similar. Interestingly, the PTB domains of SHC and IRS-1 share little sequence homology, and residues contacting the phosphotyrosine are different in the two domains (204,216).

Several SH2-containing proteins possess a second protein-protein interaction domain, which has been designated the SH3 domain. SH3 domains consist of approximately 50 amino acids, and they are present in a wide variety of signaling proteins and cytoskeletal elements. The interaction of the SH3 domain of ABL and the ABL binding proteins 3BP-1 and 3BP-2 was demonstrated by Cicchetti et al. using λGT11 expression cloning (43,44). The crystal structures of the SH3 domain of spectrin, fyn, and lck have been determined by X-ray crystallography (45,46). In addition, the crystal structures of the SH3 domains of the SRC tyrosine kinase, PLCγ, and p85 have been determined by NMR(47,48). These studies have revealed that SH3 domains consist of two 3-stranded antiparallel β-sheets, and that
the interface of these sheets forms a hydrophobic core (45). Furthermore, NMR studies of SRC bound to proline-rich peptides derived from 3BP-1 sequences indicate that the amino acid sequence PPPLPP interacted specifically with SH3 domains (48-50). Several in vitro studies later confirmed that SH3 domains bind the motif PPPXPP. For example, the SH3 domains of GRB2 were shown to bind to proline-rich sequences in the guanine nucleotide exchange factor Son-of-Sevenless (51,52). The functional importance of SH3 domains was evident from mutational studies performed by Kato et al. and others which revealed that deletions and/or substitutions of amino acids in SH3 domain of SRC increased both its catalytic activity and oncogenic potential (53-56). In addition, these mutants failed to bind p110, a known substrate of SRC (57). Hence, SH3 domains and SH2 domains both seem to be important in regulating protein-protein interactions in signal transduction via receptor tyrosine kinases.

D. Role of Ras in Signaling by RTK

Over the past ten years, evidence has accumulated that clearly demonstrates the role of Ras as a downstream component in signaling pathways from transmembrane receptors. The Ras signaling pathway
modulates important cellular processes such as transcription and protein synthesis (58-65). These transmembrane receptors are tyrosine kinases, or transmembrane receptors associated with tyrosine kinases, or receptors coupled to heterotrimeric G-proteins. Depending on the type of cell and receptor, Ras signaling can mediate either differentiation or proliferation (4, 66-67). Stimulation of receptors by ligand results in activation of guanine nucleotide exchange factors, and as a result accumulation of Ras-GTP. Subsequent to Ras activation there are increases in protein phosphorylation, phospholipid metabolism, and transcriptional activity. Ras genes are evolutionarily conserved, and thus Ras proteins seem to function as a universal relay in cell signaling.

Initial insight into the Ras signaling pathway was provided by genetic studies with the budding yeast *S. cerevisiae*. Two Ras proteins exist in the budding yeast, Ras1 and Ras2, and both seem to regulate adenylate cyclase. Nutritional signals initiate the activation of Ras by the guanine nucleotide exchange factor Cdc25 which catalyzes the exchange of GDP for GTP on Ras. Activated Ras stimulates adenylate cyclase resulting in increased levels of cAMP. The cAMP dependent protein kinases, (TPK1, 2, 3), then regulate cell growth and cell division by directly phosphorylating multiple protein substrates (68-70). The action of these kinases is to prevent cell
division by inhibiting transcription of Cln1 and Cln2 G1 cyclins and to promote cell growth by removing transcriptional repression which allows the cell to proceed from G1 to S phase once it has achieved a critical mass. As understanding of the steps in the Ras signaling pathway in the budding yeast increased, it became clear that mammalian Ras functions differently from Ras in budding yeast. Beckner et al. showed that mammalian Ras does not stimulate adenylate cyclase (71). Thus, the key question still remained, "What is the function of Ras in mammalian cells?"

Biochemical and genetic studies using mammalian cells, *Caenorhabditis elegans*, and *Drosophila melanogaster* have demonstrated that Ras is a critical downstream component of receptor tyrosine kinase mediated pathways. Mulcahy *et al.* demonstrated that microinjection of anti-Ras antibodies into mammalian fibroblasts caused the cells to become refractory in their response to serum and growth factors (72). In addition, microinjection experiments using dominant negative mutants of Ras were shown to: 1.) block Ras function in fibroblasts (73,74), 2.) block NGF-induced differentiation of neuronal PC12 cells (75), and 3.) block insulin-induced maturation of oocytes (76). In the same manner, oncogenic Ras genes were shown to induce NGF-independent differentiation of PC12 cells and growth factor independent proliferation in fibroblasts (77). Several
studies have revealed that stimulation of cells with growth factors such as EGF, PDGF, insulin, NGF, and M-CSF results in the accumulation of the active GTP-bound form of Ras (67,78-84). Activated Ras is then able to interact with effector molecules to initiate downstream signaling pathways, e.g., the Ras activated mitogenic pathway. The serine/threonine protein kinase Raf directly associates with Ras-GTP, resulting in the translocation of Raf to the plasma membrane where it becomes activated (85,86). This activation of Raf results in the initiation of a kinase cascade. MAPK kinase, the direct substrate for Ras becomes activated and in turn activates MAP kinase, several isoforms of which have been described (64,87). Activated MAP kinase subsequently translocates to the nucleus where it phosphorylates and activates transcription factors resulting in the induction of gene expression via Ras responsive elements. Several substrates for MAP kinases, including p90 ribosomal S6 kinase, cPLA2, PHAS-1, c-MYC, and ELK have been described (64,87-88). Ras therefore seems to be a key player in regulating diverse signaling pathways between cell surface tyrosine kinase receptors and the activation of transcription in the nucleus.

Functional similarities exist between mammalian Ras and Ras from both Caenorhabditis elegans and Drosophila melanogaster. In C. elegans Ras functions downstream of the receptor tyrosine kinase LET-23, a member of
the EGFR family, to control the differentiation of specific ectodermal precursor cells to a vulval rather than an epidermal fate (90). Genetic experiments have identified the components downstream of Ras in this pathway: lin-45 (Raf homologue), mpk-1/sur-1 (MAPK), and transcription factors such as lin-31. In *Drosophila*, genetic experiments have demonstrated that Ras participates in three receptor tyrosine kinase signaling pathways which regulate different aspects of development: 1.) sev-sevenless tyrosine kinase receptor; 2.) tor-torso tyrosine kinase receptor; and 3.) the DER- *Drosophila* EGFR (89). Specifically, Sev regulates the fate of the R7 photoreceptor precursor cell in each eye ommatidium. Differentiation of the tail and unsegmented head regions during embryogenesis requires activation of tor dependent kinase cascade (89). Lastly, DER tyrosine kinase receptor has been implicated to play a key role in multiple developmental steps (89).

E. Ras Effectors

Over the past several years, data has emerged which suggests that Ras has multiple effectors, molecules which preferentially bind to GTP-bound Ras. Three-dimensional structural studies of Ras have shown that when Ras
undergoes transition from its GTP-bound state to its GDP-bound state, conformational changes occur in two regions. These two regions are referred to as Switch I (residues 30-37), which overlaps the effector domain, and Switch II (residues 60-76). Three specific amino acids (Tyr 32, Thr 35, and Gly 60) found within these two regions are conserved in all GTP-binding proteins (91,92). Studies indicate that these residues are involved in binding the γ-phosphate of GTP, and thus they play an important role in the GTP-dependent mechanism which modulates the interaction of Ras with specific effectors.

Three standard methods have been applied to identify Ras effector molecules: 1.) the yeast two-hybrid system; 2.) GST-fusion assays using purified GST-Ras-GTP and cell extracts; 3.) a method of genetic screening in *S. cerevisiae* which identifies proteins that inhibit Ras-dependent activation of adenylate cyclase. Identification of Ras effector molecules is integral to understanding how Ras proteins function.

The first vertebrate proteins identified to associate preferentially with Ras-GTP were the GAPs. GAPs negatively regulate the Ras signaling pathway by accelerating the intrinsic Ras GTPase activity. GAP has a molecular weight of 120 kDA. The amino-terminus of GAP consists of two SH2 domains and one SH3 domain. Experiments suggest that GAP is not
normally a mitogenic target of Ras. For example, mutations within the Ras effector loop inhibit the interaction of GAP with Ras-GTP, yet mitogenic signaling is retained (93). However, other studies indicate that mitogenic signaling can occur via the amino-terminal domain of GAP. Specifically, microinjection into *Xenopus* oocytes of antibodies raised against the SH3 domain of GAP prevents Ras-dependent germinal vesicle breakdown (94). Additionally, overexpression of the amino-terminus of GAP results in Ras-dependent transcription from the fos promoter (95). DeClue and coworkers have shown that cotransfection of the amino-terminus of GAP and v-Src results in the transformation of NIH3T3 cells (96). Mattingly et al. demonstrated that overexpression of the SH3 domain of GAP in NIH3T3 cells inhibits muscarinic stimulated Ras activation (97).

Two proteins have been identified, p62 and p190, which associate with GAP via its amino-terminus in growth factor stimulated cells (98). The p62 protein is a cytoplasmic protein with RNA binding capability, and it has been shown to interact with GRB2, PLCγ, and Src family kinases (99). The p190 protein possesses GAP activity for the mammalian Rho protein, and is able to bind guanine nucleotides (100). In order to elucidate exactly how GAP serves as an effector for Ras, precise functional roles for p62 and p190 need to be defined.
Although several putative effectors for Ras have been delineated, only one such protein, Raf, has been clearly established as a Ras target protein in mitogenic and developmental signaling. Raf a serine/threonine kinase, exists as three separate isoforms, c-Raf-1, B-Raf, and A-Raf. A vast body of data has been generated which implicates association of Ras with Raf. In vitro studies performed by Moodie and coworkers initially demonstrated the association of Raf with GTP bound Ras (101). Subsequently, the yeast two-hybrid system was applied to confirm this interaction and to demonstrate that the Ras binding domain in Raf consists of residues 51-131(102). Vojtek and coworkers utilizing various cDNA expression libraries isolated proteins that bind to Ras-GTP (103). They succeeded in isolating a fragment of Raf which corresponds to its regulatory domain. Subsequently, several groups have demonstrated the ability of recombinant Ras to bind to the regulatory domain of Raf in vitro, and mutations in the Ras effector region seem to block this interaction (104, 105). Ras recruits Raf to the plasma membrane, and other proteins have been suggested to participate in the recruitment of Raf to the plasma membrane. This recruitment, as well as the association of Raf with Ras-GTP, and the phosphorylation of Raf, all seem to contribute to the activation of Raf.
Zhang et al. utilizing the yeast two-hybrid system to identify proteins which bind Raf, isolated a Ras-related protein called Rap1A (104). Rap1A is a homologue of Ras in the sense that it has the same core effector region. X-ray crystallography was used to generate the three-dimensional structure of the complex of Rap1A with the Ras binding domain of Raf (106). The structure revealed that Rap and Raf interact via an inter-protein β-sheet which is formed by two anti-parallel strands from the two proteins (106). These structural studies also confirmed that residues that were predicted to be involved in effector binding were indeed located at this interface and involved in binding.

Other putative Ras effectors include PI3K and MEK kinase-1 (MEKK1). PI3K is an enzyme which phosphorylates phosphotidylinositol at the D-3 position on the inositol ring. PI3K consists of two subunits, the p110 catalytic subunit and the p85 regulatory subunit. The regulatory subunit consists of one SH3 domain, two SH2 domains, and a Rho-GAP domain. While the majority of published data suggests that PI3K acts upstream or independently of Ras (107), some evidence exists to suggest that PI3K is downstream of Ras. Sjolander and coworkers detected PI3K activity in Ras immunoprecipitates of both Ras transformed and agonist treated cells (108). Subsequently, Rodriguez-Viciona et al. demonstrated in vitro binding of
Ras-GTP and p110, and that this association requires an intact effector loop (109,110). In addition, these studies provided evidence that this Ras-GTP interaction occurs in vivo and might contribute to the regulation of p110. Specifically, it was demonstrated that expression of the N17 Ras dominant inhibitory mutant in PC12 cells blocks NGF induced production of PI(3,4)P₂ and PI(3,4,5)P₃. Furthermore, coexpression of p85, p110, and Ras in COS cells resulted in increased PI(3,4)P₂ and PI(3,4,5)P₃ while in contrast expression of Raf, Rho, Rac(V12), or Ras(A38) together with p85 and p110 induced an increase in PI3K activity.

The role of MEKK-1 as a Ras effector molecule has not been studied as extensively as PI3K. MEKK-1 is an upstream regulator of the Jun nuclear kinases (JNKs). JNK kinases are also referred to as stress activated protein kinases (SAPKs). The SAPKs are related to the MAPK family however, they are activated by different extracellular stimuli (111-113). JNKs increase transcriptional activity of target genes by regulating the activity of c-Jun and ATF2 via phosphorylation. In 1995 Russell and coworkers initially demonstrated that MEKK-1 associates with Ras-GTP in vitro (114). The kinase domain of MEKK1 was shown to bind to GST-Ras(V12) in a GTP dependent manner, and this association could be disrupted by the addition of an effector domain peptide derived from the Ras effector
sequence. The relevance of the MEKK-1/ Ras interaction in vivo needs to be studied further to understand the role of MEKK-1 in Ras signaling.

PKCζ has also been postulated to be a Ras effector protein. PKCζ is a protein kinase C that is unique in the sense that it is neither Ca++ dependent nor responsive to phorbol esters (115,116). It has been demonstrated that PI(3,4,5)P3 can activate PKCζ in vitro (117). Putative substrates of PKCζ are MEK1, MEK2, and IKB (I kappa B-alpha) kinase (118,119). Initial studies in Xenopus oocytes illustrated that PKCζ is necessary for Ras dependent maturation (120). Diaz-Meco and coworkers coimmunoprecipitated Ras and PKCζ from PDGF treated NIH3T3 cells, while expression of the N17 dominant inhibitory Ras mutant inhibited PDGF stimulated PKCζ activity (120). They also demonstrated that PKCζ associates with Ras-GTP in vitro. Further studies need to be performed in order to determine if PKCζ is truly a Ras effector.

F. Ras Activators

As described previously, GAP was the first regulator identified for the Ras proteins. The fact that Ras has both a low intrinsic GDP/GTP exchange rate and a negative regulator, GAP, suggests a fixed rate of accumulation of
inactive Ras. Therefore, it was predicted that positive regulators of Ras proteins must exist in order to accelerate this low intrinsic exchange rate. The identification of guanine nucleotide exchange factors (GNEF) has been an important step in understanding how Ras proteins are activated. Since Ras proteins are conserved in lower eukaryotes, yeast genetics were initially used to identify the GNEF, Cdc25. Broek and coworkers succeeded in isolating the gene corresponding to the \textit{S. cerevisiae} cell division cycle mutant cdc25 (121). The cdc25 temperature sensitive mutant was shown to cause G1 arrest at the non-permissive temperature. This temperature sensitive mutation can be suppressed by either the presence of a high copy number plasmid carrying the gene coding for an active cAMP-dependent protein kinase (Tpk1, Tpk2, or Tpk3), or by the presence of constitutively active Ras or adenylyl cyclase (121,122). Initial genetic data suggested that Cdc25 regulates Ras and is required for its activation. Subsequent biochemical data confirmed that the Cdc25 gene product promotes the release of guanine nucleotide bound to yeast Ras and mammalian Ras (123). The Cdc25 protein has a molecular weight of \(\sim 180\) kDa. In vitro studies demonstrated that the catalytic domain of Cdc25 is approximately 450 amino acids (124). Three additional yeast proteins that share homology with Cdc25 have been isolated, Sdc25 (125) and Bud5 (126,127) from \textit{S.}
cerevisiae and Ste6 from *S. pombe* (128). Sdc25 and Ste6 have been shown to be Ras-specific GNEF's however. Experimental evidence suggests that Bud5 is a GNEF for the Rsr1 protein, a member of the Ras subfamily of GTPases, and that it is involved in the control of bud site localization in the yeast *S. cerevisiae* (129).

In 1990, four papers were published that initially identified a nucleotide exchange promoting activity for Ras in mammalian cells (130-133). In 1992 several groups began to attempt to isolate mammalian homologues of the yeast exchange factor. Figure 3 illustrates proteins that share homology with the catalytic domain of Cdc25, and thus function as guanine nucleotide exchange factors for Ras. Utilizing a method of genetic complementation of the temperature sensitive *cdc25* strain, Martengani and coworkers succeeded in isolating a partial cDNA from a mouse brain cDNA library which they referred to as *CDC25Mm/RASGRF*. In vitro studies confirmed that Cdc25 has been able to activate adenylyl cyclase in a Ras-dependent manner (134). This brain specific Cdc25 homologue was demonstrated to activate Ras both in vivo and in vitro (135). Northern blot analysis revealed the presence of the Cdc25 homologue in mouse heart and to a small extent in liver and spleen (134). Hui Cen et al. subsequently screened a mouse brain cDNA library and identified several full length
clones of Cdc25<sub>Mn</sub> (136). Additionally, degenerate oligonucleotides designed to code for all peptides present in two highly conserved regions of Cdc25, Sdc25, and Ste6 were utilized as a method to clone mammalian homologues of Cdc25. Thus, Shou <em>et al.</em> cloned a cDNA from a rat brain library which encodes p140<sup>Ras-GRF</sup>, a 140 kDa guanine nucleotide releasing factor for Ras which contains the Cdc25 catalytic domain. The Cdc25 homology domain of Ras-GRF was able to catalyze the release of GDP from Ras in vitro, but not the release of GDP from RalA (Ras related protein) or Cdc42Hs, suggesting that the p140 Ras-GRF is a guanine nucleotide releasing factor specific for Ras (137). Two groups have also succeeded in isolating homologues of Ras-GRF from human brain cDNA libraries (138,139).

Concurrently, the son of sevenless (SOS) gene was isolated and shown to encode a <i>Drosophila</i> homologue of Cdc25. Genetic experiments in <i>Drosophila</i> first demonstrated that the sos gene functions in a signal transduction pathway downstream of the sevenless receptor tyrosine kinase and the EGFR (140-142). The sos gene was first described as a dominant mutant allele referred to as SOS<sup>Jc2</sup>. Bonfini and coworkers isolated and characterized the sos gene which coded for a protein with a molecular
mSOS1
mCDC25
C3G
dSOS
STE6
CDC25
SDC25
BUD5
RALGDS

Figure 3. Proteins with homology to yeast CDC25 protein catalytic domain. Functional domains are denoted as follows:

, CDC25 homology; ■, PH domain; , Proline-rich regions; , Dbl homology; and ■■, SH3 domain (†3).
weight of ~180 kDa (143). Sequence analysis revealed that SOS protein contained a region which was 45% identical and 70% similar to the Cdc25 catalytic domain suggesting that the sos gene product is an exchange factor for Drosophila Ras. Therefore, SOS seems to play a key role upstream of Ras in the sevenless receptor tyrosine kinase cascade leading to Drosophila eye development.

Several months later, mammalian homologues of the Drosophila sos gene were isolated. Bowtell et al. screened a mouse cDNA library under low stringency using the Drosophila sos gene as a probe (144). Two mouse genes were isolated in this manner and referred to as mSOS1 and mSOS2. Primary sequence analysis showed that mSOS1 and mSOS2 share an overall 45% amino acid identity with the Drosophila SOS protein. The predicted molecular weight of these proteins is ~150 kDa. Unlike the brain specific Ras-GRF/ Cdc25Mm, northern blot analysis demonstrated that the two mouse sos genes are widely expressed both during development and in adult tissues. Subsequent genetic studies illustrated that the mSOS1 catalytic domain was able to suppress the loss of Cdc25 function in S. cerevisiae (145).

Furthermore, in vitro studies demonstrated that the GST-SOS1 catalytic domain is able to bind to the nucleotide free form of Ras, (145) in
a manner similar to that previously demonstrated for the yeast Cdc25 protein (124). The GST-SOS1 catalytic domain was also shown to catalyze guanine nucleotide exchange on Ras in vitro, but only in the presence of an excess of free guanine nucleotide (145). These data suggest that SOS recognizes nucleotide-free Ras as a reaction intermediate and Ras-GTP as a product.

Pierre Chardin and coworkers later succeeded in isolating the human SOS1 and SOS2 genes. They screened a rat brain cDNA library using degenerate oligonucleotides that were designed to code for all peptides present in two highly conserved regions of Cdc25, Sdc25, Ste6, and Drosophila SOS (146). Two distinct clones were isolated which were then used as probes to screen two human fetal brain cDNA libraries and a human skeletal muscle cDNA library. Two cDNAs were isolated and identified as hSOS1 and hSOS2, and share 69% overall amino acid identity (146). The predicted molecular weight of these proteins is approximately 150 kDa. As described previously for mSOS1, the hSOS1 catalytic domain was also able to complement the loss of Cdc25 in yeast (146). These studies also demonstrated that hSOS1 catalytic domain can activate Ras in vitro, and overexpression of full length hSOS1 in human kidney 293 cells resulted in an increase in guanine nucleotide exchange activity in cell
extracts (146). Thus, these experiments suggested that hSOS1 is a guanine nucleotide exchange factor for Ras. The key question still remained, “Are SOS proteins the major activators of Ras in vivo?” Recent published results demonstrated a specific requirement for mSOS1 in the EGF receptor signaling cascade in vivo. Genetic experiments were used to show that disruption of the msos1 gene resulted in a defective MAPK activation in response to EGF(147). In addition, these experiments illustrated a strong genetic interaction between a weak allele of the EGF receptor and a heterozygous msos1 mutation (147).

Another protein referred to as C3G (CRK-SH3 binding protein) was identified as a mammalian GNEF. CRK, like GRB2, is an adapter protein consisting of only SH2 and SH3 domains. Sequence analysis of C3G revealed that C3G has the conserved Cdc25 homology domain at its carboxy terminus (148). Antibodies against C3G recognize a protein with an apparent molecular weight of 140 kDa that is widely expressed in human adult and fetal tissues. Like SOS, C3G is also able to suppress the loss of Cdc25 function in the yeast S. cerevisiae (148). Initially, it was thought that C3G was an activator for Ras. However, the substrate specificity of C3G was examined by Gotoh et al., and results clearly demonstrated that C3G is a Rap1 activator. Rap1 is a member of the Ras
subfamily and has the same core effector region as Ras, but its function is still elusive. Recent studies have revealed that Rap1 is activated in human platelets and neutrophils in response to various agonists suggesting that Rap1 may play a role in one of the specialized functions of these cells. In vitro studies showed that C3G could stimulate the dissociation of GDP from Rap1 to a large extent but, had little effect on the dissociation of GDP from Ras or RAL (149,150). Coexpression of C3G and Rap1 in COS7 cells results in an increase in the amount of GTP bound to Rap1 whereas, SOS1 is unable to activate Rap1 (151).

Recently, a mammalian GNEF with homology to Ras-GRF, SOS, and Cdc25 was cloned and characterized from a mouse brain cDNA library, and this cDNA is referred to as Ras-GRF2 (152). Ras-GRF2 seems to be widely expressed based on northern blot analysis. The predicted molecular weight of Ras-GRF2 is ~135 kDa. In vitro studies confirmed that Ras-GRF2 functions as a Ras-specific GNEF (152). In addition as described for GRF1 (152) expression of Ras-GRF2 in kidney epithelial cells results in the activation of Ras and activation of ERK1 in response to elevated intracellular calcium levels (152). Future experiments will determine what specific extracellular signals are responsible for the activation of Ras-GRF2.
The mammalian Ras-GRF proteins and the *Drosophila* and mammalian SOS proteins possess two additional important homology domains. These two domains are arranged in tandem (Figure 4) and are designated the DBL homology (DH) domain and the pleckstrin homology domain (PH). The DBL homology domain consist of ~250 amino acids subdivided into three conserved regions (153,154). DBL is a protein which was initially isolated from a diffuse B-cell lymphoma (155). DBL shares homology with the *S. cerevisiae* cell division cycle protein Cdc24. Cdc24 acts together with the Ras-like GTP binding protein Cdc42 to regulate bud site assembly in yeast (156). Studies performed by Hart and coworkers demonstrated that baculovirus expressed DBL catalyzes the dissociation of GDP from human Cdc42 protein (157). More specifically, amino acids 640-646 (LLKELL), which are very highly conserved, appear to be required to catalyze GTP loading of Rho-like GTP binding proteins (157). The DBL homology region of SOS and Ras-GRF proteins represents only a small portion of the regions in Cdc24 and DBL (136), but this region appears to be required in all cases to catalyze GTP loading of Rho-like GTP-binding proteins. The degree of sequence similarity between the DBL homology domains in SOS/GRF proteins is somewhat low, approximately 30%.

There is no evidence thus far to suggest that SOS or
Figure 4. Domains of the Sos Of Sevenless protein. The dSOS protein can be divided into three major domains: 1.) the amino-terminal domain (NH2 domain), 2.) the catalytic domain, and 3.) the carboxyl-terminal domain (COOH-domain). The NH2-terminal domain contains the PH and the DBL homology regions. The COOH-terminal domain contains proline-rich motifs which are responsible for binding the adaptor protein GRB2. The amino acid numbers are denoted beneath the dSOS construct.
Ras-GRF proteins are able to act as activators for the Rho family proteins. However, the presence of two separate catalytic domain similarities (Cdc25 and DBL) in these guanine nucleotide exchange factors suggest that by some mechanism they may be able to coordinately regulate the activities of Ras and Rho family proteins.

The second mentioned homology domain common to all DBL family proteins is referred to as the PH domain. The PH domain was originally identified as a protein module of ~120 amino acids that shares homology with two regions of the protein pleckstrin. Pleckstrin is a protein which is the major substrate for ser/thr phosphorylation by PKC in platelets. The PH domain is present in a variety of intracellular signaling and cytoskeletal proteins. The PH domain was proposed to be involved in protein-protein interactions. More then 90 PH domain-containing proteins have been identified, including ser/thr kinases, tyrosine kinases, isoforms of phospholipase C, GAP’s, GNEF’s, and cytoskeletal elements (158). There is only one invariant amino acid in all PH domain-containing proteins, which is a conserved tryptophan near the carboxy terminus. The three-dimensional structure of the PH domains from pleckstrin (159), spectrin (160), dynamin (161-163), and SOS (164) have been determined by NMR and or X-ray crystallography analysis. Although the overall
homology shared among PH domains is low, all three structures show a common fold consisting of two orthogonal β-sheets which form a sandwich that is capped by the C-terminal α-helix at one end (165). The invariant tryptophan is found within the helix. It is noteworthy to mention that more recent structural studies of phosphotyrosine recognition sites (PTB domains) found in proteins such as IRS-1 and SHC illustrate that they share a common fold with PH domains (204). Thus, it was predicted that PH and PTB domains serve to recruit proteins close to the membrane surface where they can interact with their target proteins.

The structures revealed that there is a potential for a cluster of positively charged amino acids to form on the face of the molecule opposite from the C-terminal α-helix. This suggested that PH domains may interact with membrane surfaces via binding negatively charged phospholipids. In support of this, Hyvonen et al. performed structural studies that demonstrated the binding of Ins(1,4,5)P₃ to the PH domain of mouse spectrin (166). In addition, the PH domains of pleckstrin, T-cell specific kinase, rasGAP, and βARK were found to bind PI(4,5)P₂ (167). Mutational studies of the PH domain of pleckstrin indicated that three specific lysine residues (positions 13, 14, and 22) are important for binding PIP₂ in vitro (168). PIP₂ binds to the PH domains of pleckstrin and spectrin with
relatively low affinity (Kd~ 30 μM) (166,168). By contrast, the PH domain of PLCδ binds PIP2 with relatively high affinity (Kd~1.7 μM). PI-4,5-P2 binding to the PH domain of PLCδ seems to play a role in recruiting PLCδ to the membrane (165). Very recently, Kubiseski and coworkers demonstrated utilizing lipid vesicles containing PI-4,5-P2, that PIP2 binds with relatively high affinity (Kd~1.8 μM) to the PH domain of mSOS1 (169,170). More specifically, these studies demonstrated that a specific amino acid within the PH domain of mSOS1, Arg459, plays a critical role in mediating this interaction with PIP2 (169). All of these lipid binding studies are in vitro studies and further experimentation is required to determine if this interaction of PH domains with PIP2 plays an important role in regulation of these proteins in vivo.

Recently, the ability of various PH domains to bind to products of PI3-kinase has been investigated by several groups. PI-3,4-P2 and PI-3,4,5-P3 could provide one possible mechanism for regulated recruitment of proteins to the membrane since both of these lipids are produced in response to cell stimulation (210). PI-4,5-P2 and PI-4-P on the other hand are constitutively produced in cells (210). Initial studies of the PH domain in Akt, the Ser/Thr kinase, suggest PI-3,4-P2 binds selectively to the PH domain and that PI-3,4-P2 activates the kinase (208,209). In addition, the
PH domain of Btk protein tyrosine kinase was shown to bind to PI-3,4,5-P_3 and Ins-1,3,4,5-P_4 selectively (206,207).

Rameh, *et al.* have examined the binding of several PH domains to five different phosphoinositides (170). In these in vitro studies, radiolabeled lipids were utilized in direct binding and competitive binding experiments. The following three PH domains were shown to selectively bind PI-3,4,5-P_3: 1.) the SOS PH domain (SOS activates Ras); 2.) the Tiam-1 PH domain (Tiam activates Rac); and 3.) the Grp-1 (general receptor for phosphoinositides) PH domain (Grp-1 may activate Arf) (205). The Grp-1 protein was isolated based on its ability to bind to PI-3,4,5-P_3 in an expression cloning assay (205). The amino-terminal PH domain of Tiam-1 seems to be important for membrane localization (211-213), as mentioned above for the PH domain of PLCδ. By contrast, the PH domains of βARK, spectrin, OSBP (oxysterol-binding protein) were found to bind PI-3,4,5-P_3 weakly and showed specificity for PI-4,5-P_2 over PI-3,4-P_2 (170). Thus, PH domains seem to have specificity for different phosphoinositides, and published data suggest that their interaction with lipids may play a role in recruiting proteins to the membrane.

Furthermore, data from several labs has suggested that PH domains bind to the Gβγ complex. Initial experiments by Lefkowitz and colleagues
illustrated the binding of βARK to Gβγ complex (171,172). Subsequent studies demonstrated that PH domains from βARK, PLCγ, IRS-1, Ras-GRF, and Ras-GAP, when expressed as glutathione S-transferase fusion proteins, can reversibly bind bovine brain Gβγ subunits in vitro (173). Experiments using PH domain-containing peptides derived from βARK, PLCγ, IRS-1, Ras-GRF, and Ras-GAP were shown to behave as specific antagonists of Gβγ-mediated signaling in intact COS-7 cells (174). In addition, the PH domains of DBL, mSOS1, IRS-1, and βARK were shown to bind Gβγ subunits in vitro in a dose dependent manner (174). In these same experiments, mSOS1 was unable to bind to Gβγ subunits in vitro (174). In summary, the data generated on PH domains implies that they play a role in the assembly of membrane-associated protein complexes that initiate signal transduction cascades.

The last domain that I would like to discuss is shared by Drosophila / mammalian SOS proteins and C3G. This domain is referred to as the SH3 binding domain (see Figure 3). The SH3 domain has been described in the previous section entitled Signaling by Receptor Tyrosine Kinases. As mentioned previously, SH3 domains bind to proline rich motifs. Upon examination of the primary amino acid sequence of the SOS sequence, it was discovered that the carboxy terminus of SOS is proline-rich. In 1993
two papers were published in the same issue of Nature illustrating that the SH3 domain of GRB2 associates with the proline-rich motifs in the carboxy terminus of SOS. Egan and coworkers were able to coimmunoprecipitate GRB2 and SOS from Rat 1 cells (175). Furthermore, they performed in vitro studies, using GST-Grb2myc and various GST- mSOS1 deletion constructs which confirmed that GRB2 was binding specifically to the C-terminal domain of mSOS1 (amino acids 1,135-1,336) (175). More specifically, their studies indicated that GRB2 and mSOS1 association is dependent upon the cooperative interactions of the two GRB2 SH3 domains (175). The second paper also demonstrated the coimmunoprecipitation of mSOS1 and GRB2 from NIH3T3 cells (176). A number of research groups subsequently showed that Ras-GEF's are involved in signal transduction pathways from the cell surface to Ras proteins by demonstrating complex formation between the EGFR, GRB2, and SOS upon stimulation with EGF (146,177).

G. GRB2

The initial identification and characterization of SEM5 (C. elegans) / GRB2 (mammalian)/ DRK (Drosophila) was a major step towards understanding the mechanisms by which the Ras proteins are regulated in
response to extracellular mitogens. Genetic experiments in *C. elegans* led to the cloning of sem5 (178). The sequence of sem5 revealed the protein consisted of two SH3 domains and one SH2 domain. Genetic experiments demonstrated that SEM5 serves as an adapter protein linking tyrosine kinase receptors and Ras (179). Therefore, this discovery immediately suggested a function for the independently cloned human homologue, GRB2. GRB2 was cloned based on the ability of its SH2 domain to bind to tyrosine phosphorylated EGFR (180). As stated previously, a series of papers published in 1992-1993 demonstrated that the link between tyrosine kinase receptors and Ras occurs via the adapter protein GRB2 (51-52,146,175-177). For instance, Buday and Downward showed that upon stimulation with EGF, a complex of receptor, GRB2, and mSOS1 is formed (176). Furthermore, there was no change in the specific activity of mSOS1 after stimulation of Rat 1 cells with EGF (176). This data argued that the redistribution of mSOS1 from the cytoplasm to the membrane may be the major form of SOS regulation. Genetic experiments in *Drosophila* showed that the adapter protein DRK is required for Ras activation and also is able to bind to the Sevenless receptor and SOS in vitro (181). In conclusion, the studies in *Drosophila, C. elegans*, and
mammalian cells illustrate how the signaling cascade from tyrosine kinase receptors to Ras has been evolutionarily conserved.
CHAPTER II
INTRODUCTION

A. SOS and Cellular Signaling

Signal transmission by many receptor tyrosine kinases involves the conversion of Ras proteins from the inactive GDP-bound form to the active GTP-bound form (1-3). The Ras-GTP is able to associate with Raf protein kinase and this results in the stimulation of these and multiple other protein kinases such as MAPKs (87-88). The MAPKs catalyze the phosphorylation of many cellular proteins, resulting in their regulation. Therefore, the Ras signaling cascade modulates important cellular processes such as transcription and protein synthesis (59,88).

Stimulation with growth factors results in autophosphorylation of tyrosine kinase receptors which results in the formation of recognition sites for SH2 domain containing proteins. The formation of these complexes between tyrosine phosphorylated receptor and SH2 domain containing proteins initiates signal transduction cascades. The growth factor stimulation of tyrosine phosphorylation of membrane receptors or receptor substrates provides the appropriate tyrosine phosphate sites for binding the SH2 domain of GRB2. As a result, the rapid association of GRB2 (35,180-
182) or SOS (175,180,183) proteins to complexes containing tyrosine-phosphorylated species such as EGFR, SHC, IRS-1, and SYP has been demonstrated. For instance, competition experiments using synthetic phosphopeptides showed that GRB2 binds directly to the EGFR via phosphorylated tyrosines at positions 1068 and 1086 (184) and SHC binds directly to the EGFR via phosphorylated tyrosines at positions 1173 and 992 (184). Activation of Ras proteins by receptor tyrosine kinases is thought to result from recruitment of SOS proteins to plasma membrane receptor substrates via adapter proteins such as GRB2. As described in Chapter I, the mechanism of this recruitment involves the binding of the SH3 domains of GRB2 with the proline-rich motifs in the carboxy terminus of SOS proteins. For example, studies performed by Aronheim and coworkers suggested that the GRB2 / SOS association may have two functions. First, GRB2 may be responsible for recruiting SOS proteins to plasma membrane sites where SOS can activate Ras. Second, GRB2 binding to the carboxy terminus of SOS may alleviate the potential inhibition that the SOS carboxy terminus invokes on the SOS catalytic activity. More specifically, transient transfection of NIH3T3 cells with mSOS mutant proteins tagged with either farnesylation or myristoylation signals resulted in localization of these SOS proteins to the plasma
membrane and as a consequence were also able to activate Ras (185). Furthermore, SOS constructs which lacked the C-terminal GRB2 binding domain displayed an increase in their ability to activate Ras compared to full length SOS proteins (185). This data is consistent with their model that recruitment of SOS to the plasma membrane via GRB2 is the primary mechanism by which Ras is activated.

B. Sevenless Receptor Signaling

The sevenless gene of *Drosophila melanogaster* encodes a transmembrane protein tyrosine kinase. The sevenless receptor functions to ensure proper development of the *Drosophila* eye (90, 186). The eye consist of ~800 repeated units called ommatidia each of which contains a central core of eight photoreceptors surrounded by 12 accessory cells. During development, the R7 photoreceptor is the last of the photoreceptors to be recruited. In the *Drosophila* eye binding of BOSS (Bride of Sevenless), which is present on the neighboring R8 cells, to the sevenless receptor tyrosine kinase (SEV) on the R7 cells leads to the downstream activation of Ras. As a result, the R7 precursor cell is able to differentiate to a photoreceptor neuron. Genetic experiments have led to the discovery
that dSOS and DRK (GRB2 homologue) are involved in this signaling system and function between SEV and Ras (142, 181, 187).

In order to further understand the role of SOS in signal transduction, Karlovich and co-workers initiated an in vivo structure-function analysis of the dSOS gene product. Deletion constructs of the dsos gene were generated and transformed into flies in which the Sevenless signal is attenuated in order to assess the effects of the constructs on R7 development. Their initial experiments demonstrated that in intact flies a mutant Drosophila dSOS protein lacking the DRK/GRB2 adapter binding COOH-terminal domain (referred to as NCat) was at least as capable as the wildtype dSOS protein in promoting R7 cell development (188) (Figure 5). In these experiments a genetically sensitized background (sevE4; SosJc2/+; SOS+) was used in which the Sevenless signal is attenuated, only 17% of the ommatidia develop an R7 cell (188). In sevE4 flies no R7 cells develop, but in this sensitized background the SosJc2 allele, which encodes an overactive SOS protein, partially compensates for the loss of signal due to the sevE4 mutation (188). The NCatC and NCat constructs enhanced transmission of the Sevenless signal, increasing the population of ommatidia with R7 cells to 33% and 40%, respectively (Figure 5) (188). In contrast, the amino-terminal domain (N), the catalytic domain (Cat), the
Figure 5. SOS constructs and their effects on Sevenless signaling in a sensitized genetic background. (A) Predicted protein products of the SOS constructs. (B) Effects of SOS constructs on R7 development in a sev$^{E4};Sos^{JC2}/Sos^+$ genetic background. For each genotype, one copy of a given construct was used, except for sE(PH) which had very low amounts of expression and the results shown were obtained from flies with two copies of the construct. The eyes were scored by the pseudopupil technique for the presence of R7 cells. For each genotype, at least 2000 ommatidia were counted (Taken from reference 188, Karlovich et al.).
carboxy-terminal domain (C), and the Cat in combination with the C
domain (CatC) all decreased the fraction of R7-containing ommatidia
(Figure 5). In addition, the ability of the mutant dSOS protein (NCat) to
promote R7 cell development required the expression of an intact Sevenless
protein (see Figure 6). In these particular experiments, both NCat and
NCatC were able to promote R7 development in a sev<sup>Ex</sup> background
(Figure 6, A, B, and D). However, neither NCat or NCatC were able to
suppress the null allele sev<sup>d2</sup> which makes no sevenless protein (Figure 6,
A, C and E). Therefore, the sevenless molecule is essential for NCat
function suggesting an interaction between NCat and Sevenless which is
independent of DRK.

In addition, these dSOS deletion constructs were tested for their ability
to act as dominant negatives using a sev<sup>+/sev</sup><sup>Ex</sup> background (188). In this
genetic background the flies have a functional Sevenless signaling system
that results in an eye containing R7 cells in all ommatidia because sev<sup>Ex</sup> is a
recessive mutation. A construct acting as a dominant negative would be
expected to compete with endogenous components and disrupt the wild-type
signal resulting in ommatidia lacking R7 cells. The following constructs
were able to disrupt the wild-type signaling system to a modest extent:
sE(C), sE(N), sE(Cat), and sE(PH) (Table 1). Interestingly, the construct
Figure 6. Suppression of $sev^{E4}$, but not the null allele $sev^{d2}$ by sE(NCat) and sE(NCatC). (A) Fraction of ommatidia in which R7 cells appear in $sev^{E4}$ and $sev^{d2}$ backgrounds in the presence of sE(NCatC) and sE(NCat). For each genotype, two copies of either sE(NCatC) or sE(NCat) were used and at least 2000 ommatidia were scored. (B through E) Distal tangential sections from adult eyes. The dark structures in each facet or ommatidium are light-gathering organelles called rhabdomeres. The centrally positioned R7 rhabdomere is marked with an arrow. (B) $sev^{E4}$;sE(NCatC). (C) $sev^{d2}$;sE(NCatC). (D) $sev^{E4}$;sE(NCat). (E) $sev^{d2}$;sE(NCat) (Taken from reference 188, Karlovich et al.).
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**Table 1. Effects of SOS constructs on R7 development in a sev<sup>+</sup>/sev<sup>E4</sup> genetic background.** In each case, at least 2000 ommatidia were scored. Two copies of each construct were used in this assay except for sE(PH), for which expression was low and four copies were introduced (Taken from reference 188, Karlovich et al.).
lacking the NH$_2$-terminal domain (CatC) had a strong dominant negative effect, resulting in 70% of the ommatidia lacking R7 cells (see Table 1) (188). In a similar manner, the CatC construct failed to rescue the S. cerevisiae cdc25-5 temperature sensitive mutation (Figure 7). Transformation of the $cdc25^n$ with the CDC25 gene, or the dSOS complementary DNA fragments NCat or Cat resulted in rescue of the $cdc25^n$ strain and allowing its growth at the nonpermissive temperature (Figure 7). As a result, the ability of the Cat domain to activate Ras was lost upon the addition of the COOH-terminus onto it which is consistent with the hypothesis that the COOH-terminus may have an inhibitory influence on the catalytic domain. The results from these genetic experiments described above allow several conclusions to be made regarding the function of SOS in the developing eye of the Drosophila:

1.) The amino-terminal region of SOS was essential for its function, 2.) The carboxyl-terminal region of SOS was not essential for its function, and 3.) The COOH-terminus may play an inhibitory role. Therefore, these genetic experiments argue against a role for GRB2/DRK in the recruitment of SOS to the plasma membrane and suggest that regions within the NH$_2$-terminal domain of SOS proteins are necessary for their
Figure 7. Complementation of a *S. cerevisiae* cdc25<sup>ts</sup> mutation by SOS constructs. Growth of the cdc25-5 temperature-sensitive mutation at restrictive (34.5 °C) and permissive (26°C) temperatures was examined after transformation with the pAD54 plasmid containing a complementing fragment of the *S. cerevisiae* CDC25 gene or the SOS cDNA fragments NCat, N, Cat, or CatC. For each construct, two independent transformants are shown (Taken from reference 188, Karlovich *et al.*).
physiological function. It was these genetic experiments that initiated my thesis research project.

C. Thesis Focus and Specific Aims

The Focus of my Thesis is, "What is the Mechanism by Which SOS Activates Ras in Response to Tyrosine Kinase Receptors?" The specific aim of CHAPTER IV is to test the hypothesis proposed in the literature which postulates that the GRB2 binding domain of SOS is necessary for SOS to be recruited to the plasma membrane where it can activate Ras. In order to test the proposed hypothesis three specific questions were addressed: 1.) What are the roles of the NH₂-terminal and COOH-terminal regions of dSOS?, 2.) Is GRB2 able to bind regions in the dSOS protein that reside in the NH₂-terminal or catalytic domains?, and 3.) Are there other important structural elements within the NH₂-terminal domain of dSOS that are required for its ability to activate Ras? The specific aim of CHAPTER VI is to test the hypothesis that the amino-terminal domain of dSOS, specifically the PH and DBL regions, is important for its membrane association. In order to test the proposed hypothesis two specific questions were addressed: 1.) What is the distribution of various dSOS mutant proteins in 293 cells? and 2.) Can neomycin which binds PI-4,5-P2
strongly, inhibit the ability of various dSOS mutant proteins to associate with total membranes in 293 cells?
A. Construction of pCMV5-dSOSHA deletion constructs

The dSOS constructs were engineered using standard molecular biology techniques. Each construct was modified at its respective COOH-terminus to encode a hemagglutinin (HA) epitope extension. The 9-amino acid peptide sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala of the influenza HA which is specifically recognized by the monoclonal antibody 12CA5 (189), was added to the 3' end of the dSOS construct coding sequences. The dSOS cDNA constructs encode the following amino acids: NCatCHA, 1-1596; NCatHA, 1-1125; NCatδPHHA, 1-475 and 592-1125; PHCatHA, 433-1125; CatHA, 758-1125; CatCHA, 1-53 and 681-1596; CHA, 1-53, 681-690, and 1040-1596; NHA, 1-690; NCatδ200HA, 1-311 and 681-1125; NCatδ300HA, 1-219 and 681-1125; DBLIIIPHCatHA, 311-1125; and PHCatHA(w-->g, the conserved tryptophan in the PH domain was mutated to a glycine) (see Figures 9 and 22).
B. Cell lines and transient transfections

COS-1 cells (American Type Culture Collection RL 1650) were grown in DMEM medium containing 10% bovine calf serum, and 293 cells (American Type Culture Collection CRL 1573) were grown in DMEM medium containing 10% calf serum. These cells were maintained in a 37°C incubator at 5% CO₂. Plates of COS-1 cells and 293 cells were transfected using the CaPO₄ method according to standard protocols.

C. Immunoprecipitation

Cells (100 mm plates) were washed and lysed in 1.0 ml of an ice-cold buffer composed of 0.05 M NH₄(SO₄), 20 mM Hepes (pH 7.4), 10% glycerol, 1.0 % Triton X-100, 0.1 % Tween 20, 0.1 mM EDTA, 1.0 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl chloride, and aprotinin, pepstatin, and leupeptin at 10 μg/ml each. Lysates were clarified by centrifugation at 13,000 x g for 10 min. at 4°C. Lysates were next incubated with the desired antibody (anti-HA polyclonal) and protein A-Sepharose (Pharmacia) for approximately 16 hours at 4°C. Immunoprecipitates were washed five times in a buffer containing 0.05 M NH₄(SO₄), 20 mM Hepes (pH 7.4), 10% glycerol, 1.0 % Triton X-100, 0.1
% Tween 20, and 0.1 mM EDTA and then boiled in reducing SDS gel sample buffer for 5 min. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (190) using an electroblotting tank at 110 mA overnight.

D. Immunoblotting

Blots were blocked for a minimum of 30 minutes in 3% nonfat dry milk, 2.9% NaCl, 0.5% BSA, 100 mM Tris-HCl (pH 7.4), 0.1% Tween-20. Blots were next incubated at room temperature with gentle agitation for a minimum of 2 hours in primary antibody, which was diluted in a tris buffered saline solution (TBS) with 0.1% Tween-20. Subsequently, the blots were washed three times in TBS / 0.1% Tween-20 buffer (first wash for 15 min and last two washes for 5 min each). The blots were then incubated for 25 min with the appropriate horseradish peroxidase conjugated secondary antibody at a 1 : 5,000 dilution in TBS / 0.1% Tween-20 solution. Blots were again washed in the same manner as described above. Primary antibodies were detected by enhanced chemiluminescence (Amersham or NEN) and densitometry was performed using a Pharmacia LKB 2222-020 ultrascan XL.
E. Immunofluorescence

COS-1 / 293 cells were plated on glass coverslips and transiently transfected with the desired DNA construct via CaPO₄ mediated method. Approximately 48 hours after transfection, the cells on the coverslips were washed three times in ice-cold phosphate-buffered saline (PBS) consisting of 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 3.35 mM KCl, 170 mM NaCl, 0.68 mM CaCl₂, and 0.49 mM MgCl₂. Cells were next fixed in 4% formaldehyde in PBS for 10 min at room temperature. Subsequently, the cells were washed three times with ice-cold PBS and permeabilized with 0.5% Triton X-100 and 1.0% FBS in PBS for 15 min at room temperature. After two washings in the permeabilization buffer, cells were incubated for two hours with primary antibody (anti-HA at a dilution of 1:1,000 in permeabilization buffer) at room temperature with gentle shaking. The cells were next washed four times with ice-cold permeabilization buffer for 5 min each time with shaking and then incubated for 30 min with fluorescein isothiocyanate-conjugated anti-mouse (TAGO)(dilution of 1:1,000 in permeabilization buffer) with agitation. After incubation with the secondary antibodies, the cells were thoroughly washed, first with ice-cold permeabilization buffer (4 times) and once with PBS. Last, the coverslips were mounted on slides using 90% glycerol
plus 2.5% DABCO (1,4 diazabicyclo-(2,2,2)-octane). Immunofluorescence was visualized by digital imaging microscopy.

F. Cell labeling and Ras activation assay

Two days after transfection, the cells (60 mm plates) were washed twice with phosphate-free DMEM (Gibco) supplemented with 25 mM Hepes and 2 mM pyruvate. Subsequently the cells were labeled with carrier-free \[^{32}\text{P} \text{orthophosphate}\] (1.0 mCi / ml) (NEN) for 4 hours at 37 °C in 1.5 ml of phosphate-free DMEM supplemented with 25 mM Hepes and 2 mM pyruvate. Each plate of cells was next lysed in 0.9 ml of buffer composed of 50 mM Hepes, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mg/ml BSA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 : 10 dilution of 1742 supernatant (anti-Ras antibody). Lysates were clarified by centrifugation at 13,000 x g for 5 minutes at 4 °C. Supernatants were transferred to a new tube and combined with 0.2 ml of a detergent solution (0.5 M NaCl, 0.5% DOC, and 0.05% SDS) and 30 ul of goat-anti rat Sepharose (Sigma). After overnight incubation at 4°C on an end-over-end mixer, immune-complexes were pelleted by centrifugation at 13,000 x g for 40 seconds. The goat-anti rat Sepharose complexed with anti-Ras
antibody was next washed 8 times in a wash buffer composed of 50 mM Hepes, 0.5 M NaCl, 5 mM MgCl$_2$, 0.1% Triton X-100, and 0.005% SDS. Guanine nucleotides bound to Ras were eluted by the addition of 20 µl of elution buffer (0.075 M KH$_2$PO$_4$ pH 3.4, 5 mM EDTA, 0.5 mM GTP, and 0.5 mM GDP) to each sample and heating the samples for 3 min at 85°C. The samples were clarified by centrifugation, and 8 µl of each sample was spotted onto a sheet of polyethyleneimine cellulose (VWR). $^{32}$P]GTP and $^{32}$P]GDP were separated by developing the chromatograms with 0.65 M KH$_2$PO$_4$ (pH 3.4) (191). GTP and GDP standards were visualized with short wave UV. The radiolabeled nucleotides were visualized by autoradiography. The GTP and GDP spots on the TLC plate were excised, and the amount of $^{32}$P]orthophosphate incorporated was determined using a β counter (Beckman LS 5000 TD).

G. In vitro binding assay

*Spodoptera frugiperda* Sf9 cells were cultured in Grace’s complete medium and infection with high titer baculovirus stocks of either NCatCHA or NCatHA was carried out according to standard protocols. NCatCHA and NCatHA cDNA constructs were subcloned into the baculovirus transfer vector pVL1393 for expression in Sf9 cells. Frozen pellets of baculovirus-
infected Sf9 cells (NCatCHA, NCatHA, and wild-type) were homogenized on ice using a 2-ml homogenizer (10 strokes) in 1 ml of cold lysis buffer (phosphate-buffered saline with 10 mM NaF, 1 mM dithiothretitol (DTT), 1 mM vanadate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin, pepstatin, and leupeptin at 10 μg/ml each). Homogenates were spun in a microcentrifuge at 15,000 x g for 15 min at 4 °C. The supernatants were removed and assayed for total protein content using the Bradford method (192). Proteins from each lysate were dissolved in sample buffer, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue.

Equal amounts of protein from each lysate were incubated with 30 ul of glutathione-Sepharose (Pharmacia), which was prebound with GST or GST-GRB2, on an end-over-end mixer at 4°C for 2 hours. GST and GST-GRB2 fusion plasmid were gifts from Dr. J. Schlessinger (New York University, New York, NY). The Sepharose was pelleted by centrifugation at 15,000 x g for 2 min at 4°C. Pellets were washed twice with 20 mM Hepes, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT, twice with 20 mM Hepes, pH 7.5, 10% glycerol, 500 mM NaCl, and 1 mM DTT and once with 20 mM Hepes, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT. Proteins remaining bound to the GST or GST-GRB2 were dissolved
in SDS-PAGE sample buffer. Samples were loaded on SDS-PAGE gels (6%) and transferred to nitrocellulose filters. Filters were probed with anti-HA monoclonal antibody, and antibody bound proteins were visualized using ECL (Amershem Corp.) according to the manufacturer’s specifications.

H. Partial purification of Ras

Sf9 insect cells were cultured in Grace’s complete medium and infected with a high titer baculovirus stock of Ras (gift from Dr. Martin McMahon, DNAX, Palo Alto, CA). Frozen pellets (~1.0 ml) of baculovirus-infected Sf9 cells were homogenized on ice using a Dounce homogenizer (20 strokes) in 10.0 ml of cold lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 200 μM GDP, 1 μM DTT, and 10 μg/ml each of leupeptin and aprotinin. After homogenization, Triton X-114 was added to achieve a final concentration of 1.0% and the lysate was centrifuged at 100,000 x g at 4°C for 30 min. The supernatant was removed and separated into aqueous and detergent phases by warming to 37°C for 2 min and subsequent centrifugation at 400 x g for 2 min. Two sequential phase-separations were performed to wash the original detergent phase. The aqueous and detergent phases were next separated
and adjusted to 1.0% Triton X-114. The detergent phase was dialyzed against a buffer composed of 20 mM Tris-HCl, pH 7.5, 0.05% DOC, 50 mM NaCl, 1 mM DTT, and 5 mM MgCl₂ and then centrifuged at 10,000 x g at 4°C for 10 min. Post-translationally processed Ras proteins were purified by anion exchange chromatography from the original detergent phase on a Cellex D column (~0.5 ml). The column was equilibrated and washed with a buffer composed of 50 mM Tris-HCl, pH 7.5, 0.5% DOC, 20 mM NaCl, and 1 mM DTT. Processed Ras proteins were eluted in three 1.5 ml fractions of buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% DOC, 500 mM NaCl, and 1 mM DTT. Ras proteins were dialyzed against a buffer composed of 20 mM Tris-HCl, pH 7.5, 0.5% DOC, 50 mM NaCl, 1 mM DTT, and 5 mM MgCl₂ and stored at -70°C until use.

I. In vitro Ras activation assay

The partially purified Ras proteins were first tested for their ability to bind [³H]GDP. Ras proteins were incubated with 1.0 µM [³H]GDP in a buffer composed of 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.4 mg/ml BSA, 1.0 mM DTT, and 1.0 mM EDTA for 30 min at 30°C. The exchange was stopped by adding 1.0 ml of ice cold buffer composed of 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10.7 mM MgCl₂, 1.0 mM DTT, and 0.4
mg/ml BSA and incubation on ice for 5 min. The radioactivity bound to Ras was determined by immunoprecipitating Ras with the Ras antibody (1:10 dilution of 1742 cell supernatant) and 80 ul of Goat-anti Rat Sepharose (Sigma) in the presence of 0.5% DOC and 0.05% SDS. After incubation for 1 hour at 4°C on an end-over-end mixer, the goat-anti rat Sepharose complexed with anti-Ras antibody was next washed 3 times in a wash buffer composed of 50 mM Hepes, 0.5 M NaCl, 5 mM MgCl₂, 0.1% Triton X-100, and 0.005% SDS. The beads were drained well and resuspended in 1.0 ml of opti-fluor and the radioactivity associated with the beads was determined using a β counter (Beckman LS 5000 TD).

After demonstrating that the partially purified Ras was active, the Ras was utilized in experiments to test the ability of NCatCHA and NCatHA to catalyze the exchange of GDP for GTP on the Ras in vitro. First, frozen pellets of baculovirus-infected Sf9 cells (NCatCHA and NCatHA) were homogenized on ice using a 2-ml homogenizer (10 strokes) in 1 ml of cold lysis buffer (phosphate-buffered saline with 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM vanadate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin, pepstatin, and leupeptin at 10 μg/ml each). Homogenates were spun in a microcentrifuge at 13,000 x g for 15 min at 4°C. The supernatants were removed and assayed for total
protein content using the Bradford method (192). Lysates (~2.5 mg) were next incubated with the desired antibody (anti-HA polyclonal) and Protein A-Sepharose (Pharmacia) for approximately 16 hours at 4°C.

Immunoprecipitations were washed five times in a buffer containing 0.05 M NH₄(SO₄), 20 mM Hepes (pH 7.4), 10% glycerol, 1.0% Triton X-100, 0.1% Tween 20, and 0.1 mM EDTA and the beads were drained well. Ras was next equilibrated at room temperature for 10 min in a buffer composed of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1.0 mg/ml BSA, 1.0 mM DTT, and 0.05% sodium cholate. The binding assay was initiated by aliquoting 25 µl of the Ras mixture into tubes containing 25 µl of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1.0 mg/ml BSA, 1.0 mM DTT, 0.05% sodium cholate, 1.0 µM [³H]GTP (31.5 Ci/mmol), and the immunoprecipitated NCatCHA or NCatHA (drained beads). The final concentration of Ras in each reaction was ~ 100 nM.

The binding reactions were carried out for the indicated times (10, 30, or 60 min) at 25°C and subsequently stopped by the addition of 1.0 ml ice-cold stop buffer composed of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM MgCl₂. The radioactivity bound to Ras was determined by immunoprecipitating Ras with the Ras antibody (1:10 dilution of 1742 cell supernatant) and 60 µl of goat-anti rat Sepharose (Sigma) in the presence of
0.5% DOC and 0.05% SDS. After incubation for 1 hour at 4°C on an end-over-end mixer, the goat-anti rat Sepharose complexed with anti-Ras antibody was next washed 3 times in a wash buffer composed of 50 mM Hepes, 0.5 M NaCl, 5 mM MgCl₂, 0.1% Triton X-100, and 0.005% SDS. The beads were drained well and resuspended in 1.0 ml of opti-fluor and the radioactivity associated with the beads was determined using a β counter (Beckman LS 5000 TD).

J. Membrane fractionation experiments

293 cells (100-mm plates) were harvested approximately 60 hours after transfection and washed once with cold PBS. Cells were homogenized for 20 strokes with a motor-driven Teflon-glass homogenizer in 1.5 ml (for two 100 mm plates) of ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 255 mM sucrose, 1 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin and leupeptin at 10 μg/ml each). Homogenates were spun in a microcentrifuge at 800 x g for 5 min at 4°C to remove nuclei and unbroken cells. Next, the homogenate was spun at 100,000 x g for 25 min at 4°C. The resulting supernatant was saved as cytosol and the pellet was washed twice with a buffer composed of
0.05 M NH₄(SO₄), 20 mM Hepes (pH 7.4), 10% glycerol, 1.0% Triton X-100, 0.1% Tween 20, 0.1 mM EDTA, 1.0 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin, pepstatin, and leupeptin at 10 µg/ml each. The total membrane pellet was then resuspended in 1.0 ml of this same buffer. Total membranes were clarified by spinning at 13,000 x g for 10 min at 4°C which resulted in a Triton X-100 insoluble fraction (pellet). Protein in the cytosol and membrane fractions was quantitated using the BCA protein determination kit with BSA as the standard. HA-tagged proteins were immunoprecipitated from cytosol and membrane fractions as described previously, using anywhere from 1.0 mg to 3.0 mg of total protein.

K. Neomycin inhibition experiments

Cells were fractionated as previously described. After the 100,000 x g spin, the total membranes were resuspended in a buffer composed of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 255 mM sucrose, 1 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin and leupeptin at 10 µg/ml each). Resuspended membranes were incubated for 10 min at 4°C in the presence or absence of neomycin (15 or 40 µM). Total membranes were pelleted by spinning again at 100,000 x g for 24
min at 4°C. Supernatants were saved and used for anti-HA immunoprecipitations. The particulate fraction was then resuspended in 1.0 ml of a buffer composed of 0.05 M NH₄(SO₄), 20 mM Hepes (pH 7.4), 10% glycerol, 1.0% Triton X-100, 0.1% Tween 20, 0.1 mM EDTA, 1.0 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and aprotinin, pepstatin, and leupeptin at 10 μg/ml each. The particulate fraction was clarified by spinning at 13,000 x g for 10 min at 4°C which resulted in a Triton X-100 insoluble fraction (pellet). Protein in the cytosol and membrane (particulate) fractions was quantitated using the BCA protein determination kit with BSA as the standard. HA-tagged proteins were immunoprecipitated from cytosol and membrane fractions as described previously, using anywhere from 1.0 mg to 3.0 mg of total protein.
CHAPTER IV
CHARACTERIZATION OF FUNCTIONAL DOMAINS OF SOS

A. Specific Aims and Rationale

Ras proteins are activated primarily by specific guanine nucleotide exchange factors such as the Son of Sevenless (SOS) proteins. Activation of Ras is postulated to occur in response to plasma membrane localization of a complex containing SOS and the adapter protein GRB2. The specific aim of this Chapter is to test the hypothesis proposed in the literature which postulates that the GRB2 binding domain of SOS is necessary for SOS to be recruited to the plasma membrane where it can activate Ras (Figure 8).

The rationale that this model proposed in the literature may not be correct is based on preliminary data from Chris Karlovich and colleagues at UCLA. These genetic experiments are discussed thoroughly in Chapter II. Briefly, Karlovich et al. applied an in vivo structure-function analysis of the dSOS gene product in order to map important functional domains of the protein. These experiments in intact flies suggested that there are other important functional domains in the NH₂-terminus of dSOS and that the DRK (GRB2) binding domain was not required for dSOS function. Therefore, data generated using the Drosophila system suggest that the
Figure 8. Model for the activation of Ras by SOS in response to growth factors (Based on reference 185). SOS C-terminal sequences were shown to bind to the SH3 domains of GRB2. Therefore, it was postulated that GRB2 serves to recruit the cytosolic SOS protein to the plasma membrane. This model proposes that GRB2 binds to activated growth factor receptors via the SH2 domain of GRB2. GRB2 binding to the receptors results in recruitment of SOS protein from the cytosol to the plasma membrane where SOS is able to active Ras.
model proposed for the mammalian system (Fig. 8) involving SOS recruitment via GRB2 binding requires critical examination.

B. Construction of *Drosophila* SOS mutant proteins

*Drosophila* SOS cDNA constructs were engineered in order to evaluate the functions of its three major domains: 1.) catalytic domain, 2.) COOH-terminal domain, and 3.) the NH$_2$-terminal domain. The catalytic domain, Cat, was originally defined as the region which shares homology with the *Saccharomyces cerevisiae* Ras activator protein CDC25 (123). The domain COOH-terminal to Cat is denoted as C and consists of the characteristic proline-rich motifs which have been demonstrated to bind to the SH3 domains of GRB2 (51-52,146,175-177). The region NH$_2$-terminal to Cat is denoted as N. Hemagglutinin epitope (HA)-tagged constructs corresponding to those represented in Figure 9 were prepared and ligated into the mammalian expression vector pCMV5.
Figure 9. Schematic representation of the HA-tagged native and mutant SOS constructs employed in the experiment of Fig. 12. The amino acid numbers are denoted beneath NCatCHA.
C. Stimulation of Ras activation by expression of recombinant dSOS proteins

COS-1 cells were transiently cotransfected with the constructs listed in Figure 9 and the Ras cDNA which was also subcloned into pCMV5. Next, the cells were labeled for four hours with \(^{32}P\)orthophosphate in serum / phosphate-free media. The cells were lysed and the Ras was immunoprecipitated using an anti-Ras monoclonal antibody. Immunofluorescence studies illustrated that the Ras was expressed virtually exclusively at the cell surface and also Ras was expressed at relatively the same level in each condition as verified by immunoblotting (Figure 10). Furthermore, the HA-tagged constructs could be readily visualized by western blotting (see Figure 11) or immunofluorescence (data not shown) when transiently expressed in either COS-1 or 293 cells.

Several studies have been published which demonstrate that the catalytic domain of SOS alone, as expected, possesses intrinsic guanine nucleotide exchange activity. Banerjee and coworkers showed that the catalytic domain of dSOS (CatHA construct) is able to rescue \(S.\ cerevisiae\) with the CDC25\(^a\) mutation which is responsible for inactivating endogenous Ras exchange activity (Figure 7) (188). In addition, the catalytic domain of human SOS generated in Sf9 cells was fully effective in catalyzing the
Figure 10. Immunoprecipitation of transiently expressed Ras. Western Blot of anti-Ras immunoprecipitates with the anti-Ras antibody. COS-1 cells were transiently co-transfected with the Ras (2 μg) and the HA-tagged dSOS cDNAs (5 μg each) shown. [32P]Orthophosphate-labeled cells were then lysed, and the cleared lysate was immunoprecipitated with anti-Ras antibody as described in Materials And Methods section. Immune-complexes were separated by SDS-PAGE (12.5% acrylamide) and immunoblotted with the anti-Ras antibody. Protein bands were visualized by enhanced chemiluminescence (NEN).
Immpt:
anti-RAS

Western blot
anti-RAS

RAS
NCatCHA
NHA
NCatHA
CatHA
CatCHA
CHA

+ + + + + + + + + +
- + - - - - - - - -
- - + - - - - - - -
- - + - - - - - - -
- - - + - - - - -
- - - + - - - - -
- - - - + - - -
Figure 11. Immunoprecipitation of transiently expressed HA-tagged dSOS constructs. COS-1 cells were transiently transfected with NCatCHA, NCatHA, CHA, CatHA, and CatCHA. Forty eight hours after transfection cells were lysed in lysis buffer containing 1% Triton/0.1% Tween. Cleared lysates (500 μg protein) were incubated overnight with 20 μl of Protein-A Sepharose and 15 μl of anti-HA monoclonal antibody. Immunoprecipitates were washed five times in 1% Triton/0.1% Tween. Immune complexes were separated by SDS-PAGE and immunoblotted with the anti-HA antibody. Protein bands were visualized by enhanced chemiluminescence (NEN).
Western blot:
anti-HA
exchange of GDP for GTP on Sf9 cell-purified K-Ras in vitro (193). Interestingly, CatHA had no substantial stimulatory effect of $[^{32}\text{P}]$GTP binding to Ras when expressed in either intact COS-1 (Fig. 12, lane 5) or 293 cells (Fig. 22, lane 7). Therefore, although the catalytic domain of dSOS possesses intrinsic guanine nucleotide exchange activity, the data in Figures 12 and 22 clearly indicate that the catalytic domain alone is unable to catalyze the exchange of GDP for GTP on Ras when overexpressed along with Ras in mammalian cells.

The failure of CatHA to activate Ras in intact cells suggested that the GRB2 binding domain of SOS is required to carry out this function. Several studies published in the literature argue that GRB2 plays a role in recruiting SOS to the plasma membrane where it can activate Ras. However, the evidence for this is very limited and indirect. Unexpectedly, the CatCHA construct which contains both the catalytic and COOH-terminal domains of dSOS failed to cause Ras activation when co-expressed with Ras in the COS cell system (Fig. 12, lane 6). Surprisingly, transfection of the NCatHA cDNA construct which encodes for the complete NH$_2$-terminal and catalytic domains of dSOS resulted in an increase in the amount of $[^{32}\text{P}]$GTP bound to Ras (Fig. 12, lane 4). NCatHA seems to be even more active than NCatCHA (Fig. 12, lanes 2 and 4). However this is probably
Figure 12. Stimulation of Ras GTP/GDP exchange in intact cells by expression of recombinant dSOS constructs NCatCHA or NCatHA. (A) COS-1 cells were transiently co-transfected with the RAS (2 μg) and the HA-tagged dSOS cDNAs (5 μg each) shown. [32P]Orthophosphate-labeled cells were then lysed, and the cleared lysate was immunoprecipitated with anti-Ras antibody as described in Materials And Methods section. The positions of the GTP and GDP standards are indicated. Lane 1 represents cells that were transfected with Ras only; lane 8, with NCatCHA only. (B) Graphic representation of Ras activation by expression of NCatCHA or NCatHA in COS-1 cells. The GTP and GDP spots on the TLC plate were excised, and the amount of [32P]orthophosphate incorporated was determined using a β counter. The ratio of GTP to GDP was calculated and expressed as a percentage, and the values shown represent the average of three individual experiments with vertical bars denoting standard errors. The leftmost bar on the graph represents cells that were transfected with Ras only.
not the case because Western blotting and immunofluorescence studies using the anti-HA antibody indicated that NCatHA may be expressed to a greater extent than NCatCHA. Furthermore, as expected, transfection of either the NHA or CHA constructs in COS-1 cells had no effect on \([^{32}P]GTP\) binding to Ras (Fig. 12, lanes 3 and 7). If the Ras cDNA was omitted from the transfection, then no \([^{32}P]GTP-Ras\) could be detected (Fig. 12, lane 8). The results described above for COS-1 cells were also obtained using 293 cells although the magnitude of Ras activation by NCatHA was greater than in COS-1 cells due to the high levels of protein expression achieved in the 293 cells (Figure 22, lane 2).

D. NCatHA is unable to associate with GRB2 in vivo and in vitro

One trivial explanation to account for the data in Fig. 12 is that GRB2 is also able to bind to regions in the dSOS protein that are upstream of the catalytic domain. The ability of GRB2 to associate with dSOS constructs containing the C-terminus was first tested by using lysates of COS-1 cells which had been transiently transfected with the desired cDNA constructs. COS-1 cells were transiently transfected with either NCatCHA, CHA, or CatCHA, and GRB2 protein was immunoprecipitated using anti-GRB2 antibody. All of the HA-tagged dSOS proteins were expressed efficiently
as detected by immunoblotting with the antibody raised against the C-terminus of dSOS (Fig. 13). This antibody is referred to as the anti-dSOS COOH antibody. Immunoprecipitation of the lysates with anti-GRB2 antibody and subsequent western blotting with the anti-COOH antibody revealed that NCatCHA, CHA, and CatCHA were able to associate with GRB2 in COS-1 cells. In a similar manner, 293 cells were transiently cotransfected with either NCatCHA or NCatHA in order to determine if GRB2 could associate with NCatHA present in either the cytosolic or total membrane fractions. Immunoprecipitation of the cytosol (S) and total membrane fractions (P) with anti-HA (Fig. 14) and subsequent immunoblotting with the anti-GRB2 antibody illustrated that GRB2 is unable to associate with NCatHA in vivo. However, GRB2 is able to associate with NCatCHA presumably via the COOH-terminus (Fig. 14).

In addition, the ability of NCatHA to associate with GRB2 was examined in vitro. HA-tagged NCatC and HA-tagged NCat dSOS proteins were expressed in the baculovirus-insect cell system (Fig. 15) and assayed for their ability to associate with GRB2 using a GST-GRB2 fusion protein. Based on coomassie staining, the concentration of NCatHA protein is estimated to be ~15-20 mg/L and the concentration of NCatCHA protein is estimated to be ~2-5 mg/L. As illustrated in Fig. 16 (lanes 2 and 3) lysates
Figure 13. Association of GRB2 with NCatCHA, CatCHA, and CHA in COS-1 cells. Western Blot of NCatCHA, CatCHA, and CHA from anti-HA immunoprecipitation and anti-GRB2 immunoprecipitation. COS-1 cells were transiently transfected with NCatCHA, CatCHA, CHA, or vector alone. Forty eight hours after transfection cells were lysed in a 1% Triton/0.1% Tween buffer. For anti-HA immunoprecipitations, cleared lysate (1.0 mg) was incubated overnight with 30 μl of Protein-A Sepharose and 20 μl of the anti-HA antibody. For GRB2 immunoprecipitations, cleared lysate (1.0 mg) was incubated overnight with 30 μl GAM Sepharose and 4 μl of the anti-GRB2 antibody (UBI). Immunoprecipitats separated by SDS-PAGE (6% acrylamide) and immunoblotted with anti-dSOS-COOH antibody. Protein bands were visualized by enhanced chemiluminescence. This experiment was repeated a total of two times.
Western Blot
anti-dSOS-COOH

NCaC,CHA
CHA
CatCHA
pCMV5

anti-GRB2
immppt.

anti-HA
immppt.
Figure 14. Association of NCatCHA but not NCatHA with GRB2 in 293 cells. (A) Anti-HA western blot of NCatHA and NCatCHA immunoprecipitates from cytosol (S) and total membrane fractions (P). (B) Anti-GRB2 western blot of NCatHA and NCatCHA immunoprecipitates from cytosol (S) and total membrane fractions (P). 293 cells were transiently transfected with NCatHA (10 μg) or NCatCHA (10 μg) via the CaPO₄ mediated method. Approximately 60 hours later, the cells were homogenized in the presence of Tris/EDTA/sucrose and protease inhibitors. The homogenate was precleared and the resulting homogenate was centrifuged at 100,000 x g for 25 min at 4°C. The resulting supernatant represents the cytosol, and the pellet represents the total membrane fraction. Total protein in the cytosol and membrane fractions was determined, and HA-tagged dSOS proteins were immunoprecipitated from both fractions. Immunoprecipitates were separated by SDS-PAGE (6% acrylamide, anti-HA and 12% acrylamide, anti-GRB2 blot) and blotted using a 1/1000 dilution of the monoclonal anti-HA antibody or a 1/5000 dilution of monoclonal GRB2 antibody (Santa Cruz). Protein bands were visualized by enhanced chemiluminescence. The experiment was repeated a total of two times.
A. Western Blot
anti-HA

B. Western Blot
anti-GRB2
Figure 15. Expression of HA-tagged NCatC and NCat dSOS proteins in the baculovirus insect cell system. Sf9 cells were infected with a high titer baculovirus stock of NCatCHA and NCatHA. Cells were homogenized and total protein content of the lysates was assayed using the BCA kit (BioRad). Total protein was solubilized in sample buffer and resolved by SDS-PAGE on 6% acrylamide gels. After resolution, proteins were detected by Coomassie staining. The lanes are designated as follows: Lane 1, molecular weight markers; Lane 2, lysate (40 μg) from wildtype infected cells; Lanes 3, 5, 7, 9, 11, and 13, lysate (5, 10, 20, 60, 100, and 200 μg, respectively) from NCatCHA infected cells. Lanes 4, 6, 8, 10, 12, and 14, lysate (5, 10, 20, 60, 100, and 200 μg, respectively) from NCatHA infected cells. The positions of NCatCHA and NCatHA are indicated.
Figure 16. Association of recombinant NCatCHA but not NCatHA protein with GST-GRB2 fusion protein. Lanes 1-3 represent equal amounts of protein from total lysate of Sf9 cells infected with wild-type, NCatHA, and NCatCHA baculoviral stocks, respectively. The positions of NCatHA and NCatCHA are indicated. In lanes 4-9, equal amounts of sample were loaded, and proteins associating with GST or GST-GRB2 were detected by immunoblotting with anti-HA. The sample loaded in lane 4 represents GST-GRB2 incubated with phosphate-buffered saline alone. The sample loaded in lane 5 represents GST-GRB2 incubated with lysates from wild-type infected cells. Proteins remaining bound to the GST or GST-GRB2 were dissolved in SDS-PAGE sample buffer. Samples were loaded on SDS-PAGE gels (6%), and transferred to nitrocellulose filters. Filters were probed with anti-HA monoclonal antibody, and antibody-bound proteins were visualized by using enhanced chemiluminescence. This experiment was repeated for a total of two times.
<table>
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<th>Pellet</th>
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<td>1 2 3</td>
<td>4 5 6 7 8 9</td>
</tr>
<tr>
<td>NCatCHA</td>
<td>- - + - - + + - -</td>
</tr>
<tr>
<td>NCatHA</td>
<td>- + - - - - + +</td>
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Precipitated with:
- GST-GRB2: + + - + - + +
- GST: - - + - + -
of Sf9 cells expressing NCatHA or NCatCHA displayed immunoreactive bands at the expected mobilities, 125 and 180 kDa respectively, when electrophoresed on SDS-PAGE and subsequently immunoblotted with anti-HA monoclonal antibody. As a negative control, the wild-type Sf9 cell lysates were used and failed to display any immunoreactivity under similar conditions (Fig. 16, lane 1). Incubation of Sf9 cell lysates containing NCatCHA protein with a GST-GRB2 fusion protein but not GST alone resulted in the adsorption of the full-length dSOS protein (Fig. 16, lanes 6 and 7). However, under similar conditions the baculoviral-expressed NCatHA protein failed to associate with the GST-GRB2 fusion protein (Fig. 16, lane 9). This in vitro binding data indicate that the ability of NCatHA to cause Ras activation when expressed in COS-1/293 cells is independent of high affinity GRB2 binding.

E. Purification and in vitro activation of Ras by NCatCHA and NCatHA

One possibility for these in vitro binding results could be that the NCatHA protein was not folding properly when expressed in Sf9 cells and therefore not functional. In order to explore this possibility, the ability of NCatHA and NCatCHA to activate Ras in vitro was tested. Sf9 cells expressing Ras proteins were harvested four days after infection with a
Figure 17. Expression of Ras in the baculovirus insect cell system. Sf9 cells were infected with a high titer baculovirus stock of Ras. (A) Time course of Ras expression. An aliquot of the infected cells was taken on the indicated days after infection. Cells were homogenized and total protein content was assayed using the BCA kit (BioRad). Total protein (25 μg) was solubilized in sample buffer and resolved by SDS-PAGE on 12.5% acrylamide gels. After resolution, proteins were transferred electrophoretically and immunoblotted using an anti-Ras antibody (1742 cell supernatant). Antibody bound proteins were visualized by enhanced chemiluminescence. (B) Separation of post-translationally processed Ras proteins from unprocessed Ras proteins. Cells were homogenized four days after infection. After homogenization, Triton X-114 was added to a final concentration of 1.0%, and the lysate was centrifuged. The pellet was solubilized in sample buffer. The supernatant was removed and separated into aqueous and detergent phases. Total protein (25 μg) from both phases was solubilized in sample buffer and analyzed by immunoblot analysis as described above. Supernatant represents the aqueous phase.
WESTERN BLOTS
ANTI-Ras

<table>
<thead>
<tr>
<th>A.</th>
<th>B.</th>
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<tr>
<td>DAY 2, DAY 3, DAY 4</td>
<td>DETERGENT PHASE, PELLET, SUPERNATANT</td>
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![Western Blot Images Showing Protein Expression Over Different Days and Conditions]
Figure 18. Partial purification of processed Ras proteins. Post-
translationally processed Ras proteins were purified by anion exchange
chromatography from the original detergent phase (see Fig.17) on a
Mono Q column via FPLC (Flow Rate = 0.6 ml/min). Bound proteins
were eluted (0.5 ml) using a linear NaCl gradient (0-500 mM). Protein
concentration in the collected fractions was determined using BCA assay
(BioRad). Fractions were subsequently analyzed by SDS-PAGE (12.5%).

(A) Western blot analysis of the collected fractions using the anti-Ras
(1742 supernatant) antibody. (B) Coomassie stain of the collected fraction.
The lanes in each case are identical except the coomassie stained gel shows
the molecular weight markers to the left of Lane 1. The Lanes are
designated as follows: Lane 1, detergent phase; Lane 2, aqueous phase;
Lane3, pellet 1 (pellet obtained after initial homogenization of cells); Lane
4, pellet 2 (pellet obtained after dialysis of detergent phase, prior to
applying to column); Lane 5, flow through; and Lanes 6-25, fractions
eluted from the column.
A. Western Blot: Anti-Ras

B. Commassie Stain
three days after infection of Sf9 cells but optimal expression of farnesylated Ras occurs on the fourth day following infection (Figure 17, panel A). Approximately 80% of the total Ras protein recovered from the cell lysate is farnesylated. Ras proteins were purified by anion exchange chromatography from Sf9 lysates prepared in 1% Triton X-114. The Triton X-114 phase partition method (194) was used to separate post-translationally processed (farnesylated) Ras proteins from unprocessed Ras proteins (Figure 17, panel B). The farnesylated Ras protein was partially purified from the detergent-enriched phase and the unprocessed Ras was partially purified from the aqueous phase. The detergent phase was applied to an anion exchange column and eluted using a linear NaCl gradient (0-500 mM) in the presence of 0.5% DOC. The processed Ras began to elute at 135 mM NaCl and was completely eluted at 310.5 mM NaCl (Figure 18, panels A and B). The anion exchange chromatography step failed to significantly enhance the purity of the farnesylated Ras (Fig. 18, panel B, lane 1 and lanes 17-20). The partially purified farnesylated Ras was estimated to account for 0.7% of total Sf9 cell protein. The processed Ras was able to bind [3H]GDP efficiently, and the sensitivity of this assay could be enhanced on the order of three-fold by immunoprecipitating the Ras from the reaction instead of applying the typical method utilized in the high
literature which involves spotting the reaction on nitrocellulose (Figure 19, panel A and panel B).

The partially purified Ras was used to determine if NCatHA and NCatCHA could stimulate guanine nucleotide exchange in vitro. The assay was initiated by aliquoting the equilibrated Ras into tubes containing [3H]GTP and the NCatCHA or NCatHA (drained beads) which had been immunoprecipitated from Sf9 cells. The final concentration of Ras in each reaction was ~ 100 nM. The binding reactions were carried out for the indicated times (10, 30, or 60 min) at 25°C. The NCatHA protein stimulated the binding of [3H]GTP to the processed Ras with efficiency comparable to that of full length dSOS, NCatCHA (Fig. 20, panel A). Relatively equal amounts of NCatHA and NCatCHA were immunoprecipitated from Sf9 cells and used in the in vitro exchange assay (Fig. 20, panel B) at the time points indicated in panel A. From this western blot, it is important to point out that the amounts of both NCatHA and NCatCHA seem to decrease as increasing time of incubation with Ras. NCatHA and NCatCHA therefore seem to degrade to some extent with increasing time at 25°C. Both NCatHA and NCatCHA enhanced the guanine nucleotide exchange rate by approximately 5-fold when compared to the spontaneous exchange reaction that occurs. Furthermore, NCatHA and
Figure 19. Binding of [3H]GDP to partially purified farnesylated Ras. Ras (~0.25 µg) was incubated with 1.0 µM [3H]GDP in the presence of buffer for 30 min at 30°C. After the reaction was stopped, the amount of radioactivity associated with Ras was determined by the following two methods. (A) Graphical representation of the amount of [3H]GDP bound to Ras which was measured by the nitrocellulose filter binding assay (176). Briefly, the reaction is spotted onto nitrocellulose, and the radioactivity associated with the nitrocellulose is determined using a β counter. (B) Graphical representation of the amount of [3H]GDP bound to Ras which was determined by immunoprecipitating Ras from the reaction. Briefly, Ras was immunoprecipitated using the anti-Ras antibody (1742 cell supernatant) and goat-anti rat Sepharose. After incubation for 1 hour at 4°C, the beads were drained well and resuspended in 1.0 ml of opti-fluor. The radioactivity associated with the beads was determined using a β counter.
A. Nitrocellulose Filter Binding Assay

B. Immunooppt. of RAS using Y13259 Ab
Figure 20. In Vitro stimulation of Ras activation by NCatHA and NCatCHA. Baculovirus-infected Sf9 cells (NCatCHA and NCatHA) were homogenized, and total protein was assayed using the Bradford method. Lysates (~2.5 mg) were incubated with anti-HA antibody and Protein A-Sepharose overnight at 4°C. Immunoprecipitates were washed and drained well. The exchange assay was initiated by adding equilibrated Ras (100 nM) and 1.0 μM [3H]GTP to the immunoprecipitated NCatCHA or NCatHA. The binding reactions were carried out for the indicated times at 25°C. The Protein A-Sepharose was next pelleted, and the supernatant was removed. The radioactivity bound to Ras was determined by immunoprecipitating Ras from the supernatant (see Figure 19) and counting the radioactivity associated with the beads using a β counter. (A) Effects of immunoprecipitated NCatCHA and NCatHA on the kinetics of GTP binding to Ras. Results are representative of at least two independent experiments. (B) Anti-HA Western blot of the immunoprecipitated NCatHA and NCatCHA. After the binding reaction was carried out for the indicated times, the Protein A-Sepharose was pelleted and immune complexes were analyzed. Immune complexes were separated by SDS-PAGE and immunoblotted with the anti-HA antibody. Protein bands were visualized by enhanced chemiluminescence (NEN).
of \(^3\)H]GTP to Ras after 30 min. NCatCHA and NCatHA were both able to NCatCHA cause maximal binding catalyze the exchange of GDP for GTP on processed Ras \textit{in vitro}, confirming that they are active functional proteins.

F.Expressed dSOS proteins were not found associated with endogenous SOS in COS-1 cells

Another trivial explanation to account for the data in Fig. 12 would involve the ability of the endogenous SOS of COS-1 / 293 cells to associate with expressed NCatHA protein. As a result, GRB2 binding to the C-terminus of the endogenous SOS of COS-1 cells would result in the recruitment of NCatHA to the plasma membrane where it can activate Ras. In order to test this, COS-1 cells were transiently transfected with NCatHA and the HA-tagged protein was immunoprecipitated with the anti-HA antibody (Fig. 21). Subsequent blotting of the anti-HA immunoprecipitate with the anti-HA antibody revealed that the NCatHA protein is expressed efficiently (Fig. 21, panel A). The endogenous SOS of COS-1 cells is detected in total lysates by immunoblotting with an anti-mSOS antibody and the endogenous SOS migrates at the expected molecular weight of ~150 kDa (Fig. 21, panel B). This anti-mSos antibody does seem to recognize a
Figure 21. Absence of detectable levels of dimers formed between NCatHA and the endogenous mSOS of COS-1 cells. (A) Western blot of NCatHA from total lysate and anti-HA immunoprecipitation. COS-1 cells were transiently transfected with NCatHA or vector alone. Forty eight hours after transfection cells were lysed in a 1% Triton/0.1% Tween buffer. Total cell lysate (50 μg) was then separated by SDS-PAGE (6% acrylamide) and immunoblotted with the anti-HA antibody. For the immunoprecipitation of NCatHA, cleared lysate (1.0 mg protein) was incubated overnight with 30 μl of Protein-A Sepharose and 20 μl of anti-HA antibody. Immunoprecipitates were washed and subsequently separated by SDS-PAGE (6% acrylamide) and immunoblotted with the anti-HA antibody. Protein bands were visualized by enhanced chemiluminescence. (B) Western blot of anti-HA immunoprecipitates with the mSOS antibody (UBI). Total cell lysate (50 μg) was also blotted with the anti-mSOS antibody in order to illustrate that the mSOS antibody recognizes the endogenous SOS of COS-1 cells.
A. Western Blot
anti-HA

B. Western Blot
anti-mSOS1
fair amount of nonspecific proteins indicating that the commercially purchased antibody is not very specific. Immunoblotting of the electrophoresed anti-HA immunoprecipitates with the anti-mSOS antibody illustrated that there was no detectable dimerization occurring between NCatHA and the endogenous SOS of COS-1 cells. These results suggest that dimerization of NCatHA protein with endogenous SOS of COS-1 cells cannot explain the ability of NCatHA to activate Ras in vivo.

G. Mapping of important functional domains in the amino terminus of dSOS

Taken together, the results in the previous figures suggest that structural elements within the NH₂-terminal domain of dSOS are necessary in order for the exchange activity of Cat to act on Ras in intact COS-1 / 293 cells. Therefore, truncation and deletion mutants of the HA-tagged NCat cDNA were engineered as represented schematically in Figure 22. These dSOS constructs were designed such that two homology domains of dSOS were focused on. One region in dSOS was previously identified to contain sequence similarities to domains found in pleckstrin (158) which represent amino acids 475-592 in dSOS. In addition, another region in dSOS (~250 amino acids) which is upstream and adjacent to the PH domain
Figure 22. Modulation of Ras GTP/GDP exchange in intact cells by expression of truncation and deletion constructs of NCatHA. (A) Schematic representation of the various dSOS constructs which were tested for their ability to activate Ras in intact COS-1 and 293 cells. (B) Autoradiogram (overexposed) of the TLC plate to visualize the labeled guanine nucleotides which were bound to Ras. Human 293 cells were transiently transfected via the CaPO$_4$- mediated method with Ras (2 µg) and the HA-tagged dSOS cDNAs shown (5 µg each). Cells were labeled, and GDP and GTP associated with Ras was determined (see Figure 12). In each case, (n) represents the total number of experiments which were performed utilizing the individual construct. For each construct the $^{32}$P]GTP loading of Ras was calculated as the average percent GTP/GDP ± standard deviation. The basal level of Ras activation [1.21±0.44 (n=4)] represents those cells which were transfected with Ras only (data not shown). The value for the $^{32}$P]GTP/$^{32}$P]GDP ratio expressed as a percent in cells cotransfected with NCatHA and Ras was 29.6±3.9 (n=4), while PHCatHA and NCatδPHHA were about 60% as effective.
A. 

<table>
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<td>NCat8300HA</td>
<td></td>
<td></td>
<td></td>
<td>5.6 ± 1.9  (n = 2)</td>
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B. 

Diagram showing gel patterns for GDP and GTP with lanes 1 to 7.
Figure 23. Sequence comparison of the PH domain of dSOS with the PH domain of pleckstrin and the DBL domain of dSOS with DBL. (A) Alignment of the PH domain of dSOS with the PH domain of pleckstrin. The shaded boxes show amino acids which are identical. Three specific residues of the PH domain of pleckstrin have been demonstrated to be important in binding PIP$_2$ in vitro and are labeled with an asterik (*). The only invariant amino acid among all PH domains is a tryptophan (**). (B) Alignment of the third conserved region of the DBL domain of dSOS with the same region within the DBL domain of DBL. The shaded boxes show amino acids which are identical. The region of DBL that is most highly conserved among all members of the DBL family is labeled with shaded circles above the residues.
### A.

| Pleckstrin | -M E P K R I T F E G Y L V K K G S |
| dSOS      | G Q N C N E F I R E D S L S K L G S |
|           | V F N T W * K P M W Y V D L E D S I |
|           | G K R I W S R K V F L F D - G L |
|           | E F Y K K K - - - - - - - - - - - S D |
|           | M V L C K A N T K K Q T P S A G A T A Y D |
|           | N S P F G M I P L K G S T L T S P C Q |
|           | Y R L E E K Y F M R R V D I N D R P D |
|           | D F G K R M F V F K I T T T T K Q Q D |
|           | S D D L K - N S P E L A P R M Q O P P |
|           | H F F Q A A F L E E R D A W V R D I N |
|           | I V L T A K N A Q H K H D W M A D L L |
|           | K A I I K C I E G M V I T K S M L |

### B.

| DBL | R L R L D S Y L L E K P V Q R I T K Y Q |
| dSOS| K Y Y L P K L L E V I C H A F Y Y F |
|     | L L L K E L L K Y S K D C E G S A L L |
|     | D Y I K H L K D L S S S Q D D L E - S |
|     | K K A L D A M L D L |
|     | F E Q V Q G L H P |
was identified to contain sequence similarities to a domain found in the RHO guanine nucleotide exchange factor DBL (153, 154). In Figure 23, the PH domain of pleckstrin was aligned with the PH domain of dSOS. These two PH domains share 19% overall amino acid identity which is represented by the shaded boxes. If conservative substitutions are counted, the PH domain of dSOS is 43% similar to that in pleckstrin. In addition, a portion of the DBL domain of dSOS was aligned with a region of DBL (Figure 23). The dots mark residues in DBL which have been shown by mutation to be required for its transforming activity (157). This region within the two DBL domains shares 18% overall amino acid identity which is represented by the shaded boxes. If conservative substitutions are counted, the entire DBL domain of dSOS (250 amino acids) is 30% similar to that in DBL. The DBL domain consist of three conserved regions referred to as I, II, and III which are separated by more variable regions (153).

These dSOS cDNAs depicted in Figure 22 were cotransfected with Ras cDNA into COS-1 and 293 cells and cells were subsequently labeled with $[^{32}\text{P}]$orthophosphate as described previously. CatHA expression in 293 cells (Fig. 22, panel B, lane 7) failed to significantly enhance $[^{32}\text{P}]$GTP loading of Ras compared to transfection of Ras alone as was discovered
previously in COS-1 cells (Fig. 12). Remarkably, the PHCatHA construct consisting of the pleckstrin homology (PH) domain amino-terminal to the Cat domain greatly stimulated $[^{32}\text{P}]\text{GTP}$ loading of Ras to levels approaching those achieved with NCatHA (Fig. 22 compare lanes 2 and 4). Immunofluorescence microscopy of the transfected 293 cells revealed that all HA-tagged dSOS constructs depicted in Fig. 22 were expressed at similar levels with the exception of NCatHA which was expressed at a somewhat higher level. PHCatHA may therefore be as active as NCatHA in this system when normalized for protein expression levels. These results demonstrate for the first time a functional role for the PH domain of dSOS. Interestingly, an NCatHA construct which has the PH domain deleted was also able to effectively stimulate $[^{32}\text{P}]\text{GTP}$ loading of Ras (Fig. 22, panel B, lane 3). However, basal levels of exchange activity were observed upon deletion of the PH and DBL domains of NCatHA. Deletion of the PH domain along with the DBL III region from NCatHA also resulted in basal levels of exchange activity (Fig. 22, panel B, lanes 5 and 6).

Furthermore, addition of the region of the DBL domain (III) of dSOS which correlates to the region in DBL that has been demonstrated to be required for its transforming activity (154,157) failed to further enhance the ability of the PHCatHA construct to stimulate GTP loading of Ras (Fig.
Figure 24. Mutation of the invariant tryptophan in the PH domain of dSOS had no effect on Ras activation in intact cells. (A) 293 cells were transiently co-transfected with the Ras (2 µg) and the HA-tagged dSOS cDNAs (5 µg each) shown. $^{32}$POrthophosphate-labeled cells were then lysed, and the cleared lysate was immunoprecipitated with anti-Ras antibody (1742 supernatant). The positions of the GTP and GDP standards are indicated. Lane 1 represents cells that were transfected with Ras only. (B) Graphic representation of Ras activation. The GTP and GDP spots on the TLC plate were excised, and the amount of $^{32}$Porthophosphate incorporated was determined using a β counter. The ratio of GTP to GDP was calculated and expressed as a percentage, and the values shown represent the average of two individual experiments with vertical bars denoting standard errors. The leftmost bar on the graph represents cells that were transfected with Ras only.
24, lanes 3 and 8). Addition of this same region of the DBL domain (III) of dSOS onto the catalytic domain of dSOS failed to cause Ras activation when co-expressed with Ras in 293 cells (Fig. 24, compare lanes 5 and 7).

As a control, the catalytic domain of dSOS was fused with a sequence which specifies farnesylation and palmitoylation of Ras to its C-terminus in order to target the catalytic domain of dSOS to the membrane. Somewhat unexpectedly, this membrane targeted Cat construct (CatFAR) failed to increase GTP-loading of Ras (Fig. 24, compare lanes 5 and 6) even though CatFAR was processed and delivered to the membrane properly (see Fig. 28).

H. Mutation of the invariant tryptophan in the PH domain of dSOS

Based on the previous data, the PH homology domain of dSOS plays an important role in activating Ras in intact cells. The degree of homology shared among PH domains is actually fairly low. However, there is one invariant tryptophan located near the C-terminus of all PH domains (Fig. 23). This tryptophan was mutated to a glycine in the PHCatHA construct in order to determine if mutation of this conserved amino acid would abolish the ability of the PHCatHA to activate Ras in intact 293 cells. This mutant
construct PHCatHA (w-->g) was transiently transfected into 293 cells along with Ras. The cells were labeled in vivo with [32P]orthophosphate as previously described and the amount of [32P]GTP loading on Ras was measured. Figure 24 illustrates that the PHCatHA(w-->g) mutant stimulated [32P]GTP loading of Ras to relatively the same extent as PHCatHA (Fig. 24, compare lanes 3 and 4. Both PHCatHA and PHCatHA(w-->g) were about 60% as effective as NCatHA in stimulating GTP loading of Ras (Fig. 24, panel B).

I. Discussion

The data presented in this chapter suggest that the GRB2 binding region of dSOS is not sufficient to allow the catalytic domain of dSOS to function in COS-1 or 293 cells. Therefore, the initial simple model proposed in the literature which stresses that quantitative recruitment of SOS to the membrane via GRB2 binding is the main mechanism by which SOS can activate Ras cannot be completely accurate. More specifically, deletion of the C-terminus of dSOS resulted in a protein which could still activate Ras in the intact cell (Figures 12 and 22) but was unable to bind GRB2 (Figures 13, 14, and 16).
Our data revealed an additional requirement for the NH$_2$-terminal domain of dSOS in Ras regulation. A simple hypothesis that explains this data is that either the PH or DBL homology regions of dSOS are sufficient to allow the catalytic domain of dSOS to interact with cellular Ras proteins. Figure 22 illustrated that the absence of both of these regions in NCatHA abolishes activity, while the presence of the PH domain positioned N-terminal to the Cat domain confers high activity.

The data presented regarding the DBL domain of dSOS is not as unequivocal. Comparison of two results in Fig. 22 suggest that the DBL domain of dSOS contributes to the ability of the dSOS protein to activate Ras in vivo. First, the deletion of the PH domain from NCatHA resulted in a mutant dSOS protein which was still able to stimulate GTP-loading of Ras. Second, the deletion of the PH and DBL domains of dSOS resulted in a mutant dSOS protein which can no longer stimulate GTP loading of Ras. Three additional dSOS deletion mutants were constructed to attempt to address the relative contribution of the conserved regions which has not been determined for any DBL domain-containing protein. The first construct is referred to as DBL(III)CatHA consisting of a region of the DBL homology domain NH$_2$-terminal to the Cat domain (Fig. 24). The second construct is referred to as DBL(III)PHCatHA consisting of both a
region of the DBL homology domain and the PH domain NH$_2$-terminal to the Cat domain (Fig. 24). In the third construct the I and II conserved regions of the DBL domain were deleted from NCatHA (Fig. 22). The DBL(III)CatHA construct failed to activate Ras in the intact cell and the DBL(III)PHCatHA construct stimulated GTP-loading of Ras to the same extent as PHCatHA. However, deletion of the entire DBL and PH domains from NCatHA results in a dSOS mutant protein unable to activate Ras in the intact cell. These results suggest that neither the DBL III region alone or the DBL I and II regions in combination are sufficient to allow the Cat domain of dSOS to activate Ras in the intact cell. Therefore, all three regions of the DBL domain seem to be required for the ability of dSOS to activate Ras in vivo. From the results presented here, it can be postulated that the interaction of the PH and DBL homology regions of dSOS with other elements in the cellular complexes that regulate Ras proteins are necessary for the localization of the catalytic domain of dSOS and/or its appropriate operation in intact cells.

The PH domain is an approximately 120 amino acid motif that was first identified in pleckstrin, which is the major substrate for protein kinase C (PKC) in platelets (195). This conserved motif is present in over 90 proteins, and several of these proteins play a role in signal transduction and
cytoskeletal functions (158,165). Ligands for PH domains have been described, based on in vitro studies. These ligands include both proteins, such as the βγ subunit of heterotrimeric G proteins (171-174) and PKC isoforms (196), and lipids, such as PIP_2 (166-170) and PIP_3 (170). However, the importance of these interactions with PH domains in vivo remains unclear.

Previous studies performed by Pitcher and co-workers illustrated that a tryptophan which is invariant in all PH domain sequences is important for the function of βARK (172). The PH domain of βARK is able to interact with both G_βγ and PIP_2 at the carboxyl and amino termini, respectively (172). The interaction of these two ligands with βARK was demonstrated to be required for both membrane association and activation (172). A fusion protein consisting of the PH domain of βARK was able to inhibit G_βγ/PIP_2-dependent βARK activity in vitro (172). However, a mutant fusion protein in which the invariant tryptophan was mutated to an alanine failed to inhibit G_βγ/PIP_2-dependent βARK activity in vitro (172). This experiment provided evidence that the tryptophan residue is important for the function of βARK. Therefore, the tryptophan residue in the PH domain of the dSOS PHCatHA construct was mutated to an glycine. Figure 24 shows that this mutant protein, PHCat(w->g) is still able to activate Ras
in the intact cell, suggesting that ligand binding to the the C-terminal region of the PH domain of dSOS may not be essential for SOS function.

Several studies subsequent to ours also suggested that the PH domain of SOS plays an important functional role. Injection of the PH domain of hSOS1 into Xenopus oocytes resulted in the induction of significant rates of germinal vesicle breakdown and meiotic maturation (197). The injection of the PH domains of IRS-1, βARK, and PLCδ failed to cause germinal vesicle breakdown and meiotic maturation (197). Furthermore, the microinjected hSOS1 PH domain cooperated with insulin or coinjected normal Ras, but not progesterone, in the induction of Xenopus oocyte maturation (197). These results suggested that the PH domain of hSOS1 plays a functional role.

Recently, evidence has been provided for the ability of the PH domain of SOS to bind PIP₂ and PIP₃. Kubiseski and co-workers demonstrated that the PH domain of mSOS1 binds liposomes containing PI-4,5-P₂ in vitro with an apparent $K_d$ of 1.8 μM (169). More specifically, site-directed mutagenesis studies revealed that Arg⁴⁵⁹, a conserved basic residue, plays an important role in the association with PIP₂ (169). Subsequently, Chen et al. showed that the PH domain derived from hSOS1 is able to bind with high affinity to PIP₂ in vitro ($K_d$~1.5 μM) and that,
when microinjected into COS cells or embryo fibroblasts, the hSOS1 PH domain seems to be enriched at the plasma membrane (198). Mutation of the two residues previously shown to be critical for lipid binding (198), Lys 456 and Arg 459, to glutamic acid residues resulted in a PH-SOS protein unable to bind PIP₂ in vitro but still localized to the plasma membrane after injection into COS cells (198). These experiments published by Chen and co-workers suggested that PIP₂ binding to the SOS PH domain is not essential for its ability to associate with the plasma membrane.

Recently, our lab in collaboration with Cantley’s lab (Rameh et al.) illustrated that the SOS PH domain binds to radiolabeled PI-3,4,5-P₃ with a Kₒ value that is submicromolar (170). These experiments also illustrated a two-fold higher selectivity for binding to PI-3,4,5-P₃ over PI-4,5-P₂ (170). Thus, the authors suggest that the conversion of PI-4,5-P₂ to PI-3,4,5-P₃ by PI 3-Kinase may contribute to the recruitment of SOS to the membrane where PI 3-Kinase is activated. However, it is important to point out that the PH domain of SOS only had a two-fold preference for PI-3,4,5-P₃ over PI-4,5-P₂, suggesting that both of these lipids may play a role in recruitment of SOS to the membrane where it can activate Ras.
Chen et al. also provided evidence that the PH domain of SOS has a dominant negative effect on serum-induced activation of the Ras signaling pathway which was independent of PIP$_2$ binding (198). More specifically, COS cells were cotransfected with HA-tagged ERK2 and HA-tagged deletion mutants of SOS (PH, PH$^{KR>EE}$, and δCAT) and SOS associated MAP kinase activity was measured in vitro. The three HA-tagged SOS constructs inhibited MAP kinase activity by ~75% (198). As a control Raf-CAAX was expressed along with the PH domain. No inhibition was observed in this case, suggesting that the dominant negative effect of the PH domain of SOS is acting upstream of Ras. These experiments support the idea that multiple domains of SOS are important for its ability to activate Ras.

The data presented here also suggest that the COOH-terminal domain of the dSOS protein may encode a negative regulatory domain. This is supported by the observation that a construct consisting of the catalytic and COOH-terminal domains of dSOS failed to activate Ras in intact cells (Fig. 12). Strong support of this hypothesis is provided by our collaborator’s (Karlovich et al.) experiments, in which genetic complementation was used to show a dominant inhibitory function of CatC and physiological activity of NCat in intact flies (Figs. 5 and 7). Aronheim et al. also demonstrated
that a farnesylated full-length hSOS construct only partially complemented CDC25 activity in a cdc25 temperature sensitive strain (185). However, a farnesylated hSOS construct which lacks the COOH-terminal domain was able to completely complement CDC25 activity (185). Wang et al. have also found enhanced transforming activity of a mutant mSOS protein which lacks the COOH-terminal domain (199). From all of the above mentioned results, GRB2 has been proposed to play a role in relieving an inhibitory influence of the SOS COOH terminus.

The key result described in this Chapter is that the GRB2 binding region of dSOS is not necessary to allow the catalytic domain to function in intact COS-1 or 293 cells. Our data revealed an additional requirement for the NH$_2$-terminal domain of dSOS in Ras regulation. One possible model that can be proposed is presented in Figure 25. This model postulates that the function of the PH or DBL domains of dSOS may be to bind to components that position dSOS to interact more favorably with membrane-bound Ras, while GRB2 may play a role in relieving an inhibitory influence of the SOS COOH terminus (Fig. 25). In addition to our data and our collaborator’s genetic experiments, Wang et al. published results which implied that the GRB2 binding domain of mSOS1 is not required
Figure 25. Model for the Role of NH$_2$-terminal Domains of dSOS in Facilitating GTP/GDP Exchange on Rasin Intact Cells. Subsequent to the binding of GRB2-SOS complexes to tyrosine phosphate sites on receptor or receptor substrate proteins (left), association of PH or DBL regions in the SOS NH$_2$-terminus to unknown components (?) hypothetically position SOS to activate Ras. Alternatively, SOS-GRB2 complexes may be anchored to the membrane through binding of NH$_2$-terminal sequences prior to receptor activation and binding of GRB2 to tyrosine phosphates (not shown).
for downstream signal transduction (199). More specifically, they were successful in isolating a dominantly transforming mutant which was activated via deletion of the COOH-terminus including the GRB2 binding sites. Further analysis revealed that this mSOS1 mutant protein was unable to associate with GRB2 \textit{in vitro} but, was still found associated with the plasma membrane in the Rat1 transformed cells (199). These experiments also argue that SOS proteins do not require GRB2 binding in order to interact with the plasma membrane.

More recently, Byrne \textit{et al.} investigated both the requirement for the GRB2/SOS association and the functional role of various domains of SOS using Ras-dependent signaling pathways (200). In their system, the hSOS C-terminal domain was able to inhibit PC12 cell differentiation, NIH3T3 cell proliferation, and ERK2 activation (200) by sequestering the endogenous GRB2, which suggested that the GRB2/SOS interaction was required for Ras activation. This inhibitory effect was rescued by expression of a full-length hSOS construct or GRB2 (200).

Furthermore, Byrne \textit{et al.} examined how various SOS constructs effected NIH3T3 cell proliferation and ERK2 activation (200). Their studies provided evidence confirming our initial finding that the amino-terminal domain of SOS is important for its function and that the C-
terminal domain of SOS plays an inhibitory role. They demonstrated that a construct consisting of the exchange factor domain (EFD) of SOS, which is equivalent to our CatHA construct, failed to replenish ligand-stimulated ERK2 activation in NIH3T3 cells expressing the C-terminal domain of SOS. However, a construct comprising both the SOS N-terminal and catalytic domains (NterEFD) was able to rescue the inhibitory effects that the SOS C-terminal domain had on NIH3T3 cell proliferation and ERK2 activation (200). A construct containing the catalytic and C-terminal domains (EFDCter) was able to partially suppress the inhibitory effect of the SOS C-terminal domain on ERK2 activation but failed to restore DNA synthesis in NIH3T3 cells expressing the C-terminal domain of SOS (200). These findings suggest that the C-terminal region of SOS does contribute to the activation of Ras via growth factors, but the major signal seems to be relayed through the NH₂-terminal domain of SOS. Based on their findings, the authors proposed a model similar to ours (Fig. 25) which postulates that SOS is activated as the result of two ligand-dependent steps: 1.) the association of SOS/GRB2 complexes with RTK's or phosphotyrosine signaling proteins, such as SHC, which may lead to the initial recruitment of SOS to the membrane and 2.) a second event involving the SOS N-terminal domain that is required for SOS to activate Ras.
Strong support of our data and the conclusions drawn is provided by genetic experiments from our collaborators (188), a combination of genetic and biochemical experiments performed by Wang and co-workers (199), and the more recent data of Byrne and co-workers (200). Therefore, the striking similarity of results obtained by these independent methodologies suggest that regions within the NH\textsubscript{2}-terminal region of the SOS protein are necessary for their physiological function. The simple recruitment model which proposes that SOS is activated as a result of its recruitment to the membrane via GRB2 binding seems to be incorrect based on our findings and several other groups. The data suggest that a second unclear event must occur involving the SOS N-terminal domain in order for SOS to catalyze guanine nucleotide exchange on Ras. Strong evidence is also provided for the C-terminal domain of SOS playing an important role in the down-regulation of the Ras signaling pathway.
A. Specific Aims and Rationale

The specific aim of this Chapter is to test the hypothesis that the amino-terminal domain of dSOS, specifically the PH and DBL regions, is important for its membrane association. Several proteins containing PH domains have been demonstrated to require membrane association for their function. Evidence has also been provided which illustrates that several PH domains can bind to lipid molecules such as PIP$_2$ in vitro. Therefore, it has been proposed that the interaction of PH domains and phospholipids may be one possible mechanism by which PH-domain containing proteins are anchored to the membrane (Figure 26). The rationale to test this proposed model is based on our previous observation that either the PH or DBL homology regions of dSOS were sufficient to allow the catalytic domain of dSOS to interact with cellular RAS proteins. This will help to determine if simple recruitment of dSOS to the plasma membrane is the mechanism by which dSOS is activated. Another alternative would be that once dSOS is recruited to the plasma membrane binding of a yet unidentified component to the PH/DBL region is necessary for the ability of SOS to activate RAS in
Figure 26. Model for the role of the PH/DBL domains of dSOS in localizing dSOS to plasma membrane. The binding of lipid molecules to the amino-terminal domain of dSOS (PH and DBL regions) may be important for the association of dSOS with the plasma membrane where it can activate Ras.
the intact cell. In order to test the proposed hypothesis two specific questions were addressed: 1.) What is the distribution of various dSOS mutant proteins in 293 cells? and 2.) Can neomycin inhibit the ability of various dSOS mutant proteins to associate with total membranes in 293 cells?

B. Distribution of dSOS mutant proteins in 293 cells

293 cells were transfected with the various HA-tagged dSOS mutant proteins and cytosolic and total membrane fractions were prepared in order to determine if membrane association of a specific construct is sufficient for its ability to activate RAS. 293 cells were transiently transfected with the following HA-tagged dSOS mutant cDNA constructs: NCat, NCat(δPH), NCat(δ200), NCatC, CatC, C, Cat, and Cat-tagged with the RAS farnesylation sequence (refer to Figs. 9 and 22). Cells were homogenized and initially centrifuged at a low speed in order to remove nuclei and unbroken cells. Next, the homogenate was centrifuged and separated into cytosolic and total membrane fractions. Protein in the cytosol and membrane fractions was quantitated and equal amounts of HA-tagged dSOS proteins were immunoprecipitated using a polyclonal anti-HA antibody. Immunoprecipitates were separated by SDS-PAGE and
subsequently immunoblotted using a monoclonal anti-HA antibody (Fig. 27).

The data from these fractionation experiments suggest that the amino-terminal domain of dSOS is important for the ability of dSOS to associate with cell membranes. This is supported by the finding that constructs that lack the amino terminus, CatC and C, are predominantly cytosolic (Figure 27). More specifically, 90% of the expressed CatC is found in the cytosol and 85% of the expressed C is found in the cytosol (Figure 28). Three of the constructs, NCatHA, NCatδPHHA, and NCatδ200HA were relatively equally distributed among the cytosolic and total membrane fractions (Figs. 27 and 28). In addition, the Cat domain alone was expressed and found predominantly associated with the cytosolic fraction (Fig. 27). These observations suggest that the amino-terminal domain of the dSOS protein plays a role in its association with the total membrane fraction in transiently transfected 293 cells.

These fractionation experiments also provide evidence to support the hypothesis that the C-terminal domain of SOS plays an inhibitory role. The data suggest that the COOH-terminus may inhibit membrane association since NCatC, CatC and C seem to be predominantly cytosolic whereas removal of the COOH-terminus (NCat) results in a 5-
Figure 27. Distribution of dSOS mutant proteins in 293 cells. (A) Anti-HA western blot of NCatHA, NCatCHA, NCatδPHHA, NCatδ200HA, CatCHA, and CHA immunoprecipitates from cytosol (S) and total membrane fractions (P). (B) Anti-HA western blot of CatHA and CatFARHA immunoprecipitates from cytosol (S) and total membrane fractions (P). 293 cells were transiently transfected with the indicated HA-tagged dSOS constructs and cytosol and total membrane fractions were prepared (see Fig. 14). Total protein in the cytosol and membrane fraction was determined and HA-tagged dSOS proteins were immunoprecipitated from both fractions. Immunoprecipitates were separated by SDS-PAGE and blotted using a 1/1000 dilution of the monoclonal anti-HA antibody. Protein bands were visualized by enhanced chemiluminescence.
fold increase in the amount associated with the total membrane fraction (Figs. 28). Therefore, it can be postulated that GRB2 may be regulating the localization of dSOS as previously discussed in Chapter IV, but through an indirect mechanism involving relief of repression of the COOH-terminal inhibitory effects on the amino-terminus.

The data from these fractionation studies in conjunction with the data from the in vivo RAS activation experiments together suggest that localization of dSOS to the membrane is not sufficient for its ability to activate RAS. The construct lacking half of the DBL domain and the entire PH domain (Ncat8200) was found associated with total membranes (Figs. 27 and 28) but failed to activate RAS in the intact 293 cells (Fig. 22). In addition, tagging of the Cat domain with the RAS farnesylation sequence resulted in a significant increase in the amount of Cat associated with total membranes (~90%). However, this construct also failed to activate RAS in the intact cell (Figs. 27 and 28). The presence of an intact DBL or PH domain therefore seems to be required for the ability of dSOS to activate RAS, but is not necessary for dSOS to be associated with the total membranes.
Figure 28. Graphical representation of the amount of dSOS mutant proteins present in the soluble and particulate fractions of transfected 293 cells. The data shown in Figure 27, along with data from repeated experiments, were quantitated using a scanning densitometer. The data is expressed as a ratio of the numerical value obtained from densitometric scanning of the expressed protein bands in the soluble or particulate fractions to the sum of the total expressed protein in both fractions. The ratio obtained represents the percentage of the indicated protein in either the soluble or particulate fraction. The total number of experiments (n) which were performed utilizing the individual constructs are listed as follows: NCatHA, n=13; NCatCHA, n=4; NCatδPHHA, n=14; NCatδ200HA, n=4; CatCHA, n=5; CHA, n=3; and CatFARHA, n=5.
soluble fraction
particulate fraction

% PROTEIN

NCaHA  NCaCHA  NCa6P3HHA  NCa6300HHA  CaCHA  CHA  CaFARHA
C. Release of dSOS mutant proteins from total membranes upon treatment with neomycin

293 cells were transiently transfected with either NCatHA or NCatδPHHA cDNA constructs and total membranes were prepared as described in the previous section. Total membranes were next resuspended in the presence or absence of neomycin (15 or 40 μM) and incubated at 4°C for 10 min on an end-over-end rotating mixer. Total membranes were again pelleted, and the supernatants were saved to determine the quantity of the dSOS proteins released from the membranes. The HA-tagged dSOS proteins were immunoprecipitated from the cytosol, total membrane, and the supernatant after neomycin treatment (released fraction) using a polyclonal anti-HA antibody. Immunoprecipitates were separated by SDS-PAGE and subsequently immunoblotted using a monoclonal anti-HA antibody (Fig. 29).

The data presented in Figures 29 and 30 suggest that lipid binding to the dSOS amino-terminus may play a role in the association of NCatHA and NCatδPHHA with total membranes in transfected 293 cells. Treatment of prepared total membranes (particulate fraction) with 15 μM neomycin
Figure 29. Release of NCatHA and NCatδPHHA from total membranes upon treatment with neomycin. Cells were fractionated as previously described in Fig. 27. Total membranes were resuspended and incubated for 10 min at 4°C in the presence or absence of neomycin (40 μM). Total membranes were again pelleted and the resulting supernatant (Releas.), along with the cytosol (S) and total membrane pellet (P), was analyzed for indicated HA-tagged dSos constructs as described previously in Fig. 27. Anti-HA immunoprecipitates were separated by SDS-PAGE and blotted using the monoclonal anti-HA antibody. Protein bands were visualized by enhanced chemiluminescence.
Neomycin (40µM)
Figure 30. Graphical representation of the amount of NCatHA and NCatδPHHA released from total membranes upon neomycin treatment. The data shown in Figure 26, along with data from a repeated experiment, were quantitated using a scanning densitometer. The data is expressed as a ratio of the numerical value obtained from densitometric scanning of the expressed protein bands in the soluble, particulate, or released (after neomycin treatment) fractions to the sum of the total expressed protein in all fractions. The ratio obtained represents the percentage of the indicated protein in either the soluble, particulate, or released fraction. Standard errors are denoted by vertical bars. The absence of error bars indicates that the experiment was only performed once.
soluble fraction
particulate fraction
released
resulted in only a small percentage of the NCatHA being released (10%) and no release of the NCatδPHHA (Fig. 30). However, treatment of prepared total membranes with 40 μM neomycin resulted in a significant percentage of the NCatHA and NCatδPHHA being released, ~44% and 38% respectively (Fig. 30). Neomycin therefore seems to release both NCatHA and NCatδPHHA from total membranes presumably via binding the PIP₂ lipid molecules. Published in vitro studies suggest that PIP₂ is able to bind to the SOS PH domain (169, 170). Our neomycin experiments suggest that PIP₂ may be interacting with regions in the amino-terminus of dSOS other than the PH domain.

D. Discussion

The data presented in this Chapter suggest that the amino-terminus of dSOS is important for its ability to associate with total membranes in transfected cells. Deletion of the PH domain from NCatHA resulted in a dSOS mutant protein which is still able to associate with the particulate fraction suggesting that additional regions in the NH₂-terminus may contribute to the association of dSOS with total membranes (Figs. 27 and
28). In addition, deletion of the PH domain and half of the DBL domain resulted in a dSOS mutant protein which was still capable of associating with the particulate fraction in transfected 293 cells (Figs. 27 and 28). The CatHA construct in which the entire NH₂-terminus is deleted failed to associate with the particulate fraction significantly (Fig. 27). Tagging of the CatHA construct with the RAS farnesylation sequence resulted in a significant increase in association of the CatHA dSOS mutant protein with the particulate fraction. Taken together, these results suggest that regions upstream of the Cat domain of dSOS contribute to the ability of dSOS to associate with the particulate fraction in intact cells.

The fractionation experiments and the in vivo RAS activation experiments together illustrate that the amino-terminal domain of dSOS plays an important role in its function. The amino-terminus is necessary for membrane association and RAS activation in intact cells. The aminoglycoside antibiotic neomycin was used to determine if PIP₂ is contributing to the association of the dSOS constructs NCatHA and NCatδPHHA with the membranes prepared from 293 cells. Neomycin is an antibiotic that exhibits a specific affinity for PI-4-P and PI-4,5-P₂ (214,215). This is believed to result from a strong ionic interaction between the cluster of six primary amino groups on the neomycin and the
several negatively charged phosphate groups on the lipid molecules. In vitro studies utilizing bilayer vesicles consisting of PI-4,5-P₂ revealed that 10 μM neomycin binds greater than 50% of the PIP₂ in the bilayer (215).

The NCatHA construct was released from total membranes upon treatment with neomycin (Figs. 30 and 31). Therefore, one possible explanation for this result is that neomycin may be binding the cellular PIP₂, and therefore prevents the PIP₂ from associating with the PH domain of NCatHA. The ability of neomycin to release NCatδPHHA from the total membranes was also tested. Surprisingly, NCatδPHHA was also released from the total membranes upon neomycin treatment, suggesting that multiple regions in the amino-terminus of dSOS may be capable of binding PIP₂. Upon careful examination of the entire DBL domain, regions were found that contained charged basic residues. Therefore, one possibility is that PIP₂ or some another negatively charged molecule may be able to associate with the DBL domain of dSOS and anchor it to the membrane where it can activate RAS. These neomycin experiments and the published in vitro studies demonstrating PIP₂ binding to the SOS PH domain suggest that lipid association with the SOS amino-terminus may be one mechanism by which SOS is associated with the membrane.
In a similar manner, Wang et al. used Rat1 cells (Rat1pMFGmSos1.2A) which were infected with the retroviral vector carrying the msos1 gene to determine the localization of the SOS proteins (199). This cell line expressed both a transformation-associated protein referred to as p135\textsuperscript{5msos1}, which lacks the COOH-terminus, and endogenous SOS1 proteins (196). Fractionation of these cells revealed that the endogenous and transforming SOS1 proteins (COOH-terminal truncation) were found relatively to the same extent in the soluble and particulate fractions. In this system, it is important to point out that expression levels achieved are relatively high. In our system which utilized transiently transfected 293 cells, the NCatHA construct was also found relatively equally distributed among the soluble and particulate fractions at high and low expression levels. However, NCatCHA in the transfected 293 cells was found predominantly in the soluble fraction but, as increasing amounts of NCatCHA are expressed, more seems to become associated with the particulate fraction (data not shown). Therefore, our data seems to be in agreement with the data presented by Wang and co-workers.

While our studies suggest that the amino-terminus of dSOS is important for membrane association and RAS activation in intact cells, individual studies by Aronheim et al. (185) and Quilliam et al. (201)
provide evidence that the targeting of hSOS to the membrane is the primary mechanism leading to activation of the RAS signaling pathway. Aronheim and co-workers generated modified hSOS constructs: the full length SOS containing either the v-SRC myristoylation signal or the Ha-RAS farnesylation and palmitoylation signal and a C-terminally truncated SOS containing the same membrane targeting sequences. These modified and unmodified SOS constructs were transfected into HeLa cells and fractionation studies along with immunofluorescence studies confirmed that only the modified constructs were targeted to the membrane (185). These results are inconsistent with results from our experiments, Wang et al. experiments, and Karlovich et al. experiments which clearly show that both full-length and C-terminally truncated mSOS and dSOS constructs are found associated with the total membrane fraction without being artificially targeted (188,199). Furthermore, these constructs were transiently transfected into NIH3T3 cells and tested for their ability to stimulate the RAS signaling pathway. Only the modified constructs were able to activate AP-1 and ERK1 (185). Analysis of NIH3T3 cells stably transfected with the modified and unmodified constructs revealed that only the modified constructs resulted in neoplastic transformation. Interestingly, the modified SOS construct lacking the C-terminus was much more efficient in
transformation then the modified full-length SOS construct, supporting the
previously mentioned hypothesis that the C-terminal domain of SOS may
play an inhibitory role (185). The modified SOS constructs were also
capable of suppressing the \textit{cdc25} temperature-sensitive phenotype, whereas
the unmodified constructs failed to suppress the \textit{cdc25} phenotype (185).
These results are inconsistent with genetic studies performed by Karlovich
\textit{et al.} which demonstrated that the wild-type dSOS and a dSOS construct
lacking the COOH-terminus were both able to suppress the temperature-
sensitive \textit{cdc25} phenotype (188). These artificial membrane localization
studies suggest that targeting of SOS to the plasma membrane appears to be
the primary mechanism leading to activation of the RAS signaling pathway,
suggesting that the accessibility of SOS to RAS is the rate limiting step in
signal transduction. The authors therefore proposed a model suggesting
that upon activation of growth factor receptors, SOS is targeted to the
membrane via GRB2 which results in RAS activation.

In summary, the model in which the main mechanism for
recruitment of SOS is through GRB2 cannot completely explain how SOS
activates RAS in the intact cell. Our results show that a construct lacking
the GRB2 binding domain is found associates to a significant extent with
the particulate fraction and also can activate RAS. Furthermore, a
construct that lacks the GRB2 binding domain, PH domain, and half of the DBL domain also associates with the particulate fraction although this construct fails to activate RAS in the intact cell. In a similar manner, when the Cat domain is modified with a membrane targeting sequence, it is targeted to the membrane but unable to activate RAS. All of these results suggest that regions upstream of the Cat domain are important for the ability of SOS to activate RAS in the intact cell. Karlovich and co-workers also showed using genetic experiments that a dSOS construct lacking the GRB2 binding domain was fully active whereas, a construct lacking the NH$_2$-terminus had a dominant negative effect (188). In addition, Wang et al. illustrated that a MSOS construct lacking the GRB2 binding domain was capable of transforming Rat1 cells and also could associate with the particulate fraction in these cells (199). However, genetic studies in both worms and flies provide evidence that GRB2 proteins function as rate limiting positive elements in the RAS signaling pathway. However, the mechanism by which GRB2 operates is unclear. Thus, the proper presentation of SOS to RAS seems to involve multiple intermolecular interactions in which both the NH$_2$ and COOH-terminal domains of SOS participate.
CHAPTER VI
CONCLUSIONS AND FUTURE WORK

A. Possible Mechanisms for SOS Activation

Ras proteins are critical downstream components of receptor protein tyrosine kinases. The mechanism by which SOS activates Ras remains unclear. Three possible models have been proposed for the mechanism by which SOS activates Ras. The first model postulates that allosteric activation of SOS may occur upon association with the complex of activated receptor and GRB2. However, in vitro studies have shown that when the GRB2 SH2 domain is bound to a phosphotyrosine peptide derived from the EGFR, there is no change in the affinity of the SH3 domain of GRB2 for proline-rich SOS derived peptides (217). These data suggest that allosteric activation of SOS via GRB2 is not the main mechanism by which SOS can activate Ras.

The second model proposes that phosphorylation of SOS via protein tyrosine kinases may activate its exchange activity. SOS has multiple phosphorylation sites, but there are no detectable phosphotyrosines. Upon simulation with growth factors, SOS becomes phosphorylated on serine and threonine residues an event that can be detected by its decreased mobility
on SDS polyacrylamide gels (52,177,218,219). Several groups have confirmed that SOS is phosphorylated in vitro and in vivo by MAP kinase (220-222). Very recently, p90 RSK-2 kinase has been shown to phosphorylate SOS in vitro and in vivo after treatment of PC12 cells with EGF (223). In both cases, no change in SOS exchange activity has yet been demonstrated upon its phosphorylation. Phosphorylation of SOS seems to occur with a time course which lags behind Ras activation and MAP kinase activation (219,224). The published data suggest that phosphorylation of SOS does not seem to activate Ras but, instead, may inhibit Ras as a result of the dissociation of SOS from GRB2 or the dissociation of the SOS-GRB2 complex from the receptor (225-227).

Two findings have led to the development of the last model: 1.) the failure of growth factors to induce changes in SOS activity (176) and 2.) the detection of complexes consisting of either receptor/GRB2/SOS or SHC/GRB2/SOS (176). The last model proposes that the translocation of SOS to the membrane via GRB2 results in its ability to activate Ras simply through a proximity effect. Limited evidence for this model is provided by two related studies (see Chapter V Discussion) in which the GNEF’s SOS and CDC25 were targeted to the plasma membrane via an artificial
localization signal (185,201). As a result, SOS/CDC25 were targeted to the plasma membrane and were able to activate the Ras signaling pathway.

B. Summary of Findings

Our experimental observations along with those of others in the field argue that the simple recruitment model proposed to explain SOS activation is not completely accurate. Our data clearly show that the amino-terminal domain of SOS plays an important role in its ability to activate Ras based on the findings that a construct lacking the NH$_2$-terminus, CatCHA, was unable to activate Ras in intact cells whereas, a construct lacking the C-terminal GRB2 binding domain was fully capable of activating Ras in the intact cell. Furthermore, analysis of the NH$_2$-terminal domain revealed that either the PH and/or DBL domains of dSOS are required for GTP loading of Ras. Our data illustrate that proper localization of the catalytic domain of SOS to the membrane is insufficient to cause activation of Ras. More specifically, deletion of both the PH and DBL (III) regions from NCatHA results in a construct that is targeted to the membrane but is unable to cause GTP loading of Ras. In the same manner, the CatFAR construct which consists of the catalytic domain fused to the Ras membrane targeting sequence failed to activate Ras but was found associated with the
particulate fraction. Our findings suggest that in addition to the interaction of SOS with GRB2, there is an unknown interaction involving the NH₂-terminus of SOS which is important for its function. Therefore, the proper presentation of SOS to Ras seems to be achieved through multiple intermolecular interactions involving both the amino and carboxy-terminal domains of the protein.

C. Future Directions

1. Is there a specific ligand for the PH and or DBL domain of SOS?

   Our data demonstrates a functional role for the PH and DBL domains within the dSOS protein. It can be postulated that the function of the PH or DBL regions of dSOS is to bind membrane components that position dSOS to better interact with membrane-bound Ras. In vitro data show that both PIP₂ and PIP can bind to the PH domain of SOS, suggesting that lipid may be the unidentified membrane component that positions dSOS to interact with cellular Ras (169,170). However, recent data published by Chen et al. illustrate that mutations in the PH domain of SOS that abolish lipid binding do not effect the ability of the PH domain to associate with the plasma membrane (198). Thus, identification of potential ligands for the PH
domain of SOS will help to shed light on the mechanism by which SOS activates Ras.

Several experiments can be proposed to determine if there is an important physiological ligand for the PH domain of SOS. One possibility would be to screen an expression library using radiolabeled SOS PH domain as a probe. In addition, total membranes could be prepared from radiolabeled cells, and incubated with the fusion proteins GST-NCat and GST-NCatδPH. This experiment may reveal if any radiolabeled membrane proteins are associating with SOS via its PH domain. More specifically, 293 or COS-1 cells would be radiolabeled, stimulated with EGF, and subsequently fractionated into cytosol and total membranes. The prepared total membranes would next be incubated with NCat and NCatδPH recombinant proteins in order to determine if any membrane proteins are found preferentially associated with recombinant NCat protein versus NCatδPH. In the same type of experiment, prepared total membranes from radiolabeled cells could be incubated with a fusion protein consisting of only the SOS PH domain, GST-PH. These above experiments may help to reveal if there is a protein ligand for the PH domain of SOS that is important for the ability of SOS to activate Ras.
2. Coordinated regulation of Rho/Rac and Ras activities

Members of the Rho family of small GTP-binding proteins include Cdc42, Rac, and Rho (8). Each of these Rho-family members induces unique morphological changes, that involve rearrangement of the F-actin, when microinjected into Swiss 3T3 fibroblasts (228). More specifically, microinjection of a constitutively active Cdc42 induces filopodia, Rac induces membrane ruffles, and Rho induces stress fibers (228). Studies have illustrated that Rho, Rac, and Cdc42 regulate a signal transduction pathway in Swiss 3T3 cells linking extracellular signals to the organization of the actin cytoskeleton (229-231). In addition, one member of the family can activate another: Cdc42 can activate Rac which can then activate Rho (228).

The presence of two catalytic domains in DBL family members suggests that they may act as multifunctional proteins linking signaling pathways. SOS has both a CDC25 homology domain that encodes the Ras GEF activity and the DBL homology domain that potentially encodes Rho GEF activity. Bar-Sagi et al. through both microinjection of recombinant proteins and by analysis of cell lines transformed by Ras demonstrated that activated Ras proteins are capable of inducing membrane ruffling (232). Subsequently, Ridley et al. illustrated that both Rac and Rho proteins are
required in order for Ras to stimulate reorganization of the actin cytoskeleton suggesting that Ras is acting upstream of Rac and Rho (230). Expression of activated Rac along with RAF-CAAX in NIH3T3 cells results in an increase in transforming activity compared to each transfected individually (233). This indicated that Rac and MAPK pathways cooperate to cause cellular transformation. Through the use of Ras mutant proteins, Joneson et al. recently provided evidence that Ras controls both membrane ruffling and MAPK activation by distinct effector systems (234). Data therefore has been emerging supporting the idea that the Ras and Rho/Rac signaling pathways are linked.

Several experiments can be proposed to test if SOS plays a role in linking the Ras/Rho signaling pathways. One intriguing question is whether SOS can exhibit exchange activity towards Rho family proteins. This can be easily tested by looking at the ability of the DBL domain of SOS to catalyze exchange on GST-Rho, GST-Rac, and GST-Cdc42 in vitro. Another possibility is that SOS may serve to regulate Rho family members and not actually catalyze exchange. Recently, a Cdc42/Rac interactive binding (CRIB) motif has been identified which consists of approximately 16 amino acids (236). Upon examination of the dSOS sequence, there is a potential CRIB motif which is located within the DBL homology domain.
Therefore, it would be feasible to determine if SOS is able to associate with Rac, Rho, or Cdc42 and determine if this association is nucleotide dependent. I have recently constructed a SOS GST-DBL fusion protein. Preliminary results from experiments performed by Dr. Andrew Cherniack in this laboratory suggest that the DBL domain of SOS is able to associate with Rac in vitro. More specifically, the SOS GST-DBL protein was incubated with lysates prepared from COS-1 cells overexpressing myc-Rac, i.e., Rac with a myc epitope at the NH₂-terminus. In addition, 3T3L1 adipocytes could be used as a model system to determine if insulin triggers the association of SOS with Rac in vivo. If a complex of SOS and Rac is observed, one question that should be addressed is what is the phosphorylation state of SOS. The presence of multiple domains (DBL and PH domains) in several DBL-like GNEFs suggest a possibility for merging of signaling pathways and/or an additional level of regulation for individual GNEF activities.

In order to begin to delineate a role for the DBL domain of SOS, I have recently tested whether expression of NCatHA and NCatδPHHA have an effect on actin organization. As illustrated in Figures 31 and 32, preliminary results suggest that both of these dSOS constructs cause reorganization of the actin cytoskeleton when overexpressed in COS-1
cells, as compared to cells that were mock transfected with pCMV5. In Figure 31 (panels A-D) COS-1 cells were mock transfected with the pCMV5 vector, and the presence of long stress fibers was detected by actin staining using rhodamine-labelled phalloidin. Both NCatHA (panels E and G) and NCatδPHHA (panels I and K) were successfully expressed in COS-1 cells as detected by incubation with the polyclonal anti-HA antibody followed by staining with FITC-conjugated goat anti-rabbit IgG. Staining of the cells expressing NCatHA and NCatδPHHA with rhodamine-labelled phalloidin revealed that both of these SOS constructs seem to affect the actin cytoskeleton (Figure 31, panels E,H,J, and L). More specifically, expression of NCatHA and NCatδPHHA seems to result in both a decrease in the number of long stress fibers in the cell and the formation of actin foci in the cytoplasm. Both of these events suggest that expression of NCatHA or NCatδPHHA in COS-1 cells causes a reorganization of the actin cytoskeleton, most likely via an event involving the DBL domain of SOS.
Figure 31. Reorganization of the Actin Cytoskeleton by NCatHA and NCatδPHHA. COS-1 cells were plated on coverslips and transiently transfected with either vector alone (pCMV5), NCatHA, or NCatδPHHA. Approximately 48 hours after transfection cells were fixed and permeabilized as described in the Methods chapter. Cells were subsequently incubated for two hours with the anti-HA antibody. The cells were next washed and incubated with FITC-conjugated goat anti-rabbit IgG and rhodamine labelled phalloidin. Cells were mounted and immunofluorescence was visualized by digital imaging microscopy. Panels A-D, COS-1 cells were mock transfected with pCMV5 and stained with rhodamine labeled phalloidin. Panels E-H, COS-1 cells were transfected with NCatHA and expression was detected by anti-HA; FITC-conjugated goat anti-rabbit IgG staining (panels E and G). The same cells shown in panels E and G were also examined for actin filaments using rhodamine-labelled phalloidin (panels F and H respectively). Panels I-L, COS-1 cells were transfected with NCatδPHHA (panels I and K) and expression was detected as described above for NCatHA. The same cells shown in panels J and L were also examined for actin filaments using rhodamine-labelled phalloidin. The results are representative for two independent experiments and ~150 cells were scored for each condition.
rhodamine-labelled phalloidin

A

B

C

D

pCMV5
anti-HA

rhodamine-labelled phalloidin

NCatHA
anti-HA  rhodamine-labelled phalloidin

NCatδPHHA
Figure 32. Graphical Representation of the Percent of COS-1 cells Expressing NCatHA and NCat8PHHA which Exhibit Reorganization of the Actin Cytoskeleton. Quantitation of the data presented in Figure 31. More specifically, for each transfection condition, ~150 cells were scored for two phenotypes: 1.) the presence of long stress fibers and 2.) disappearance of long stress fibers and the formation of foci of actin near the periphery of the cell. The results are a summary of two independent experiments.
% of Cells with long stress fibers

% of Cells with reorganized areas of actin
Presently, I am testing if any of the following dSOS constructs have an effect on the actin cytoskeleton: NHA, CatCHA, NCatδ200HA, and NCatCHA. Preliminary results suggest that the NH$_2$-terminal domain alone of dSOS exhibits the phenotype described for both NCatHA and NCatδPHHA, whereas expression of the dSOS construct which deletes the NH$_2$-terminal domain does not seem to display this phenotype. However, the experiment with the NHA and CatCHA constructs has only been performed once. Initial results suggest that SOS may play a role in reorganization of the actin cytoskeleton presumably via the DBL domain. Therefore, it would be interesting to determine if this is occurring in a Ras-independent or Ras-dependent manner.

3. Determination of the three-dimensional structure of domains of SOS

Ultimately, in order to understand the specific mechanism by which GNEF's promote nucleotide exchange on Ras, detailed structural studies need to be performed. Several labs have demonstrated that GNEFs catalyze nucleotide exchange by stabilizing the apoRas intermediate (235). Using Ras mutants, residues critical for interaction with GNEF's have been identified. More specifically, NMR analysis of the Ras mutant protein Ras(E69N), a mutant that is unable to associate with CDC25, indicated that
no structural changes were occurring outside the switch 2 region which suggest that GNEFs interact with the switch 2 region of Ras (236,237). In addition to understanding the residues in Ras involved, it is important to understand how the domains in SOS contribute to the activation of Ras. Detailed structural studies of the DBL, PH, and CDC25 homology domains of SOS independently and in combination may aid in understanding the mechanism by which SOS activates Ras, and potentially, Rho family members.
CHAPTER VII

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