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STUDIES OF THE INTERACTIONS OF RAS PROTEIN WITH ITS REGULATORS

by

YING JU SUNG

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

STUDIES OF THE INTERACTIONS OF RAS PROTEIN WITH ITS REGULATORS

by

Ying Ju Sung

Adviser: Yu Wen Hwang

Ras proteins structural elements required for interacting with their regulators were determined. Dominant negative ras mutants were used to achieve this goal. Specifically, two types of mutants were employed: (1) H-ras(N116Y), (N116I), (K117E); (2) H-ras(G60A). These two classes of mutants affect two different functional domains in ras. Mutants v-H-ras(N116I) and v-H-ras(K117E) form stable complexes with guanine-nucleotide exchange factor (GEF), thereby inhibiting guanine-nucleotide exchange. In contrast, the G60A mutant primarily affects the interactions with effectors.

In the first part of this thesis Sdc25p-C, SDC25 C domain gene product, a ras GEF, was used to differentiate H-ras and K-ras guanine-nucleotide exchange activities and binding affinities. H-ras and K-ras differ extensively in their C-termini. Therefore, the interaction of H-ras and K-ras' C-terminal domain with Sdc25p-C was investigated. Studies using ras chimeras showed that H-ras and K-ras interact with Sdc25p-C in a highly selective manner. Ras p21' hypervariable C-terminal domain is the main determinant for ras•Sdc25p-C complex formation. These studies represent the first functional assignment of the C-terminal domain.

The second part of this thesis focused on characterizing H-ras-(G60A). This residue is conserved in all regulatory GTPases, and is critical for GTP-induced conformational change. Mutating Gly-60 to Ala hindered the GTP-induced conformational change of ras, abolished ras' biological activity, and reduced the GTPase (both intrinsic and GAP-stimulated) of ras. This mutation also substantially decreased the ras-Raf interaction. Interestingly, rasGAP interaction was not affected by the mutation. Ras(G60A) is a dominant negative mutant against viral (oncogenic) H-ras. The inhibition was found to be due to the limitation of Raf. These studies established that Raf is likely to be the direct cellular target of ras protein.

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List of Abbreviations

ANS, 8-anilino-1-naphthalene sulfonate
cAMP, cyclic AMP
CDC25, cell division cycle gene product in *Saccharomyces cerevisiae*
DTT, dithiothreitol
ERK, extracellular signal-regulated kinase or MAPK
EGF, epidermal growth factor
EF-Ts, elongation factor Ts
EF-Tu, elongation factor Tu
ERK, extracellular-signal-regulated kinase or MAPK
G60A, Gly-60 to Ala mutation
G83A, Gly-83 to Ala mutation
G226A, Gly-226 to Ala mutation
GAP, GTPase activating protein
GEF, guanine nucleotide-exchange factor
GDP, guanosine diphosphate
Grb2, growth factor receptor-bound protein 2
Gpp(NH)p, guanyl-5'-yl imidodiphosphate
GST, glutathione-S-transferase
GTP, guanosine triphosphate
GTPase, guanosine triphosphatase
GVBD, germinal vesicle breakdown
c-H-ras, cellular H-ras
v-H-ras, viral H-ras
IGF, insulin-like growth factor
MAPK, mitogen-activated protein kinase
MAKAP, MAPK-activated protein kinase
MAPKK, mitogen-activated protein kinase kinase or MAPK kinase
MAPKKK, mitogen-activated protein kinase kinase kinase
MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase or MAPK kinase or ERK kinase
NGF, nerve growth factor
PC12 cells, pheochromocytoma cells
PC-PLC, phosphatidylcholine-specific phospholipase C
PCR, polymerase chain reaction
PDGF, platelet-derived growth factor
PI(3)K, phosphatidylinositol 3' kinase
PI-PLC, phosphatidylinositol-specific phospholipase C
PKC, protein kinase C
PLA2, phospholipase A2
PLCγ, phospholipase Cγ
PMSF, phenylmethyl sulphonyl fluoride
c-Raf, cellular Raf
v-Raf, viral Raf
RBD, ras binding domain
RSK, ribosomal S6 kinase
SDC25, suppressor of the CDC25 gene product in *Saccharomyces cerevisiae*
Sdc25p-C, C-domain gene product of SDC25

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2 domain, Src-homology 2 domain
SH3 domain, Src-homology 3 domain
TKR, tyrosine kinase receptor

Chapter I. Introduction

Ras proteins are ubiquitous among eukaryotic organisms and their amino acid sequences are highly conserved. They have been shown to be involved in regulating cell growth (Barbacid, 1987; Broach and Deschenes, 1990), cellular differentiation (Beitel et al., 1990; Balch, 1990; Benito et al., 1991; Chardin, 1991; Kremer et al., 1991; Satoh et al., 1987), and carcinogenesis (Bos, 1989; Gibert and Harris, 1988; Nori et al., 1991; Smith et al., 1986). Studies in which ras proteins were micro-injected into quiescent fibroblasts have shown that they are potent mitogens (Mulcahy et al., 1985). Similar studies using rat pheochromocytoma cells (PC12 cells) and embryonic neurons reveal that ras proteins induce terminal differentiation similar to that induced by NGF (Bar-Sagi and Feramisco, 1985; Borasio et al., 1989). In *Saccharomyces cerevisiae*, RAS is linked to the production of cAMP and resulted in the mating activities (Fedor-Chaiken et al., 1990; Field et al., 1990; Fukui et al., 1986; Nadin-Davis et al., 1986). In *Caenorhabditis elegans* the ras homologue, let-60, is required for vulva development (Beitel et al., 1990; Han and Sternberg, 1990). In *Xenopus* oocytes, ras proteins promote the breakdown of the nucleus or germinal vesicle (GVBD), an external indication that the oocytes are maturing (Birchmeier et al., 1985). In *Drosophila melanogaster*, ras proteins are involved in eye development (Simon et al., 1991). This evidence clearly suggests that ras proteins play a dynamic role in various cellular functions. However, the exact role ras proteins play in regulating normal cell growth, mitogenic signal transduction, and cellular differentiation is still not well understood.

Ras proteins bind guanine-nucleotides and belong to a class of proteins collectively called regulatory GTPases (Bourne et al., 1991). Despite

their functional diversity, all regulatory GTPases share a common mode of action. Regulatory GTPases are inactive when bound to GDP, and are activated by binding to GTP which induces a conformational change in the molecule, promoting its ability to interact with a target protein (Figure 1). Interaction with the target leads to GTP hydrolysis and returns the regulatory GTPase to the GDP-bound inactive state (Figure 1).

A review of ras structure and function pertinent to my studies is presented below.

I. The Structure of Ras Proteins

Mammalian ras proteins, termed p21, are composed of 189 amino acids with a mass of about 21 kDa (Bourne et al., 1990; Hall, 1990). The ras family consists of three members: H-ras, K-ras, and N-ras. All three share extensive sequence homology (Figure 2) (Barbacid, 1987; Lowy and Willumsen, 1993). The N-terminus of these proteins are virtually identical, while the C-terminus contains a hypervariable region (from residue 165 to 185) followed by another conserved motif consisting of a cysteine followed by two aliphatic amino acids. It has been suggested that the hypervariable region is involved in interacting with some unique cellular components. In Chapter II, the role ras' C-terminal domain plays in recognizing specific guanine-nucleotide exchange factors will be addressed.

Ras Primary Structure:

Comparing the amino acid sequence of ras p21 with other regulatory GTPases revealed three regions with remarkable similarities (Halliday, 1984; Dever et al., 1987). Each region is characterized by a set of invariant

amino acids. For ras p21, these residues are located at positions 10-16, 57-60 and 116-119 as depicted in Figure 2. According to X-ray crystallographic models, these regions form the guanine-nucleotide binding pocket (Pai et al., 1990; Pai et al., 1989).

The first consensus domain, residues 10-16, (GXXXXGK) forms a loop surrounding the pyrophosphate moiety of the bound GDP that is essential for nucleotide binding and GTP hydrolysis. The second conserved region, residues 57-60, (DXXG) is situated near the end of the γ phosphate moiety. Residues within this region may help form the part of the binding pocket that accommodates the γ-phosphate of GTP. The third region, residues 116-119, (NKXD) interacts with the guanine ring and determines the nucleotide binding specificity. The importance of these domains in guanine-nucleotide binding, GTP hydrolysis, base specificity, and conformational change has been extensively documented (De Vos et al., 1988; Feig and Cooper, 1988; Feig et al., 1986; Hwang et al., 1989c; Hwang et al., 1989b; Hwang and Miller, 1987; Jurnak, 1985; la Cour et al., 1985; Milburn et al., 1990; Pai et al., 1990; Pai et al., 1989; Sigal et al., 1986; Walter et al., 1986; Zhong et al., 1995).

In addition to these generally conserved nucleotides binding motifs, the ras family possess two other conserved motifs, EXSAK and CAAX, which are shared by only a subset of the entire regulatory GTPase family. There is no direct evidence to show that EXSAK is involved in the binding of the guanine base. Nevertheless, it has been proposed that these residues assist in the binding, or dissociation of the guanine-nucleotide from ras p21 (Wittinghofer and Pai, 1991). P21 proteins are known to be associated with

the inner surface of the plasma membrane (Willumsen et al., 1984) through posttranslational modifications at their C terminal end. The CAAX motif, a signal for protein polyisoprenylation (Gutierrez et al., 1989), is one of the features required for ras' membrane localization and biological function (Gutierrez et al., 1989; Hagag et al., 1986; Schafer et al., 1989).

Ras Secondary and Three Dimensional Structures:

Figure 3 shows the overall topology of the secondary structure elements of the ras polypeptide chain and their connecting loops. Ras p21 has a central β-sheet consisting of six strands, five α-helices, and ten interconnecting loops. In this structure, loop1 (residues 10 to 15), loop2 (residues 26 to 36), and loop4 (residues 59 to 64) comprise the active site of the molecule which surrounds the γ-phosphate of GTP.

The guanine base of the nucleotide is bound in a hydrophobic pocket through interactions with the conserved sequence motifs ^{116}NKXD and ^{143}EXSAK (Figure 2 and 3). NKXD is therefore defined as the guanine ring binding domain. The side chain carbamoyl group of Asn116, has been proposed to form hydrogen bonds to the side chain of Thr144 and to the main chain oxygen atom of Val14 (Figure 4) (Tong et al., 1991; Pai et al., 1989). This indicates that the main function of Asn116 in ras p21 is to bring the three elements, ^{10}GAGGVGKS, ^{116}NKCD, and ^{143}ETSAK together (Figure 2). A similar function is performed by the amino group of Lys117 (Figure 2), which links ^{10}GAGGVGKS and ^{116}NKCD by binding to the main chain carboxyl group of Gly 13 (Figure 4) (Tong et al., 1991; Pai et al., 1989).

It should be pointed out that most of the crystallization studies on ras,

used a truncated protein produced by mutating Lys167 (Figure 2) to a stop codon; this eliminates the mobile elements on the surface of the protein that prevent the full-length molecule from being packed into a crystal (Scherer et al., 1989).

Regulatory GTPases require at least one divalent cation complexed directly to the phosphoryl oxygens for catalytic activity. The precise role of the metal ion in the mechanism of regulatory GTPases has not been clarified. Magnesium ions also play an important part in the interaction of guanine-nucleotides with ras p21. X-ray crystallographic studies have shown that nucleotide bound p21 contained a Mg^{2+} which was coordinated to β and γ-phosphate oxygen atoms of GTP, two molecules of water, and the hydroxyl groups from the side chain of Ser17 and Thr 35 (Figure 5) (Pai et al., 1990).

GTP-induced Conformational Change:

Switching from the GDP to GTP-bound conformation enables regulatory GTPases to react with their regulator and effector molecules. In the case of ras p21, its affinity to GAP increases a hundred fold when the protein is bound to GTP (Vogel et al., 1988). The elements involved in this structural change have been elucidated by comparing the structures of ras•GDP and ras•GTP complexes. It appears that the rearrangement in ras p21 is confined to loop 2 (residues 30 to 38), and loop 4-helix α_2-loop 5 (residues 60 to 76) (Figures 2 and 3) (Pai et al., 1989; Milburn et al., 1990; Stouten et al., 1993). The DXXG motif forms a sharp flexible turn (loop 4) connecting β-strand 3 and α-helix 2 of H-ras p21 (De Vos et al., 1988; Pai et al., 1989). This sharp turn requires a Gly residue because Gly exhibits

much broader range of phi and psi dihedral angles than other amino acids (Richardson, 1981). The GTP induced conformational change appears to originate near residues 59-60 and then propagates to other regions of p21, primarily, residues 30-38 (loop 2, the effector region, switch I) and 60-76 (loop 4 and helix 2, switch II) (Pai et al., 1989; Milburn et al., 1990; Stouten et al., 1993). These conformational changes not only involve the movement of protein domains, but also include a massive reorientation, particularly, in loop 4. This reorientation redirects the amide group of Gly-60 from a residue in helix 2 (possibly Glu-63) to the γ-phosphate of GTP, forcing part of helix 2 to relax into an extended loop 4 and to rotate along its own helix axis. Presumably, these changes place the helix 2 and loop 2 regions in a favorable position for target binding and GTP hydrolysis (Pai et al., 1989; Milburn et al., 1990; Stouten et al., 1993). These results are consistent with the findings that loop 2 and helix 2 are important for interacting with rasGAP and the *Raf* gene product, two putative targets of ras p21, in a GTP-dependent manner (Bollag and McCormick, 1991; Marshall, 1993; Moodie et al., 1993; Van Aslet et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993).

This analysis is also in accord with conformational studies of EF-Tu and heterotrimeric G proteins. Although they possess only modest sequence similarities, the X-ray structures of H-ras p21 and the EF-Tu G-domain are nearly superimposable (Valencia et al., 1991; Jurnak et al., 1990); therefore, the GTP-induced conformational changes in EF-Tu should mimic those found in H-ras p21. It has been shown that the amino acid in EF-Tu corresponding to the H-ras Gly-60 residue, Gly-83 of EF-Tu from *Escherichia coli* (Jurnak, 1985; la Cour et al., 1985), and Gly-84 of EF-Tu

from *Thermus thermophilus* (Berchtold et al., 1993) and *Thermus aquaticus* (Kjeldgaard et al., 1993), reorients in response to GTP and initiates molecular rearrangements parallel to those found in H-ras p21. These changes subsequently extend to other domains of EF-Tu and allow the protein to bind aminoacyl-tRNA and to ribosomes (Berchtold et al., 1993; Kjeldgaard et al., 1993). Despite the fact that the GTP-induced conformational changes in heterotrimeric G-proteins are inherently more complex than that of H-ras (and EF-Tu), the basic mechanism appears to be similar. That is, the conserved glycine (Gly-199 in α subunit of transducin), again, plays a pivotal role in the GTP-induced conformational change (Noel et al., 1993; Lambright et al., 1994).

The importance of this conserved Gly residue has been examined in $G_{s\alpha}$ and *E. coli* EF-Tu by substituting the invariant glycine with alanine, a change that should drastically reduce the ability to rearrange around the Gly residue. In $G_{s\alpha}$, the G226A mutation does not affect the strength of GDP/GTP binding nor does it affect the interaction of Gsα with its receptor (Miller et al., 1988; Lee et al., 1992a). However, the mutation does block GTP-induced subunit dissociation and prevents the activation of adenylyl cyclase (Miller et al., 1988; Lee et al., 1992a). The dissociation of α from βγ subunits is known to be the essential step preceding adenylyl cyclase activation (Gilman, 1987). The loss of GTP-induced subunit dissociation has been taken as evidence that Gsα(G226A) can no longer switch into an active form in the GTP-bound state (Miller et al., 1988; Lee et al., 1992a). The G83A mutation in EF-Tu, the counterpart of Gsα(G226A), affects EF-Tu similarly: abolishing GTP-induced aminoacyl-tRNA binding while not affecting EF-Tu's ability to bind GDP/GTP and EF-Ts (Hwang et al., 1989a). The

importance of this conserved glycine at position 60 of ras in the GTP-induced conformational change will be addressed in Chapter III.

Ras Mutants:

One of the most important and interesting properties of *ras* genes are their ability to transform cells. The transforming activity of ras p21 can be greatly enhanced by single amino acid mutations at critical locations. Naturally occurring mutations have been localized to codons 12, 13, 59, 61, and 117 (Bos et al., 1985; Dhar et al., 1982; Reddy et al., 1982; Reynolds et al., 1987; Tabin et al., 1982; Taparowsky et al., 1983; Taparowsky et al., 1982; Tsuchida et al., 1982). Mutagenesis studies have also shown that mutations at positions 10, 63, 116, 119, or 146 cause ras to become oncogenic (Clanton et al., 1987; Fasano et al., 1984; Cunningham et al., 1990; Sigal et al., 1986; Walter et al., 1986). In general, these mutants fall into two biochemical categories: (1) mutants with an enhanced rate of GDP dissociation and (2) mutants with decreased GTPase activity. These mutations, which affect either GDP dissociation or GTP hydrolysis, have a significant effect on the biological function of ras protein and regulation of the ras activity.

II. The Interaction of Ras Proteins with GEFs

Being a member of the family of regulatory GTPases, ras p21 binds guanine-nucleotides and functions as a molecular switch in mediating a diverse array of cellular processes (Figure 1). Regulatory GTPases are only able to interact with their targets and exert their cellular effects when they are bound to GTP. This interaction is accompanied by hydrolysis of the γ-phosphate of GTP which deactivates the regulatory GTPase. Reactivation requires the exchange of the bound GDP with

exogenous GTP, a process referred to as guanine-nucleotide exchange. Since the activity of regulatory GTPases depend upon the state of the bound guanine-nucleotide, it is clear that the activity of regulatory GTPases are maintained by balancing guanine-nucleotide exchange and GTP hydrolysis (Bourne et al., 1990; Bourne et al., 1991).

Regulatory GTPases commonly have a very low intrinsic rate of guanine-nucleotide exchange. This insures that they are active only when needed, because the ligand for their activation, GTP, is abundant intracellularly (Friesen et al., 1975; De S.Otero, 1990). The low intrinsic rate of guanine-nucleotide exchange can be greatly accelerated by GEF. GEFs are usually highly specific and are essential for the function of regulatory GTPases (Bourne et al., 1990; Bourne et al., 1991). Inhibiting the GEF specific for a particular regulatory GTPase will generally shut down the cellular pathway mediated by that regulatory GTPase (Feig and Cooper, 1988; Hwang et al., 1989c; Powers et al., 1989; Jung et al., 1994).

Several ras p21 GEF's have been identified (Downward, 1992; Lowy and Willumsen, 1993; Polakis and McCormick, 1993). The first ras exchange factor discovered, the cell division cycle gene product CDC25, was found in the yeast *Saccharomyces cerevisiae* (Robinson et al., 1987; Broek et al., 1987; Daniel et al., 1987). CDC25 plays a crucial role in the RAS/adenylyl cyclase pathway, which controls the production of cAMP, an initial signal for the onset of cell division in *S. cerevisiae* (Boy-Marcotte et al., 1989). Subsequently, another ras p21 GEF, Sdc25p-C, was found in *S. cerevisiae*. Sdc25p-C has been shown to stimulate guanine-nucleotide exchange *in vitro* in both *S. cerevisiae* and human ras proteins (Crechet et al., 1990).

Other proteins which have been identified as ras exchange factors include BUD5 (Chant et al., 1991) and LTE1 (Wickner et al., 1987) from *S. cerevisiae*, the STE 6 protein from the fission yeast *Schizosaccharomyces pombe* (Hughes et al., 1990), the SOS protein from *Drosophila melanogaster* (Hwang et al., 1989b), and the mammalian and human homologs SOS, mSOS1 and mSOS2 (Bowtell et al., 1992), hSOS1 and hSOS2 (Chardin, 1991), a brain-specific mammalian homologue of CDC25 termed Ras-GRF from rat (Shou et al., 1992), $CDC25^{Mm}$ from mouse, and CDC25h from human (Martegani et al., 1992) *etc.* All these proteins share certain sequence similarities to the catalytic domain of CDC25; these similarities are confined in three domains spanning about 200 amino acid residues as shown in Figure 6 (boxed regions 1, 2, 3). The catalytic domain of these factors have been shown to be indispensable for guanine-nucleotide exchange activity (Downward, 1992; Lowy and Willumsen, 1993; Polakis and McCormick, 1993).

It has been demonstrated that *E coli* EF-Ts stimulated guanine-nucleotide exchange goes through an intermediate consisting of EF-Tu and EF-Ts (EF-Tu's specific GEF) (Miller and Weissbach, 1977; Hwang and Miller, 1985; Romero et al., 1985). The EF-Tu•EF-Ts complex is subsequently dissociated by an exogenous guanine-nucleotide that releases EF-Ts, resulting in guanine-nucleotide exchange. Sdc25p-C-stimulated guanine-nucleotide exchange appears to involve a similar pathway: first a ras•Sdc25p-C binary complex forms, then guanine-nucleotide exchange occurs (Mistou et al., 1992; Hwang et al., 1993). The formation of ras and exchange factor complexes have also been demonstrated for other GEF's (Baroni et al., 1992; Munder and Furst, 1992; Lai et al., 1993; Liu et al., 1993);

therefore, it is likely that the guanine-nucleotide exchange promoted by other GEF's may also involve a similar mechanism as shown in Figure 7.

EF-Tu, GDP/GTP, and EF-Ts exist in a state of dynamic equilibrium. EF-Ts and GDP/GTP compete against each other for binding to EF-Tu (Miller and Weissbach, 1977; Hwang and Miller, 1985). The interaction between H-ras, GDP/GTP and Sdc25p-C also shows similar characteristics, as Sdc25p-C competes against GDP/GTP for binding to H-ras (Hwang et al., 1993). This normal equilibrium can be disrupted by removing guanine-nucleotide (Miller and Weissbach, 1977; Lai et al., 1993) or by introducing a mutation into the regulatory GTPase that reduces GDP/GTP binding. Amino acid substitutions in the conserved GDP/GTP binding domain have been shown to stabilize GTPase•GEF complexes (Hwang et al., 1989c; Feig and Cooper, 1988; Munder and Furst, 1992; Hwang et al., 1993; Chen et al., 1994; Jung et al., 1994; Zhong et al., 1995). In some cases, the GTPase•GEF complex is so stable that mutants exhibit a dominant negative phenotype against their normal cellular counterpart (Lowy and Willumsen, 1993; Polakis and McCormick, 1993; Nuoffer and Balch, 1994).

Regions of Ras Involved in GEF Binding:

The structural requirements of ras for binding its respective GEFs are still not completely understood. A theoretical analysis (Valencia et al., 1991) has suggested that the most likely domains for interacting with GEF are loops L8 and L10 (Figure 2). Part of this hypothesis has been supported by a mutagenesis study using mutants in the guanine ring binding domain of EF-Tu that confer a dominant negative phenotype. Many of the

mutations affecting EF-Ts binding were localized to residues 154-200 of EF-Tu, a region comprising L10 (Hwang et al., 1992). Nevertheless, other studies seeking to determine the GEF-binding domains of ras have revealed that residues 62-69 (Mosteller et al., 1994; Howe and Marshall, 1993), 73-76 (Mistou et al., 1992; Mirisola et al., 1994; Quilliam et al., 1994), and 103-108 (Segal et al., 1993) are also important. Most of these experiments used different GEFs to probe the sites on ras that are important for their interactions. Therefore, it is possible that different GEFs may interact with ras through different domains. In the following chapter, Sdc25p-C was used as a model to show that it can discriminate between different ras p21 based on their C-terminal domain and possibly the loop L8 of ras p21.

III. The Interaction of Ras with GAPs

A cytoplasmic factor in *Xenopus* oocytes that stimulated GTP hydrolysis in the cellular form of ras p21 but not its oncogenic form was identified in 1987 (Trahey and McCormick, 1987). This factor, termed ras GTPase Activator Protein (rasGAP), had a molecular weight of 120 kDa. In mammalian cells two GAPs have been identified to date: one is rasGAP, the other is NF-1 (neurofibromin-1), a protein with a molecular weight of 250 kDa (Xu et al., 1990). In addition, GAPs have also been isolated from *Drosophila* (Ggap) and yeasts (IRA-1 and IRA-2) (Tanaka et al., 1990; Tanaka et al., 1991).

Figure 8 shows the structure of the GAP family in schematic form. These proteins share a about 250 residues of homology in their C terminal region; this region is responsible for stimulating GTPase activity (Marshall

et al., 1989). In addition to a hydrophobic amino terminus, rasGAP also contain features that distinguish it from other structures in the GAP family: a SH2 (Src homology 2) domain which is flanked by two SH3 (Src homology 3) domains (Figure 8) (Vogel et al., 1988). SH2 domains are often found in conjunction with SH3 domains. In such a configuration these two domains control the binding of phosphorylated tyrosine residues embedded within a specific peptide sequence. It has been demonstrated that the SH2 and SH3 domains of rasGAP are involved in its interaction with non-receptor tyrosine kinases such as src, fps, abl, and some cytosolic proteins like phospholipase C-γ and the *crk* oncogene (Sadowski et al., 1986; Koch et al., 1991).

GAPs interact only with the GTP-bound form of ras p21 and stimulate its GTPase activity. The catalytic domain for GAP activity is conserved among all GAPs (Figure 8). It has been shown that the isolated catalytic domain of rasGAP is able to stimulate the GTPase activity of ras p21, but the kinetics differ from that of full length of rasGAP (Gideon et al., 1992; Marshall et al., 1989). Therefore, rasGAP may contact ras protein at a region distal to its catalytic domain; the significance of this possibility is however, unknown.

Regions of Ras Involved in GAP Binding:

Two regions of ras p21, residues 30-38 (switch I domain) and 60-76 (switch II domain), are essential for the rasGAP interaction (Bar-Sagi and Feramisco, 1985; Srivastara et al., 1989; Trahey and McCormick, 1987). Analysis of ras mutants revealed that mutating the effector region (switch I domain) renders the protein unable to respond to rasGAP (Cale's et al.,

1988; McCormick, 1989; Adari et al., 1988; Farnsworth et al., 1991). In addition, rasGAP also interacts with the switch II domain of ras, since mutating amino acid residues around this area strongly influence the binding affinity between ras and rasGAP (Srivastara et al., 1989; Krengel et al., 1990). Moreover, it has been shown that the neutralizing ras antibody, Y13-259, which recognizes an epitope within amino acids 63-73 impaired GAP activity *in vitro* (Trahey and McCormick, 1987). In a study of chimeric mutants of ras and rap1a (ras-related small regulatory GTPase), mutants composed of the switch I domain of ras and the switch II domain from rap1a bound to rasGAP but had no catalytic activity (Hata et al., 1990; Zhang et al., 1990). Therefore, the switch II region in ras is likely to encode the domain which will respond to rasGAP stimulated GTPase activity. As mentioned earlier in this chapter, both of these regions undergo a conformational change in response to GTP binding (Milburn et al., 1990; Pai et al., 1990; Schlichting et al., 1990).

The Role of GAP: A Regulator or An Effector:

Two contradictory hypotheses for the biological function of rasGAP have been proposed. From mutational analyses of the ras effector domain, rasGAP was suggested to act as a downstream effector of ras (Hall, 1990). This hypothesis is also supported by patch clamp studies showing that rasGAP and ras p21 cooperated in inhibiting heterotrimeric G protein-mediated coupling of muscarinic receptors to atrial K^+ channels. In particular, the SH2 and SH3 domains in the amino terminus of rasGAP mediated this effect (Martin et al., 1992). On the contrary, genetic studies of rasGAP homologs in yeast and *Drosophila*, suggest that GAP may be a negative feedback controller of ras rather than a downstream target (Gaul

et al., 1992; Tanak et al., 1989; Wang et al., 1991). For example, in *S. cerevisiae,* RAS proteins are generally inactive, but they accumulate in their GTP-bound state in the absence of either of the *IRA* gene products (IRA-1 and IRA-2), causing an overproduction of cAMP (Tanaka et al., 1990). In addition, it has been observed that ras•GTP accumulates after stimulating the T cell antigen receptor or erythropoietin receptor (Downward et al., 1990). These data correlate well with the rapid decrease in the level of GAP activity measured in stimulated cell extracts (Torti et al., 1992). Furthermore, it has also been found that the binding of rasGAP to the activated EGF receptor leads to a small decrease in its activity (Serth et al., 1992). *NF*-1 is a tumor suppressor gene whose misregulation is responsible for type I neurofibromatosis (Cawthon et al., 1990; Wallence et al., 1990). Evidence that the NF-1 protein negatively regulates ras p21 comes from a study of tumor cell lines isolated from NF-1 patients showing that the expression and the activity of NF-1 protein are reduced. This reduction causes ras p21 to preferentially stay in an active GTP-bound state, although the activities of ras protein and rasGAP remain normal (DeClue et al., 1992; Basu et al., 1992). These observations support the view that GAP directly modulates ras and that a decrease in GAP activity, due to certain extracellular stimuli, contributes to the activation of ras protein.

The catalytic activity of GAP is apparently regulated through tyrosine phosphorylation. It has been shown that rasGAP is phosphorylated on tyrosine in response to PDGF and EGF, or the oncogenic products v-*src* or v-*fps* (Ellis et al., 1990; Kaplan et al., 1990; Liu and Pawson, 1991; Molloy et al., 1990; Reedijk et al., 1990). This phosphorylation enables rasGAP to interact with other macromolecules, such as a protein complex consisting of the

PDGF receptor, phospholipase C-γ, phosphatidylinositol 3' kinase, and the Raf proto-oncoprotein (Kaplan et al., 1990; Kaplan et al., 1987; Meisenhelder et al., 1989; Wei et al., 1992) resulting in the redistribution of their subcellular localization. However, the binding of rasGAP to the PDGF receptor does not require the presence of ras p21. The effects of rasGAP tyrosine phosphorylation as well as its association with the proteins mentioned above are not known; however one might expect this protein complex to generate a multiple signals.

Two tyrosine-phosphorylated proteins, p62 and p190, have been found in anti-rasGAP immunoprecipitates of the stimulated cells. The cDNAs encoding p62 and p190 have been cloned (Wong et al., 1992; Settleman et al., 1992b). P62 was found to share some sequence similarity with heterogeneous nuclear ribonuclear protein (hnRNP); it can bind DNA and mRNA implying that it may be involved in RNA processing (Wong et al., 1992). The C terminus of p190 was found to share 40% homology to the GAP for rho (a ras-related small regulatory GTPase) (Settleman et al., 1992b). Indeed, recombinant p190 showed specific GAP activity for members of the rho family (Settleman et al., 1992a). This suggests that the association of p190 and rasGAP in growth factor stimulated cells may couple the ras and rho signalling pathways. However, the actual functions of these proteins are not yet known.

IV. The Interaction of Ras with Raf Protein

Recently, biological and biochemical evidence has shown that Raf protein is an effector of ras p21 in its signal transduction pathway. Several lines of evidence have shown that oncogenic Raf protein can bypass the

cellular requirement for a functioning ras (Smith et al., 1986; Noda et al., 1983); dominant negative mutations of Raf (kinase-defective Raf mutants) as well as Raf antisense constructs can suppress transformation induced by oncogenic ras protein (Kolch et al., 1991). The formation of a ras•Raf protein complex has been demonstrated both *in vivo* and *in vitro* (Koide et al., 1993; Moodie et al., 1993; Smith et al., 1986; Finney et al., 1993; Hallberg et al., 1994; Reddy et al., 1982). In addition, genetic studies showed that *Drosophila* Raf is necessary for both the sevenless receptor tyrosine kinase- and ras-stimulated phenotypes in eye development (Dickson et al., 1992). Moreover, ras and Raf proteins are both required for signaling through Let-23 (an EGF-like receptor tyrosine kinase) in *C. elegans* (Han et al., 1993; Sternberg and Horvitz, 1991). These data provide a clear link between Raf activation and ras p21. Nevertheless, the precise mechanism by which ras p21 activates Raf protein is not known.

Raf was originally identified as the oncogenic component of murine sarcoma virus 3611 (Rapp et al., 1983). In vertebrates, three members of the *Raf* family have been identified: c-*Raf*, B-*Raf*, and A-*Raf* (Avruch et al., 1994). All these three genes are functionally classified as proto-oncogenes because specific mutations can render them oncogenic. *Raf* genes encode proteins of 64-74 kDa that have intrinsic kinase activity towards serine and threonine (Rapp et al., 1983). The c-Raf-1 protein is ubiquitously expressed; in contrast, the A-Raf protein is more restricted in its tissue distribution; B-Raf protein is particularly highly expressed in brain and testis (Storm et al., 1990).

Members of the Raf family contain a carboxyl-terminal kinase

domain and an amino-terminal regulatory region. As shown in Figure 9, three distinct regions of homology among the Raf family have been identified; they are termed CR-1, CR-2, and CR-3, respectively. In the case of the c-Raf-1 protein, the CR-1 domain (residues 62-196) contains a zinc-finger motif (C-X2-C-X9-C-X2-C) spanning residues 151 to 168 and a negative autoregulatory domain implicated in ras-Raf interactions (Daum et al., 1994). The CR-2 domain (residues 255-268) is rich in serine and threonine residues and contains the major phosphorylation sites *in vitro*. Presumably this region also contributes to negative autophosphorylation (Daum et al., 1994). The CR-3 domain (residues 330-627) represents the kinase domain of the protein (Daum et al., 1994). The CR-3 domain has approximately 30% sequence homology with a variety of cellular protein kinases including serine/threonine protein kinases and tyrosine protein kinases.

v-Raf oncoproteins are constitutively active because their N-terminal regulatory region is absent (Avruch et al., 1994). It has been demonstrated that the c-Raf amino-terminal residues 1-275 [c-Raf(1-275)] acts as a dominant inhibitor of protein tyrosine kinase-stimulated mitogenesis and MAP kinase activation (Kolch et al., 1991; Troppmair et al., 1994). Therefore, the amino terminus of Raf protein may function either to suppress the activity of the kinase domain or to regulate the interaction of the catalytic domain with substrates.

It is known that the N-terminal regulatory domain of c-Raf-1 protein physically interacts with ras p21. This interaction is specific for the GTP bound form of ras; however, ras does not activate Raf directly (Moodie et al., 1993; Koide et al., 1993; Vojtek et al., 1993; Leevers et al., 1994; Stokoe et al.,

1994). Deleting residues 53-132 in c-Raf-1 abolished its kinase activity in ras activated cells (Pumiglia et al., 1995). It has been further demonstrated that the binding of Raf to ras is mediated by at least two distinct domains on c-Raf-1: residues 55-132 and 128-198 (Scheffler et al., 1994; Bruder et al., 1995; Zhang et al., 1993; Ghosh et al., 1994). The zinc-binding motif within CR-1 domain of c-Raf-1 protein has been suggested to be important for the ras-Raf interaction, because replacing Cys168 by Ser greatly reduced its interaction with ras (Bruder et al., 1992; Zhang et al., 1993). In contrast, using a yeast two-hybrid screening system, the zinc finger sequence was shown not to be essential for ras to bind to Raf (Vojtek et al., 1993). Moreover, a c-Raf-1 fragment containing residues 55 to 132 appears to have full ras binding ability (Pumiglia et al., 1995).

The ras-Raf interaction appears to be transient, resulting in the localization of Raf to the membrane where it is activated by a yet unknown mechanism (Leevers et al., 1994; Stokoe et al., 1994). Nevertheless, c-Raf-1 protein has been shown to form a stable complex with MEK-1 and directly mediate its activation within this complex *in vitro* (Jelinek et al., 1994).

Regions of Ras Involved in Raf Binding:

Residues 30-38 and 60-76 of ras have been implicated as the binding sites for Raf protein (Figure 2) (Van Aslet et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993; Shirouzu et al., 1994; Koide et al., 1993). These two sites are in the same region as the rasGAP binding domain. The binding sites may overlap in this region but are not necessarily identical. As a result, Raf binding to GTP-bound ras may protect it from rasGAP inactivation. Therefore, it is unlikely that rasGAP and Raf simultaneously

bind to an activated ras molecule. These questions have been addressed in Chapter III.

Ras-Raf Interactions with 14-3-3 Proteins:

14-3-3 proteins play a diverse role in a wide variety of cellular functions such as the regulation of protein kinase C and exocytosis. These proteins were first identified as a series of very abundant 27 to 30 kDa proteins in brain tissue (Moore and Perez, 1968). *14-3-3* belongs to a growing gene family of at least 16 members (Fu et al., 1993), which are expressed in most tissues (Isobe et al., 1991; Leffers et al., 1993) and show remarkable evolutionary conservation extending to both lower eukaryotes and plants (Hirsch et al., 1992; Ford et al., 1994; Martens et al., 1992). It has recently been found to form complexes with the amino terminal part of Raf using the two hybrid system (Freed et al., 1994; Irie et al., 1994; Fu et al., 1994). Two members of this family, 14-3-3ζ and 14-3-3β, were actually shown to be involved in activating Raf protein during *Xenopus* oocytes maturation (Fantl et al., 1994). However, Raf activation by 14-3-3 proteins alone *in vitro* has not yet been demonstrated and the interactions among ras, Raf and 14-3-3 proteins are still unclear. Presumably, after ras p21 is activated, the Raf•14-3-3 protein complex is recruited to the plasma membrane by its association with *ras*•GTP *via* the N-terminus of Raf. The presence of 14-3-3 protein in the complex may facilitate Raf kinase activation, which then activates the downstream targets.

V. The Ras Signaling Pathway

Biochemical and genetic studies have indicated that activating ras p21 is an essential early step in the signalling cascade initiated by receptor

tyrosine kinases. The earliest evidence in support of this notion came from experiments showing that injecting neutralizing antibodies blocked the ability of growth factor to induce DNA synthesis (Mulcahy et al., 1985). Similarly, microinjecting anti-ras antibody or expressing dominant negative ras mutants inhibited the capacity of NGF to induce neurite extension in PC12 cells (Hagag et al., 1986; Szeberenyi et al., 1990). Moreover, expressing these mutants in either PC12 cells or in fibroblasts has been shown to block the activation of serine-threonine kinases, such as Raf and MAP kinase (mitogen-activated protein kinase), in response to NGF, PDGF, or insulin (Wood et al., 1992). The MAPK (MAP kinase) cascade is a major signalling system by which cells transduce extracellular stimuli into intracellular responses. The role of ras proteins in transducing signals from receptor tyrosine kinases appears to be highly conserved. For example, in *Drosophila*, activation of the sevenless receptor tyrosine kinase is required for the proper specification of R7 photoreceptors in eye development, and the activation of ras is a crucial early event in this signal pathway (Simon et al., 1991). In *C. elegans*, a receptor tyrosine kinase encoded by the *let*-23 gene and a ras protein encoded by the *let*-60 gene are required for inducing vulva formation (Beitel et al., 1990; Han and Sternberg, 1990). Furthermore, it has been demonstrated that constitutively active ras p21 can substitute for receptor tyrosine kinase activity in the development of *C. elegans* vulva precursor cells and *Drosophila* R7 photoreceptor cells (Han et al., 1993; Dickson et al., 1992).

Ras proteins function as a molecular switch passing signals from cell surface receptors that trigger cell growth and differentiation to intracellular effector molecules. The biochemical basis for this switch

function involves the cycling of ras p21 between active GTP-bound and inactive GDP-bound states. In normal quiescent cells, ras p21 exists primarily in its inactive GDP-bound form. Upon stimulation by a variety of mitogenic and differentiation signals such as EGF, PDGF, NGF, interleukin, and granulocyte/macrophage colony-stimulating factor; a rapid and transient elevation of ras•GTP levels are observed (Dhar et al., 1982). Thus, the GDP/GTP cycle provides an efficient regulatory mechanism for ras modulation in signal transduction pathways. A defect or the inappropriate operation of this cycle can significantly perturb downstream events in the network. For example, mutations found in oncogenic forms of ras impair their GTPase activity, which leads to a chronic activation of the downstream MAP kinase cascade.

A group of proteins containing SH2 and SH3 domains have been implicated as adapters that mediate ras signalling. The activation of receptor protein tyrosine kinase by growth factors cause the receptor to auto-phosphorylate at several tyrosine residues, permitting its interaction with the SH2 domains of those adapter proteins. For example when Grb2 (growth factor receptor-bound protein 2) becomes tyrosine phosphorylated it can interact with the tyrosine kinase receptor through its SH2 domain and with SOS *via* its SH3 domains (Schlessinger, 1993; Downward, 1994; Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993). These reactions cause GEF to be recruited to the plasma membrane, and ultimately results in the conversion of ras p21 from its inactive, GDP-bound form to the active, GTP-bound state.

Ras•GTP may also help to recruit Raf protein to the plasma

membrane, where its kinase activity is activated by an as yet unidentified mechanism. Activated Raf protein then initiates a cascade of highly conserved protein kinases by phosphorylating and activating MEKs (mitogene-activated protein kinase/extracellular signal-regulated kinase kinases) (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992; Macdonald et al., 1993) which then phosphorylate and activate two tyrosine/threonine isoenzymes p42MAPK and p44MAPK (Lee et al., 1992b; Anderson et al., 1991). Subsequently, the activated MAPK further catalyzes the phosphorylation of multiple substrates: groups of transcription factors that change gene transcription and other molecules such as the RSK (ribosomal S6 kinase) isoenzymes (Rsk-1 and Rsk-2), MAPKAP-1 kinase (MAP kinase-activated protein kinase 1) and PLA2 (phospholipase A2) (Avruch et al., 1994).

Figure 10 shows a simplified version of the ras signalling world. The transmission of signals are initiated at the cell surface by a wide variety of ligand-receptor interactions. Through a series of phosphorylation events, extracellular signals are connected from the tyrosine kinase cascade to the serine/threonine kinase pathway. As a result, changes in gene expression as well as protein synthesis occur. Regulating the ras cycle, plays crucial role in this complicated cellular signalling process. Nevertheless, many details of the interaction between ras with its upstream and downstream regulators are still unknown despite the numerous relationships that have been established.

VI. Dominant Negative Ras Mutants

Mutants which are functionally defective but capable of inhibiting the

activity of normal cellular genes are defined as dominant negative mutants (Herskowitz, 1987). Dominant negative ras mutants have been used extensively to probe the cellular roles of ras. As shown in Figure 1, there are two possible ways that the ras GDP/GTP regulation cycle can be disrupted. One is to interfere with ras' interaction with GEF and the other is to perturb the interaction of ras with its effectors. Thus, two classes of ras dominant negative mutants can be differentiated. The first class inhibits cellular ras, but not oncogenic ras by perturbing guanine-nucleotide exchange. Since cellular ras can be down regulated by GAP, it is more susceptible to guanine-nucleotide exchange perturbations than oncogenic ras, which possesses little GTP hydrolysis activity. Ras mutants S17N, G15A, D57Y, and N116I (or N116Y) (Feig and Cooper, 1988; Chen et al., 1994; Powers et al., 1989; Hwang et al., 1993; Ogiso et al., 1990) belong to this class. The second class of dominant negative mutants inhibit oncogenic ras by interferring its interaction with downstream effectors. An example of this class of mutants is the Q61L mutation in the non-processed or truncated ras form (Vogel et al., 1988; Michaeli et al., 1989; Medema et al., 1991).

During the process of elucidating the role of the conserved asparagine and lysine in the NKXD region of EF-Tu in *E. coli*, it was found that overexpressing EF-Tu mutants N135I and K136E, inhibited the growth of their host cells (Hwang and Miller, 1987). Genetic and biochemical evidence have shown that this growth inhibition results from sequestering EF-Ts by the mutant EF-Tu (Hwang and Miller, 1987). These results have been extended to the ras protein (Hwang et al., 1993). Biological assays indicate that ras mutants N116I or N116Y can revert the transformed phenotype induced by over-expressing c-H-ras in fibroblasts. These

mutants, however, have little or no effect on v-H-ras transformed cells (Ogiso et al., 1990). The ability to suppress c-H-ras, coupled with an inability to suppress v-H-ras, indicates that mutants N116I and N116Y must exert their effects by sequestering the essential upstream element required for the proper functioning of cellular ras protein. According to the model developed for GTPases, ras' immediate upstream element is the GEF (Figure 1). This hypothesis is supported by the fact that both mutants N116I, and N116Y form stable complexes with Sdc25p-C and inhibit the activity of Sdc25p-C *in vitro* (Hwang et al., 1993). In addition, the ras mutant, G15A, also has been shown to form a stable complex with GEF at physiological concentrations of guanine-nucleotides which reduce GEF recycling (Chen et al., 1994). The ability of the N116I mutant to form stable GEF complexes has been utilized to study the interaction of ras and GEF (see Chapter II)

Ras^T, a truncated (at amino acid 186) yeast Ras1 mutant that harbors an additional Q61L mutation, is one of the well studied second type of dominant negative mutants (Gibbs et al., 1989). Ras^T has an enhanced affinity for GAP and exhibits its dominant negative activity against v-H-*ras* (Gibbs et al., 1989). RasGAP negates this phenotype (Gibbs et al., 1989). Recently, another second type of ras dominant negative mutant has been discovered (Chapter III). This mutant contains a glycine to alanine change at position 60, which hinders ras' GTP-induced conformational change, and requires proper membrane attachment to function. Gly-60 of ras p21 is located in the conserved DXXG domain of regulatory GTPases (Dever et al., 1987). As previously described, this residue is part of the guanine-nucleotide binding pocket. The importance of this conserved Gly residue

has been examined in $G_{s\alpha}$ and *E. coli* EF-Tu by substituting the invariant glycine with alanine, a change that should drastically reduce the ability to rearrange around the Gly residue. In both cases, the GTP-induced conformational change was blocked by the Gly to Ala mutation.

Ras p21 acts as molecular switch to transmit extracellular messages to internal cellular effectors in signal transduction pathways. In order to understand the mechanism of this molecular switch in signal transduction, my thesis focuses on determining the essential structural elements in ras proteins that interact with their regulators. In particular, chapter II, will address the role ras' C-terminal domain plays in recognizing specific guanine-nucleotide exchange factors. Likewise, chapter III, will focus on two aspects of the importance of the conserved glycine at position 60. First, its role in mediating GTP-induced conformational changes will be discussed. Subsequently, the dominant negative effect of the ras G60A mutation will be described.

Figure 1. The ras GDP/GTP cycle.

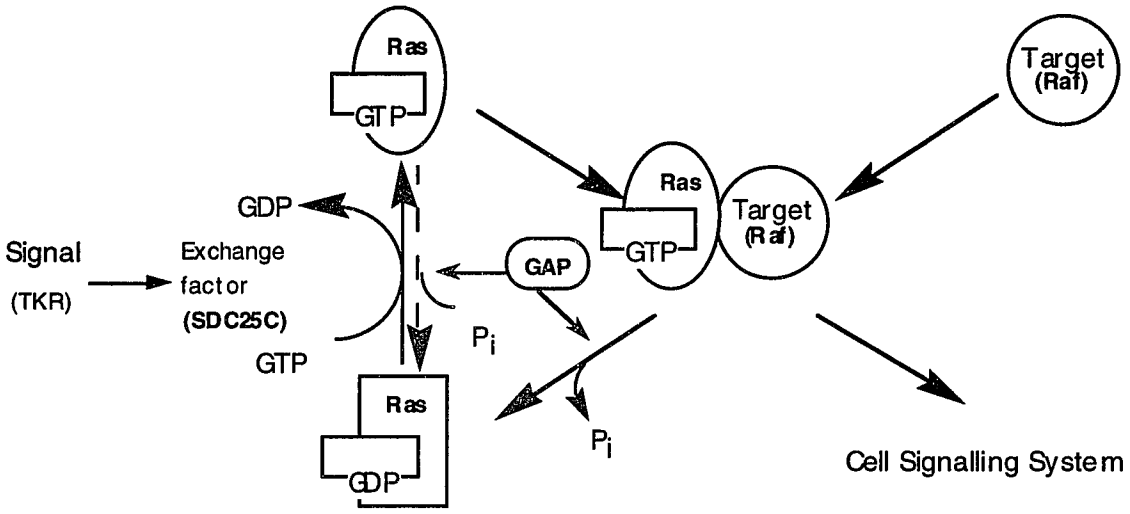


Figure 2. The amino acid sequences and structure of human H-, K-, and N-ras.

Symbols: "-" indicates an identical amino acid residue, and "*" indicates a sequence gap. Conserved motifs which have been shown to be involved in various aspects of ras function are underlined. The secondary structure elements: α=alpha helix, β=βeta sheet, L=unstructured loop, were assigned according Pai, *et. al.* (1989) (Pai et al., 1989).

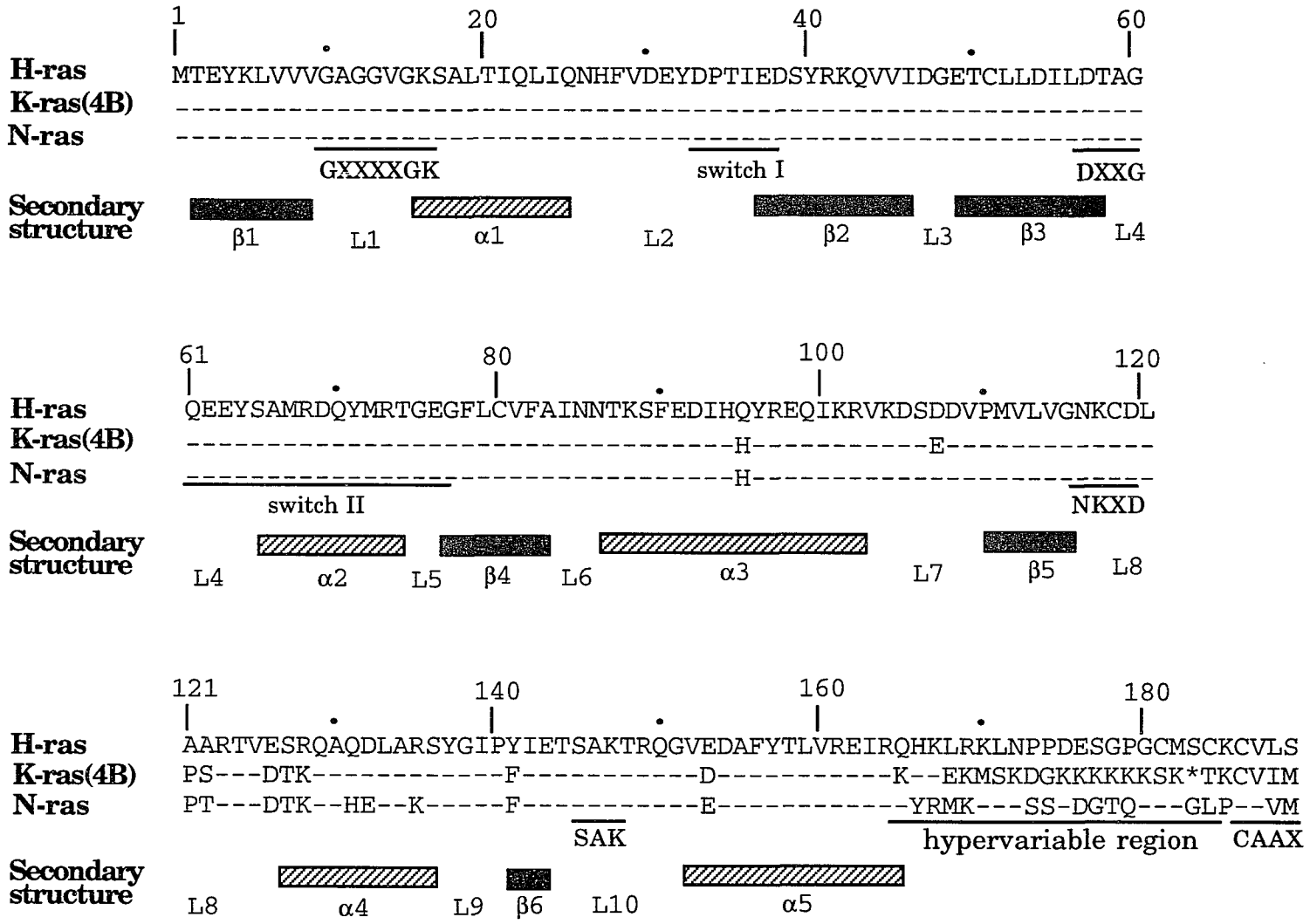

1
20
40
60
H-ras
K-ras(4B)
N-ras
MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG
GXXXXGK
switch I
DXXG
Secondary structure
β1
L1
α1
L2
β2
L3
β3
L4
61
80
100
120
QEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDL
switch II
NKXD
L4
α2
L5
β4
L6
α3
L7
β5
L8
121
140
160
180
AARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKLNPPDESGPGCMSCKCVLS
PS---DTK----------F----------D----------K--EKMSKDGKKKKKKSK*TKCVIM
PT---DTK--HE--K-----F----------E----------YRMK---SS-DGTQ---GLP--VM
SAK
hypervariable region
CAAX
L8
α4
L9
β6
L10
α5

Figure 3. Conformation of ras p21•GTP.

The topology of the amino acid chain of ras p21•GTP was modified from Valencia *et al.*, (Valencia *et al.*, 1991).

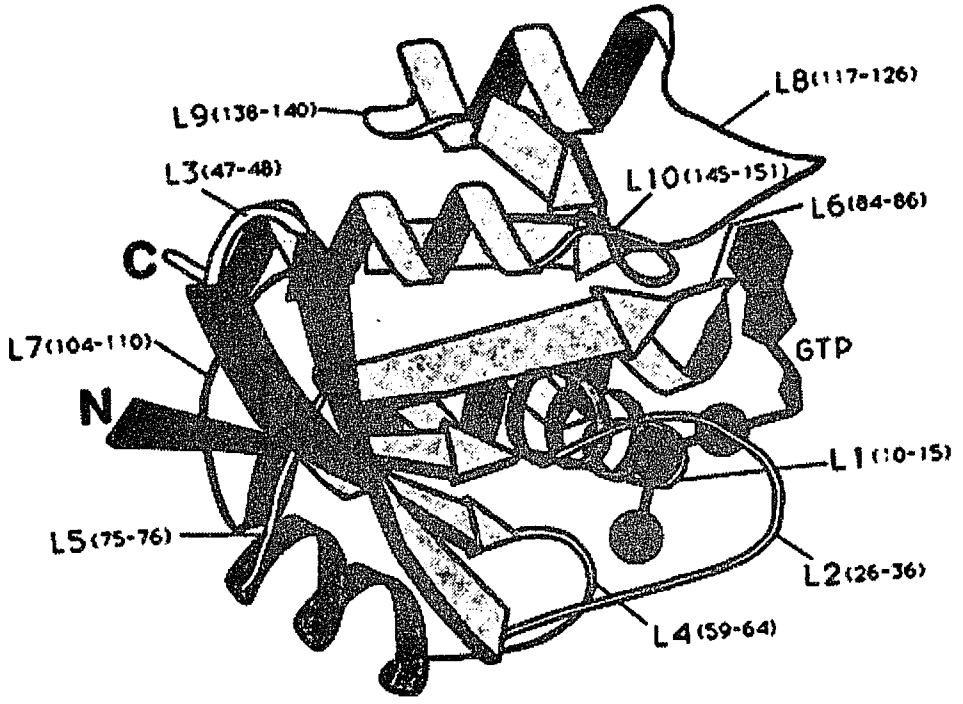


Figure 4. Interaction of ras p21 with the GTP analogue, GppNHp.

From: Whittinghofer and Pai, (1991) TIBS 16:382.

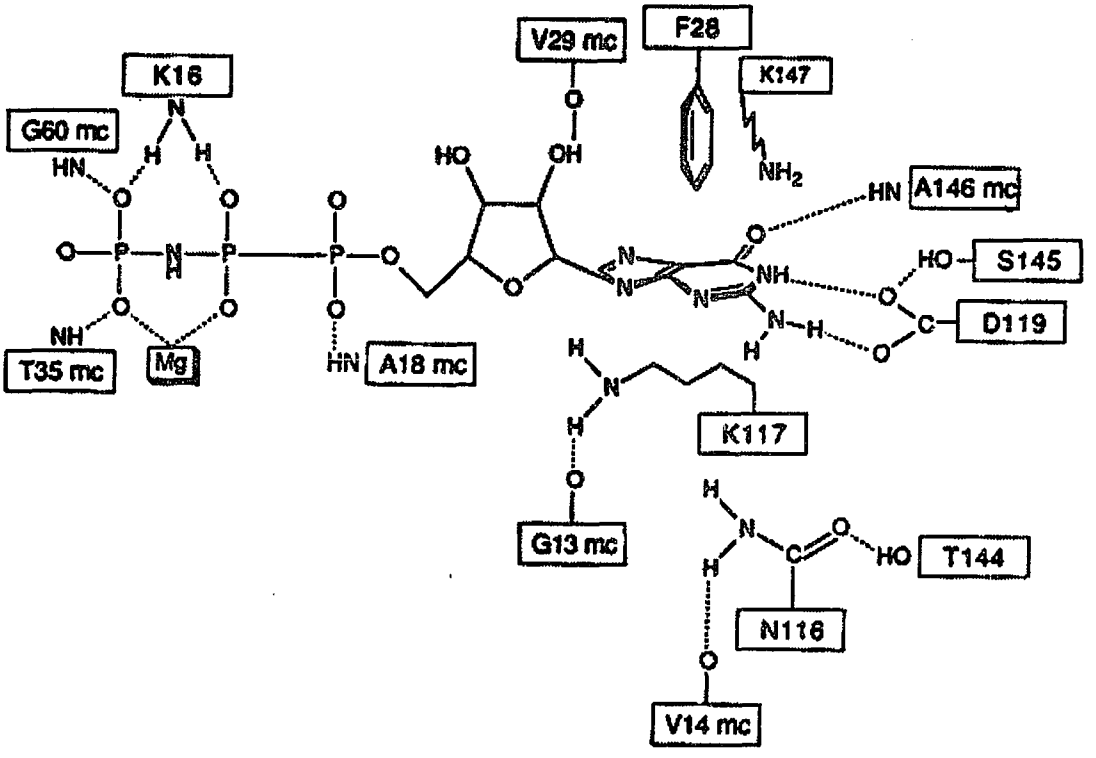


Figure 5. Coordination of Mg^{2+} with ras p21 and other ligands.

From: Pai *et al.*, (1990) EMBO J. 9:2351.

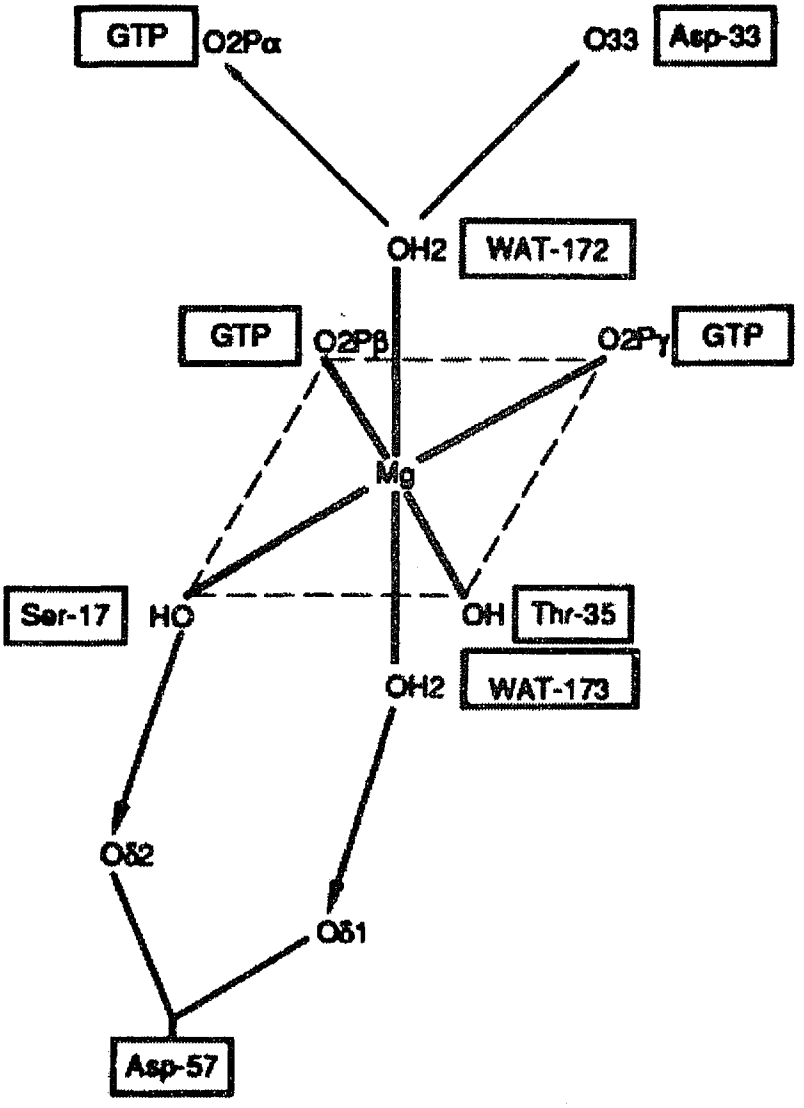


Figure 6. Comparison the protein structures among GEFs.

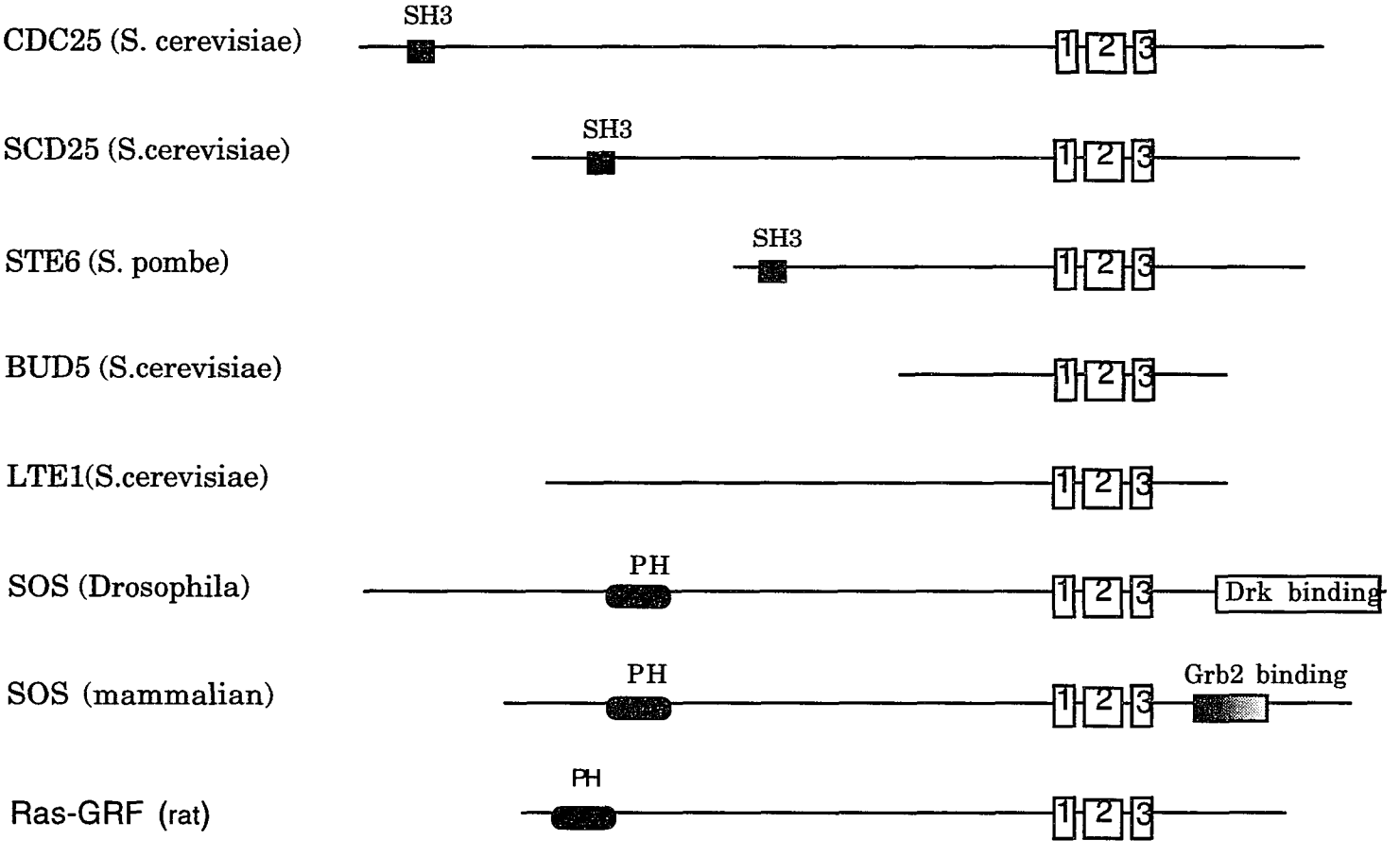


Figure 7. Proposed model of GEF catalyzed GDP/GTP exchange for ras.

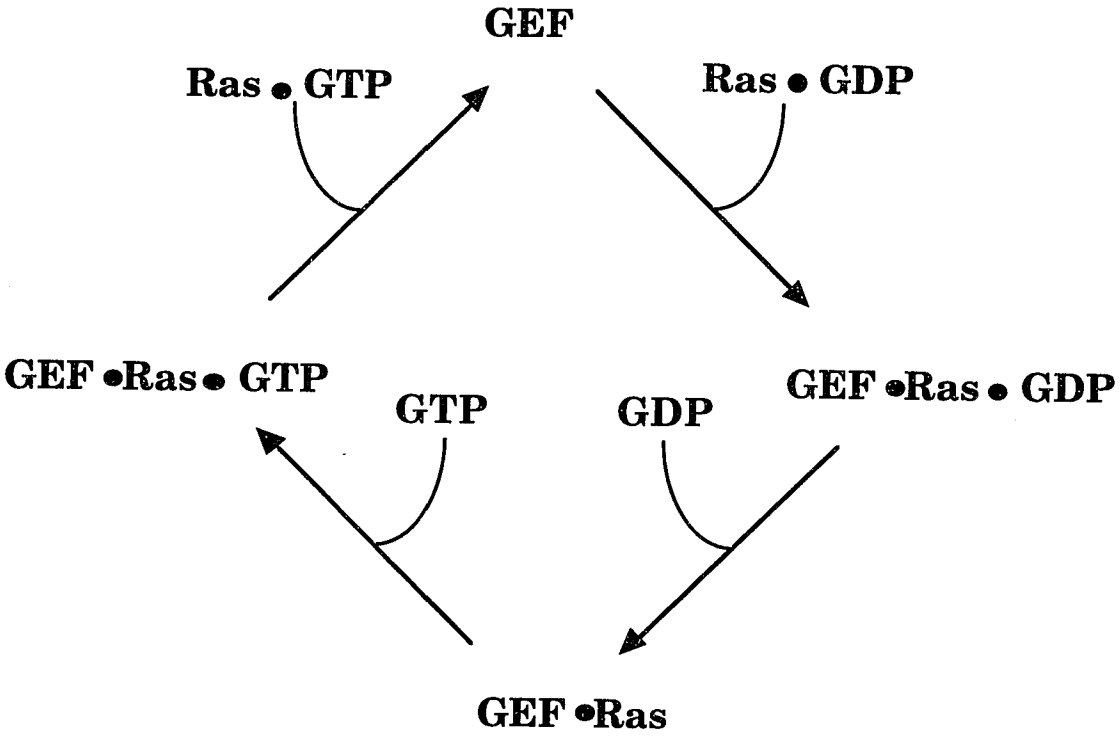


Figure 8. Comparison protein structures of GAP family.

Solid sections represent the conserved core catalytic domain among these proteins.

The cross-hatched sections represent external homology among IRA-1, IRA-2, and NF-1.

Relative amino acid residues are numbered for each protein.

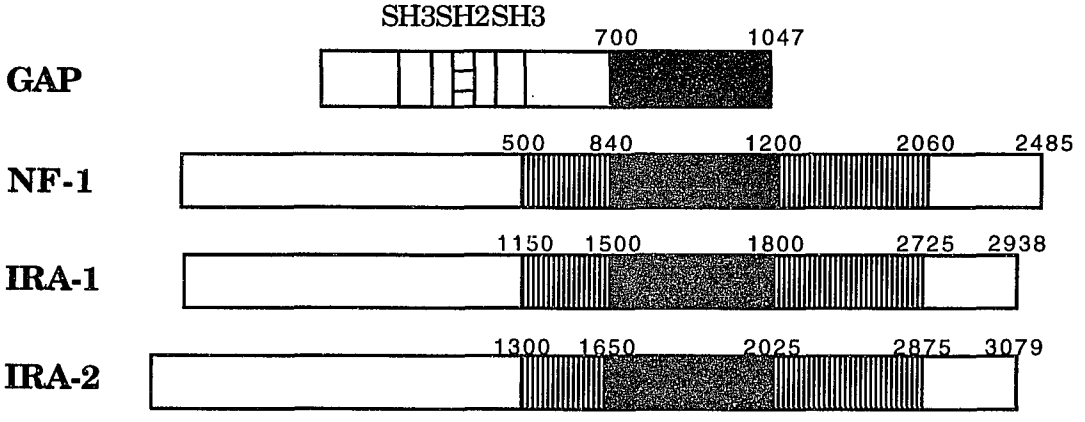


Figure 9. Protein Structure of Raf protein.

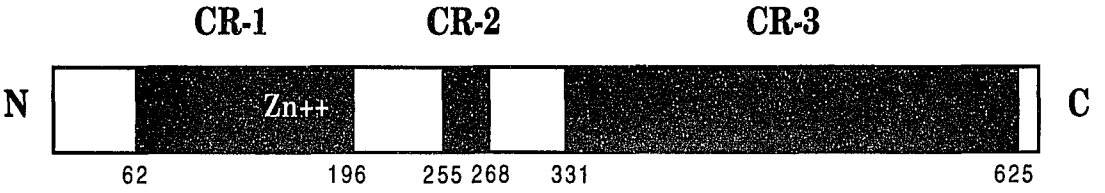
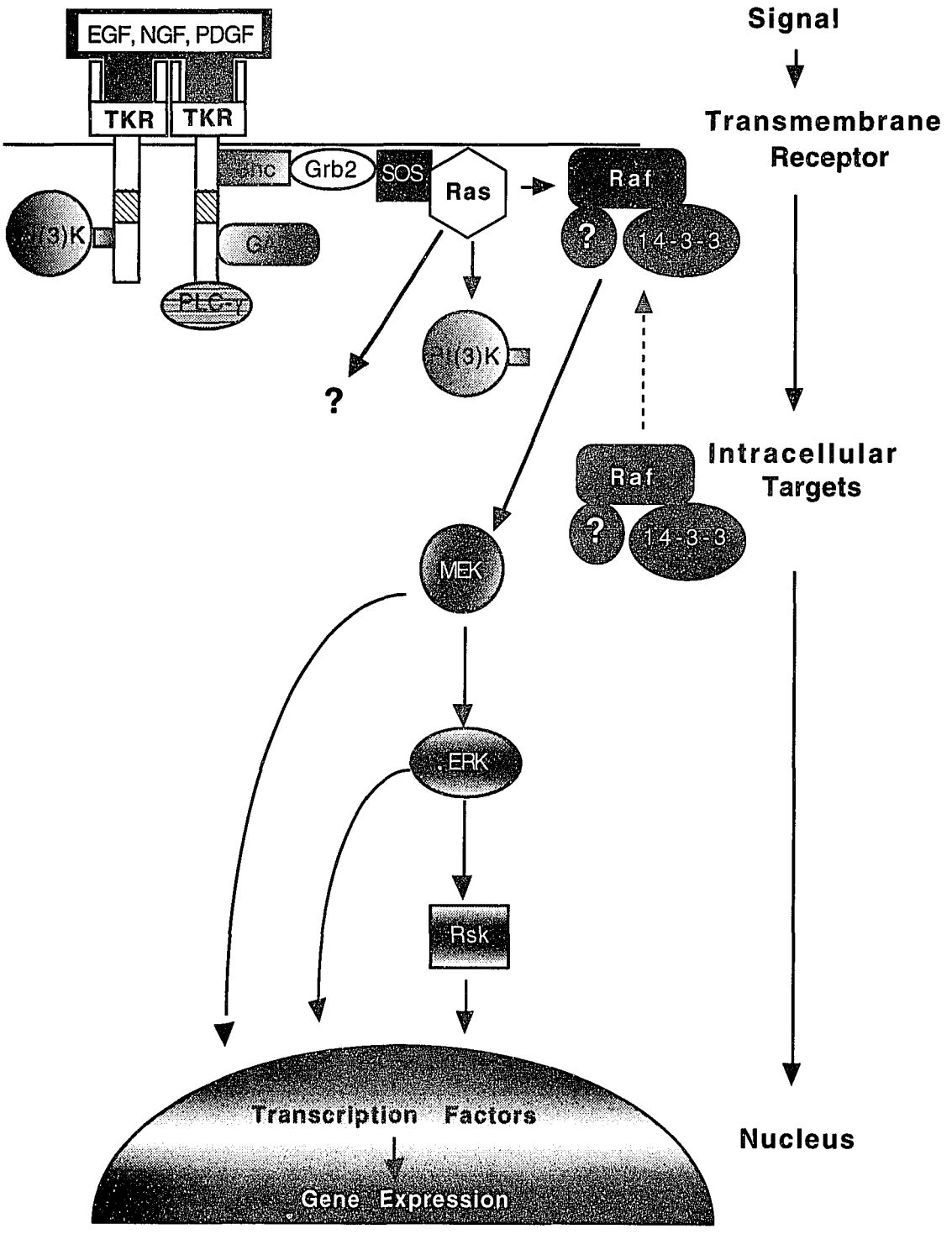


Figure 10. Ras signalling pathway in mammalian cells.



Chapter II. The Interaction of H-Ras and K-Ras with the Guanine-Nucleotide Exchange Factor, Sdc25p-C

Abstract:

The interaction of H-ras and K-ras with guanine-nucleotide exchange factor Sdc25p-C (*SDC*25 C-domain gene product) was examined using a combination of guanine ring binding domain mutations (N116I and K117E), chimeric ras constructs, and C-terminal deletion mutants. The interaction of different ras constructs with Sdc25p-C was analyzed by the following three assays: direct Sdc25p-C binding, inhibition of Sdc25p-C activity by ras p21 harboring a guanine ring binding domain mutation, and Sdc25p-C-stimulated guanine-nucleotide exchange. It was found that while H-ras could interact with Sdc25p-C, K-ras was unable to do so. This differential effect was conferred mainly by residues in the C-terminal domain. In fact, the ability of H-ras to respond to Sdc25p-C was abolished when the C-terminal domain of H-ras was replaced with the corresponding fragment from K-ras. The polylysine stretch in the hypervariable domain was subsequently identified as the major determinant for Sdc25p-C recognition. In addition, sequences in the first 138 residues of ras p21 were also found to contribute to Sdc25p-C binding and activity. These sequences include residues 121 to 128, a region comprising the C-terminal portion of loop L8 and the N-terminal end of α4. Our findings support the possibility that the region around loop L8 of p21 is also involved in Sdc25p-C-stimulated guanine-nucleotide exchange.

Results:

The interaction of truncated H-ras(K117E, 166) with Sdc25p-C. The region of H-ras p21 required for Sdc25p-C-stimulated guanine-nucleotide exchange has been localized to the first 166 residues of H-ras p21 (John et al., 1989; Mistou et al., 1992). We confirmed this by showing that Sdc25p-C binding, a prerequisite step for guanine-nucleotide exchange, was not affected by C-terminal end truncation. We routinely use H-ras harboring guanine ring binding domain mutations, such as N116I and K117E, to perform Sdc25p-C binding assays because quantitative ras•Sdc25p-C complex formation can be readily achieved with this type of mutation (Hwang et al., 1993). Figure 11 shows a schematic diagram of the structure of various ras constructs used in these studies. Figure 12 shows that removing the last 23 amino acids as in the mutant H-ras(K117E, 166) (termination at codon 167) had little or no effect on the ability of H-ras(K117E) to bind Sdc25p-C. In addition, no significant difference in Sdc25p-C binding between the intact and the truncated H-ras(K117E) was found at several different Sdc25p-C concentrations (Table 1). Quantitative ras•Sdc25p-C complex formation was achieved with a 10-fold molar excess of Sdc25p-C for both the intact and truncated mutants (Table 1). Both GDP and GTP compete with Sdc25p-C for binding to ras p21 and causes ras•Sdc25p-C complexes to dissociate (Figure 12). Therefore, the amount of GDP or GTP required to dissociate the ras•Sdc25p-C complex was taken as an index of their stability. The amount of GDP required for promoting complete complex dissociation was estimated to be about 0.3 mM for H-ras(K117E) and 0.25 mM for H-ras(K117E, 166). This result indicates that the complex formed by the truncation mutant was nearly as stable as the

one involving the intact H-ras(K117E). Similar results were obtained when GTP was used as the competing ligand (Table 2). The affinity of ras for GTP is about 3-fold higher than its affinity to GDP (John et al., 1988); in accord with this, we found that only 0.1 mM and 0.09 mM of GTP were required to dissociate the complexes of H-ras(K117E) and H-ras(K117E, 166), respectively (Table 2). Based on these observations, we concluded that all of the necessary structural requirements for Sdc25p-C binding and guanine-nucleotide exchange stimulation are contained within the first 166 residues of H-ras.

Sdc25p-C binding of different ras constructs. Intact K-ras did not bind Sdc25p-C under the conditions used to measure H-ras binding (Table 2). Even when up to four times as much Sdc25p-C was added (Sdc25p-C/ras molar ratio 40:1) K-ras still failed to bind. This observation indicates that Sdc25p-C can distinguish H-ras from K-ras; thus, the structural differences between H-ras and K-ras should provide clues for understanding the interaction of ras and Sdc25p-C. The major difference between H-ras and K-ras lies in the C-terminal region with the most prominent difference being localized near the C-terminus termed the hypervariable domain (Figure 2). To examine the properties of ras containing different C-domains, we constructed chimeric HK-ras(N116I) and HK-ras(K117E); these mutants consisted of residues 1-138 of H-ras and residues 139-188 of K-ras 139 (Figure 11). Besides about 20 amino acid residues near the C-terminus (except the last four amino acids), the chimeric HK-ras' differ from the H-ras at positions 141, 153 and 165 (Figure 2). Chimeric HK-ras mutants were then subjected to complex formation analysis in a gel filtration column. In contrast to H-ras(N116I) or H-

ras(K117E), which readily form complexes with Sdc25p-C, neither chimeric HK-ras(N116I) nor HK-ras(K117E) bound Sdc25p-C, even when it was present in a 40 molar excess (Table 1 and 2).

To test whether the C-terminal hypervariable domain was directly involved in inhibiting Sdc25p-C binding, two C-terminal truncation mutants, HK-ras(K117E), HK-ras(K117E, 183) (terminating at codon 184) and HK-ras(K117E, 171) (terminating at codon 172), were constructed and examined (Figure 11). HK-ras(K117E, 183), whose last 5 amino acid residues were removed, was unable to bind to Sdc25p-C; however, when the last 17 amino acid residues of HK-ras(K117E) were removed [HK-ras(K117E, 171)]•Sdc25p-C complexes were formed (Table 2). We subsequently compared the Sdc25p-C binding of HK-ras(K117E, 171) and H-ras(K117E) by titrating them with Sdc25p-C. The results showed that the truncated protein HK-ras(K117E, 171) bound nearly as well as the full-length H-ras(K117E) (Table 1). The concentration of GTP required for promoting HK-ras(K117E, 171)•Sdc25p-C complex dissociation was estimated to be about 0.05 mM. This value is about half that required for dissociating the H-ras(K117E)•Sdc25p-C complex (Table 2).

Interestingly, replacing the last 50 amino acid residues of K-ras(N116I) by the corresponding fragment from H-ras (Figure 11) rendered the resulting chimera capable of binding to Sdc25p-C (Table 2). By performing a titration experiment with different concentrations of Sdc25p-C as described in Table 1, it was found that a quantitative complex formation of KH-ras(N116I)•Sdc25p-C was achieved with an approximate 10-fold molar excess of Sdc25p-C. However, the complex thus formed does

not appear to be as stable as H-ras(N116I)•Sdc25p-C in the presence of guanine-nucleotide (Table 2). The KH-ras(N116I)•Sdc25p-C complex was readily dissociated with GTP at concentration about 0.5 mM. This value is about 20 fold lower than that required for dissociating the H-ras(N116I)•Sdc25p-C complex (Hwang et al., 1993).

The inhibition of Sdc25p-C-stimulated guanine-nucleotide exchange by different ras constructs. H-ras(N116I) inhibits Sdc25p-C-stimulated guanine-nucleotide exchange by forming stable H-ras(N116I)•Sdc25p-C complexes. We utilized this property to examine the effect incorporating different C-terminal domains had on the ras N116I mutant constructs' ability to act as Sdc25p-C inhibitors. The N116I mutants used in this assay were expressed as GST-ras fusion proteins. Fusing ras to GST not only simplifies the protein purification procedure, but also partially overcomes the solubility problem of ras mutants containing K-ras C-terminal ends. The structural integrity of ras p21 was not affected by its fusion to GST; we observed that GST-H-ras(N116I) was as effective as H-ras(N116I) in inhibiting the Sdc25p-C-stimulated guanine-nucleotide exchange reaction.

As expected, the ability to inhibit Sdc25p-C-stimulated guanine-nucleotide exchange matched Sdc25p-C binding. For example, H-ras(N116I) readily bound Sdc25p-C (Table 2) and was a potent inhibitor of Sdc25p-C (Figure 13A). On the other hand, K-ras(N116I) did not bind Sdc25p-C (Table 2) and had very little inhibitory effect on Sdc25p-C (Figure 13A). The potency of Sdc25p-C inhibition by H-ras(N116I) was drastically reduced by replacing the H-ras C-terminal domain with the corresponding

fragment from K-ras, while the low inhibitory activity of K-ras(N116I) was greatly enhanced by substituting the K-ras C-terminal region with its counterpart from H-ras (Figure 13A). Nevertheless, the potency of chimeric KH-ras(N116I) inhibition never reached the level of H-ras(N116I) and it could be suppressed by GTP at concentrations much lower than that required for suppressing H-ras(N116I) (Figure 13B). This observation is consistent with the finding that KH-ras(N116I) forms less stable complexes with Sdc25p-C than H-ras(N116I) (Table 2).

Guanine-nucleotide exchange of different ras constructs stimulated by Sdc25p-C. Next, we examined the Sdc25p-C-stimulated guanine-nucleotide exchange of the different ras constructs. In agreement with the Sdc25p-C binding and inhibition assays, the presence of the K-ras C-terminal domain strongly suppressed Sdc25p-C-stimulated guanine-nucleotide exchange (Figure 14). The intact K-ras and the chimeric HK-ras had barely detectable levels of Sdc25p-C-stimulated guanine-nucleotide exchange activity. In contrast, the HK-ras(171) truncation mutant, in which the polylysine region was deleted, retained substantial Sdc25p-C-stimulated guanine-nucleotide exchange activity. Although this mutant did not fully regain Sdc25p-C-stimulated activity, these data still demonstrate that the polylysine region of K-ras prevents the interaction with Sdc25p-C. We have also examined the truncated HK-ras(166) in this assay. The results were essentially the same as HK-ras(171) (data not shown). Unexpectedly, Sdc25p-C-stimulated guanine-nucleotide exchange was not associated with KH-ras (Figure 14) despite its ability to bind to Sdc25p-C (Table 2). This appears to be an inherent property of K-ras, because K-ras(171), a K-ras lacking the last 17 amino acid residues, also

exhibited low Sdc25p-C-stimulated guanine-nucleotide exchange activity as well.

Discussion:

In this study, we demonstrated that ras' C-terminal hypervariable domain and conserved CAAX box, the signal for C-terminal modification (Lowy and Willumsen, 1993), are not required for its interaction with Sdc25p-C. However, we showed that the ability of H-ras to interact with Sdc25p-C was abolished when the C-terminal domain (from residue 139 to C-terminus) of H-ras was replaced with the corresponding fragment from K-ras (Figure 11, 13 and Table 2). We further determined that the K-ras C-terminal effect could be attributed to the polylysine stretch in the K-ras hypervariable domain. This was demonstrated by the HK-ras(K117E, 171) truncation whose Sdc25p-C binding activity was comparable to H-ras (Table 2); the amount of GDP/GTP needed to dissociate the HK-ras(K117E, 171)•Sdc25p-C complex was estimated to be about half that required to dissociate the H-ras(K117E) complex (Table 2). Therefore, small differences between the C-terminal ends of H-ras and K-ras have profound effects on the binding of Sdc25p-C.

Replacing the K-ras C-terminal domain by its counterpart from H-ras enables the chimera to bind Sdc25p-C. Although KH-ras(N116I) and H-ras(N116I) appeared to bind Sdc25p-C similarly, less GTP was required to dissociate the KH-ras(N116I)•Sdc25p-C complex than was needed to dissociate the H-ras(N116I)•Sdc25p-C complex (Table 2). Unfortunately, the GDP/GTP binding affinity to the N116I mutants cannot be reliably

measured (Der et al., 1988; Walter et al., 1986). If one assumes that both K-ras(N116I) and H-ras(N116I) bind GDP/GTP equally, then the lower amount of GTP required to dissociate the KH-ras(N116I) complex implies the complex is less stable. Therefore, we would predict that KH-ras(N116I) should be a less potent inhibitor. As shown in Figure 13A, this appears to be the case. This interpretation also agrees with the observation that lower amounts of GTP were needed to suppress the inhibition of Sdc25p-C activity by KH-ras(N116I) than by H-ras(N116I) (Figure 13B). The basis for the different biochemical properties of H-ras and KH-ras must lie in their sequence differences. Within the first 138 amino acid residues of ras, there are seven amino acid differences. They are at positions 95, 107, 121, 122, and 126-128 (Figure 2). The majority of them are located just C-terminal to the guanine ring binding domain (residues 121 to 128 of ras p21) (Figure 2), a region assigned as loop L8 and helix α4 of ras p21 (Pai et al., 1989). It has been proposed that loops L7, L8, L10, the helix α2 or/and the distal part of helix α3 may be involved in factor-stimulated guanine-nucleotide exchange (Valencia et al., 1991). The findings in this study are consistent with that proposal.

The failure of Sdc25p-C to stimulate KH-ras is intriguing (Figure 14). Apparently, Sdc25p-C binding does not always lead to guanine-nucleotide exchange. Sdc25p-C-stimulated guanine-nucleotide exchange is a multiple-step process consisting of distinct events such as Sdc25p-C binding (or release), a conformational change that relays the binding signal (from either Sdc25p-C or guanine-nucleotide), and guanine-nucleotide release (or binding) (Hwang et al., 1992). From a thermodynamic point of view, in order to release GDP, the energy of

Sdc25p-C binding to ras must be sufficient to overcome the activation energy of GDP release which includes protein conformational change and hydrogen bond breakage between ras and GDP. Based on the stability of KH-ras(N116I)•Sdc25p-C, it is likely that the binding energy of Sdc25p-C to KH-ras is not sufficient to trigger the next event; therefore, Sdc25p-C readily dissociates from KH-ras without achieving the release of guanine-nucleotide. Again, this observation points to the importance of the seven non-conserved amino acid residues in Sdc25p-C-stimulated guanine-nucleotide exchange. The data for K-ras(171) are also consistent with this picture (Figure 14). However, the results using the truncated K-ras are in direct contrast to a study in which CDC25 was found to be active on the truncated K-ras protein (Chevallier-Multon et al., 1993).

Removing the last 17 or 23 amino acid residues of HK-ras re-established Sdc25p-C-stimulated guanine-nucleotide exchange. However, neither HK-ras(171) nor HK-ras(166) fully reached the Sdc25p-C-stimulated exchange activity of H-ras. There are three amino acid residue differences between HK-ras(166) and H-ras(166) (Figure 2 and 11). This implies that residues 141, 153, or 165 are also important for Sdc25p-C-stimulated guanine-nucleotide exchange. Taken together, these data show that the Sdc25p-C-stimulated guanine-nucleotide exchange may involve the region of ras spanning residues 121 to 166. Previously, many mutations affecting EF-Ts binding were mapped in the region of EF-Tu corresponding to ras residues 126 to 166 (Hwang et al., 1992). However, this finding does not rule out the involvement of other ras domains in GEF-stimulated guanine-nucleotide exchange. As mentioned in the introduction, several studies have suggested that residues 61-69, 75-78, and 103-108 of ras are also

important (Mistou et al., 1992; Mosteller et al., 1994; Howe and Marshall, 1993; Verrotti et al., 1992; Mirisola et al., 1994; Quilliam et al., 1994; Segal et al., 1993).

v-H-ras harboring the N116I mutation or its sibling, N116Y, are dominant negative mutants that are capable of suppressing cellular H-ras but not mutated H-ras *in vivo* (Ogiso et al., 1990). The pattern of inhibition is consistent with the view that suppressing c-H-ras is caused by sequestering the guanine-nucleotide exchange factor. This hypothesis was verified *in vitro* using an Sdc25p-C-stimulated guanine-nucleotide exchange assay (Hwang et al., 1993). Interestingly, it was found that efficient suppression of H-ras in fibroblast cells required the presence of the C-terminal domain of H-ras in the dominant negative mutant (Ogiso et al., 1993). Apparently, the guanine-nucleotide exchange factor that activates H-ras in fibroblast cells is H-ras specific and can distinguish ras p21 based on its C-terminal domain. Furthermore, in accord with the *in vitro* inhibition data, H-ras(N116Y) was also found to be a more effective suppressor than KH-ras(N116Y) *in vivo* (Ogiso et al., 1993).

Post-translational processing at the C-terminal end of ras p21 has been suggested to be important for its interaction with some GEFs (Mizuno et al., 1991; Orita et al., 1993). Although we have exclusively used unprocessed ras proteins in this study, our findings of Sdc25p-C interaction specificity are unlikely to be due to the lack of C-terminal protein processing. Similar mutants displayed the same ras species specificity in fibroblasts (Ogiso et al., 1993), which should process ras proteins properly. In addition, we have shown that deleting the last 23

amino acid residues, including the CAAX motif, did not affect the interaction of ras with Sdc25p-C. Finally, Rab1 harboring the corresponding mutation of N116I, Rab1(N121I), was shown to be a potent inhibitor of endoplasmic reticulum to Golgi transport (Tisdale et al., 1992). As with ras, the inhibitory effect of Rab1(N121I) did not require C-terminal end processing (Tisdale et al., 1992). In addition, the inhibitory effect of Rab1(N121I) was eliminated when the C-terminal 35 amino acids were substituted with the corresponding fragment from Rab5 (Tisdale et al., 1992). The C-terminal region of Rab proteins have been implicated in Rab subcellular localization (Chavrier et al., 1991; Brennwald and Novick, 1993). Therefore, Rab protein localization may be a direct outcome of the interaction of Rab with its guanine-nucleotide exchange factor. This is in agreement with the findings that the localization of Rab5, Rab9, and ARF require guanine-nucleotide exchange (Helms and Rothman, 1992; Soldati et al., 1994; Ullrich et al., 1994).

Evidence suggests that the polylysine region of K-ras is important for membrane association of K-ras p21 (Hancock et al., 1990). Obviously, the polylysine region's role in guanine-nucleotide exchange factor recognition must not interfere its role in membrane association. In the light of *in vivo* and *in vitro* suppression of guanine-nucleotide exchange activity, we have concluded that guanine-nucleotide exchange may be ras species specific. In line with this argument, the guanine-nucleotide exchange factor smgGDS has been shown to prefer K-ras over other ras proteins (Mizuno et al., 1991; Orita et al., 1993). However, it is not known whether smgGDS represents a K-ras specific exchange factor or not. It should be noted that ras-GRF, SOS1, and CDC25 from *Saccharomyces cerevisiae* do not appear

to exhibit ras species specificity in stimulating guanine-nucleotide exchange *in vitro* (Shou et al., 1992; Orita et al., 1993; Liu et al., 1993; Chevallier-Multon et al., 1993). Therefore, it is not clear how these factors achieve ras species specific suppression *in vivo* (Ogiso et al., 1993). We also do not know how N-ras responds to Sdc25p-C. However, if the involvement of loop L8 in Sdc25p-C-stimulated guanine-nucleotide exchange is correct, then N-ras should behave like K-ras because the sequence of N-ras around loop L8 resembles K-ras rather than H-ras (Figure 2). A similar argument can be applied to K-ras(4A) as well (Lowy and Willumsen, 1993). Although Sdc25p-C is not a *bona fide* guanine-nucleotide exchange factor for mammalian ras p21, it can distinguish different forms of ras p21 similar to the cellular GEF in fibroblasts. Therefore, we believe that Sdc25p-C will serve as a valuable tool for probing the molecular mechanism of guanine-nucleotide exchange.

Materials and Methods:

Mutant and clone construction. The scheme for constructing H-, K- and chimeric *ras* mutants was as described (Ogiso et al., 1993). K-*ras* used in this study was the 4B form (Ogiso et al., 1993). All of the *ras* constructs used in this study contain Arg-12 and Thr-59 mutations unless stated otherwise. *E. coli* expression vectors pA-*ras,* for producing ras proteins under the control of a T7 RNA polymerase promoter, were constructed as described (Hwang et al., 1993; Sung et al., 1995). H-*ras*(166), a truncated *ras* mutant, was constructed by oligonucleotide site-directed mutagenesis (Kunkel et al., 1987) using the oligonucleotide $^{5'}$CCGCAGTTAATGCTGC$^{3'}$ that introduces a stop codon at position 167

(Figure 11). A similar protocol was used to construct truncated K- and chimeric HK-*ras* deletions 183 (terminating at codon 184), 171 (terminating at codon 172), and 166 (terminating at codon 167) using oligonucleotides $^{5'}$ATTACACACTATGTCTTT$^{3'}$, $^{5'}$TACCATCTTAGCTCATC$^{3'}$, and $^{5'}$TCTTTTCTTAATGTTTTG$^{3'}$, respectively (Figure 11). The GST-ras fusion proteins were constructed in a modified pGEX3 vector (Pharmacia) using the unique ClaI and XhoI sites as described (Sung et al., 1995).

Protein purification. All non-fusion *ras* clones were expressed in the bacteria strain BL21(DE3) (Studier and Moffatt, 1986). This strain lacks the *ompT* gene, which is required for preparing ras containing the K-ras polylysine domain (Lowe et al., 1991). The non-fusion KH-ras, K-ras(171) and HK-ras(171) were purified similarly to H-ras as previously described (Hwang et al., 1993). Bacteria BL21(DE3) harboring the pA-*ras* plasmids were cultured at 37°C in YT medium (1% Bacto-tryptone, 0.5% yeast extract and 0.5% NaCl) supplemented with 50 μg/ml of ampicillin. When the cell density reached an OD_{600} of 1, IPTG was added to a final concentration of 0.5 mM to induce ras p21. The cells were cultured for an additional 3 to 4 hours and subsequently harvested by centrifugation at 6,000xg. Ras p21 was purified by slightly modifying an established procedure (Satoh et al., 1987). All steps of the purification were carried out at 0-4°C. The bacterial pellet was resuspended in 1/50 the original volume of buffer A (50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 150 mM KCl; 1 mM DTT; 1 mM EDTA; 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM GDP) and sonicated 5 times (1 min. each sonication) with a Branson cell disrupter. Cell debris was removed by centrifugation at 30,000 xg for 30 min. The resulting supernatant was centrifuged at 100,000 xg for 100 min. to obtain a clear

supernatant. The proteins in the supernatant were then further fractionated by precipitation with ammonium sulfate at 0°C. Ras p21 was localized in the 30-45% ammonium sulfate. Proteins in this cut were dissolved in 5 ml of buffer B (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 10 μM GDP), and then dialyzed against 1 L of buffer B overnight at 4°C. Contaminating proteins were subsequently removed from ras p21 by a combination of ion-exchange chromatography and gel filtration chromatography. First, the dialysate was applied to a 1.5 x 15 cm fast flow Q Sepharose ion-exchange column previously equilibrated with buffer B. Proteins were eluted with 0-0.4 M NaCl linear gradient in buffer B and collected in 5 ml fractions. Ras p21 elution was monitored by GDP exchange and gel electrophoreses (15% SDS-PAGE gel). Fractions containing the highest concentration of p21 were pooled, and concentrated by ammonium sulfate precipitation. The precipitate was redissolved in 1ml of buffer B and applied to a 2.5 x 90 cm P-60 gel filtration column . Proteins were eluted from the column in buffer B. The p21 containing fractions were pooled, concentrated by ultrafiltration on an Amicon Centricon 10 filtration unit, and stored at -80°C.

The non-fusion K-ras and HK-ras were purified by non-denaturing conditions as follows. Bacterial pellets were initially resuspended in 1/50 of their original volume of a buffer containing 50 mM PIPES, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF, and 10 μM GDP, and then sonicated with 5 x 30 second bursts with a Branson cell disrupter. Cell debris was removed by centrifugation at 30,000x g for 30 minutes and then at 100,000x g for 100 minutes. The unwanted proteins in the supernatant were removed by ammonium

sulfate precipitation (at 40% saturation, 0 °C) and centrifugation at 10,000x g for 10 minutes. The subsequent supernatant was applied to a 2.5 x 20 cm Phenyl-Sepharose column and eluted with buffer D (50 mM PIPES, pH 7.5, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10 μM GDP) and a descending ammonium sulfate gradient starting from 1.5 M. The K-ras and HK-ras appeared in fractions corresponding to 0.95 M to 0.6 M ammonium sulfate respectively. The ras fractions were pooled and dialyzed against one hundred volumes of 0.2x strength buffer D overnight. The dialysate was then applied to a 2.5 x 20 cm CM-Sepharose column in buffer D, and eluted with an ascending KCl gradient from 0 to 1 M. Under these conditions ras eluted at KCl concentrations between 0.2-0.3 M. The ras proteins were concentrated in an Amicon Centriprep 10 and stored at -70 °C until use.

GST-ras fusion proteins were expressed in *E. coli* strain MV1190 and purified as described below. All of the following procedures were performed at 4 °C. A bacterial pellet from a 1 liter culture was resuspended in 20 ml of buffer E (1X PBS, pH7.4, 1 mM DTT, 0.5 mM PMSF, 1 % Trition X-100, and 10 μM GDP) and sonicated 10 times (30 seconds, each sonication) with a Branson cell disrupter. Cell debris was removed by centrifugation at 12,000 x g for 10 minutes. The resulting supernatant was incubated with 3 ml of PBS and buffer E equilibrated glutathione-Sepharose 4B for 6 hours. The resin was recovered by centrifugation at 2,000 x g for 10 min. and then washed with 60 ml of buffer G without Triton. GST-ras was eluted from the resin by incubating the protein-bound resin in 10 ml of elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH8 and 10 μM GDP) for 2 hours. The buffer containing GST-ras protein was then switched to buffer B using an Amicon Centriprep-10 concentrator. The purified protein was stored at -70

°C until it was used.

Sdc25p-C was expressed from the vector pTTQ-*SDC*25 (Mistou et al., 1992) and purified as described (Crechet et al., 1990).

Guanine-nucleotide exchange and Sdc25p-C inhibition. The exchange reactions were carried out in a 100 µl reaction mixture consisting of 25 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 50 mM KCl, 0.5 mg/ml bovine serum albumin, 50 pmole ras protein, 4 pmole Sdc25p-C, and 5 mM [^{3}H]-GTP (sp. act. 2 Ci/mmole). The reactions were started by adding [^{3}H]-GTP and were allowed to proceed for 5 minutes at 30 °C before quantifying the amount bound to nitrocellulose membrane (Hwang et al., 1993). Sartorius 0.1 µm membranes were used for the time-course study of Sdc25p-C-stimulated guanine-nucleotide exchange on deleted ras mutants, and 0.45 µm membranes (type HA, Millipore) were used for all of the other studies. The inhibition assays were performed exactly as the exchange reaction except that 50 pmole substrate (c-H-ras) and inhibitor GST-ras mutant were added. The percent inhibition was calculated using the following equation:

$$\frac{\text{CPM(Sdc25p-C+Substrate)-CPM(Sdc25p-C+Substrate+Inhibitor)}}{\text{CPM(Sdc25p-C+Substrate)-CPM(Substrate+Inhibitor*)}} \times 100$$

* Since the nucleotide binding of the inhibitor was too weak to detect (Hwang et al., 1993), we only measured the nucleotide binding of substrate alone here.

One hundred percent inhibition was defined as the value obtained from the reaction without the addition of Sdc25p-C. The suppression of Sdc25p-C inhibition by GTP was determined by measuring the release of GDP from prebound GDP•ras complexes as follows. c-H-ras p21 was pre-labeled with [^{3}H]-GDP (sp. act. 4 Ci/mmole) to equilibrium, and then used as a

substrate. The assay was similar to the exchange reaction but contained 20 pmole of [^{3}H]-GDP•c-H-ras and 100 pmol of inhibitor throughout all experiments. Reactions were initiated by adding the indicated amounts of cold GTP and then processed as the guanine-nucleotide exchange reactions. All data points represent average of at least three independent measurements whose values fall within a range of 10%.

In vitro labeling and Sdc25p-C binding assay. *In vitro* RNA transcription reactions were performed by using T7 RNA Polymerase Transcription Pac-Kits^{TM} (Epicentre Technologies). The pA-H-*ras* clones linearized by a restriction enzyme XhoI were used templates for transcription. The procedure provided by the manufacture was followed exactly. The RNA products were subjected to ethanol precipitation and the resulting pellets, dissolved in diethyl pyrocarbonate-treated water, were then used for translation. *In vitro* translation and [^{35}S]- or [^{3}H]- methionine (sp.act. > 800 Ci/mmol) labeling was carried out in an *Escherichia coli* S30 extract system obtained from Promega Corp. Two micrograms of H-*ras* RNA was used in a typical 20 µl translation reaction. The extent of [^{35}S]- or [^{3}H]- methionine incorporation was determined by trichloroacetic acid precipitation and the quality of products were determined by autoradiography following electrophoresis on a 15% SDS-polyacrylamide gel.

Complex formation and dissociation assays were performed as described (Hwang et al., 1993) using a Pharmacia gel filtration column (Superdex 75 10/30) attached to an HPLC. The percent of complex formation was estimated as follows. Complete complex formation was

assumed when additional Sdc25p-C no longer caused appreciable changes in the gel filtration elution profile. The ratio of complexed labeled p21 to total p21 (fraction number 24 to 29) under these conditions was calculated and used as the base value for 100% complex formation. The ratio of complexed ras relative to total ras p21 at other concentrations of Sdc25p-C was determined in a similar manner. These values were then used to calculate the percent of complex formation at a given concentration of Sdc25p-C by dividing with the value of 100% complex formation. All data points represent the average of at least 3 independent measurements whose values fall within a range of 5%.

Table 1. Complex formation of H-ras(K117E) species at different molar ratios of Sdc25p-C to ras p21

Molar Ratio	Percent of Complex Formation		
Sdc25p-C/ras	H-ras(K117E)	H-ras(K117E, 166)	HK-ras(K117E, 171)
1	12	8	5
2	37	34	28
4	72	67	62
8	93	91	85
10	100	100	100
20	100	100	100

The method for determining the percentage of complex formation is described in Materials and Methods. All data points represent the average of 3 independent measurements whose values fall within a range of 5%.

Table 2. Summary of ras•Sdc25p-C complex formation and dissociation

Ras Species	Sdc25p-C Binding	[GTP] Required for Complex Dissociation
H-ras	+*	0.3 μM
H-ras(N116I)	+	10 mM
K-ras(N116I)	–	NA
HK-ras(N116I)	–	NA
KH-ras(N116I)	+	0.5 mM
H-ras(K117E)	+	0.1 mM
H-ras(K117E, 166)	+	0.09 mM
HK-ras(K117E)	–	NA
HK-ras(K117E, 183)	–	NA
HK-ras(K117E, 171)	+	0.05 mM

Sdc25p-C binding was determined by the ability of each ras species to form a complex with Sdc25p-C as shown in Figure 2.

+, binding and -, no binding.

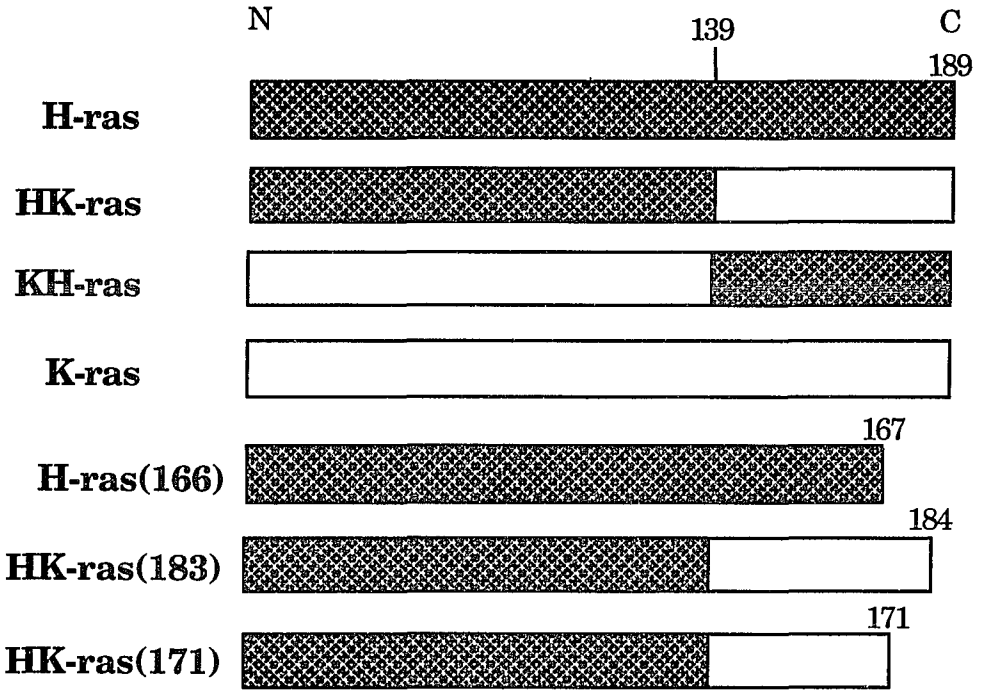
The GTP concentration required to dissociate complex was estimated as the minimal amount of GTP that caused complete complex dissociation.

NA: not applicable (no complex observed).

*Binding of the wild type H-ras to Sdc25p-C is much less efficient than guanine ring binding domain mutants as noted previously (Hughes et al., 1990).

All data points represent the average of 2 independent measurements whose values fall within a range of 1%.

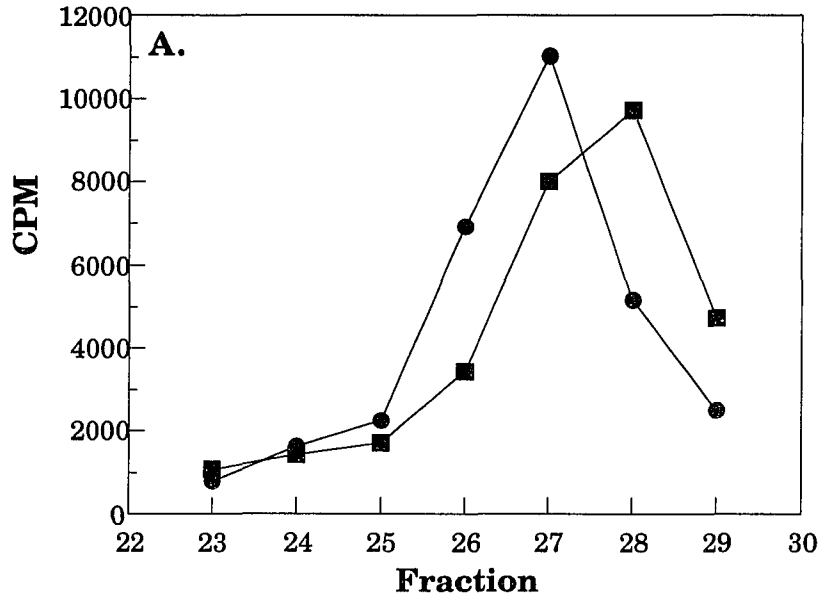
Figure 11. Ras and ras mutant proteins.

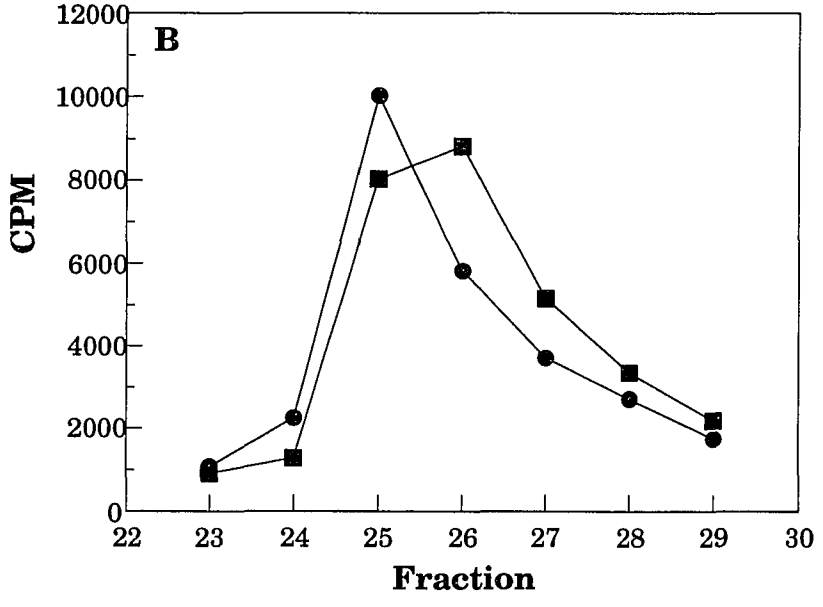


The protein structure of wild-type and various mutant ras proteins are shown. The size of each protein in amino acid residues is indicated at the C-terminal end. The N-terminus of the C-terminal domain that is switched in chimeras, amino acid 139, is also indicated.

Figure 12. Interaction of intact and C-terminal truncated forms of H-ras(K117E) with Sdc25p-C and GDP.

Complex formation and dissociation was performed by gel filtration with a mixture consisting of [^{35}S]-labeled truncated H-ras(K117E, 166) and [^{3}H]-labeled intact H-ras(K117E) (used as an internal control). Complex formation was taken as the elution of all or part of the radioactivity to earlier fractions on the column. (A) labeled proteins alone (B) labeled proteins plus Sdc25p-C, (C) labeled proteins plus Sdc25p-C in the presence 0.1 mM GDP and (D) labeled protein plus Sdc25p-C in the presence of 0.25 mM GDP. (●), H-ras(K117E) and (■), H-ras(K117E, 166).


A.
12000
10000
8000
6000
4000
2000
0
CPM
22
23
24
25
26
27
28
29
30
Fraction


B
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CPM
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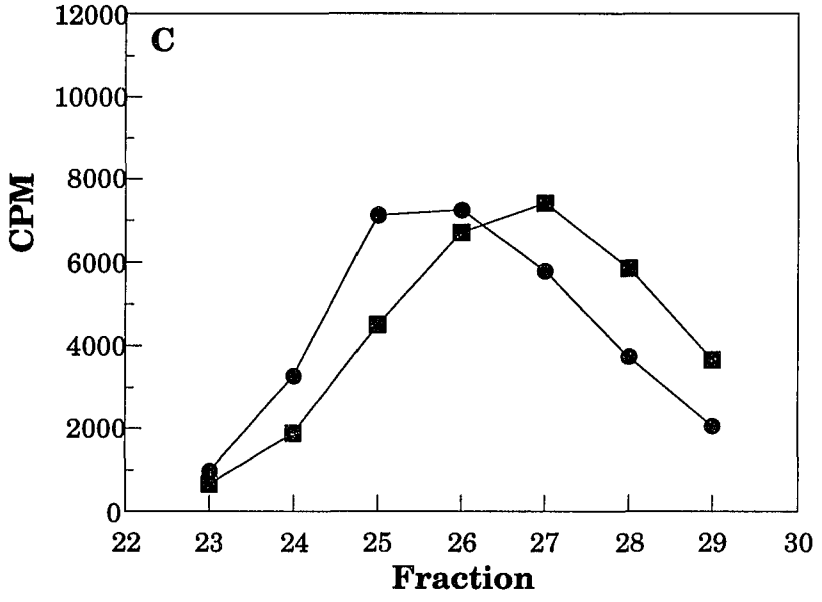
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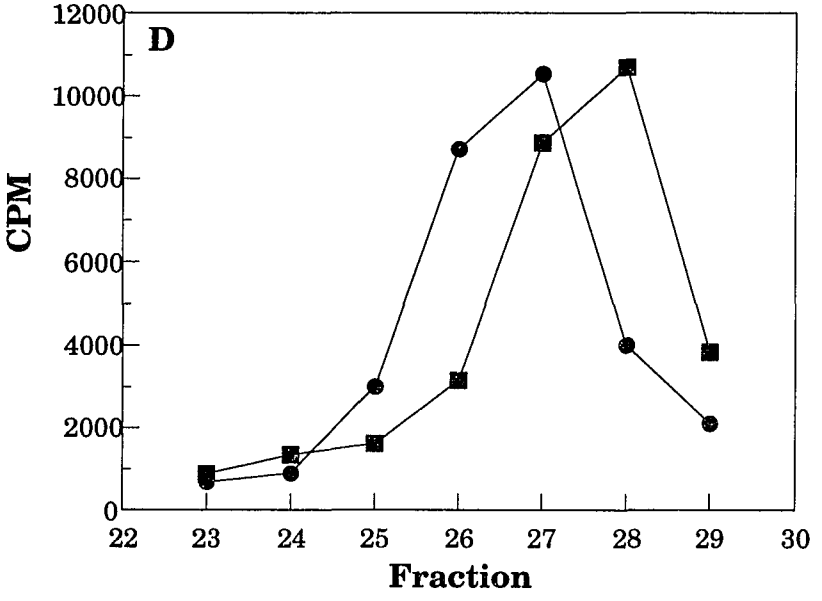
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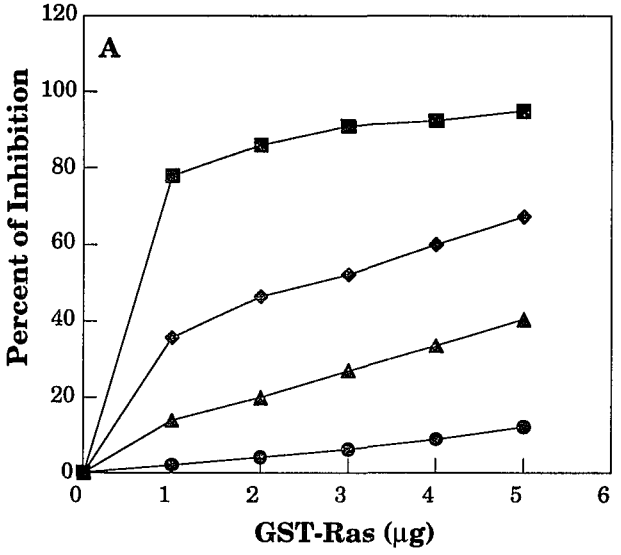
Fraction

Figure 13. Inhibition of Sdc25p-C-stimulated guanine-nucleotide exchange by ras mutants (A) and suppression of Sdc25p-C inhibition by GTP (B).

A. Sdc25p-C inhibition was determined in an exchange reaction as described in Material and Methods. (■), GST-H-ras(N116I); (◆), GST-KH-ras(N116I); (▲), GST-HK-ras(N116I) and (●), GST-K-ras(N116I).

B. The suppression of Sdc25p-C inhibition was determined by GDP release as described in Materials and Methods. (■), GST-H-ras(N116I); (◆), GST-KH-ras(N116I) and (●), GST-K-ras(N116I).


A
Percent of Inhibition
120
100
80
60
40
20
0
0
1
2
3
4
5
6
GST-Ras (μg)

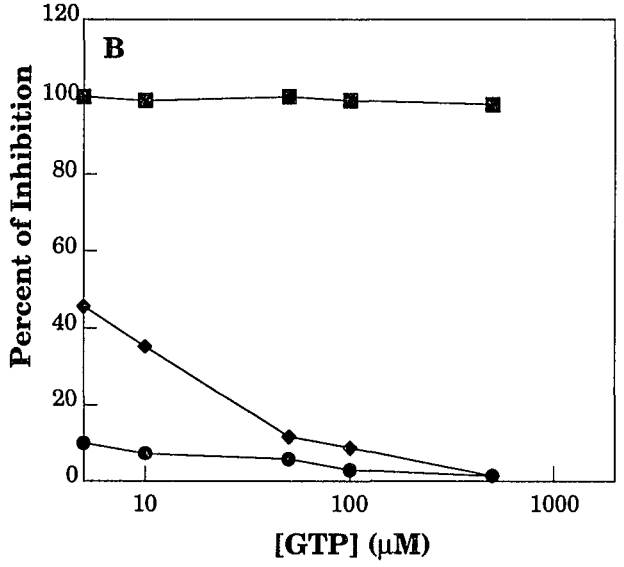
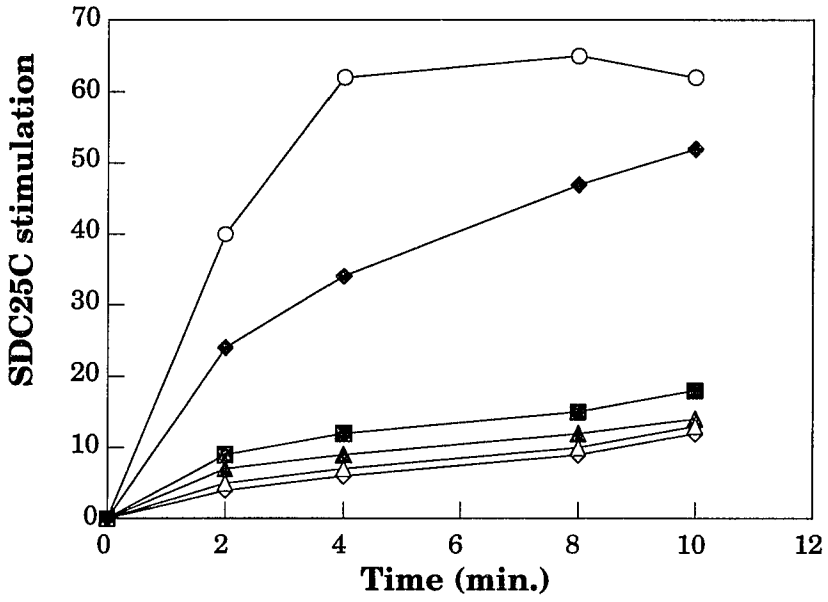

B
Percent of Inhibition
120
100
80
60
40
20
0
10
100
1000
[GTP] (μM)

Figure 14. Time course of Sdc25p-C-stimulated guanine-nucleotide exchange.

Sdc25p-C-stimulated guanine-nucleotide exchange was performed as described in the Material and Methods using [^{3}H]-GTP as a tracer. The SDC25 stimulation was defined as the difference in CPM (between with and without Sdc25p-C) divided by the CPM at equilibrium. (○), H-ras;; (◇), K-ras ; (■), HK-ras ; (▲), KH-ras; (△), K-ras (171); and (◈), HK-ras(171).



Chapter III. Mutagenesis of H-ras p21 at Glycine-60 Residue, a Conserved Amino Acid in GTP-regulatory Proteins

Abstract:

The function of Gly-60, the conserved glycine in the DXXG domain of v-H-ras and c-H-ras, was examined by site-directed mutagenesis. It was found that while the G60A (Gly-60 to Ala substitution) mutation had little effect on the interaction of H-ras with guanine nucleotides, it completely abolished the biological activity of v-H-ras. The G60A mutation also had little effect on the interaction of H-ras with Sdc25p-C (a guanine-nucleotide exchange factor) and rasGAP. However, the G60A mutation did lower the ability of H-ras to bind Raf. The G60A mutant was found to be a potent inhibitor of v-H-ras-induced oocyte maturation. The dominant negative phenotype of H-ras(G60A) required proper attachment to the plasma membrane, because introducing a second intragenic mutation, C186S, completely eliminated its dominant negative effect. Using a competition assay, we found that the N-terminal 275 residues of Raf efficiently countered the inhibition of H-ras(G60A) in oocytes; in contrast, the full length rasGAP was completely ineffective. These observations suggest that the G60A mutant inhibits v-H-ras by sequestering Raf in *Xenopus* oocytes. GTP enhanced the fluorescence emission of complexes consisting of H-ras and the fluorescent dye 8-anilino-1-naphthalene sulfonic acid. This enhancement was not observed in the G60A mutation; therefore, the GTP-induced conformational change of H-ras, a process required for its activities, is impaired by the G60A mutation. The aberrant structure of the G60A mutant is likely the basis for its dominant negative effect.

Results:

The interaction of the G60A mutants with GDP and GTP. The apparent K_Ds for the GDP and GTP complexes of the wild type p21 and the G60A mutants were determined by equilibrium binding (Table 3). The effect of the G60A mutation on the GDP/GTP binding of H-ras depended on its H-ras background. In v-H-ras; it enhanced both GDP and GTP binding approximately 2-fold; however, it slightly reduced c-H-ras's affinity for each nucleotide. In agreement with these K_D values, v-H-ras(G60A) exhibited a slower uncatalyzed GDP/GTP release rate than the wild type v-H-ras; whereas, c-H-ras(G60A) released GDP/GTP faster than its wild type counterpart (Figure 15A and 15B). Nevertheless, the difference was small and is unlikely to have much physiological significance. The uncatalyzed guanine-nucleotide exchange rate was also determined. Again, we did not detect significant differences between the wild type and the G60A mutants (Figure 16A and 16B). Since the dissociation rate of $G_{s\alpha}$(G226A) is high even at high Mg^{+2} concentrations (Lee et al., 1992a), we performed GDP/GTP exchange and dissociation reactions of G60A mutants at different concentrations of free Mg^{+2} ion ranging from 0 to 5 mM. Removing Mg^{+2} ion gradually accelerated the rate of both GDP and GTP exchange and release; however, rate enhancements were parallel in both the wild type p21 and G60A mutants, an observation that contrasts with the $G_{s\alpha}$(G226A) mutant. Overall, these results indicate that the stability and kinetics of H-ras p21 GDP and GTP binding were not significantly altered by the G60A mutation.

Biological activity of the G60A mutant. The biological activity of wild

type v-H-ras and the G60A mutant was determined by their ability to transform NIH 3T3 cells. Cells were transfected with pSV_{neo} vectors harboring three constructs (c-H-*ras*, v-H-*ras*, and v-H-*ras*(G60A)); the potency of transformation was scored as the number of foci formed. The G60A mutation was found to completely abolish the transforming activity of v-H-ras (Table 4). That is, its transforming activity was indistinguishable from that of the control vector, or c-H-*ras*. Introducing viral or mutagenic H-ras p21 readily induced GVBD in *Xenopus* oocytes (Birchmeier et al., 1985); thus, we also analyzed the biological activity of purified H-ras p21 in *Xenopus* oocytes. Under our assay conditions, oocytes injected with 46 ng of v-H-ras matured within 24 hours; while v-H-ras(G60A) was completely devoid of GVBD induction activity (Table 5). In addition, we observed that GVBD could not be detected even when 92 ng of the G60A mutant was injected and the incubation maintained for 96 hours. In addition, prebinding GTP *in vitro* was unable to restore the GVBD induction activity to the G60A mutant (Table 5).

The effects of the G60A mutation on its interaction with Sdc25p-C. The ability of the G60A mutants to form complexes with Sdc25p-C under equilibrium conditions was analyzed by gel-filtration. H-ras p21 was labeled with [^{35}S]-methionine *in vitro* and then used as a tracer for this analysis. As shown in Figure 17A and 17B, v-H-ras(G60A) bound Sdc25p-C and formed ras•Sdc25p-C to the same extent as the v-H-ras. Approximately 35% of v-H-ras and 30% v-H-ras(G60A) were complexed when a 20-fold molar excess of Sdc25p-C was used. In addition, the amounts of GTP or GDP required for complex dissociation were also similar; 0.2 μM GTP completely dissociated both v-H-ras and the G60A mutant complexes

(Figure 17A and 17B). These results suggest that the G60A mutation does not alter p21's ability to interact with Sdc25p-C. In contrast, H-ras harboring the guanine ring binding domain mutation, K117E, formed ras•Sdc25p-C complexes in much higher yield and required much more guanine nucleotide to dissociate the complex (Figure 17C) (Hwang et al., 1993). Likewise, no major difference in Sdc25p-C binding and in dissociation by GDP or GTP were found when c-H-ras and c-H-ras(G60A) were analyzed. A steady-state kinetic study revealed that the apparent K_ms of Sdc25p-C-stimulated guanine-nucleotide exchange for the wild type and the G60A mutants were comparable (Table 6). This result is in accord with the equilibrium Sdc25p-C binding data. However, the rate of Sdc25p-C-stimulated exchange was reduced by the G60A mutation, particularly when it was in a c-H-ras background (Figure 18A and 18B). We found that apparent Vmax of Sdc25p-C-stimulated GDP exchange was reduced by 5 and 10 fold in v-H-ras(G60A) and c-H-ras(G60A), respectively, as compared to their wild type equivalents (Table 6).

The effects of the G60A mutation on GTP hydrolysis. In order to further characterize the G60A mutant, the GTPase activity of wild type c-H-ras and c-H-ras(G60A) were compared. The wild type c-H-ras contains a low intrinsic GTPase activity, and GTP hydrolysis can be greatly accelerated by rasGAP (Bollag and McCormick, 1991). A time course study of GTP hydrolysis in the presence or absence of rasGAP showed that the G60A mutation drastically reduced the intrinsic and GAP-stimulated GTPase activity of c-H-ras (Figure 19). Nevertheless, the rate of GTP hydrolysis by c-H-ras(G60A) was still greater than that of p21 with v-H-ras background. Since the G226A mutation of $G_{s\alpha}$ eliminated the GTPase

activity at nanomolar but not millimolar concentrations of Mg^{+2} (Lee et al., 1992), we also determined the intrinsic and GAP-stimulated GTPase at various Mg^{+2} concentrations ranging from 0.1 mM to 5 mM. However, no significant effect on the rate of GTP hydrolysis was observed.

The effects of the G60A mutation on rasGAP interaction. We analyzed the interaction of c-H-ras and rasGAP by measuring the K_m of GAP-stimulated GTP hydrolysis. Surprisingly, the steady-state kinetics revealed that c-H-ras(G60A) had a greater affinity for rasGAP than its wild type counterpart. The apparent K_m of GAP-stimulated GTP hydrolysis was 8.9 μM for c-H-ras and 4.4 μM for c-H-ras(G60A); this represents a two-fold increase in rasGAP affinity (Table 7). We also examined the effect of the G60A mutation on the interaction of v-H-ras with GAP. Because v-H-ras exhibits very low intrinsic and GAP-stimulated GTPase activities, a competition method (Vogel et al., 1988) rather than a direct GTP hydrolysis measurement was employed to analyze this interaction. Ras in its GTP-bound state is able to compete against other forms of ras for rasGAP. This leads to an apparent reduction in rasGAP activity. The reduction in rasGAP activity can be used as an indicator for assessing p21 and rasGAP interactions (Vogel et al., 1988). Thus, we measured the strength of the GAP-inhibiting ability of wild type v-H-ras•GTP and v-H-ras(G60A)•GTP. As shown in Figure 20, v-H-ras(G60A)•GTP's inhibitory activity against rasGAP was slightly greater than v-H-ras•GTP over a range of competitor concentrations. This observation suggests that v-H-ras(G60A) has a higher affinity for rasGAP than wild type v-H-ras. The GDP-bound forms of v-H-ras and v-H-ras(G60A) were used as controls; both were less effective than the GTP-bound form in reducing rasGAP activity (Figure 20). The steady-

state inhibition constants of v-H-ras•GTP and v-H-ras(G60A)•GTP in the GAP-stimulated reaction were determined to be 69 μM for v-H-ras•GTP and 51 μM for v-H-ras(G60A)•GTP at a rasGAP concentration of 2.8 nM (Table 7). Again, the G60A mutation was found to enhance the binding H-ras p21 to rasGAP. Therefore, we concluded that G60A mutation abolished GTPase activity of H-ras p21 without significantly altering its ability to interact with rasGAP.

The effects of the G60A mutation on Raf interaction. We subsequently examined the ability of H-ras(G60A) to interact with Raf protein, the putative ras effector which binds ras in a GTP-dependent manner (Van Aslet et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). A GST-fusion clone containing the N-terminal 275 residues of Raf (including the *ras* binding domain) was used in these assays. Briefly, GST-Raf was allowed to interact with [^{35}S]-methionine labeled ras•GTP and then the complexes were coprecipitated using glutathione-Sepharose. The coprecipitated [^{35}S]-labeled ras was then analyzed on 10% tricine-polyacrylamide gel electrophoresis and quantified. As expected, the ras-Raf interaction was GTP-dependent. GTP greatly enhanced Raf binding activity of ras (Figure 21 and Table 8). We found that about 10.9% v-H-ras•GTP but less than 0.1% v-H-ras•GDP were coprecipitated. In contrast, only 3.4% v-H-ras(G60A)•GTP was coprecipitated by GST-Raf (Figure 21 and Table 8). The binding of Raf to the G60A mutant still depended on GTP; that is, v-H-ras(G60A)•GTP binding to Raf was substantially greater than v-H-ras(G60A)•GDP (Table 8). We also measured ras-Raf binding by coprecipitating GST-Raf and purified ras p21 complexed with [γ-^{32}P]-GTP. The results obtained in this study were essentially the same as those in the

preceding experiment which showed that Raf binding activity of v-H-ras was reduced approximately three fold by the G60A mutation (Table 8). We also tested the ability of c-H-ras to bind to Raf in this assay. Since c-H-ras exhibits an intrinsic GTPase activity, it was not surprising that only about half of it coprecipitated with Raf compared to v-H-ras (Table 8). Nevertheless, c-H-ras(G60A) exhibited a Raf binding activity similar to that of v-H-ras(G60A) (Table 8).

The dominant negative effects of the G60A mutants. Despite the G60A mutant's failure to transform NIH 3T3 cells or to induce GVBD in *Xenopus* oocytes, it is not biologically inert. When v-H-ras(G60A) was co-injected with v-H-ras, it strongly inhibited v-H-ras's ability to induce GVBD (Figure 22A). In the range of protein concentrations used for the injection (23-92ng), the duration and extent of inhibition were shown to depend upon the molar ratio of v-H-ras(G60A) to v-H-ras. At a molar ratio of 0.25, v-H-ras(G60A) transiently inhibited v-H-ras activity; while at a molar ratio of 2, v-H-ras(G60A) completely inhibited v-H-ras for up to four days after injection (Figure 22A). As a control, we tested the ability of c-H-ras, the cellular *ras* form which by itself has no GVBD induction activity to counter v-H-ras; and the result was negative (Figure 22A). Therefore, not every ras form that lacks the ability to induce GVBD exhibits a dominant negative effect against v-H-ras. c-H-ras harboring the same G60A mutation also displayed a dominant negative phenotype as v-H-ras(G60A), but its potency was less than that of v-H-ras(G60A) (Figure 22B).

To rule out the possibility that the inhibition of v-H-ras by v-H-ras(G60A) was due to its toxicity to oocytes, we examined the effects of

insulin, IGF-I, IGF-II, and PC-PLC on oocytes injected with v-H-ras(G60A), or a combination of v-H-ras and v-H-ras(G60A). Each of the IGFs is capable of inducing oocyte maturation by itself (El-Etr et al., 1979; Maller and Koontz, 1981); PC-PLC may also play pivotal role in ras--mediated mitogenic signalling (Cai et al., 1993). Adding insulin, IGF-I, or IGF-II to the oocyte incubation buffer readily promoted oocyte maturation regardless of whether the oocyte was injected with v-H-ras(G60A) (Table 9). Similarly, co-injecting PC-PLC with a mixture of v-H-ras and v-H-ras(G60A) eliminated the dominant negative phenotype of v-H-ras(G60A) (Table 10). PI-PLC, an agent which cannot induce GVBD, was used as a co-injection control and was found to be ineffective against v-H-ras(G60A) (Table 10). These results indicate that the dominant negative phenotype of v-H-ras(G60A) is not caused by its toxicity to oocytes. Therefore, we conclude that v-H-ras(G60A) exhibits its dominant negative effect by competing against v-H-ras for a common essential cellular factor.

The identification of the cellular target sequestered by v-H-ras(G60A). Since the v-H-ras used in the oocytes injection studies was the GDP-bound form, there are two ways that v-H-ras(G60A) could interfere with it: one, at the level of guanine-nucleotide exchange and the other, at the level of ras and effector interaction. The possibility that v-H-ras(G60A) could disrupt v-H-ras guanine-nucleotide exchange was tested by using v-H-ras precharged with GTP. As shown in Figure 23, v-H-ras(G60A) was equally effective against v-H-ras⊛GDP or v-H-ras⊛GTP. This observation demonstrates that v-H-ras(G60A) does not alter v-H-ras guanine-nucleotide exchange. Similarly, we did not observe any effect on the potency of v-H-ras(G60A) inhibition when v-H-ras(G60A) was precharged with either GTP

or GDP prior to injection. Therefore, these data suggest that GEF is not the target of v-H-ras(G60A).

Next, rasGAP was tested as the possible cellular target sequestered by v-H-ras (G60A). If rasGAP is limiting, its addition to a mixture of v-H-ras and v-H-ras(G60A) should allow v-H-ras to regain its GVBD induction activity. The human full length rasGAP was used in this study and found to have no effect on v-H-ras(G60A) (Table 11). The molar ratio of rasGAP to v-H-ras(G60A) used in these experiments approaches stoichiometry. To exclude the possibility that the rasGAP preparation used in this assay was not biologically active, a control experiment with Ras^T was performed. As shown in Table 11, incorporating rasGAP in the injection mixture readily reversed the dominant negative phenotype of Ras^T. These results show that rasGAP was biologically active; therefore, it is unlikely to be the cellular element sequestered by v-H-ras(G60A). We then tested Raf under similar conditions. The GST-Raf fusion protein used in this assay was the same as the one used in the ras-Raf binding experiment; it does not include the catalytic domain of Raf protein and is inactive in GVBD induction (Table 11). In contrast to rasGAP, the N-terminal fragment of Raf protein was highly effective in countering v-H-ras(G60A). Only a 0.05 molar ratio of GST-Raf to v-H-ras(G60A) was required to completely suppress the effect of v-H-ras(G60A) (Table 11). This observation suggests that the cellular component sequestered by v-H-ras(G60A) is Raf. We have also tested the effect of Raf on the dominant negative phenotype of Ras^T; Raf was not able to reverse the inhibition imposed by Ras^T (Table 11).

The requirement of membrane attachment for suppressing the

activity of the G60A mutation. Post-translational modification of ras near its C terminus is required for membrane localization and biological activity (Gutierrez et al., 1989; Schafer et al., 1989). To address whether membrane attachment is required for the dominant negative phenotype of v-H-ras(G60A), an intragenic C186S mutation was introduced into v-H-ras(G60A). Mutating Cys at position 186 to Ser is known to block the membrane attachment of ras (Willumsen et al., 1984), but does not alter the Raf binding affinity of v-H-ras(G60A) *in vitro*. As shown in Table 8, both v-H-ras(G60A, C186S) and v-H-ras(G60A) bound Raf roughly about one-third that of v-H-ras. However, v-H-ras(G60A, C186S) completely abolished the dominant negative effect of v-H-ras(G60A) (Table 5 and 11). These data indicate that the sequestering of Raf by v-H-ras(G60A) requires proper membrane attachment.

The effects of the G60A mutation on the GTP-induced fluorescence enhancement of H-ras and ANS complex. The fluorescent dye ANS has been used to distinguish GTP and GDP-bound forms of EF-Tu. In that study, EF-Tu•GTP was found to enhance the fluorescence of ANS to a greater extent than EF-Tu•GDP (Crane and Miller, 1974). We adapted this method to probe different conformations of various p21s. As is the case with EF-Tu, adding H-ras to ANS caused a blueshift in the wavelength of maximum dye emission from approximately 500 nm to 440 nm. In addition, the intensity of emission around 440 nm was guanine-nucleotide dependent. GTP greatly enhanced fluorescence emission around this wavelength; the effect of GDP was less dramatic. Therefore, we measured the fluorescence emission of GTP and GDP-bound p21 at 440 nm. GTP-bound v-H-ras produced a fluorescence emission which was about 9 fold

greater than that of GDP-bound v-H-ras and the enhancement persisted over a wide range of ANS concentrations (10 to 160 μM). In contrast to v-H-ras, the v-H-ras(G60A) mutant did not exhibit an emission enhancement induced by GTP (Figure 24). This result suggests that the GTP and the GDP-bound forms of the v-H-ras(G60A) mutant possess similar conformations. Nevertheless, the v-H-ras(G60A) mutant displayed an emission intensity that was identical to neither v-H-ras•GTP form nor v-H-ras•GDP (Figure 24).

Discussion:

The X-ray structures of regulatory GTPases suggest that these proteins utilize the Gly residue (Gly-60 in H-*ras* p21) in the DXXG motif as a hinge for GTP-induced conformational changes. This residue reorients in response to GTP and forms a direct bond with the γ-phosphate of GTP. This event subsequently triggers a larger scale molecular rearrangement and allows the protein to assume a proper conformation for target interaction and GTP hydrolysis (Pai et al., 1990; Milburn et al., 1990; Berchtold et al., 1993; Kjeldgaard et al., 1993; Noel et al., 1993; Lambright et al., 1994). Glycine is uniquely suitable for this structural role because it exhibits much broader range of phi and psi dihedral angles than other amino acids (Richardson, 1981). Accordingly, these models imply that GTP-induced activities, such as effector interactions or GTP hydrolysis, may be impaired if the Gly residue is substituted by other amino acids. This hypothesis has been supported by studies of Gsα(G226A) and EF-Tu(G83A) mutants (Miller et al., 1988; Lee et al., 1992a; Hwang et al., 1989a) and further corroborated by the *ras*(G60A) mutant studies presented here.

We constructed and examined v-H-ras and c-H-ras proteins that harbor the G60A mutation, a mutation corresponding to Gsα(G226A) and EF-Tu(G83A). Although the G60A mutation had little or no effect on either GDP or GTP binding, intrinsic GDP and GTP exchange and dissociation, it completely abolished the ability of v-H-ras to transform NIH 3T3 cells and to induce GVBD in *Xenopus* oocytes (Table 4 and 5). This observation is consistent with the finding that the Gly-60 to Asp mutation of H-ras was inactive in yeast (Mosteller et al., 1994). There was no significant difference between the wild type p21 and the G60A mutants in their interactions with GDP or GTP; therefore, it is unlikely that the defect in the biological activity associated with the G60A mutation can be attributed to structural perturbations at the guanine nucleotide binding site. The lack of biological activity also cannot be attributed to the failure of v-H-ras(G60A) to exchange GDP for GTP because pre-binding GTP to v-H-ras(G60A) did not restore GVBD induction in the oocyte injection assay (Table 5). In addition, the interaction of the G60A mutant with one of the guanine-nucleotide exchange factors, Sdc25p-C, appeared to be normal. Gly-60 itself is unlikely to directly participate in effector interaction; therefore, in light of the studies of Gsα(G226A) and EF-Tu(G83A) (Miller et al., 1988; Lee et al., 1992a; Hwang et al., 1989a), we postulate that the G60A mutation eliminates the biological activity of v-H-ras by preventing the GTP-bound H-ras p21 from switching into the active conformation. This postulate is supported by our observations.

The interaction of H-ras p21 with Sdc25p-C was not altered by the G60A mutation under both steady-state and equilibrium conditions. The K_ms of the Sdc25p-C-stimulated reactions for the wild type p21 and the

G60A mutants were essentially the same (Table 6). We also did not detect a significant difference between the wild type and the G60A mutant in forming H-ras•Sdc25p-C complexes (Figure 17). This result establishes that Gly-60 of H-ras is not part of Sdc25p-C binding domain. Nevertheless, the guanine-nucleotide exchange promoted by Sdc25p-C was notably decreased by the mutation (Table 6 and Figure 18). This result is consistent with earlier findings that mutations at residues around Gly-60, such as at positions 61, 62 and 63, change the rate but not the K_m of the Sdc25p-C-stimulated reaction (Mistou et al., 1992). Because these residues are either close to or at the guanine nucleotide binding pocket; therefore the reduction in the rate of guanine-nucleotide exchange was not surprising. Our results also suggest that the uncatalyzed (or EDTA-promoted) and Sdc25p-C-stimulated guanine-nucleotide exchange may involve different mechanisms because the G60A mutation affected the Sdc25p-C-stimulated exchange rate (Figure 15) but not the intrinsic (or EDTA-stimulated) exchange rate (Figure 16).

The GTP-induced conformational change of H-ras p21 was examined by the fluorescent dye ANS. ANS has been used successfully to probe the structural differences between the GDP and GTP-bound forms of EF-Tu (Crane and Miller, 1974). GTP enhances the fluorescence emission of the EF-Tu•ANS complex by permitting more ANS to bind to EF-Tu. The fluorescence emission of the H-ras•ANS complex was similar to EF-Tu in that it was also enhanced by GTP (Figure 24). The increase in emission intensity may be due to either more ANS binding or a higher quantum yield. Our current data are unable to distinguish these two possibilities. The enhancement of fluorescence emission was completely abolished by the

G60A mutation (Figure 24). This observation offers direct evidence to support the hypothesis that the GTP-induced conformational change in ras is impaired in the G60A mutant. Despite the fact that it does not possess a GTP-induced emission enhancement, the G60A mutant (either GDP or GTP form) consistently displays a much higher emission intensity than H-ras•GDP. The interpretation of this observation is not clear. It may be that the G60A mutant is locked in a structure which allows either more ANS binding or higher quantum yield. It is apparent however that, the structure of H-ras(G60A) is different from that of H-ras•GDP or H-ras•GTP.

The intrinsic and GAP-stimulated GTPase activities of c-H-ras p21 were drastically reduced by the G60A mutation (Figure 19); however, in contrast to the Gsα(G226A) mutant, the GTPase activity of c-H-ras(G60A) was not restored by adding Mg^{+2}. Since the Gly-60 residue is unlikely to serve as the nucleophile for attacking γ-phosphate of GTP, the reduction in GTPase suggests that the G60A mutation prevents the proper positioning of residues required for GTP hydrolysis. GTP-induced conformational changes occurs primarily in two regions of H-ras p21, residues 30-38 (switch I) and residues 60-76 (switch II) (Figure 2). Since the G60A mutation blocked GTP-induced activities, it is likely that some or all of the residues in switch I and II may be affected by the G60A mutation. It should be noted that while GTP-enhanced ANS emission was blocked by the G60A mutation (Figure 24), the rasGAP binding and to some extent the Raf binding activity of the G60A mutant still depended on GTP (Figure 20 and 21). Therefore, it is clear that not every aspect of the GTP-induced conformational change is abolished by this mutation.

In its GTP-bound state, Gsα resists cleavage by trypsin at Arg-232 (Miller et al., 1988; Lee et al., 1992a); however, both the GDP-bound Gsα and the GTP-bound form of Gsα(G226A) are sensitive to trypsin. This suggests that the region at or near Arg-232 of Gsα (the corresponding region of switch II) cannot assume an activated (and trypsin-resistant) conformation when Gly-226 is mutated (Miller et al., 1988; Lee et al., 1992a). This conclusion was corroborated by the observation that the GTP-dependent intrinsic fluorescence of Trp-234 was eliminated by the G226A mutation (Lee et al., 1992a). In EF-Tu, GTP-binding accelerates the rate of trypsin cleavage at Arg-59 (Douglass and Blumenthal, 1979); however, this rate was not altered by the G83A mutation (Hwang et al., 1989a). This result suggests that the G83A mutation does not affect the GTP-induced conformational changes at or near Arg-59 of EF-Tu, a region corresponding to the switch I domain (Bourne et al., 1990; Bourne et al., 1991; Berchtold et al., 1993; Kjeldgaard et al., 1993). At present, we do not know which switch region is affected by the G60A mutation. Nevertheless, the findings of Gsα(G226A) and EF-Tu(G83A), suggest that the failure of switch II to rearrange may be the key consequence of the G60A mutation. If this speculation is correct, it implies that Raf binding requires the cooperation of two switch regions; while, the binding of rasGAP may only need switch region I (effector domain). Since the G60A mutation eliminated the biological activity of v-H-ras p21, it also implies that switch II region is one of the essential domains for the biological function of v-H-ras p21. Our speculation is supported by a mutagenesis study at Tyr-64 of H-ras (Nur-E-Kamal et al., 1992); but, it is in direct contrast to the finding that part of the switch II region can be deleted without affecting the biological activity of v-H-ras (Willumsen et al., 1986). In contrast to the ras and Sdc25p-C

interaction, the interaction of ras with the CDC25 gene product is altered by mutations in residues 62 to 69 (Mosteller et al., 1994). Our conclusion implies that the G60A mutation may affect the interaction of ras with CDC25 or other guanine-nucleotide exchange factors. This possibility is currently under study.

Interestingly, the G60A mutation reduced the binding of ras to Raf about three fold but not to rasGAP (Figure 20, 21, and Table 7, 8). Although both require the GTP-form of H-ras to bind, our observation suggests that the rasGAP and Raf binding sites do not completely overlap. The ability to differentiate Raf from rasGAP offers the possibility that one may be able to determine the essential amino acids required for rasGAP and Raf binding using the G60A mutant. The fact that the biological activities of ras are affected by v-H-ras(G60A)'s sequestration of Raf (Table 11), supports the hypothesis that Raf is the direct cellular target of ras. Recruiting Raf to the membrane has previously been postulated as ras' sole function (Leevers et al., 1994; Stokoe et al., 1994). However, this hypothesis cannot fully explain why the G60A mutant bound Raf substantially well (Table 8 and Figure 21) but was biologically inactive (Table 4 and 5). The dominant negative phenotype of v-H-ras(G60A) was completely inhibited by Raf at a molar ratio of one Raf per twenty v-H-ras(G60A) (Table 11); therefore, it is clear that if no other events except membrane recruitment were needed for Raf activation, the G60A mutant should have been biologically active. These observations lead to the hypothesis that there are at least two distinct ras-Raf interactions: the first interaction recruits Raf to the membrane; Raf is then activated through a subsequent interaction and the assistance of other factors, such as the 14-3-3 protein (Burbelo and Hall, 1995). If this is the

case, G60A must block the second step of ras-Raf interaction. In this regard, the G60A mutant will provide an unique tool for studying ras and Raf interactions and its subsequent activation. It should also be pointed out that the dominant negative phenotype of Rap1 (Noda, 1993), which suppresses v-K-ras, may share a similar mechanism with v-H-ras(G60A). Membrane attachment is clearly required for the second interaction because the C186S mutation, a mutation that abolishes ras membrane association, inhibited the biological activity of v-H-ras and the dominant negative effect of v-H-ras(G60A) (Table 11). The ras binding domain (RBD) in Raf has been shown to include residues 55-132 (Scheffler et al., 1994). Recently, another site in the cysteine rich region, residues 128-198 of Raf, also has been shown to have RBD properties (Ghosh and Bell, 1994; Brtva et al., 1995). The essential nature of this second RBD was demonstrated with the Raf C186S mutant; this mutant binds ras but cannot be activated by signals through the ras pathway (Bruder et al., 1995; Zhang et al., 1993; Avruch et al., 1994). The discovery of two distinct RBDs offers the intriguing possibility that each of the two ras-Raf interactions may be mediated through different RBDs. Alternatively, components within the membrane may trigger Raf kinase activity after the ras-Raf complex is recruited to the membrane. Since the G60A mutant is impaired in its GTP-induced conformational change, it is possibile that G60A•GTP and Raf protein complex is not properly positioned in the membane, enabling it to interact with other factors.

Heterotrimeric G-proteins, EF-Tu and H-ras p21 appear to share a fundamental molecular mechanism for GTP-induced conformation changes, a mechanism that may be generally conserved in all other

regulatory GTPases. Since each protein interacts with different cellular components and performs different functions, such a mechanism has evolved to fit different formats. For example, similar conformational changes are required to dissociate α from $\beta\gamma$ subunits in heterotrimeric G-proteins, for the binding of aminoacyl-tRNA binding in EF-Tu, and for the rasGAP and Raf interaction in H-ras p21. Since the G60A mutation is able to prevent the GTP-induced conformational change and distinguish between rasGAP and Raf, it will provide a useful tool for elucidating the molecular mechanism of GTP-induced conformational change and effector interactions.

Materials and Methods:

Mutant construction. The G60A mutants were constructed by oligonucleotide site-directed mutagenesis as described by Kunkel and co-workers (Kunkel et al., 1987). Single-stranded DNA from M13 phage clones containing the entire c-H-*ras* or v-H-*ras* were used as templates for the mutagenesis. The mutagenic primers used were $^{5'}$CTTCTTGAGCTGC TGTG$^{3'}$ for the c-H-*ras* clone, and $^{5'}$CTTCTTGAGCTGTTGTG$^{3'}$ for the v-H-*ras* clone. Subsequently, the H-*ras* fragment was subcloned into the eukaryotic expression vector pSV_{neo} and used to transform NIH3T3 cells (Ogiso et al., 1990). *Escherichia coli* pHR expression vectors, for producing H-ras proteins under the control of the T7 RNA polymerase promoter, were constructed as described (Hwang et al., 1993). These plasmids were converted to pA-H-*ras* clones by replacing the PstI-HindIII(2) fragment of the parent pHR vector (Hwang et al., 1993) with an oligonucleotide linker containing a SalI site, 38 residues of polyA track, and a XhoI site,

respectively. The resulting plasmid contained unique SalI and XhoI sites 3' to the H-ras coding sequence which were used to produce RNA by *in vitro* run-off transcription. The C186S mutation was introduced into the v-H-*ras*(G60A) by PCR amplification. The 5' PCR primer, $^{5'}$ACCGGAATTCAAAATTAAGGAGGATCC$^{3'}$, included a BamHI site (underlined) in the ribosome binding region and part of the T7 promoter sequence from the cloning vector pND1 (Cunningham et al., 1990; Hwang et al., 1993). The 3' PCR primer, $^{5'}$GAGAGAGTCGACCCTCACCTGGTGT CAGGACAGCAC*AGA*CTTGCAGCTCATGCA$^{3'}$, contained a sequence that is complementary to the last nine codons of the H-*ras* gene but included a serine codon substitution at residue 186 (italics). A SalI site (underlined) was included in this primer to facilitate subsequent cloning. PCR produced a fragment of about 700 bps which was subsequently restricted by SalI and BamHI, and then cloned into the unique BamHI-SalI site of the pA-H-*ras* vector (Hwang et al., 1993). The resulting G60A mutants were confirmed by DNA sequencing.

Protein purification. The procedures for H-ras protein induction and purification were described in Chapter 2. Sdc25p-C was expressed from the vector pTTQ-*SDC*25 (Mistou et al., 1992) in an *E. coli* host and purified as described by Créchet, et al. (Crechet et al., 1990). Ras^T was supplied by Dr. Jackson Gibbs of Merck Company. The full length human rasGAP was obtained from Dr. Gideon Ballog of Onyx Pharmaceutical.

Biological assays. NIH 3T3 cell transformation was performed as previously described (Ogiso et al., 1990); foci were scored two weeks after transfection. Experiments involving *Xenopus* oocytes were performed as

follows. Large *Xenopus laevis* females (10-12 cm) were obtained from Nasco (Fort Atkinson). To accelerate oogenesis the frogs were injected with 35 U of chronic gonadotropin from pregnant mare's serum three days prior to removing the oocytes. Fragments of ovary were surgically removed from the frogs following low temperature anesthetization. Fully developed stage VI oocytes (Dumont, 1972) were manually dissected and maintained in Barth's medium (Gurdon et al., 1985). During isolation, most of the outer layer surrounding the oocytes (theca) was removed, but follicular cells remained essentially unaltered. The dissected oocytes were allowed to recover for at least 8 hours at 19°C before use. Proteins were diluted in a buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 1 mM DTT) and then injected (in a total volume of 46 nl) into the vegetal hemisphere of the cytoplasm using a nanoliter injector (Model A2033XVY, World Precision Instruments Inc.). Injected oocytes, usually in a group of 10-20, were incubated at 19°C and GVBD was scored at various time points. GVBD was initially scored by the appearance of a white spot in the animal hemisphere and further confirmed by manually dissecting the oocytes fixed in 5% (w/v) trichloroacetic acid. Insulin, IGF-I and IGF-II were obtained from GIBCO, BRL; PI-PLC and PC-PLC were obtained from Boehringer Mannheim.

Guanine nucleotide binding, exchange and dissociation. GDP and GTP dissociation constants were determined by equilibrium binding and calculated from Scatchard plots as described (Manne et al., 1984). GTP and GDP exchange reactions were performed using the following procedure. Two micrograms of purified p21 were incubated at 30°C in 100 µl of exchange buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 10 mM 2-mercaptoethanol, 10 mg/ml bovine serum albumin and 5 µM

[^{3}H]-GDP (sp. act. 2 Ci/mmole) or [^{3}H]-GTP (sp. act. 2 Ci/mmole). The concentration of free Mg^{+2} in the reaction mixture was controlled by adding either EDTA or $MgCl_2$. At the indicated times, 20 µl aliquots of the reaction mixture were withdrawn and the radioactivity was determined by a nitrocellulose membrane binding assay (Miller and Weissbach, 1974). Percent of saturation was calculated by dividing the filter bound radioactivity at the given time by the radioactivity obtained from the equilibrium binding. GTP and GDP dissociation experiments were performed by first labeling H-*ras* p21 with either [^{3}H]-GDP (sp. act. 2 Ci/mmole) or [^{3}H]-GTP (sp. act. 2 Ci/mmole) to equilibrium and then chasing with a 500-fold excess of cold guanine-nucleotide. Sdc25p-C-stimulated guanine-nucleotide exchange and kinetic parameters were determined as previously described (Hwang et al., 1993).

In vitro H-ras p21 labeling and Sdc25p-C binding assay. Ras proteins were labeled in *E. coli* S30 lysate (Promega) in the presence of [^{35}S]-methionine as described in Chapter II. Sdc25p-C binding to labeled p21 was performed in a Pharmacia Superdex 75 HR 10/30 column exactly as described in Chapter II as well (Hwang et al., 1993).

Measurement of GTPase activity. GTPase activity was measured by monitoring the release of the γ-phosphate group from [γ-^{32}P]-GTP as follows. Fifty pmole of c-H-ras p21 was labeled to equilibrium with [γ-^{32}P]-GTP (sp. act. 1,500 Ci/mmole) in the presence of EDTA. After labeling, the concentration of $MgCl_2$ in the reaction mixture was brought up to 10 mM and the [γ-^{32}P]-GTP bound p21 was separated from the unbound nucleotides by filtration through a Sephadex G25 column. GTP hydrolysis was initiated

by adding 0.4 pmole (4 nM) of rasGAP to 10 pmole (0.1 μM) of labeled p21 in a total volume of 100 μl reaction mixture (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 mM KCl and 5 mM DTT). The mixtures were incubated at 30°C and at the specified times, 10 μl aliquots were withdrawn and free phosphate quantified following isobutanol-benzene extraction (Crystal et al., 1974). Free phosphates in the reaction mixture were first converted to molybdate complexes by adding 0.3 ml of 1 mM potassium phosphate (pH 7.4) and 0.15 ml of 5% ammonium molybdate (dissolved in 4 N H_2SO_4) to the samples. Subsequently, the molybdate-phosphate complex was extracted from the reaction mixture with 0.6 ml of isobutanol and benzene (1:1 mixture). The radioactivity in the organic phase was determined by liquid scintillation counting and used to calculate the rate of GTP hydrolysis. A reaction mixture minus rasGAP protein was similarly prepared and used to determine the intrinsic hydrolysis rate. The steady-state inhibition constant of GAP-stimulated GTP hydrolysis was determined as follows. Two sets of five reaction mixtures were prepared containing (1, 2, 3, 4, 5 μM) c-H-ras •[γ-^{32}P]-GTP in a total volume of 30 μl containing 20 mM Na-Hepes, pH7.5, 2 mM $MgCl_2$ and 2.8 nM rasGAP. One of the two sets included 50 μM inhibitor (either v-H-ras•GTP or v-H-ras(G60A)•GTP). Reactions were initiated by adding rasGAP and were allowed to proceed for 5 min at 30 °C. The extent of GTP hydrolysis was determined by isobutanol-benzene extraction. The apparent K_m and V_{max} values were extrapolated from Lineweaver-Burk plots. To determine the apparent K_i, an additional set of reactions containing substrate, 2.8 nM rasGAP and 50 μM of inhibitor (either v-H-ras•GTP or v-H-ras(G60A)•GTP) were included. Each data point (either with or without inhibitor) was corrected for intrinsic GTPase activity before calculating the K_i .

Preparation of GST-Raf fusion protein. The GST fusion protein containing the 275 residue N-terminal domain of Raf was constructed as follows. The N-terminal Raf domain DNA was prepared by PCR from a baculovirus expression clone containing the full-length human c-*Raf* gene (Bonner et al., 1986). The following primers, $^{5'}$TTCCTT*ATCGAT*GGA GCACATACAGGGAGCT$^{3'}$ and $^{5'}$TTCCTT*CTCGAG*TCACATCCTGCTG TCCACAGGCAG$^{3'}$ containing ClaI and XhoI sites (italic), respectively, were used for PCR amplification. PCR produced a fragment of about 900 bps which was subsequently confirmed to be the human *Raf* gene by DNA sequencing. The PCR product was restricted by ClaI plus XhoI and then cloned into the unique ClaI-XhoI site of a modified pGEX3 vector (Pharmacia). The modification was made by inserting an oligonucleotide linker containing ClaI and XhoI cutting sites, $^{5'}$CAATCGATGGCCGCCCTCGAGAATT$^{3'}$, into the BamHI-EcoRI site of pGEX3. The GST-Raf fusion protein was expressed in *E. coli* strain MV1190 and purified as described below. All of the following procedures were performed at 4 ^{o}C. A bacterial pellet from a 1 liter culture was resuspended in 20 ml of buffer G (1X PBS, pH7.4, 1 mM DTT, 0.5 mM PMSF, and 1 % Triton X-100) and sonicated 10 times (30 seconds, each sonication) with a Branson cell disrupter. Cell debris was removed by centrifugation at 12,000 x g for 10 minutes. The resulting supernatant was incubated with 3 ml of PBS and PBS pre-equilibrated glutathione-Sepharose 4B for 6 hours. The resin was recovered by centrifugation at 2,000 x g for 10 min. and then washed with 60 ml of buffer G without Triton. GST-Raf was eluted from the resin by incubating the protein-bound resin in 10 ml of elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH8) for 2 hours. The buffer

containing GST-Raf protein was then switched to a buffer containing 50 mM Tris-HCl pH7.4, 150 mM NaCl and 1 mM DTT using an Amicon Centriprep-10 concentrator. The purified protein was stored at -70 °C until it was used.

Raf and ras binding assay. [^{35}S]-labeled H-ras p21 was complexed with guanine nucleotides (GDP or GTP) at 30 °C to equilibrium in a 35 µl binding solution containing 25 mM Tris, pH 7.5, 2 mM $MgCl_2$, 100 mM NH_4Cl, 0.2 mg/ml BSA, 0.5 mM GDP/or GTP, and 5 mM EDTA for 5 minutes. The reaction was terminated by adding 20 mM $MgCl_2$. Complex formation between ras and Raf was determined by mixing the GDP or GTP equilibrated [^{35}-S]-labeled ras (5.6 pmol) with 2.8 nmol of GST-Raf fusion protein in 200 µl of Raf binding buffer composed of 50 mM Hepes, pH 7.5, 100 mM KCl, 20 µM $ZnCl_2$, 5 mM $MgCl_2$, 1 mg/ml BSA. After incubating on ice for 1 hour, 20 µl of glutathione-Sepharose beads (12.5% gel bed) was added to the reaction mixture and the incubation (with gentle shaking) was continued for another hour at 4 °C. Free ras and GST-Raf were removed by washing the beads 5 times with 1 ml of ice-cold washing buffer (50 mM Hepes, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 20 µM $ZnCl_2$, and 0.1% Triton-X100). Ras•GST-Raf complexes were then eluted from the washed beads by resuspending them in 30 µl of PAGE loading buffer. The amount of coprecipitated [^{35}S]-labeled ras protein was analyzed by autoradiography following electrophoresis on a 10% Tricine-polyacrylamide gel. The amount of precipitated ras was quantified by scanning densitometry using IP Lab Gel, a image analysis program from Signal Analytics Corporation. Alternatively, complex formation was examined using [γ-^{32}P]-GTP labeled ras as described by Chuang, *et. al.* (Chuang et al., 1994). Purified ras was

labeled with [γ-^{32}P]-GTP (sp. act. 4500 Ci/mmol) to equilibrium. The labeled ras (2 pmole) was then allowed to react on ice with GST-Raf (2 nmole) for one hour in 200 µl of Raf binding buffer. Subsequently, 20 µl of glutathione-Sepharose beads were added to the reaction mixture and the incubation (with gentle shaking) was continued for additional hour at 4 °C. The bound ras was then separated from the reaction mixture by passing the mixture through a glass fiber filter GF/B obtained from Millipore. The filter was washed three times with 2 ml of ice-cold washing buffer and radioactivity on the filter was quantified by liquid scintillation counting. For the control, the above experiment was repeated, except that GST was used instead of GST-Raf. The data presented were the average of three independent experiments and fell within a range of 15%.

Fluorescence measurement. Fluorescence emission of the H-ras-ANS complex was performed by titrating a constant amount of H-*ras* (complexed either with GDP or GTP) with 10 µM to 160 µM ANS as previously described (Crane and Miller, 1974). Fluorescence was measured in a Perkin-Elmer luminescence spectrometer (model LS 50B) with an excitation wavelength of 350 nm (slit width 2.5 nm) and a detection wavelength of 440 nm (slit width 7.5 nm). The measurements were conducted at room temperature in a buffer containing 50 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$. The concentration of H-ras was set at 2 µM. For each increment of dye concentration, the dye was allowed to bind to p21 for two minutes before measuring. At least ten readings were taken over a period of 2 to 3 minutes for each dye concentration. The readings were corrected for the background (dye only) before plotting.

Table 3. The apparent GDP/GTP dissociation constants of various H-ras p21 species

H-ras p21	K_D(GDP) (x10^{-8}M)	K_D(GTP) (x10^{-8}M)
c-H-ras	1.3	0.6
v-H-ras	4.0	1.5
c-H-ras(G60A)	1.8	1.4
v-H-ras(G60A)	1.8	1.0

The data presented were the average of four independent experiments and fell within a range of 10%.

Table 4. NIH 3T3 Cells Transforming Activity of various H-*ras* p21 Species

H-*ras* p21	foci/μg DNA
$pSV2_{neo}$, vector	0.1
c-H-*ras*	0.2
v-H-*ras*	123
v-H-*ras*(G60A)	0.1

Focus-forming activity was examined in NIH3T3, 18A cells. 18A is a cell line transformed by a LTR-linked rat c-H-*ras* proto-oncogene.

The data presented were the average of two independent experiments and fell within a range of 15%.

Table 5. GVBD induction in *Xenopus* oocytes

	Percent GVBD after		
Injected Material	12 hrs	18 hrs	24 hrs
buffer	0 (0/52)	0 (0/52)	0 (0/52)
c-H-ras	0 (0/55)	0 (0/55)	0 (0/55)
v-H-ras	38 (21/56)	79 (44/56)	100 (56/56)
v-H-ras(G60A)	0 (0/85)	0 (0/85)	0 (0/85)
v-H-ras(G60A)•GTP	0 (0/23)	0 (0/23)	0 (0/23)
v-H-ras(G60A, C186S)	0 (0/40)	0 (0/40)	0 (0/40)

Oocyte injection was performed on oocytes obtained from four individual female frogs. The results were combined together. Numbers inside parenthesis indicate the number of mature and total oocytes, respectively. Unless indicated otherwise, the amount of ras p21 used for each oocyte was 46 ng.

Table 6. Apparent Sdc25p-C-stimulated Guanine-Nucleotide Exchange Kinetic Parameters

H-ras p21	K_m (μM)	V_{max} (pmol•min^{-1})
c-H-ras	2.1	1.64
v-H-ras	2.2	2.42
c-H-ras(G60A)	3.5	0.17
v-H-ras(G60A)	3.1	0.47

The data presented were the average of four independent experiments and fell within a range of 15%.

Table 7. Apparent Kinetic Parameters of GAP-Stimulated GTP Hydrolysis

H-ras p21	K_m (μM)	V_{max} (pmole•min^{-1})	K_i(μM)
c-H-ras•GTP	8.9	9.0×10^{-3}	ND
c-H-ras(G60A)•GTP	4.4	0.2×10^{-3}	ND
v-H-ras•GTP	ND	ND	69
v-H-ras(G60A)•GTP	ND	ND	51

ND: not determined.

The data presented were the average of two independent experiments and fell within a range of 10%.

Table 8. Direct demonstration of the interaction of ras and Raf by coprecipitation

H-ras p21	Percent of *Ras* Coprecipitated by GST	GST-Raf
[^{35}S]-v-H-ras•GTP	<0.1	10.9
[^{35}S]-v-H-ras•GDP	ND	<0.1
[^{35}S]-v-H-ras(G60A)•GTP	0.1	3.4
[^{35}S]-v-H-ras(G60A)•GDP	ND	0.9
v-H-ras•[^{32}P]-GTP	1.3	24.6
v-H-ras(G60A)•[^{32}P]-GTP	1.1	8.3
c-H-ras•[^{32}P]-GTP	1.0	14.1
c-H-ras(G60A)•[^{32}P]-GTP	1.6	9.2
c-H-ras(G60A, C186S)•[^{32}P]-GTP	0.8	9.0

ND: not determined.

The data presented were the average of three independent experiments and fell within a range of 5%.

Table 9. Effects of growth factors on the suppression activity of the v-H-ras(G60A) mutant

Growth Factor Treatment	Control	Injected Material v-H-ras	v-H-ras + v-H-ras(G60A)
Barth's medium	0% (0/30)	100% (30/30)	0% (0/30)
Insulin 24 mM	100% (30/30)	100% (30/30)	100% (30/30)
IGF-I 13 nM	100% (30/30)	100% (30/30)	100% (30/30)
IGF-II 13 nM	100% (30/30)	100% (30/30)	100% (30/30)

The results were combined from the experiments were performed on oocytes obtained from three individual female frogs. The amount of ras p21 used was 46ng throughout the experiments. The ratio of mature to the total number of oocytes used in the experiments was indicated inside parenthesis. GVBD was scored 19 hours after injection.

Table 10. Suppressing the G60A dominant negative effect by co-injecting PI-PLC and PC-PLC into oocytes

	Control	PI-PLC (75μU)	PC-PLC (25μU)
Buffer B	0% (0/30)	0% (0/30)	100% (30/30)
v-H-ras	100% (30/30)	ND	ND
v-H-ras(G60A)	0% (0/30)	0% (0/30)	100% (29/29)
v-H-ras+v-H-ras(G60A)	0% (0/30)	0% (0/30)	100% (30/30)

ND: not determined.

Oocytes obtained from two individual female frogs were used in this experiment, and the results were combined together. The amount of each ras p21 used was 46ng. The ratio of mature to the total number of oocytes used in the experiments is indicated inside parenthesis. GVBD was scored 19 hours after injection.

Table 11. Effects of rasGAP and Raf on the suppression activities of the G60A mutant

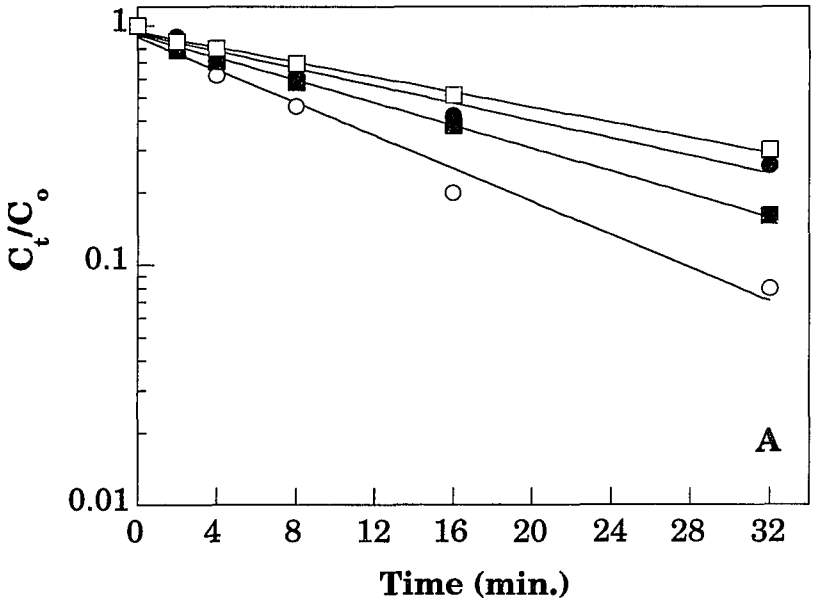
Injected Material	Percent GVBD	Injected Material	Percent GVBD
v-H-*ras* 46ng	100 (40/40)	v-H-*ras* +v-H-*ras*(G60A) (1:1)	0 (0/40)
v-H-*ras*(G60A) 46ng	0 (0/40)	v-H-*ras* +v-H-*ras*(G60A, C186S)) (1:1)	0 (0/40)
v-H-*ras*(G60A, C186S) 46ng	0 (0/40)	v-H-*ras* +Ras^T (1:1)	0 (0/30)
Ras^T 46ng	0 (0/30)	v-H-*ras* +v-H-*ras*(G60A)+GAP (1:1:0.9)	0 (0/40)
*ras*GAP 100ng	0 (0/40)	v-H-*ras* +Ras^T+GAP (1:1:0.9)	100 (30/30)
GST-Raf 100ng	0 (0/40)	v-H-*ras* +v-H-*ras*(G60A)+GST-Raf (1:1:0.05)	100 (40/40)
GST 50ng	0 (0/30)	v-H-*ras* +Ras^T+GST-Raf (1:1:1)	0 (0/30)
		v-H-*ras* +v-H-*ras*(G60A)+GST (1:1:1)	0 (0/30)

Oocytes were obtained from four individual female frogs. Percent GVBD numbers (A/B) indicate the number of mature and total oocytes, respectively. Materials were injected at molar ratios of (N1:N2).

Figure 15. Intrinsic guanine nucleotide dissociation of H-ras p21 species.

Guanine nucleotide dissociation was measured by labeling p21 with [^{3}H]-GDP (A) or [^{3}H]-GTP (B) to equilibrium then followed by chasing with 500-fold excess of cold respective guanine-nucleotides. The chase reactions were carried out in the presence of 0.5 mM free Mg^{+2} ion at 30 ^oC and quantified by membrane filtration. C_o represents the amount of GDP (or GTP) at time zero and C_t represents the amount of GDP (or GTP) at the indicated time point.

(□), c-H-ras, (○), v-H-ras, (■), c-H-ras(G60A), and (●), v-H-ras(G60A).


1
0.1
0.01
C_t/C_o
A
0
4
8
12
16
20
24
28
32
Time (min.)

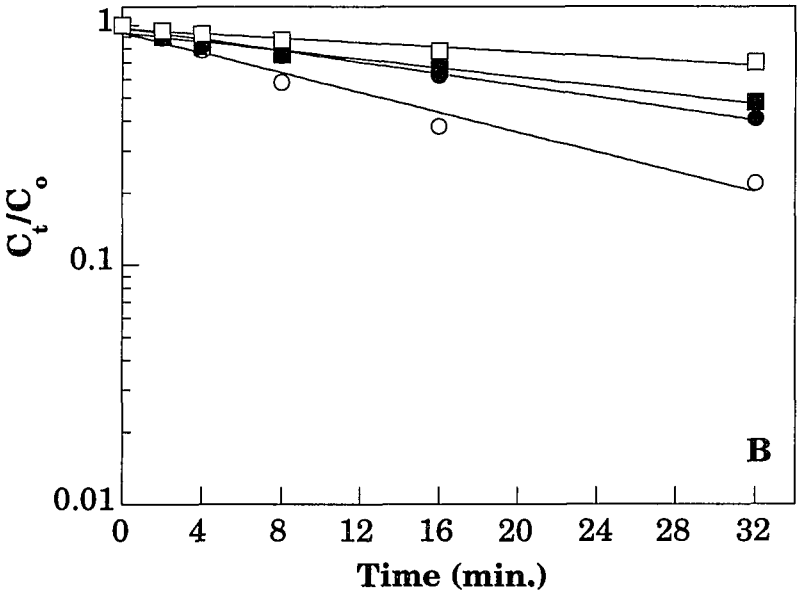
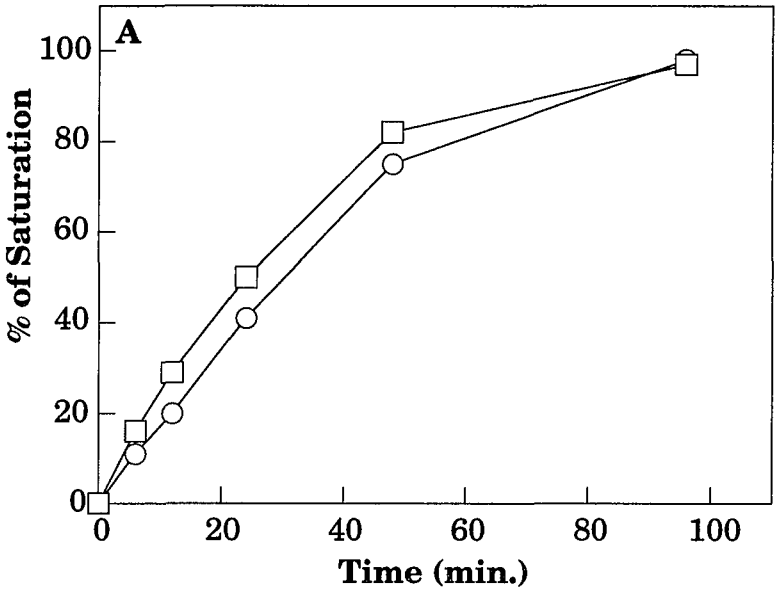

1
0.1
0.01
C_t/C_o
B
0
4
8
12
16
20
24
28
32
Time (min.)

Figure 16. Intrinsic GDP exchange of H-ras p21 species.

GDP exchange reactions were performed in the presence of 0.5 mM free Mg^{+2} ion as described in the Materials and Methods.

A. c-H-ras (○) and v-H-ras(G60A) (□).

B. v-H-ras (●) and v-H-ras(G60A) (■).


A
100
80
60
40
20
0
% of Saturation
0
20
40
60
80
100
Time (min.)

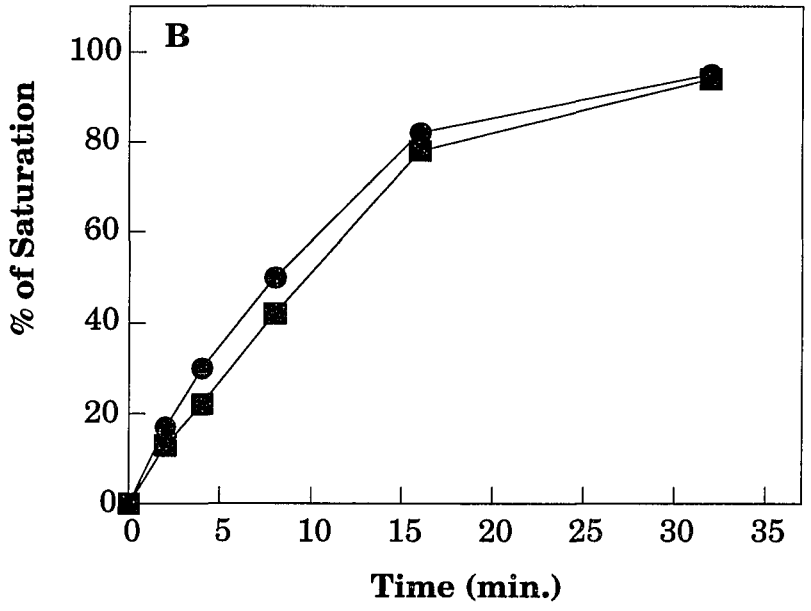

B
100
80
60
40
20
0
% of Saturation
0
5
10
15
20
25
30
35
Time (min.)

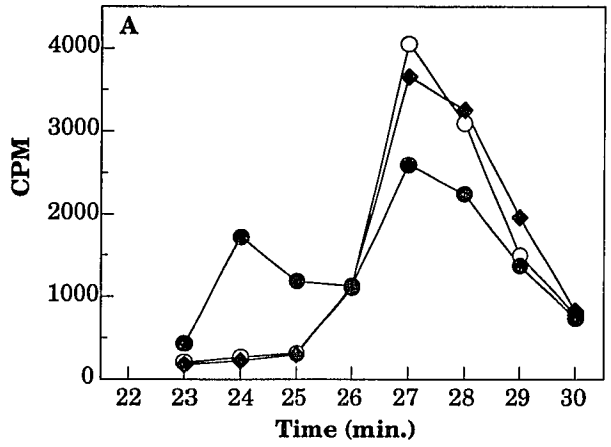
Figure 17. Interaction of H-ras species with Sdc25p-C and GTP.

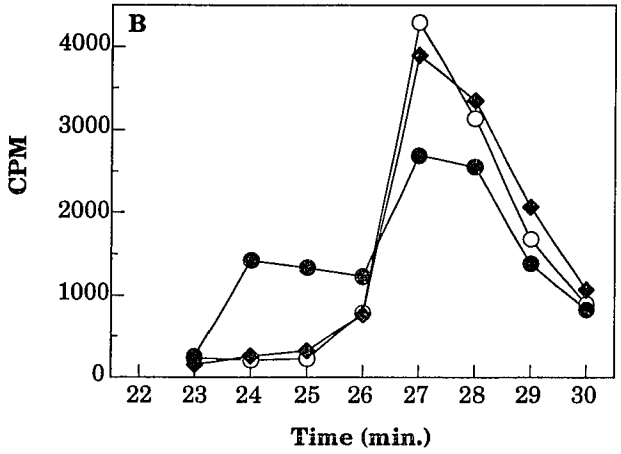
Experiments were performed by mixing 0.2 pmole of labeled H-ras with either 26 pmole (A and B) or 13 pmole (C) of Sdc25p-C followed by elution in a Superdex 75 HR column.

A. v-H-ras control (○), v-H-ras plus Sdc25p-C (●) and v-H-ras plus Sdc25p-C in the presence of 0.2 μM GTP (◆).

B. v-H-ras(G60A) control (○), v-H-ras(G60A) plus Sdc25p-C (●) and v-H-ras(G60A) plus Sdc25p-C in the presence of 0.2 μM GTP (◆).

C. v-H-ras(K117E) control (○), v-H-ras(K117E) plus Sdc25p-C (●) and v-H-ras(K117E) plus Sdc25p-C in the presence of 0.1 mM GTP (◆).


A
CPM
4000
3000
2000
1000
0
22 23 24 25 26 27 28 29 30
Time (min.)


B
CPM
4000
3000
2000
1000
0
22 23 24 25 26 27 28 29 30
Time (min.)

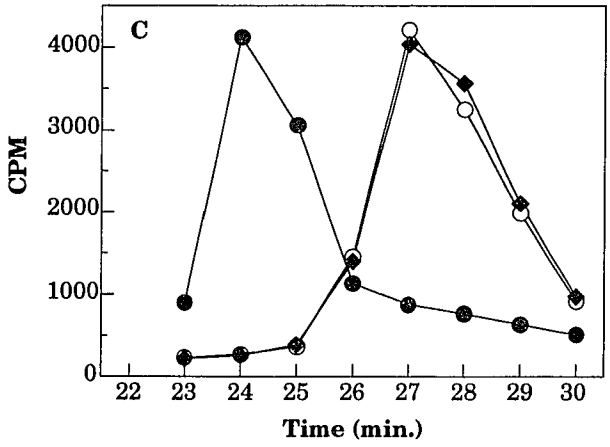
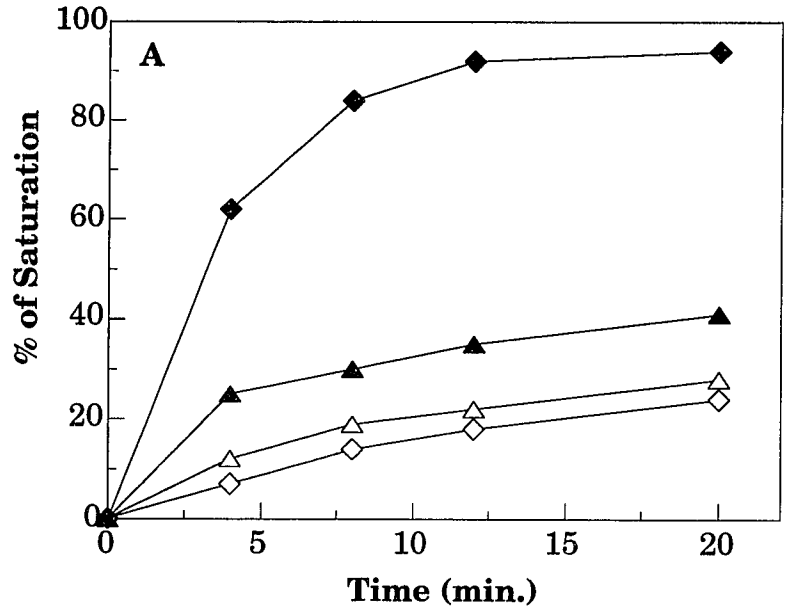

C
CPM
4000
3000
2000
1000
0
22 23 24 25 26 27 28 29 30
Time (min.)

Figure 18. GDP exchange of H-ras and H-ras(G60A) stimulated by Sdc25p-C.

Sdc25p-C-stimulated GDP exchange was performed in the presence of 2 mM free Mg^{+2} ion as described in Materials and Methods.

(A). c-H-ras control (◇), c-H-ras(G60A) control (△), c-H-ras plus Sdc25p-C (◆) and c-H-ras(G60A) plus Sdc25p-C (▲).

(B). v-H-ras control (○), v-H-ras(G60A) control (□), v-H-ras plus Sdc25p-C (●) and v-H-ras(G60A) plus Sdc25p-C (■).


A
100
80
60
40
20
0
% of Saturation
0
5
10
15
20
Time (min.)

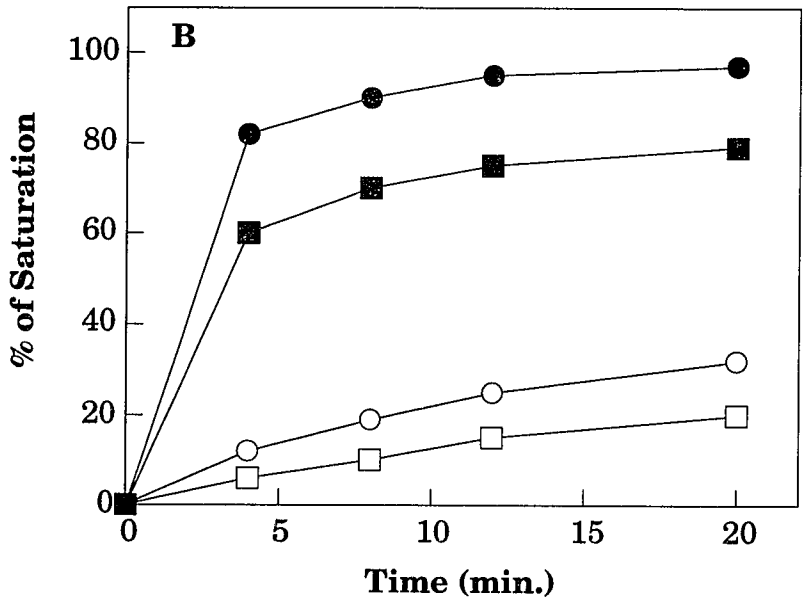

B
100
80
60
40
20
0
% of Saturation
0
5
10
15
20
Time (min.)

Figure 19. Intrinsic and GAP-stimulated GTP hydrolysis of various H-ras p21 species.

GTP hydrolysis measurements were carried out by monitoring the release of ^{32}P from [γ-^{32}P]-GTP as described in Materials and Methods. Four nanomolar full length human rasGAP was used in the experiment. c-H-ras control (○), c-H-ras plus rasGAP (●), c-H-ras(G60A) control (△), c-H-ras(G60A) plus rasGAP (▲), v-H-ras (□) and v-H-ras plus rasGAP (■).

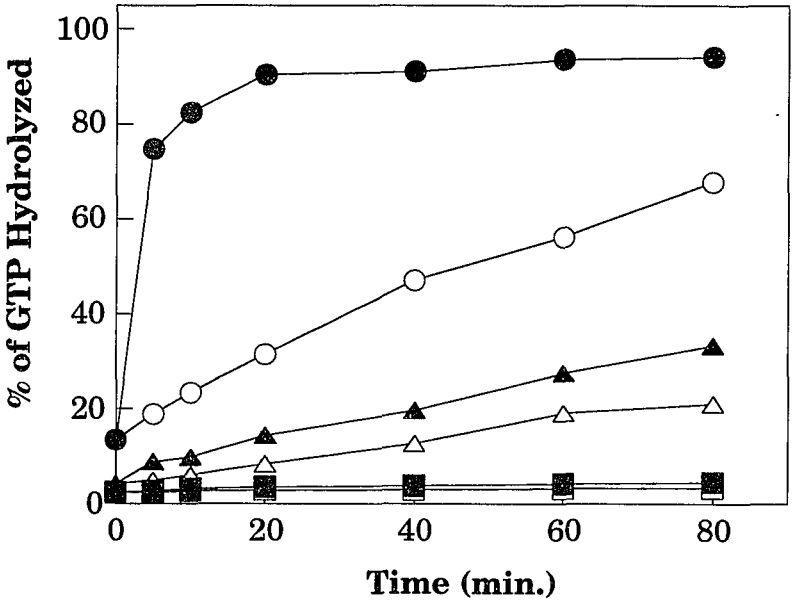


Figure 20. Inhibition of rasGAP activity by v-H-ras and v-H-ras(G60A).

The experiments were performed by measuring the GAP-stimulated GTP hydrolysis of c-H-ras •[γ-^{32}P]-GTP in the presence of indicated amounts of competitor H-ras p21. Constant amounts of full length human rasGAP (2.8 nM) were used throughout experiments. Competing H-*ras* species were v-H-ras•GDP (●), v-H-ras(G60A)•GDP (■), v-H-ras•GTP (○), and v-H-ras(G60A)•GTP (□).

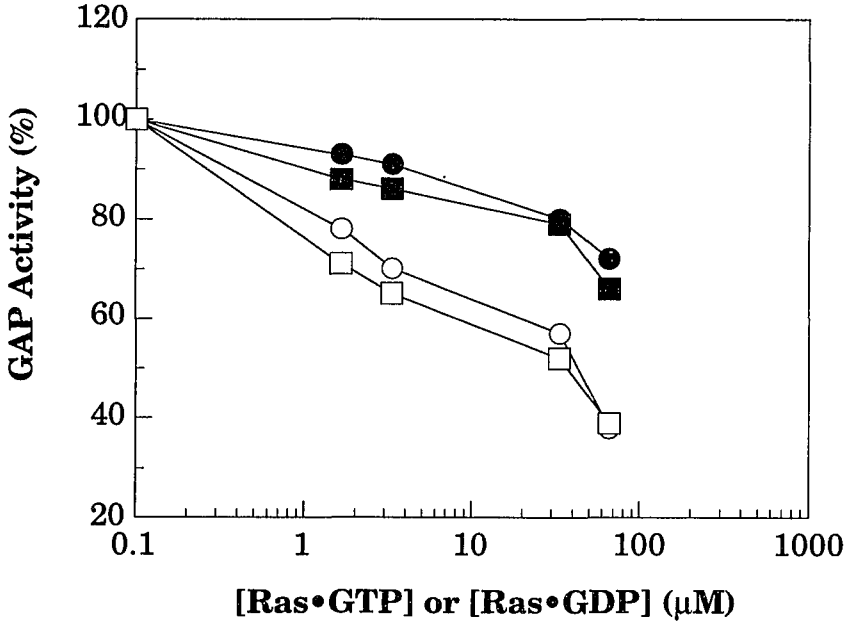


Figure 21. The interaction of ras and Raf.

Coprecipitation of [^{35}S]-labeled ras and GST-Raf by glutathione-Sepharose was performed in the presence of the following components: lane 3, v-H-ras•GTP plus GST-Raf ; lane 4, v-H-ras•GTP plus GST; lane 5, v-H-ras•GDP plus GST-Raf; lane 6, v-H-ras(G60A)•GTP plus GST-Raf; lane 7, v-H-ras(G60A) plus GST and lane 8, v-H-ras(G60A)•GDP plus GST-Raf. The starting materials for precipitation, v-H-ras•GTP and v-H-ras(G60A)•GTP, are shown in lane 1 and lane 2, respectively.

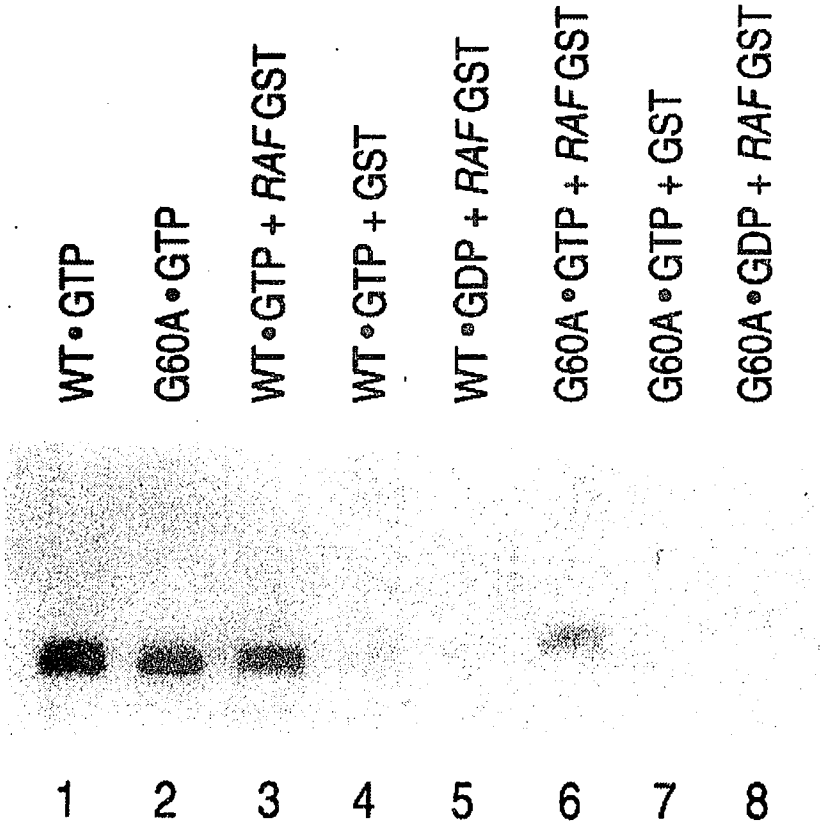


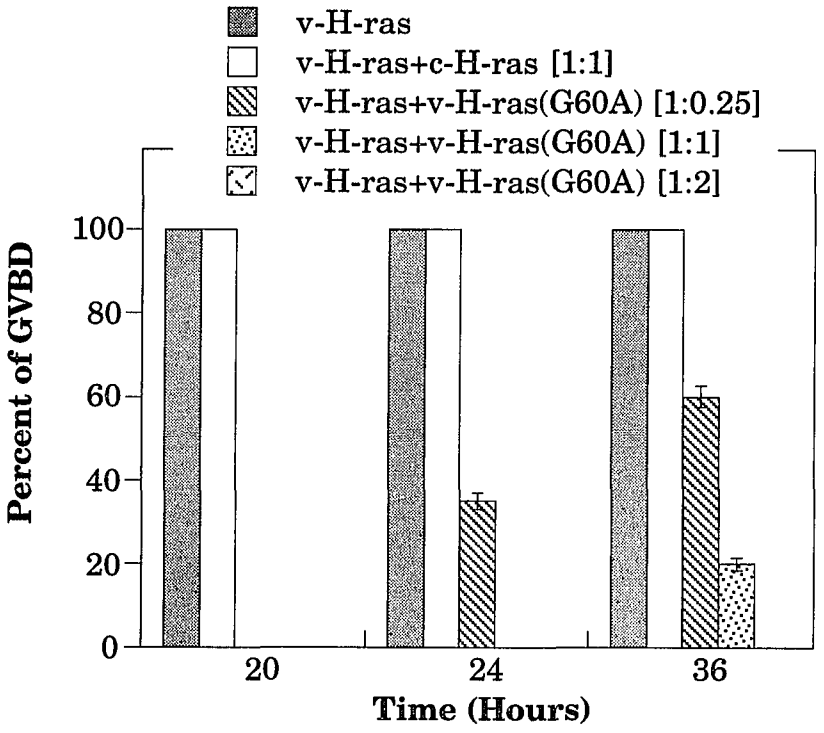
Figure 22. Dominant negative effect of G60A mutants in *Xenopus* oocytes.

Various concentrations of G60A H-ras proteins were coinjected with 46 ng of wild type p21 into oocytes. GVBD was scored 19, 24 and 36 hours after injection. The molar ratio of wild type p21 to G60A mutant is indicated inside the brackets. The results were taken from the average of four individual experiments performed on four different *Xenopus*.

A. Wild type v-H-ras co-injected with v-H-ras(G60A).

B. Wild type v-H-ras co-injected with c-H-ras(G60A).

(A)


v-H-ras
v-H-ras+c-H-ras [1:1]
v-H-ras+v-H-ras(G60A) [1:0.25]
v-H-ras+v-H-ras(G60A) [1:1]
v-H-ras+v-H-ras(G60A) [1:2]
Percent of GVBD
100
80
60
40
20
0
20
24
36
Time (Hours)

(B)

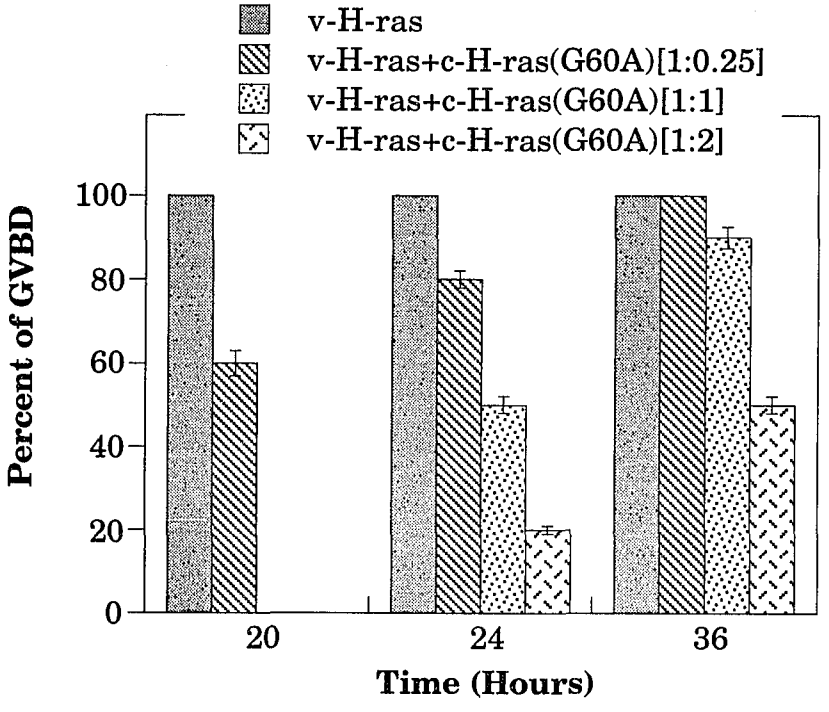

v-H-ras
v-H-ras+c-H-ras(G60A)[1:0.25]
v-H-ras+c-H-ras(G60A)[1:1]
v-H-ras+c-H-ras(G60A)[1:2]
Percent of GVBD
100
80
60
40
20
0
20
24
36
Time (Hours)

Figure 23. Suppression of GTP-bound wild type ras p21 by the G60A mutant.

Various concentrations of GDP bound v-H-ras(G60A) mutant were co-injected with 46 ng of GDP or GTP bound wild type p21 into oocytes. GVBD was scored 24 hours after injection. The molar ratio of wild type p21 to G60A mutant is indicated inside the brackets. Results were taken from the average of thirty oocytes from two individual frogs.

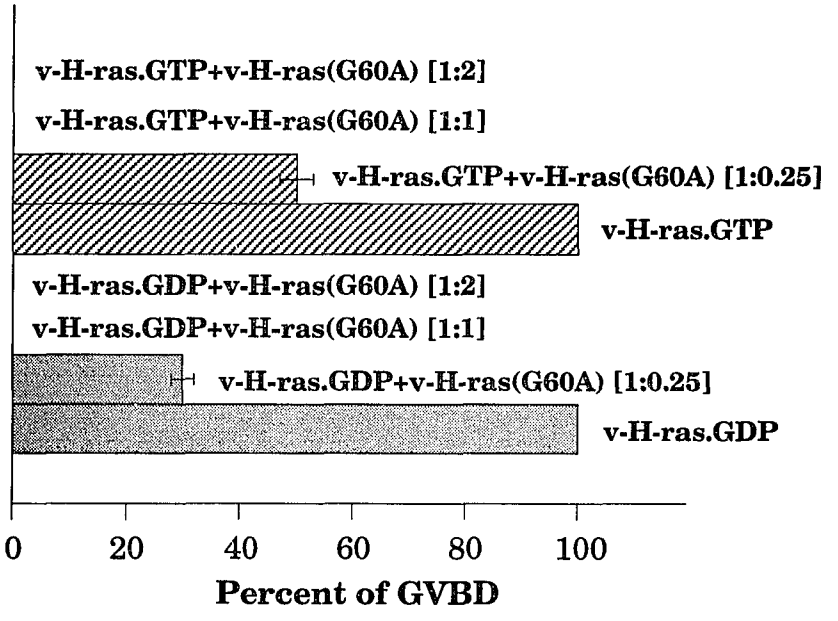
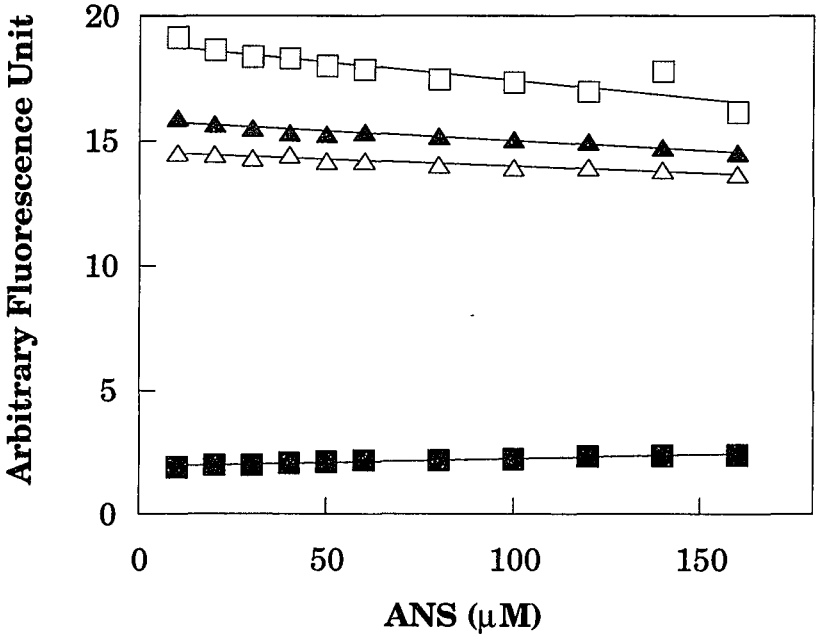


Figure 24. Fluorescence emission of ras•guanine-nucleotide•ANS ternary complexes.

ANS titration was performed as described in Materials and Methods. Symbols used in the Figure are v-H-ras•GDP (■), v-H-ras(G60A)•GDP (▲), v-H-ras•GTP (□), v-H-ras(G60A)•GTP (△).



Conclusion

In the first part of this thesis (Chapter II), an absolute requirement for the guanine nucleotide binding domain (amino acid residues 1-166) of ras p21 in mediating Sdc25p-C binding and guanine nucleotide exchange was demonstrated. In particular, it was shown that elements in the hypervariable C-terminal domain were necessary for ras and Sdc25p-C to form a stable complex. These data show that H-ras and K-ras are highly selective in their interactions with GEFs. These results also suggest that other non-conserved residues in the 121-166 region of ras are involved in the selection process as well.

In the second part of this study (Chapter III), a novel ras dominant negative mutant, v-H-ras(G60A), which inhibited v-H-ras activity was characterized. Biochemical studies showed that the properties of the G60A H-ras mutation were similar to comparable mutations in EF-Tu and Gsα. This suggests that the conserved glycine plays the same functional role in all three of these regulatory GTPases. My results also demonstrate that the biological activity of H-ras requires a proper conformational state. Finally, c-raf-1 is likely to be the direct cellular target of H-ras.

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