Characterization of a Mammalian Exchange Factor, ralGDS

by

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Honors Bachelor of Science, Mathematics
University of Utah, 1987

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

at the
Massachusetts Institute of Technology
September 1994

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18 July 1994

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In memory of Pat Farrell, and for all my teachers.

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ABSTRACT

The low molecular mass GTPases are important regulators of many cellular processes, including cell growth and differentiation, cytoskeletal organization, and protein trafficking. The cycling of these proteins between their inactive GDP-bound and active GTP-bound forms is controlled by at least two classes of regulators: GTPase activating proteins (GAPs) and guanine nucleotide dissociation stimulators (GDSs). In this thesis, I describe the cloning and characterization of a mammalian guanine nucleotide dissociation stimulator, ralGDS, that was cloned by virtue of its similarity to yeast genes that regulate Ras. ralGDS catalyzes nucleotide release from the RalA and RalB GTPases, but not from closely related gene products. I also present data that suggest that the specificity of interaction between GTPase and GDS is regulated by residues in theswitch II region of the GTPase structure.

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Think I'm just Happy
-Kurt Cobain

ACKNOWLEDGEMENTS

The best part of my scientific training was interacting with the interesting, talented, and diverse group of people who flock to work with Dr. Robert Weinberg. I am grateful to all of you! I have especially benefited from the training and direction of Charlie Albright, Sean Egan, and Dennis Templeton. Many thanks, also, for the constant feedback and attention from the other members of the Weinberg Ras group: Jeff Settleman, Sang-Ho Park, Joana Liu, Lauren Foster, Mary Brooks, Peter Sicinski, and David Shaywitz. I am also grateful to the Weinberg lab's collection of former Ras scientists: Andrew Sizeland, Riki Perlman, and Rene Medema.

Thank you to the members of my thesis committee: David Housman, Paul Matsudaira, Iswar Hariharan, Peter Kim, and my advisor, Bob Weinberg. I have greatly appreciated your interest and your guidance through the years.

My early scientific training was guided by Paul Nicholes, Stanley Marcus, Ted and Tucker Gurney, and Simon Tavaré. Thank you.

Thanks also to my friends, Ed and Laura Wall, Eric and Becky Rebentisch, Luther and Kit Giddings, Sean Becker, Mark Edlund, David McKean, Greg Beitel and Annette Elton, and Les Dethlefsen, for helping me get away from the science once in a while, for listening, and for giving words of encouragement and wisdom.

Special thanks are due the former inhabitants of what is now known as Grand Gulch for leaving something beautiful and spiritually redeeming.

Most of all, I am grateful to Susan, Rachel, David, and my parents, Lu and Bonnie. For everything.

My doctoral work was supported by a National Science Foundation Graduate Fellowship and by NIH Training Grant 5T32-CA0941.

CONTENTS

CHAPTER 1

Introduction:

The regulation of Ras proteins and their relatives

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The *ras* genes have been extensively studied since the identification of transforming alleles in 1982 (Tabin et al., 1982; Reddy et al., 1982). Since then, numerous researchers have tried to understand how *ras* oncogenes disrupt normal proliferative signaling (reviewed in Barbacid, 1987). The work described in this thesis is a small part of that very large effort to understand how *ras* (and its relatives) function in cellular signaling.

The study of Ras has been an exciting and dynamic field in recent years. Literally hundreds of reports have been written on Ras and numerous recent reviews cover every imaginable aspect of its function, including proteins related to Ras (Hall, 1990a; Valencia et al., 1991), Ras structure/function (Bourne et al., 1991); Wittinghofer and Pai, 1991); Polakis and McCormick, 1993), and regulation (Bourne et al., 1990; Evans et al., 1991; Lowy et al., 1991; Downward, 1992a; Downward, 1992b; Downward, 1992c; Satoh et al., 1992; McCormick, 1993; Feig, 1993; Medema and Bos, 1993; Lowy and Willumsen, 1993; Boguski and McCormick, 1993).

This thesis focuses on ralGDS, a guanine nucleotide dissociation stimulator (GDS; also called an exchange factor) for the Ras-related RalA and RalB GTPases. To set the stage for this work, I will briefly describe the identification and characterization of *ras* genes. Then I will describe the many related proteins that have been identified. Finally, I will review what we know about proteins that regulate the GTPase cycle.

The identification of the *ras* genes

The study of *ras* genes began with the identification of the H-*ras* and K-*ras* genes as the transforming factors of the Harvey and Kirsten murine sarcoma viruses (Harvey, 1964; Kirsten and Mayer, 1967; DeFeo et al., 1981; Ellis et al., 1981). Scientists interested in the etiology of cancer originally turned their attention to viruses because, they reasoned, if they could discover how a virus with only a few genes caused the development of a tumor, they might discover important determinants of normal and malignant cell growth (Logan and Cairns, 1982). This reasoning proved essentially correct, and the study of tumor viruses yielded numerous genes that regulate the proliferation of cells (Varmus, 1989).

Transforming *ras* genes were subsequently isolated from human tumors (Chang et al., 1982; Shih and Weinberg, 1982; Parada et al., 1982). These oncogenic alleles were shown to differ from their normal cellular counterparts by the alteration of a single amino acid (Tabin et al., 1982; Reddy et al., 1982). Mutant *ras* genes have been identified in many human tumors (reviewed in Bos, 1989).

These highly conserved 21 kDa proteins tightly bind GTP and GDP (Scolnick et al., 1979; Shih et al., 1980), possess intrinsic GTP hydrolytic activity (McGrath et al., 1984; Sweet et al., 1984), and are associated with the plasma membrane (Willingham et al., 1980; Willumsen and Christensen, 1984). By analogy to G proteins, *ras* gene products were thought to act as molecular switches: biologically active when GTP-bound and inactive when GDP-bound (Gibbs et al., 1985).

Ras-related proteins

The identification of mutated *ras* genes in human tumors aroused considerable interest. In the dozen years since, at least fifty related proteins have been identified. These low molecular mass GTPases regulate numerous cellular functions, including proliferation, differentiation, cytoskeletal organization, protein transport, and secretion (Hall, 1990a; Bourne et al., 1990; Valencia et al., 1991; Boguski and McCormick, 1993).

The low molecular mass GTPases form four families: Ras, Rho, Rab, and Ran (see Fig. 1). Some workers also include the Arf (ADP-ribosylation factor) family members in the Ras superfamily. Proteins in the Ras family (described further below) appear to regulate growth control and differentiation. Rho (*ras* homologous) family members, including RhoA, RhoB, RhoC, Rac1, Rac2, CDC42Hs (also known as G25K), and TC10, regulate cytoskeletal organization. The genes in the Rab family regulate vesicular transport and secretion. At least twenty-four members of the Rab family have been identified, including the mammalian Rab1A, Rab1B, Rab2, Rab3A, Rab3B, Rab3C, Rab3D, Rab4, Rab5, Rab6, and Rab7 proteins (reviewed in Valencia et al., 1991). Members of this family have very different effector loop sequences, suggesting that these proteins interact with a diverse set of downstream agents (Valencia et al., 1991). Ran and TC4 are related nuclear GTPases involved in regulating mitosis (Bischoff and Ponstingl, 1991). The *Schizosaccharomyces pombe* gene *spi1* is a member of this family and is involved in coordination of the completion of DNA synthesis with onset of cell division (Matsumoto andBeach, 1991).

The Ras superfamily has been discussed in several reviews (see, for example, Valencia et al., 1991; Boguski and McCormick, 1993). Since the

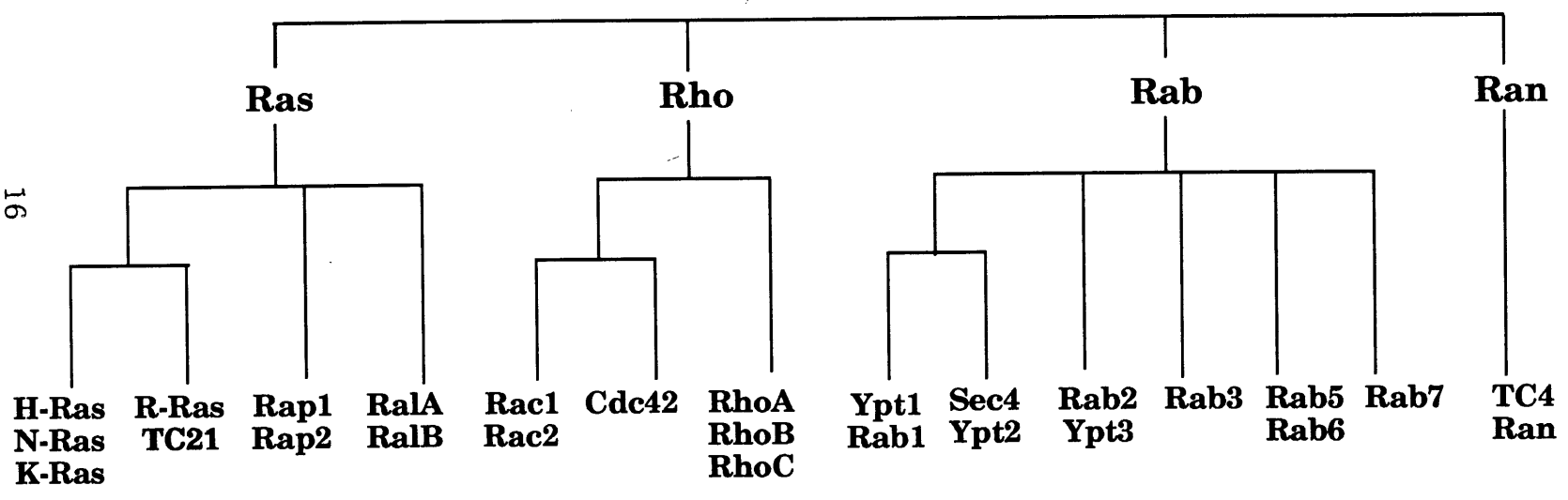

Fig. 1. The Ras-related GTPases. Adapted from Valencia et al., 1991.

work in this thesis focuses on Ras and two of its immediate family members, RalA and RalB, I will further describe only the members of the Ras family. These closely related proteins (50 - 60% identical to Ras) comprise the Ras (H-ras, K-ras, and N-ras), Rap (Rap1A, Rap1B, Rap2A, Rap2B), R-ras (R-ras and TC21), and Ral (RalA and RalB) subfamilies.

Ras. The discovery of the H-*ras* and K-*ras* genes is described above. N-*ras* was isolated from a neuroblastoma cell line (Hall et al., 1983; Shimizu et al., 1983). Oncogenic forms of these proteins display similar biological effects.

ras genes have been identified in organisms other than mammals. In the yeast *Saccharomyces cerevisiae*, the *RAS1* and *RAS2* genes activate adenylate cyclase (Broek et al., 1985; Uno et al., 1985). In the fission yeast *S. pombe*, the *ras1* gene (also known as *ste5*) is not required for viability but is essential for mating (Fukui et al., 1986; Nadin-Davis et al., 1986). In nematodes, the *let-60 ras* gene is required for viability and functions in the inductive signaling pathway that initiates vulva formation (Han and Sternberg, 1990; Beitel et al., 1990). In *Drosophila*, the *Dras1* gene appears to mediate signaling by the Sevenless receptor tyrosine kinase (Simon et al., 1991). Studies of Ras function in these organisms have made critical contributions to our understanding of Ras regulation and signal transduction.

Rap. The first gene identified in this family was the *Drosophila ras3* (*Dras3*) gene (Neuman-Silberberg et al., 1984). This gene has since been renamed *Rap1* (Hariharan et al., 1991). Human *rap1A* and *rap2A* were cloned by Pizon and coworkers (1988) by screening a library with the *Drosophila Rap1* gene. The human and fly genes are 88% identical (Hariharan et al., 1991). *rap1A* was subsequently identified as the v-K-*ras* reverting gene, K-*rev*1 (Kitayama et al., 1989) and as a small G-protein found in brain dubbed *smg* p21 (Kawata et

al., 1988). The Rap proteins share approximately 50% amino acid sequence identity with the Ras proteins (Pizon et al., 1988).

In *Drosophila*, the *Roughened* mutation affects the *Rap1* gene (Hariharan et al., 1991). Flies heterozygous for this mutation have rough eyes, and a substantial fraction (54%) of the ommatidia in these rough eyes are missing the R7 photoreceptor (Hariharan et al., 1991). Since Ras signaling is required for the differentiation of the R7 cell (Simon et al., 1991), these observations suggest that Rap is an antagonist of Ras function in R7 development.

In *S. cerevisiae*, the *rap* homolog *BUD1* (also known as *RSR1*) functions in bud site selection (Bender and Pringle, 1989; Chant and Herskowitz, 1991). Mutations in *BUD1* lead to random budding. *BUD1* and *CDC24* gene products interact functionally and possibly physically. The Cdc24 protein is a putative exchange factor for Cdc42, a member of the Rho family (Hart et al., 1991). CDC24 and CDC42 function in the formation of the bud (Chant et al., 1991). Thus, in *S. cerevisiae*, a Rap protein may regulate the action of a Rho protein, CDC42.

R-ras. The human R-*ras* gene was cloned by low-stringency screening of a library with a v-Ha-*ras* probe (Lowe et al., 1987). R-ras is 55% identical to H-ras (Lowe et al., 1987) and is stimulated by rasGAP (Garrett et al., 1989). Mutant R-*ras* does not transform cells (Lowe and Goeddel, 1987). R-ras was recently to shown to interact via its C-terminus with Bcl2, a protein that regulates apoptosis (Fernandez-Sarabia and Bischoff, 1993). The mammalian TC21 and *Drosophila Dras2* genes are also members of the R-*ras* subfamily (Valencia et al., 1991).

Ral. *ralA*, like *rap1A*, was cloned by screening a library with a *ras* oligonucleotide (Chardin and Tavitian, 1986). Polakis et al. (1989) subsequently cloned *ral* while purifying GTP-biding proteins. Human *ralA* and a new *ral* gene, *ralB*, were identified by screening a human library with the simian *ralA* probe (Chardin and Tavitian, 1989). RalA and RalB proteins are 85% identical. The function of the *ral* genes is unknown. Mutant *ral* genes corresponding to activated *ras* alleles do not transform fibroblasts (Frech et al., 1990).

A possible clue to Ral regulation comes from recent work on Ras. Several groups using a yeast two-hybrid system to screen for genes whose products interact with the Ras protein unexpectedly found *ralGDS* and a *ralGDS*-like gene (S. Demo and L. Williams, personal communication; the original cloning and characterization of the *ralGDS* gene is described in Chapter 2 of this thesis). At least one of these groups has shown that the affinity of ralGDS is greater for the GTP-bound form of Ras than for the GDP-bound form and that this interaction requires an intact Ras effector domain (S. Demo and L. Williams, personal communication), consistent with the idea that Ras regulates ralGDS, which would activate Ral. The significance of the interaction between Ras and ralGDS is unknown.

Clearly, much work will be required before we can understand the function and regulation of Ral. How will this be done? For Ras, the effort took numerous workers and more than a decade of weaving together studies of mammalian cells and tumors, yeast, flies and worms. Unfortunately, *ral* genes have not been cloned or detected in non-mammalian organisms. Thus, without the benefit of genetic systems to help dissect Ral signaling, I expect that the

most successful approaches in this field will focus on identifying proteins that interact with Ral and its regulators (ralGDS and ralGAP).

Regulatory proteins

The low molecular mass GTPases act as molecular switches, cycling between their active GTP-bound and inactive GDP-bound forms (Fig. 2). This cycling is regulated by at least two classes of enzymes. GTPase activating proteins (GAPs) inactivate the GTPase by stimulating GTP hydrolysis. Exchange factors, which are also known as guanine nucleotide dissociation stimulators (GDSs), activate GTPases by accelerating the release of bound GDP, thereby allowing GTP to bind since it is in molar excess in the cell. Changes in both exchange factor and GAP activity have been observed in cells stimulated in a variety of ways (Downward et al., 1990a; Gibbs et al., 1990; Li et al., 1992; Zhang et al., 1992; Medema et al., 1993; Buday and Downward, 1993b). I will briefly describe these two classes of regulators.

GTPase Activating Proteins (GAPs). The study of Ras regulators began as an effort to understand the consequences of oncogenic mutations. Using the DNA transfection assay, researchers identified a mutated H-*ras* gene in the EJ cell line. *ras* genes mutated at positions 12, 59, and 61 were subsequently identified in other human tumors (reviewed in Barbacid, 1987). The identification of these mutant alleles raised two important questions. What effect did these single amino acid changes have on Ras function? How did these changes in Ras function contribute to oncogenesis?

Early analyses of oncogenic forms of Ras revealed that these mutants possessed reduced rates of GTP hydrolysis *in vitro* (McGrath et al., 1984; Sweet et al., 1984; Gibbs et al., 1985; Colby et al., 1986). These observations

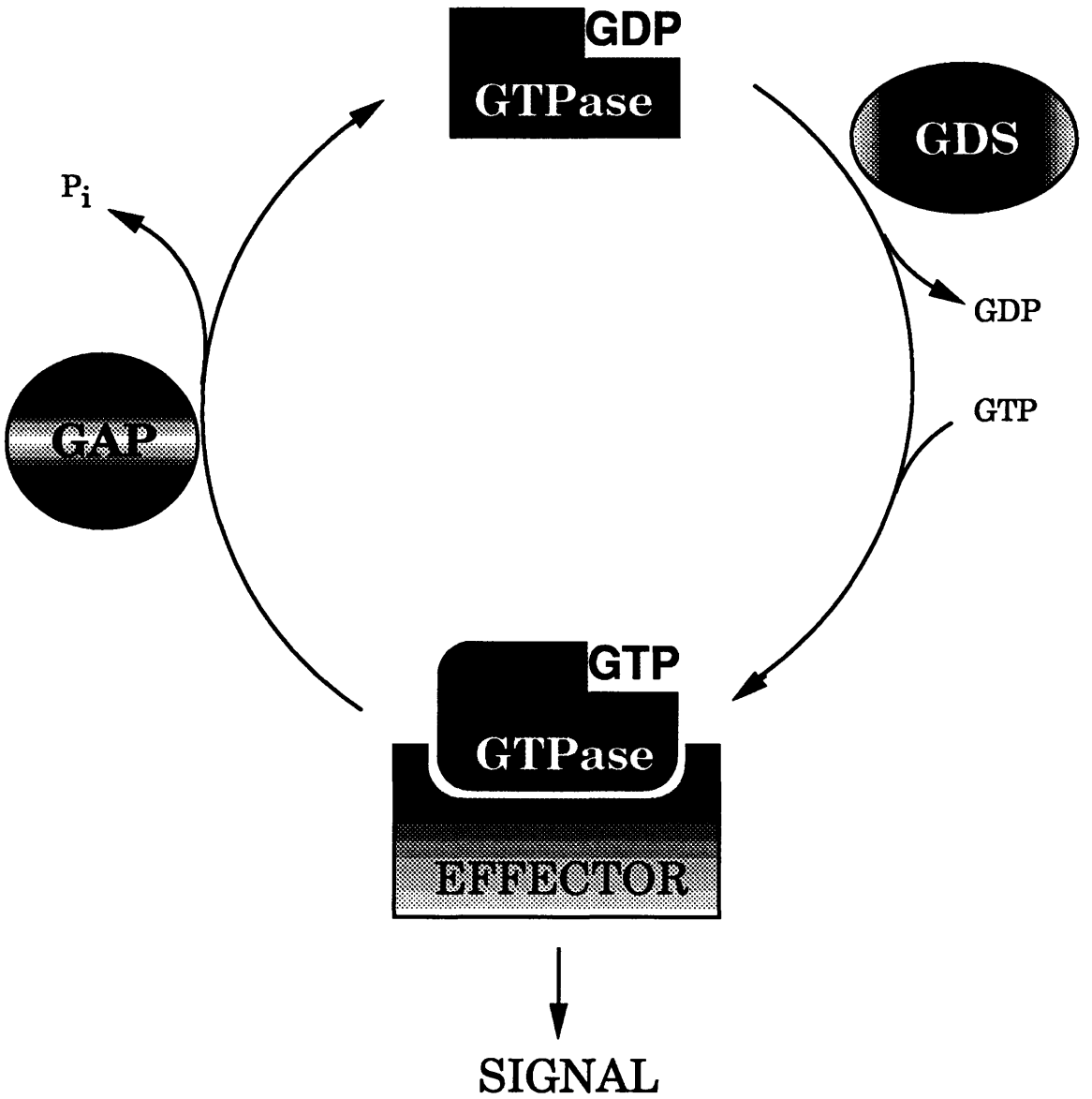

Fig. 2. The GTPase cycle. The cycling of GTPases between their active GTP-bound and inactive GDP-bound forms is regulated by GAPs and GDSs.

lead to a model in which the GTP-bound form of the Ras protein is active and oncogenic mutations, which impair the GTPase activity, render a greater fraction a cell's Ras molecules GTP-bound and active.

Subsequent biochemical studies of mutant Ras proteins, however, undermined the tight correlation between GTPase rate and transforming potential (Der et al., 1986; Lacal et al., 1986; Trahey et al., 1987). In one study, no correlation was found between GTPase activity and transforming potential in seventeen mutant proteins with substitutions at amino acid 61 (Der et al., 1986). All of the mutant proteins displayed similarly lower rates of GTP hydrolysis (eight- to ten-fold lower) than wild-type Ras but the transforming potential of these mutants varied by over 1000-fold. These data suggest that decreased GTPase activity is not sufficient to explain the oncogenic potential of Ras mutants.

Similarly, Lacal and coworkers (1986) reported that the Thr-59 Ras mutant possesses a GTPase activity comparable to the wild-type protein, but has a potent transforming activity, comparable to the Lys-12 mutant. These data suggest that decreasing the intrinsic rate of GTP hydrolysis of Ras proteins is not necessary for oncogenic transformation.

Trahey et al. (1987) reported similar discrepancies: while Val-12 and Asp-12 mutants differ in GTPase activity (12% and 43% of wild-type, respectively), the mutant gene products possess comparable transforming potential. Thus, by 1987 the failure to correlate GTPase activity and transforming potential led some researchers to conclude that the properties of Ras proteins measured *in vitro* did not adequately account for their effects *in vivo* (Trahey and McCormick, 1987). It was later shown that some oncogenic mutants with wild-type rates of GTPase hydrolysis (e.g., Thr-59) have

substantially increased rates of nucleotide release that allow quick reactivation of the GDP-bound form of Ras (Feig and Cooper, 1988).

Clearly, activating mutations should increase the amount of active (GTP-bound) Ras in the cell. One group (Gibbs et al., 1987) tested whether activating mutations actually increased the amount of GTP-bound Ras *in vivo* by expressing normal and oncogenic Ras in yeast and determining the relative proportions of GDP and GTP bound. They reported that wild-type yeast Ras1 and Ras2 proteins were bound entirely to GDP, while an increased proportion of the activated Ras1 and Ras2 mutant proteins were GTP-bound (16% ± 4% and 33% ± 10%, respectively). Surprisingly, a substantial fraction (38% ± 10 %) of the wild-type mammalian Ras expressed in yeast was GTP-bound, comparable to the fraction (33% ± 10 %) of GTP bound to activated yeast Ras2 (Gibbs et al., 1987). These observations were the first demonstration that activating mutations increased the amount of GTP-bound Ras *in vivo*, although it was unclear why so much mammalian Ras was GTP-bound.

Trahey and McCormick (1987) solved this puzzle by identifying GAP, a protein that interacts with Ras to determine the amount of Ras·GTP in the cell. GAP stimulates GTP hydrolysis by normal but not mutant Ras proteins. rasGAP activity was also demonstrated in mammalian cell extracts (Trahey and McCormick, 1987) but not yeast cells (Sigal, 1988), explaining why so much of the mammalian Ras in yeast cells was GTP-bound.

The identification of rasGAP activity answered the question of what oncogenic mutations do to *ras* gene products. Trahey and McCormick (1987) concluded their seminal Science paper with the observation that "the major role of Asp12 and Val12 mutations is to prevent interaction with GAP, and that the effects of these mutations on intrinsic GTPase activities measured in

vitro are not biologically significant." The discovery of GAP also inaugurated the study of Ras-regulating proteins.

rasGAP's ability to convert Ras to its inactive, GDP-bound form suggests that rasGAP is an upstream, negative regulator of Ras (Trahey and McCormick, 1987). rasGAP may also function downstream of Ras since it binds the Ras effector domain (Adari et al., 1988; Calés et al., 1988). Currently, the status of rasGAP as a biologically relevant Ras effector is controversial (for reviews of the conflicting evidence, see Hall, 1990b; Wigler, 1990; Medema and Bos, 1993).

Numerous GAPs for Ras proteins have been identified. In mammalian cells, rasGAP (also called p120GAP) and neurofibromin, the product of the NF1 gene, stimulate the GTPase activity of Ras (Boguski and McCormick, 1993). *S. cerevisiae* contains two rasGAPs, IRA1 and IRA2, which have catalytic domains homologous to mammalian rasGAP (Tanaka et al., 1990). In *Drosophila*, Gap1 appears to negatively regulate Ras1 activity in R7 photoreceptor development (Gaul et al., 1992).

GAPs for other GTPases have also been identified. A rapGAP has been identified (Rubinfeld et al., 1991). This protein is not related to rasGAP. ralGAP activity has been identified in brain lysates but the protein responsible for this activity has not been identified (Emkey et al., 1991).

A group of related proteins, including Bcr, n-chimaerin, p190, Grb1/p85, 3BP-1, and a *Drosophila* protein whose gene lies in the Rotund locus, appear to act as rhoGAPs (reviewed in Boguski and McCormick, 1993). rabGAP activities have been identified for Ypt1 (Tan et al., 1991) and Sec4 (Walworth et al., 1992), and a GAP specific for Rab6 has been cloned (Strom et al., 1993).

This protein, called GYP6, is not related to other known GAPs (Strom et al., 1993).

Exchange Factors. The deactiavating effects of GAPs on GTPases in the cell are counterbalanced by the activating effects of guanine nucleotide dissociation stimulators (GDSs). GDS proteins, also known as exchange factors, stimulate the release of GDP from Ras-related proteins, thereby allowing GTP to bind. These proteins appear to mediate upstream signals to the GTPase.

Guanine nucleotide exchange activities were first demonstrated for factors in mammalian cell extracts (West et al., 1990; Wolfman and Macara, 1990; Downward et al., 1990b). It was later shown that yeast *SDC25* and *CDC25* gene products, which activate Ras *in vivo*, could stimulate guanine nucleotide release from Ras (Créchet et al., 1990; Jones et al., 1991). A *Drosophila* rasGDS, known as Son of sevenless (Sos), was identified by its role in receptor tyrosine kinase signaling (Simon et al., 1991; Bonfini et al., 1992). Sos also has been shown to have GDS activity (Egan et al., 1993; Chardin et al., 1993; Buday and Downward, 1993a; Liu et al., 1993). Several other exchange factors have been isolated by virtue of their amino acid similarity to known GDSs, such as Cdc25 and Sos (Shou et al., 1992; Wei et al., 1992; Bowtell et al., 1992; Albright et al., 1993).

Many of the exchange factors identified to date are related to Cdc25 (see Chapter 2 of this thesis). This family of GDS proteins now includes at least ten members: the *S. cerevisiae* proteins Cdc25 (Camonis et al., 1986; Broek et al., 1987; Robinson et al., 1987), Sdc25 (Boy-Marcotte et al., 1989), Bud5 (Chant et al., 1991; Powers et al., 1991), and Lte1 (Wickner et al., 1987); the *S. pombe* protein Ste6 (Hughes et al., 1990), the *Drosophila* protein Sos (Simon et al.,

1991); (Bonfini et al., 1992), and the mammalian proteins RasGRF (also known as $CDC25^{Mm}$; Martegani et al., 1992; Shou et al., 1992; Wei et al., 1992), ralGDS (Albright et al., 1993), and Sos1 and Sos2 (Bowtell et al., 1992). Each of these Cdc25-like proteins is specific for the GTPases in a subfamily of the Ras family. Cdc25, Ste6, Sos, and RasGRF are GDSs for Ras proteins. Sdc25 acts on Ras *in vitro* and, when activated by removing the N-terminus, can replace Cdc25 *in vivo*. Bud5 is the exchange factor for the *S. cerevisiae* Rap homolog, Bud1. ralGDS acts on Ral proteins. The GTPase target for Lte1 is unknown.

A second group of posssible exchange factors, the Dbl family, has also been identified. This family includes Dbl (Eva et al., 1988), Vav (Adams et al., 1992), Bcr (Hariharan and Adams, 1987), RasGRF (Shou et al., 1992), Ect2 (Miki et al., 1993), and the *S. cerevisiae* protein Cdc24 (Miyamoto et al., 1987). Dbl is not related to members of the Cdc25 family of exchange factors but has been shown to catalyze nucleotide exchange from a Rho protein, CDC42Hs (Hart et al., 1991). Because Dbl acts on a Rho protein, it was originally postulated that this unique group of proteins might be the exchange factors for the Rho and Rac GTPases (for review, see Boguski and McCormick, 1993). Recently, however, Vav has been shown to act as a GDS for Ras (Gulbins et al., 1993). It is currently unkown whether proteins in this group other than Dbl and Vav actually possess GDS activity, and possible GTPase targets have not been identified.

Another exchange factor, smgGDS, is not related to either Cdc25 or Dbl. Unlike the Cdc25-related GDSs, which stimulate nucleotide release from non-prenylated forms of their targets, smgGDS only reacts with isoprenylated forms of its targets (Mizuno et al., 1991). smgGDS also differs from the Cdc25-

related GDSs, which are specific for GTPases within a subfamily, in its broad specificity. smgGDS acts on specific targets from several families: Rap1A, Rap1B, K-Ras, RhoA, and Rac1 (Kaibuchi et al., 1991; Mizuno et al., 1991; Hiraoka et al., 1992).

Another class of GDS proteins acts on members of the Rab family. Two rabGDSs have been cloned. The *S. cerevisiae* protein Dss4 is a GDS for Sec4 and, to a lesser extent, Ypt1 (Moya et al., 1993). Dss4 does not act on the related GTPase, Rab3A. The mammalian Mss4 protein stimulates nucleotide release from Sec4, Ypt1, and Rab3A (Burton et al., 1993). These proteins are not related to other GDS proteins, but they do share two short but highly conserved stretches of amino acid sequence (Burton et al., 1993).

The final class of GDSs is specific for members of the Ran/TC4 family. The mammalian Rcc1 protein is a GDS for Ran (Bischoff and Ponstingl, 1991) Rcc1 was originally identified by its role in regulating chromosome condensation (Ohtsubo et al., 1987). The *S. pombe* protein Pim1 is homologous to Rcc1 and is involved in the coupling the end of DNA synthesis with the onset of mitosis (Matsumoto and Beach, 1991). The Rcc1/Pim1 GDSs are not related to other classes of GDS proteins.

Concluding remarks

The *ras* genes, which were first identified as retroviral oncogenes, have been studied extensively. Numerous Ras and Ras-related proteins have been identified in a variety of species. These small GTPases act as switches in regulating many important cellular processes.

The study of Ras and related proteins led to the identification of several proteins that regulate GTPase cycling. These regulatory proteins include

GAPs and GDSs. GAPs deactivate GTPases by stimulating GTP hydrolysis. GDSs activate GTPases by stimulating nucleotide exchange.

In this thesis, I explore the function and regulation of GDS proteins. Chapter 2 describes the cloning and characterization of a mammalian exchange factor, ralGDS. In Chapter 3, I examine the features of a GTPase that allow specific activation by its GDS protein. In the Appendix, I present work describing how the Ras exchange factor, Sos, relays information from the upstream signaling machinery to Ras.

Acknowledgments

I would like to thank Susan Demo for sharing her unpublished observations on the interaction between Ras and ralGDS.

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CHAPTER 2

Characterization of a guanine nucleotide dissociation stimulator for a *ras*-related GTPase

Preface

This chapter represents work I did with Dr. Charles F. Albright, Joana Liu, and Maja Vito in the laboratory of Professor Robert A. Weinberg. I was involved in all phases of this work. My contributions to this study include cloning and sequencing the ralGDS PCR fragment, assisting in the screening of libraries to isolate cDNA clones, performing the sequence comparisons of Cdc25 family members, assisting in immunoprecipitation studies of the ralGDS protein, generating and characterizing ralGDS baculoviruses using transfer vectors prepared by Dr. Albright, and working out the purification of recombinant ralGDS with Dr. Albright. I was involved in critically interpreting data and assisted in the preparation of the manuscript and figures.

This chapter was previously published in the January 1993 issue of the European Molecular Biology Organization Journal, vol. 12, pp.339-347.

The EMBO Journal vol.12 no.1 pp.339 - 347, 1993

Characterization of a guanine nucleotide dissociation stimulator for a *ras*-related GTPase

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Communicated by P.A.Sharp

***ras*-related GTPases participate in signaling for a variety of cellular processes. The GTPases cycle between a GTP-bound active state and a GDP-bound inactive state. This cycling is partially controlled by guanine nucleotide dissociation stimulators (GDS, also known as exchange factors). We report on the molecular cloning of cDNAs encoding a new mammalian GDS protein, using sequences derived from the yeast *ras* GDS proteins as probes. The encoded protein stimulates the dissociation of guanine nucleotides from the *ras*-related *ralA* and *ralB* GTPases at a rate at least 30-fold faster than the intrinsic nucleotide dissociation rate. This new GDS, *ralGDS*, is at least 20-fold more active on the *ralA* and *ralB* GTPases than on any other GTPase tested, including other members of the *ras* family (H-*ras*, N-*ras*, K-*ras*, R-*ras*, *rap1a* and *rap2*), members of the *rho* family (*rhoA*, *rhoB* and *CDC42*-Hs) and members of the *rab* family (*rab3a* and *ypt1*). While the *ralGDS* protein is phosphorylated on serine residues, we find no evidence that phosphorylation affects the activity of insect cell-expressed *ralGDS* towards the *ralA* or *ralB* GTPase. The 3600 nucleotide *ralGDS* mRNA and the 115 kDa protein were found in all tissues and cell lines examined.**

Key words: *CDC25*/*ral*/*ralGDS*/*ras*

Introduction

Low molecular mass GTPases constitute a family of proteins that participate in signaling for many cellular functions including cell cycle progression, differentiation, cytoskeletal organization, protein transport and secretion (Bourne *et al.*, 1990; Hall, 1990). The most well known of these are the proteins encoded by the *ras* gene family. In performing their various cellular functions, GTPases cycle between a GTP-bound form and a GDP-bound form (Bourne *et al.*, 1991). The ratio of these two forms is largely controlled by two classes of enzymes: guanine nucleotide dissociation stimulators (GDS, also known as exchange proteins), which accelerate the release of the bound GDP allowing GTP binding (Lowy *et al.*, 1991) and GTPase activating proteins (GAP), which stimulate the hydrolysis of the bound GTP by GTPases. We focus here on the GDS proteins. It is thought that by stimulating GTP binding, the GDS proteins convey an incoming, activating signal to the GTPases.

Most GDS genes can be assigned to one of two gene families. The *CDC25* family consists of the *Saccharomyces cerevisiae* genes *CDC25* (Camonis *et al.*, 1986; Broek *et al.*, 1987; Robinson *et al.*, 1987), *SDC25* (Boy-Marcotte *et al.*, 1989), *BUD5* (Chant *et al.*, 1991; Powers *et al.*, 1991) and *LTE1* (Wickner *et al.*, 1987), the *Schizosaccharomyces pombe* gene *ste6* (Hughes *et al.*, 1990), the *Drosophila melanogaster* gene *Sos* (Simon *et al.*, 1991; Bonfini *et al.*, 1992) and the mammalian genes *CDC25*^{Mm} (Martegani *et al.*, 1992), *Ras-GRF* (Shou *et al.*, 1992), m*Sos-1* and m*Sos-2* (Bowtell *et al.*, 1992). The proteins encoded by these genes are known or suspected to interact with the *ras* GTPase, with the exception of *BUD5* and *LTE1*. The most likely target of *BUD5* is *RSR1* (Chant *et al.*, 1991), the probable *S.cerevisiae* homologue of *rap1* (Bender and Pringle, 1989).

A second class of GDS proteins consists of the mammalian genes *dbl* (Eva *et al.*, 1988), *vav* (Adams *et al.*, 1992), *bcr* (Hariharan and Adams, 1987), *Ras-GRF* (Shou *et al.*, 1992) and the *S.cerevisiae* gene *CDC24* (Miyamoto *et al.*, 1987). The *dbl* protein has GDS activity for the *CDC42*-Hs GTPase (Hart *et al.*, 1991) while the target GTPases of the related proteins are still unknown. Two other GDS genes do not fit in one of these two families. One of these proteins, *smgGDS*, has GDS activity for *rhoA*, K-*ras4b*, *rap1a*, *rap1b* and *rac1* GTPases (Kaibuchi *et al.*, 1991; Mizuno *et al.*, 1991; Hiraoka *et al.*, 1992). A second protein, *rcc1*, has GDS activity for the *ran* GTPase (Bischoff and Ponstingl, 1991).

The various GDS proteins appear to be important regulators of GTPase function. They and GAPs are potential targets for receiving signals that increase the GTP-bound fraction of the GTPases. Thus, increasing GDS activity or decreasing GAP activity can increase the amount of the GTPase binding GTP. There is evidence from the *ras*-signaling pathway that both mechanisms are used. In T-cell signaling, it appears that inhibition of GAP activity is largely responsible for *ras* activation (Downward *et al.*, 1990). In fibroblasts, *ras* activation by growth factors appears due to increased GDS activity (Gibbs *et al.*, 1990; Zhang *et al.*, 1992). In PC-12 pheochromocytoma cells, both an increase in GDS activity and an increase in GAP activity are observed in response to NGF treatment (Li *et al.*, 1992).

In vitro assays and genetic analysis also suggest that GDS proteins are themselves subject to regulation. In one case where rasGDS activity was demonstrated *in vitro*, this activity depended on the presence of phosphatase inhibitors, suggesting that phosphorylation of the GDS protein or a regulatory factor was needed for activity (Wolfman and Macara, 1990). The phenotypes of various mutant GDS genes also suggest that the activity of GDS proteins is regulated. Deletions and point mutations in the *CDC25* gene (Broek *et al.*, 1987; Kim and Powers, 1991), and in the *SDC25* gene (Damak *et al.*, 1991), deletions in the *dbl* gene (Eva *et al.*, 1988; Ron *et al.*, 1988) and a mutation in the *Sos* gene (Rogge *et al.*, 1991; Bonfini *et al.*, 1992) all

display phenotypes consistent with increased GDS activity. As a consequence, GDSs appear to serve as signal transducers that link still poorly characterized upstream signals to the various GTPases.

Our goal is to understand how mammalian GDS proteins are regulated by extracellular signals. Toward this end, we decided to isolate genes encoding mammalian GDS proteins. To do so, we exploited a polymerase chain reaction (PCR) strategy to generate GDS probes using regions conserved between the *CDC25* and *ste6* proteins, two GDSs known to regulate *ras* proteins in *S.cerevisiae* and *S.pombe*, respectively. Among the clones isolated in this fashion was one that encodes a mammalian protein having great similarity with the yeast exchange proteins and able to catalyze nucleotide dissociation on a mammalian GTPase.

Results

Isolation of a mammalian GDS cDNA

Degenerate primers corresponding to conserved regions of *CDC25* and *ste6* were designed and used to PCR-amplify total cDNA from 3T3/ΔXB, a mouse cell line. These genes

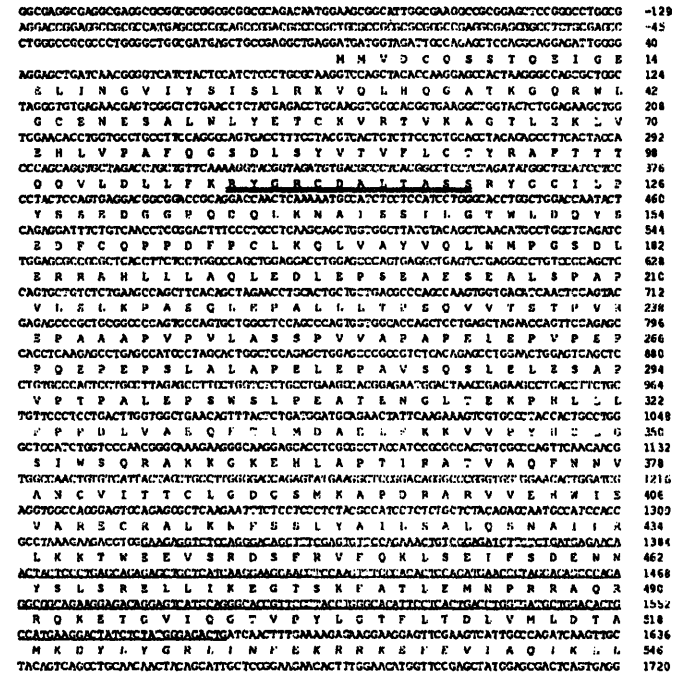

Fig. 1. Nucleotide and predicted protein sequence of *ralGDSa*. The nucleotide and predicted protein sequence for the *ralGDSa* are shown. The underlined sequence is the original fragment isolated by PCR amplification. The predicted *ralGDSb* protein sequence differs from the predicted *ralGDSa* protein sequence in several respects: the amino terminus contains an additional 55 amino acids of sequence MVQRMWAEASGPIGGPEPL-FPGSRRSRSVWDAVRLEVGVPDSCPVVLHSPTQLDPDLPRLE (SSTQ...), the 12 amino acids doubly underlined are deleted, amino acids 241 and 242 are deleted, the dipeptide PA is added after amino acid 221 and several conservative substitutions are made.

derive from two species of yeast that are as related to each other as yeast are to mammals (Moreno *et al.*, 1991). Consequently, we expected sequences conserved between these proteins to be functionally important and therefore conserved in their mammalian homologues. When the major product of this reaction was cloned and sequenced, it was found to have significant similarity to a number of known or suspected GDS proteins (data not shown). This cDNA product was used as a probe to isolate the entire cDNA from a 3T3 library. Its sequence is shown in Figure 1. We have designated this cDNA as *ralGDSa* for reasons that will be discussed.

Northern blot analysis revealed a single mRNA of ~3600 nucleotides (Figure 2A). This size is in good agreement with the size of the cDNA isolated by us. Northern blot analysis of total RNA from rat tissues revealed that the gene was transcribed in all tissues examined (Figure 2B). Furthermore, this mRNA has also been found in all of the 21 mammalian cell lines examined (data not shown).

The structure of the cDNA is unusual in that the first ATG codon in the long open reading frame is preceded by three ATG codons in other reading frames (Figure 1). Each of the three out-of-frame ATG codons is followed by a stop codon before the in-frame ATG is reached. In addition, the 5′ non-coding region is unusually long (212 nucleotides) and has a high percentage of G-C base pairs (80%). Most mammalian mRNAs have 5′ non-coding regions between 20 and 100 nucleotides and <10% have upstream ATG codons (Kozak, 1987).

A second cDNA clone from a rat fibroblast cDNA library was isolated using the coding region of *ralGDSa* as a probe. While the reading frame encoded a sequence of 895 amino acids, which was 99% homologous with the protein encoded by the previously cloned mouse cDNA, this rat cDNA had an unrelated 5′ non-coding region of 201 nucleotides. The rat cDNA also encoded an additional 55 amino acids at the N-terminus, an internal addition of two amino acids, internal deletions of two and 12 amino acids and several conservative substitutions. We have designated this cDNA as *ralGDSb*. To test the possibility that the two cDNAs represent alternatively spliced transcripts from the same gene, Northern blot analysis was performed with probes specific to the mouse 5′ non-coding region and the rat 5′ non-coding region. This analysis revealed that both regions are part of an ~3600 nucleotide mRNA and both are found in the same cell line (data not shown).

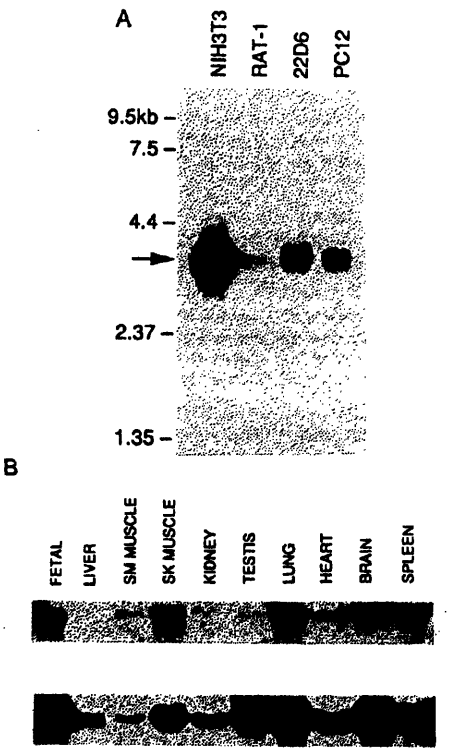

Fig. 2. Northern blot analysis of *ralGDS*. (A) Poly(A) mRNA from the stable cell lines NIH3T3, Rat-1, 22D6 (Alt *et al.*, 1981) and PC12 were analyzed by Northern blot analysis. The arrow indicates the location of the 3600 nucleotide mRNA found in all cell lines. (B) Total RNA from the indicated rat tissues were analyzed by Northern blot analysis. The upper panel was hybridized with a probe for *ralGDSa* and the lower panel was hybridized with a probe for α-tubulin to estimate the relative amount of *ralGDS* mRNA in each sample. A signal corresponding to the 3600 nucleotide mRNA with the *ralGDSa* probe could be seen in all the lanes on prolonged exposure.

Relationship of ralGDS to other members of the CDC25 family

When the predicted GDS protein sequence was used to search the protein sequence database, we found that the sequence of *ralGDS* was similar to those of proteins belonging to the *CDC25* family of GDS proteins and to several proteins rich in glutamate, serine and proline. Regions of proteins rich in proline, glutamate, serine and threonine residues, known as PEST sequences, have been found in several proteins with short half-lives (Rogers *et al.*, 1986). PEST sequences are clustered in two regions of the *ralGDS* sequence: amino acids 196–318 contain 56% PEST residues and amino acids 557–728 contain 51% PEST residues.

To explore further the similarity of *ralGDS* with other members of the *CDC25* family, we used the MACAW computer program (Schuler *et al.*, 1991), which finds statistically significant blocks of continuous amino acids shared between multiple protein sequences. We found six blocks of sequence similarity present in most members of the *CDC25* family (Figure 3). Block 1 was missing from *CDC25^{Mm}*, *BUD5* and *LTE1*. *LTE1* was also missing block 6 and contained only part of block 5. Previous work with *CDC25* (Camonis *et al.*, 1986; Broek *et al.*, 1987), *SDC25* (Boy-Marcotte *et al.*, 1989), *CDC25^{Mm}* (Martegani *et al.*, 1992) and *Ras-GRF* (Shou *et al.*, 1992) suggests that blocks 2 through 6 are necessary and sufficient for GDS activity. Interestingly, only *ralGDS*, *Sos*, m*SOS-1* and m*Sos-2* have extensive coding regions to the C-terminal side of these similar regions.

Sizing of the ralGDS gene product

To determine whether our cDNA would produce a protein of the same size as that produced *in vivo*, anti-*ralGDS* sera were produced. A portion of the *ralGDSa* cDNA including conserved blocks 1–6 was introduced into a bacterial expression vector and the resulting protein was used to immunize rabbits. When serum from an immunized rabbit and its preimmune serum were incubated with a lysate of

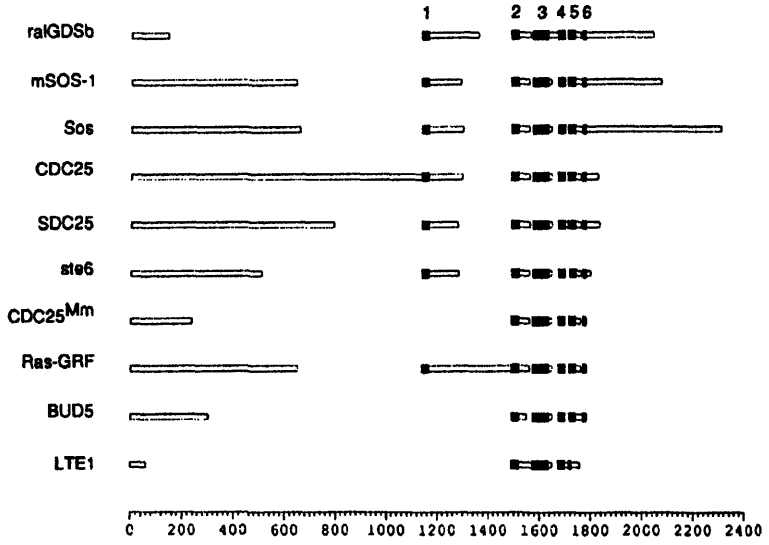

Fig. 3. Sequence comparison of *ralGDS* with the *CDC25* family. The computer program MACAW (Schuler *et al.*, 1991) was used to identify blocks of conserved sequence between *CDC25* family members. Each protein is illustrated as a horizontal box with the gene name to the left of the box. The ruler at the bottom of the figure represents the number of amino acids. Hatched regions indicate blocks which represent statistically significant similar regions found in seven or more of the proteins. These blocks are numbered above the diagram. The hollow boxes represent the protein sequence of unrelated regions of these proteins.

mouse cells metabolically labeled with [^{35}S]methionine, a protein of 115 kDa was detected in the immunoprecipitate with the immune serum, but not with the preimmune serum (Figure 4). When *ralGDSa* was synthesized by *in vitro* transcription/translation, a protein of 113 kDa was produced (Figure 4). Since the plasmid used to direct *in vitro* transcription lacked 5′ and 3′ non-coding sequences, the predicted reading frame is capable of generating a protein of virtually the same size as that produced *in vivo*. The *ralGDS* proteins of 113–115 kDa apparent mass are larger than the 95–99 kDa mass predicted by the primary sequence.

The small size discrepancy between the protein made by *in vitro* translation and the protein immunoprecipitated from cell lines probably results from both the alternative splicing of exons and post-translational modification. For instance, *ralGDSb* contains an additional 43 amino acids, relative to *ralGDSa*, which leads to an apparent increase in protein mass of 1 kDa (data not shown). Post-translational modifications, which will be discussed later, also lead to an apparent increase in mass of 2 kDa. For these reasons, we believe both the *ralGDSa* and *ralGDSb* cDNAs are authentic and full length.

Stimulation of nucleotide release by ralGDS

In order to determine the target GTPases of our GDS protein, several GTPases were tested with *ralGDSb* in a filter-binding assay. GTPases were purified from GTPase-expressing *Escherichia coli*, labeled by binding with radioactive guanine nucleotides and the extent of nucleotide dissociation was determined in the presence or absence of purified *ralGDSb*. These tests showed that the *ralGDSb* is highly specific for the *ralA* and *ralB* GTPases (Figure 5). Under conditions where 90% of the GDP bound to *ralA* or *ralB* was released, the *ralGDSb* had no apparent effect on other GTPases of the *ras* subfamily (H-*ras*, N-*ras*, K-*ras-4b*, R-*ras*, *rap1a* and *rap2*), the *rho* subfamily (*rhoA*, *rhoB and CDC42*-Hs) and the *rab* subfamily (*rab3a* and *YPT1*). Since we would be able to detect a 10% change in the bound nucleotide in our assay, we can estimate that the *ralGDS* is at least 20-fold more active on the *ralA* and *ralB* GTPases than any other GTPase tested. No differences in the GTPase specificity or specific activity were observed between *ralGDSa* and *ralGDSb* (data not shown).

The purified *ralGDSb* was able to stimulate significantly the release of guanine nucleotides from the *ralB* GTPase *in vitro* (Figure 6A). Using high levels of purified *ralGDSb* we saw a >30-fold increase in the GDP dissociation rate relative to reactions without *ralGDSb*. Even at the highest level of *ralGDSb* protein tested, the GDP dissociation rate continued to be proportional to the amount of *ralGDSb* added to the reaction. We suspect that the maximum rate of stimulation of nucleotide release is much greater than 30-fold.

We have also shown that the reaction we are following represents nucleotide dissociation and not an alternative reaction, such as proteolysis of the GTPase, by demonstrating that *ralGDSb* stimulates nucleotide association to the same extent that it stimulates nucleotide dissociation (Figure 6B). In these reactions, the *ralB* GTPase with bound, non-radioactive GDP is incubated with free, radioactive guanine nucleotides. The amount of radioactive guanine nucleotide bound to *ralB* is then determined following incubation in the presence or absence of *ralGDSb*. *ralGDS* stimulates nucleotide release from the GDP-bound form of the *ral* GTPase ~3-fold more effectively than from the GTP-bound form (data not shown).

Purification of ralGDS protein

To obtain large amounts of purified *ralGDS*, we used baculovirus vectors that express the recombinant GDS proteins at levels corresponding to 15–25% of the total

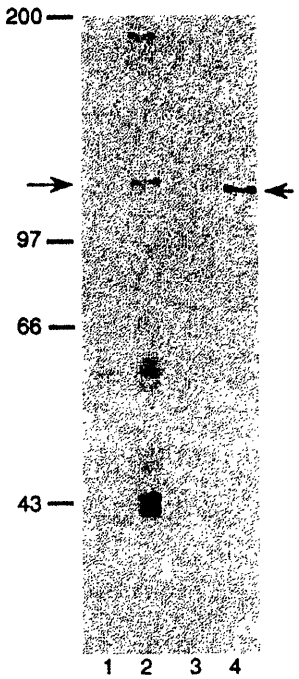

Fig. 4. Immunoprecipitation of *ralGDS*. The cell line MOLT-4 was metabolically labeled with [^{35}S]methionine and extracted protein was immunoprecipitated with either preimmune (lane 1) or immune (lane 2) serum. The 115 kDa protein representing *ralGDS* is indicated by the arrow to the left of the figure. *In vitro* transcription of pralGDSa/pBSK-2, followed by *in vitro* translation in the presence of [^{35}S]methionine and immunoprecipitation was performed with preimmune serum (lane 3) or immune serum (lane 4). The 113 kDa band representing *ralGDSa* is indicated by the arrow on the right of the figure. Other specific proteins are also seen following immunoprecipitation of protein from radiolabeled cell lines, such as the 190 kDa protein. We do not know whether these proteins cross-react with the serum or precipitate due to association with *ralGDS*.

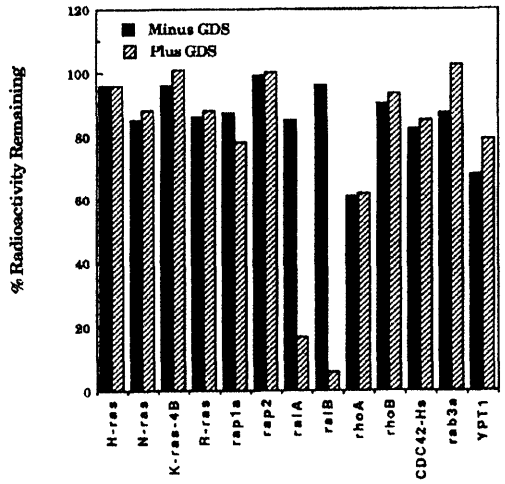

Fig. 5. Effect of *ralGDSb* on nucleotide dissociation rates of several GTPases. GTPases were loaded with radiolabeled nucleotides and incubated with or without *ralGDSb*. The products were separated by filtration and the filter-bound radioactivity was determined. Background filter-bound nucleotide levels were subtracted from the total filter-bound radioactivity and these values were normalized to the initial amount of filter-bound nucleotide. Data points represent the average of 2–4 assays, but are representative of several assays.

soluble protein of infected insect cells. *ralGDS* was purified from infected insect cells using sequential chromatography on a hydroxyapatite column, a gel filtration column and an ion exchange column. Samples of the starting material and purified fractions, which were analyzed by SDS–PAGE, are shown in Figure 7. During the purification, *ralGDS* was tracked by monitoring GDS activity, using the *ralA* GTPase as a substrate and by Coomassie staining of SDS gels. In all cases, the location of GDS activity and the 113–115 kDa protein were coincident, suggesting that the measured activity was indeed due to the *ralGDS* protein.

Unexpectedly, the protein eluted as two separate peaks from the hydroxyapatite column (data not shown). The protein that eluted at a lower phosphate concentration, which we designate peak I, migrates slightly faster on SDS gels than the protein that elutes at the higher phosphate concentrations, which we designate peak II (Figure 7). Both peaks eluted with an apparent molecular mass of 200 kDa on the gel filtration column. Furthermore, the specific activities of the purified proteins were similar (Table I). Based on data that we discuss below, we suspect that the peak II protein is more phosphorylated than the peak I protein.

Modification of ralGDS

The slight differences in mobility on SDS gels and the increased affinity for hydroxyapatite resin displayed by the protein in peak II relative to peak I, were consistent with the hypothesis that *ralGDS* is a phosphoprotein. To test this hypothesis directly, COS-7 cells were transiently transfected with an expression vector for *ralGDSa*, metabolically labeled with [^{35}S]methionine or [^{32}P]orthophosphate and *ralGDS* was immunoprecipitated. This experiment revealed that the *ralGDS* contains covalently bound phosphate (Figure 8).

To determine the nature of the phosphorylation, immunoprecipitated protein was treated with calf intestinal alkaline phosphatase, which hydrolyzes phosphomonoester linkages. Phosphatase treatment of immunoprecipitated *ralGDS* removed all detectable ^{32}P-labeled protein, but did not decrease the signal from ^{35}S-labeled protein (Figure 8). The phosphatase action was largely inhibited by orthophosphate, a competitive inhibitor of alkaline phosphatase. Interestingly, phosphatase treatment also resulted in a slight increase in the mobility of *ralGDS* on SDS gels, suggesting that peak I and peak II differ in the extent of phosphorylation. This shift in mobility was also seen when the purified peak II protein was treated with phosphatase (data not shown).

To analyze further the nature of the phosphate linkages, ^{32}P-labeled protein was purified, subjected to acid hydrolysis and the resulting products were separated by two-dimensional electrophoresis. This analysis revealed the presence of products that comigrated with phosphoserine and orthophosphate (data not shown). Phosphotyrosine could not be detected by Western blotting with antibodies that were specific to phosphotyrosine using *ralGDS* immunoprecipitated from several normal and tyrosine kinase-transformed mammalian cell lines (data not shown).

Phosphatase treatment of peak I and peak II did not, however, result in any change in the specific activity of the

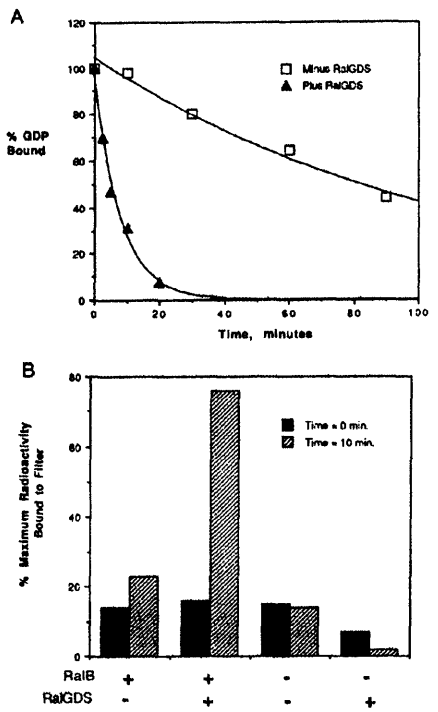

Fig. 6. *In vitro* activity of *ralGDS* towards the *ralB* GTPase. (A) The amount of [^{3}H]GDP-bound *ralB* GTPase was determined in a filter binding assay in the presence or absence of *ralGDSb* as indicated. The curves drawn represent the best-fit curves assuming first-order kinetics for nucleotide dissociation. The k_{off} values are 0.004/min without *ralGDSb* and 0.054/min with *ralGDSb*. Background levels of radiolabeled nucleotides were determined in separate incubations and these values were subtracted from the total radioactivity bound to the filter to determine the GTPase bound nucleotide. Data points represent the average of two assays and are representative of several replicates. (B) The effect of *ralGDSb* on the nucleotide association rate was determined by filter binding assay in the absence or presence of *ralB* and *ralGDSb* as indicated. Data points represent the average of two assays. The maximal binding was determined by allowing the reaction to reach equilibrium.

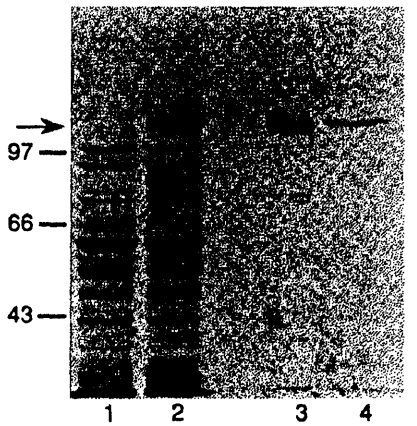

Fig. 7. Purification of *ralGDS* from recombinant baculovirus-infected insect cells. Samples of lysates from wild type baculovirus infected insect cells (lane 1), *ralGDSa* baculovirus infected cells (lane 2) and purified fractions from peak I (lane 3) and peak II (lane 4) were separated by SDS–polyacrylamide gel electrophoresis and identified by Coomassie staining. The 115 kDa *ralGDSa* protein is indicated with an arrow. Minor bands of smaller size in the purified preparations represent proteolytic fragments.

Table I. Summary of *ralGDS* purification

Sample	Volume (ml)	Protein (mg)	Activity (%)	Specific activity
Lysate	100	208	100	0.48
Peak I				
Hydroxyapatite	45	41	40	0.97
Gel filtration	28	14.8	25	1 70
Mono Q	2.25	6.7	14.3	2.17
Peak II				
Hydroxyapatite	50	11	18	1.64
Gel filtration	35	2.9	6	2.06

purified *ralGDS* (data not shown). This is consistent with the specific activities of peak I and peak II being very similar and with the notion that the observed phosphorylation of *ralGDS* does not control GDS activity.

Discussion

We report on the isolation and characterization of mammalian cDNAs that encode a protein that stimulates guanine nucleotide release for the *ral* GTPase. This protein is a new member of the *CDC25* family of GDS proteins. Two very similar *ralGDS* cDNAs, probably derived from alternatively-spliced exons, were isolated. If the internal deletion of 12 amino acids in *ralGDSb*, relative to *ralGDSa*, represents an additional alternative splicing event, then more transcripts may exist. Variant forms of other mammalian GDS cDNAs have also been found. The *CDC25^{Mm}* gives rise to at least two mRNAs and the sequence of the smaller mRNA is very similar to the sequence of the *Ras-GRF* gene (Martegani *et al.*, 1992; Shou *et al.*, 1992). Likewise, there are at least two homologues of the *D.melanogaster Sos* gene in mammals (Bowtell *et al.*, 1992). Since we have found no difference in the substrate specificity or specific activity of the two forms of the *ralGDS*, we speculate that these subtle variants may respond to distinct regulatory signals.

The physiological role of the *ralGDS* is unclear since little is known about the function of the *ral* GTPase in the cell. The *ral* GTPase was initially identified based on the similarity of its gene sequence to those of other low molecular mass GTPases. Subsequently, it was found by purifying and analyzing proteins that bind GTP (Chardin *et al.*, 1986; Polakis *et al.*, 1989). The *ral* GTPase is widely distributed in mammalian tissues (Olofsson *et al.*, 1988) as is *ralGDS*. Transfection of vectors expressing the *ralGDS* into mammalian cells did not result in transformation, but appeared to give rise to cell lines having altered cellular morphology or motility (unpublished observations). These phenomena are being studied in the hope that they will provide insight into the *in vivo* function of the *ral* GTPase.

Our *in vitro* studies revealed the *ralGDS* shows at least 20-fold more stimulation of the nucleotide dissociation rate for the *ralA* and *ralB* GTPases than for any other GTPase tested. Since the *ralA* and *ralB* GTPases differ primarily in

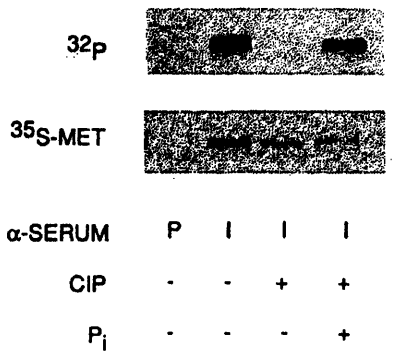

Fig. 8. Phosphatase treatment of COS cell-expressed *ralGDSa*. Transiently transfected COS-7 cells were metabolically labeled with [^{32}P]orthophosphate (upper panel) or [^{35}S]methionine (lower panel) and extracted proteins were immunoprecipitated with preimmune (P) or immune (I) serum. Some samples were incubated with calf intestinal phosphatase (CIP) and with or without 100 mM phosphate (Pi) as indicated. All samples were then separated by SDS gel electrophoresis and developed by fluorography.

their C-terminal regions (Chardin and Tavitian, 1989), the specific recognition by the *ralGDS* of the *ral* GTPases is unlikely to involve their C-terminal regions. The extent of *ralGDS* stimulation was at least 30-fold higher than the intrinsic nucleotide dissociation rate. Since only 5% of a cellular GTPase is typically loaded with GTP (Downward *et al.*, 1990; Gibbs *et al.*, 1990), the *in vivo* activity of GAPs must typically exceed that of corresponding GDSs by as much as 20-fold. We have no direct measure of either of these *in vivo* activities acting on the *ral* GTPases. However, we note that rasGAP can stimulate GTP hydrolysis of the *ras* GTPase by 100 000-fold *in vitro* (Gideon *et al.*, 1992). A corresponding activity by a ralGAP, taken together with the above calculation, suggests that the observed 30-fold stimulation of nucleotide release *in vitro* is a gross underestimate of the true intrinsic activity.

Our work suggests that the *ralGDS* has activity in the absence of additional proteins, post-translational modifications and cofactors. This basal activity is, however, relatively low in comparison to the sensitivity of our assay. About 1 μg of *ralGDS* protein is required to release 50% of the nucleotide from the *ral* GTPase in our standard assay. This is very similar to the amount of *Ras-GRF* needed to perform a similar amount of catalysis (Shou *et al.*, 1992), but is ~20-fold more that the amount of SDC25 protein needed to perform this amount of catalysis (Verrotti *et al.*, 1992). In comparison, <10 ng of rasGAP would catalyze the hydrolysis of 50% of the *ras*-bound GTP to GDP in the same assay. The low basal activity of GDS proteins and their relatively low intracellular levels probably explain the difficulty in detecting and purifying these proteins.

The low basal activity of the *ralGDS* protein provides ample opportunity for upregulation. In fact, the phenotypes of cells bearing mutations in *CDC25, SDC25* and *Sos* genes suggest that this family of GDS proteins may be activated by mutations. Such mutations may inactivate negative regulatory signals or amplify positive regulatory signals. Given the finding that phosphatase inhibitors were needed to maintain rasGDS activity in lysates (Wolfman and Macara, 1990), a simple model envisions the inactivation of negative regulatory regions by direct phosphorylation of the GDS protein. Our data with *ralGDS* does not, however, provide any support for this model since we found no difference in the activity of phosphorylated and unphosphorylated *ralGDS*. These findings do not exclude the possibility that other critical residues that were not phosphorylated in our preparation are involved in controlling *ralGDS* activity. More likely, the phosphorylation of *ralGDS* is used to control other features of signaling, such as its intracellular localization or association with other proteins. Determining if *ralGDS* contains negative regulatory regions and how they function *in vivo* may provide insight into signaling through the *CDC25* family of proteins.

Materials and methods

PCR cloning of a fragment of ralGDS

Approximately 3 μg of mRNA from 3T3/ΔXB, a transformed mouse cell line (Jackson and Baltimore, 1989), was denatured at 70°C for 10 min and reverse transcribed at 42°C for 1 h in a 20 μl volume reaction containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 units RNAsin (Promega), 2.5 mM DTT, 1 mM dNTPs (Pharmacia), 200 units Superscript reverse transcriptase (Gibco-BRL) and 200 ng random hexamers (Pharmacia). 2 μl of the reverse transcription reaction mixture were then amplified by PCR with the 100 pmol of oligonucleotides 5-1HI (5'-GGG-GGATCCCA(T/C)(A/C)GN(T/C)TN(A/C)GNAA(A/G)ACNTGG-3') and 3-3RI (5'-GGGGAATTCC(G/T)(T/C)TT(A/G)TC(A/G)AA(A/G)T-T(A/G/T)AT-3') for 30 cycles (1 min at 95°C, 1 min at 50°C, 2 min at 72°C) with Vent polymerase (New England Biolabs) according to manufacturer's instructions. Oligonucleotides 51HI and 3-3RI were based on the *ste6* protein sequences 766–772 and 836–841, respectively (Hughes *et al.*, 1990). The PCR products were purified using Prep-A-Gene (Bio-Rad) and 5% of the product was amplified for an additional 30 cycles. The major product of 300 bp was isolated, digested with *Bam*HI and *Eco*RI and cloned into pBS$^+$ (Stratagene).

cDNA cloning and DNA sequence analysis

The cloned PCR product was labeled with [α-^{32}P]dCTP and used to screen a cDNA library from mouse 3T3 fibroblasts in lambda zap (Stratagene) using standard conditions (Sambrook *et al.*, 1989). Three independent cDNA fragments were identified from a library of ~10^6 phage. Since preliminary DNA sequencing and Northern blot analysis suggested that only a partial cDNA was isolated, a cDNA library from the mouse pre-B cell line, 22D6, was screened using a [α-^{32}P]dCTP labeled DNA probe corresponding to nucleotides 744–1195. Five independent lambda phage were isolated from ~10^6 phage and the cDNA inserts were cloned into pBSK (Stratagene). Restriction mapping was consistent with all of the clones representing partial cDNAs from a single mRNA. Two of the clones from the 3T3 library, representing nucleotides 744–3361 and one clone from the 22D6 library, representing nucleotides −212 to 2679, were sequenced by the dideoxy-chain termination method using reagents from United States Biochemical Corp. Sequencing was done on some clones created by nested deletions and with some sequence-derived oligonucleotides. DNA sequence data was assembled using programs from the University of Wisconsin Genetics Group (Devereux *et al.*, 1984) and protein sequence databases were searched at NCBI using the Blast network search (Altschul *et al.*, 1990). These cDNA clones were used to reconstruct a full-length cDNA in pBSK, designated pralGDSa/pBSK. A rat clone, *ralGDSb*, was obtained by screening a cDNA library from rat 208F fibroblasts. A single positive clone from 4×10^5 plaques was isolated and sequenced as before.

Northern blot analysis

Poly(A) mRNA was prepared from cell lines harvested in mid-log phase growth according to the proteinase K–SDS method (Badley *et al.*, 1986). Approximately 5 μg of mRNA were separated by electrophoresis, transferred to nitrocellulose membrane and processed by standard methods. The blot was hybridized with a [α-^{32}P]dCTP-labeled probe corresponding to nucleotides 744–1195 of *ralGDSa*. Total RNA from rat tissues was prepared as described (Chirgwin *et al.*, 1979). Approximately 40 μg of total RNA from each tissue were separated and probed as above.

Generation of antibodies

Rabbits were immunized with a fusion protein that consisted of 60 kDa of *ralGDSa*. A fragment corresponding to nucleotides 163–1820 was

generated by PCR amplification of pralGDSa/pBSK using the oligonucleotide primers 5'-CGGGATCC ATGGCTACC TGCAAGGTG CGCACA-3' and 5'-GCGAATTC AGTCAGTCA CTCCAGCGC TTGACAATG-3'. This DNA fragment was digested with *Bam*HI and *Eco*RI and ligated into pET-5a (Novagen). The resulting vector, pralGDSa/pET-5a, was transformed into BL2 (DE3)pLysS (Novagen) and protein was produced following IPTG induction. Inclusion bodies were prepared and further purified by SDS gel electrophoresis. Gel slices containing ~200 μg of protein were injected into rabbits on a monthly schedule (Harlow and Lane, 1988). Sera were collected and stored.

Recombinant baculovirus production

Recombinant baculovirus vectors for *ralGDSa* and *ralGDSb* were constructed by first inserting the cDNAs into the transfer vector, pEV55 (Miller *et al.*, 1986; Miller, 1987). The 5' non-coding region of *ralGDSa* was removed by oligonucleotide directed site-specific mutagensis. Single-stranded DNA containing uracil was made from pralGDSa/pBSK and mutated using the oligonucleotide 5'-CGAGGTCGA CCGTATCGG ATCCATGGT AGATTGCCA GAGCT-3' and the Mutagene Kit (Bio-Rad) to create pralGDS/pBSK-2. This oligonucleotide created a *Bam*HI site upstream of the initiator methionine. This vector was used for *in vitro* transcription with the T7 RNA polymerase and *in vitro* translation with rabbit reticulocyte lysates according to the manufacturer's instructions (Promega). The *ralGDSa* coding sequence was removed from this vector by digesting with *Aat*II, which cuts one nucleotide after the stop codon, attaching *Eco*RI linkers and digesting with *Eco*RI and *Bam*HI. The resulting DNA fragment was ligated into pEV55, which had been digested with *Bgl*II and *Eco*RI to create pralGDSa/pEV55. The *ralGDSb* transfer vector was made by digesting pralGDSb/pBSK with *Nco*I, which cleaves at the initiator codon, attaching *Bam*HI linkers, digesting at *Aat*II, which cleaves one nucleotide after the stop codon, attaching *Eco*RI linkers and digesting the resulting DNA with *Bam*HI and *Eco*RI. This DNA was ligated into pEV55, which had been cut with *Bgl*II and *Eco*RI to generate pralGDSb/pEV55. Recombinant baculoviruses were produced by cotransfecting *Spodoptera frugiperda* Sf 9 cells with the corresponding transfer vectors and linear AcNPV DNA (Invitrogen) and purified by plaque assay (Summers and Smith, 1987).

Purification of GTPases

Low molecular mass GTPases were purified from *E.coli*, overexpressing these proteins (H-*ras*, N-*ras*, K-*ras4b*, *rap1a*, *rap2*, *rhoA*, *rhoB*, *ypt1* and *rab3a*) or obtained in purified form (*CDC42*-Hs) as previously described by Settleman *et al.* (1992). An expression vector for R-*ras* was constructed by PCR amplification of cDNA from HT1080 cells. The oligonucleotide primers included *Nde*I and *Bam*HI sites to facilitate cloning of the PCR product into the expression vector pET-5c. The authenticity of the clone was confirmed by DNA sequencing.

Assays for GTPase nucleotide dissociation and association

For quantifying GDS activity during protein purification, the release of [α-^{32}P]GTP from *ralA* or *ralB* was quantified by a nitrocellulose filtration assay. Approximately 2 μg of purified *ralA* or *ralB* were incubated in 200 μl of exchange buffer [50 mM Tris (pH 7.5), 10 mm EDTA, 5 mM $MgCl_2$ and 1 mg/ml BSA] with 10 μCi/ml [α-^{32}P]GTP (3000 Ci/mmol) for 20 min at 37°C. This reaction was quenched with 200 μl of stop exchange buffer [50 mM Tris (pH 7.5), 15 mm $MgCl_2$ and 1 mg/ml BSA] and diluted into 6 ml of reaction buffer [20 mM Tris (pH 7.5), 2 mM $MgCl_2$, 1 mg/ml BSA, 0.1 mM DTT and 0.1 mM GDP]. This mixture can be used for several days if it is stored on ice. The dissociation reaction was initiated by combining 5–20 μl of column fraction or buffer with 80 μl of the reaction mixture containing [α-^{32}P]GTP-labeled *ral* at 37°C. The reaction was quenched after 10 or 20 min with 0.5 ml of stop buffer [50 mM Tris (pH 7.5) and 10 mM $MgCl_2$] and immediately filtered through nitrocellulose filters (BA85, 0.45 μM, Schleicher and Schuell). The filter was washed with 10 ml of stop buffer and the radioactivity remaining on the filter was determined. This assay is linear with time for at least 30 min and is linear with respect to the amount of GDS protein until ~50% of the bound nucleotide is removed.

For testing the the ability of *ralGDSa* to catalyze nucleotide dissociation with other GTPases, 5 μl of G-protein (*ralA*, *ralB*, H-*ras*, N-*ras*, *rap1a*, *rap2*, *R-ras*, *CDC42*-Hs, *rab3a and ypt1*) representing 0.5–5 μg were equilibrated with 7 μCi [^{3}H]GDP (11.4 Ci/mmol) and 15 μl of exchange buffer as previously described. This reaction was quenched with 15 μl of stop exchange buffer and added to 700 μl of reaction buffer. 100 μl of [^{3}H]GDP-bound GTPase was incubated with 5 μl of purified *ralGDSb* (1 mg/ml) or buffer for 10 min at 37°C and processed as above. Some GTPases were not present in adequate amounts to make the [^{3}H]GDP assays possible. In these cases (*rhoA*, *rhoB and* K-*ras4b*), 10 μCi of [α-^{32}P]GTP were used.

For measuring the association of nucleotides with *ralB*, 2 μl (0.5 mg/ml) of purified *ralB* were combined with 48 μl of 25 mM Tris (pH 7.5), 1 mg/ml BSA, 0.2 μM GDP, 100 μM AMP-PNP and 2 mm $MgCl_2$ for 5 min at 37°C to ensure that all active *ralB* contained a bound GDP. 1 μl of [γ-^{35}S]GTPγS (0.5 mCi/ml, 250 μM) was added to this mixture followed by the addition of 1 μl of purified *ralGDSb* (1 mg/ml) or buffer. At the designated times, the reactions were quenched with 250 μl of stop buffer and filtered as above.

Purification of ralGDS

Trichoplusia ni High Five cells (Invitrogen) were seeded at a density of $\sim 2\times10^7$ cells per 150 mm dish and infected with ~1–5 PFU recombinant baculovirus per cell. Cells from ~50 dishes were harvested 3 days after infection, washed once with phosphate-buffered saline and stored at −80°C until use. Frozen cell pellets were resuspended and homogenized in 100 ml ice-cold lysis buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin and 10 μg/ml leupeptin]. The lysate was clarified by centrifugation for 1 h at 100 000 *g* and applied to a hydroxyapatite (Bio-Rad) column (2.5 cm×10 cm) that had been equilibrated with lysis buffer. The column was washed with 50 ml of lysis buffer and eluted with a linear gradient from 0–50% buffer B (lysis buffer with 600 mM KP_i) over 250 ml, a linear gradient from 50–100% buffer B over 50 ml and 100% buffer B for 50 ml. 5 ml fractions were collected and 5 μl were used to locate *ralGDS* activity in a 20 min reaction as previously outlined. *ralGDS* activity eluted as two symmetric peaks at ~100 mM P_i and at 600 mM P_i. The peaks were pooled separately and concentrated. The low phosphate pool (peak I) was diluted 2-fold with lysis buffer, Triton X-100 was added to 0.002% and the sample was concentrated 10-fold by ultrafiltration (Amicon YM-30) to ~4 ml. The high phosphate pool (peak II) was concentrated by ammonium sulfate precipitation. 250 mg of solid ammonium sulfate was added to each millilitre of sample, equilibrated for 30 min and the precipitate collected by centrifugation for 20 min at 10 000 *g*. The ammonium sulfate pellet was dissolved in 4 ml of gel filtration column buffer (lysis buffer, 50 mM NaCl and 0.02% Triton X-100). Each peak was individually applied to a gel filtration column (Sepharose CL-4B, 2.5×90 cm) and 4 ml fractions were collected at a flow rate of ~25 ml/h. *ralGDS* activity was located by assaying 10 μl aliquots for 10 min and *ralGDS* protein was localized by Coomassie staining of SDS gels. Both peak I and peak II eluted from the gel filtration column as single symmetric peaks with an apparent size of 200 kDa when apoferritin and BSA were used as standards. Active fractions from the gel filtration column were pooled and further purified with a Mono Q column (Pharmacia). The column was developed with a linear gradient from 0–1 M NaCl in lysis buffer at 0.4 ml/min. 0.5 ml fractions were collected and assayed as above. *RalGDS* eluted as a single symmetric peak at ~350 mM NaCl. Active fractions were pooled and stored at 4°C for several weeks or quick frozen and stored at −70°C.

COS-7 cell expression and immunoprecipitation

An expression vector for COS-7 cells was made by ligating the *Bam*HI to *Eco*RI DNA fragment, which contained *ralGDSa* coding sequence and was prepared as previously described in construction of the baculovirus transfer vector, into pSVE, which had been digested with *Bam*HI and *Eco*RI. 10 μg of the resulting vector, pralGDSa/pSVE or pSVE were transfected using the DEAE-dextran technique (Lopata *et al.*, 1984) into COS-7 cells, which had been seeded on the previous day at 2.5×10^6 cells per 100 mm dish. Two days later the cells were labeled for 3 h in methionine-free media with 10% dialyzed calf serum and 200 μCi/ml Trans-^{35}S-label (ICN) or in phosphate-free medium with 5% dialyzed serum and 200 μCi/ml [^{32}P]orthophosphate (NEN). The cells then were washed twice with cold PBS, harvested by scraping and lysed for 30 min on ice in PBS containing 1% Triton X-100, 1 mM EDTA, 5 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Insoluble material was removed by centrifugation at 390 000 *g* for 10 min and the resulting lysate was precleared by incubation with 40 μl of protein A–agarose (Biomd) for 30 min. The beads were removed by centrifugation and 10 μl of immune or preimmune sera were added for 45 min. 40 μl of protein A–agarose were added and the samples were rotated for 45 min. The beads were collected and washed four times with 50 mM Tris (pH 7.5), 0.1% Triton X-100, 1 mM EDTA, 150 mm NaCl, 1 mM Na_3VO_4 and 5 mM β-glycerophosphate. Samples that were treated with phosphatase were washed 2 more times with 20 mM Tris (pH 8.0), 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, incubated with 20 units of calf intestinal alkaline phosphatase (2500 units mg^{-1}, Sigma) for 20 min at 37°C with or without 100 NaP_i and washed twice with 50 mM NaP_i. Samples were then heated with SDS sample buffer, separated by SDS gel and analyzed by fluorography.

Acknowledgements

We thank Jasper Rees and Richard Hynes for the 3T3 library, Marjorie Oettinger, David Schatz and David Baltimore for the 22D6 library, Xiao Fan Wang for the 208F library and Jeffrey Settleman for the total RNA blot. We thank A.Hall, R.Cerione, A.Wittinghofer, I.Leroscy, J.de Gunzburg, A.Tavitian, P.Chardin and N.Segev for generously providing plasmids for GTPase expression in *E.coli*. We thank Jeffrey Settleman, Piotr Sicinski, Sean Egan and David Shaywitz for a critical reading of the manuscript. C.F.A. is supported by American Cancer Society Fellowship PF-3350, B.W.G. is supported by NIH Training Grant 5T32-CA0941. R.A.W. is an American Cancer Society Professor. This work was supported by a NIH Outstanding Investigator Grant 35-CA39826.

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Received on September 11, 1992; revised on October 7, 1992

CHAPTER 3

Residues in the switch II region of the Ral GTPase are required for interaction with its exchange factor, ralGDS

ABSTRACT

The exchange factor for the Ral GTPases, ralGDS, catalyzes nucleotide release from the RalA and RalB proteins but not from closely related GTPases. This study examines the amino acid residues of Ral that allow its exchange factor to distinguish it from closely related gene products. We constructed chimeric Ral/Ras GTPases and tested the ability of Ral and Ras exchange factors to catalyze guanine nucleotide release from these chimeric molecules. We report that residues 60 - 87 in Ral (corresponding to amino acids 49 - 76 in Ras) are necessary for the stimulation of nucleotide release from Ral by its exchange factor. These residues include the switch II region, the conformation of which differs dramatically between the GDP- and GTP-bound forms of the GTPase. We constructed a mutant Ras protein carrying the switch II region of Ral that is responsive to ralGDS. These data suggest that amino acids in the switch II region are important determinants of exchange factor recognition of GTPases.

INTRODUCTION

The Ras-related low molecular mass GTPases regulate a number of diverse cellular functions: cell cycle progression, cellular differentiation, cytoskeletal organization, protein transport, and secretion (Bourne et al., 1990; Hall, 1990). These proteins act as molecular switches, cycling between their active GTP-bound and inactive GDP-bound forms. A number of proteins that regulate Ras and its relatives have been identified (for review, see Boguski and McCormick, 1993; Lowy and Willumsen, 1993). These regulatory proteins include GTPase activating proteins (GAPs), which inactivate the GTPase by stimulating GTP hydrolysis, and exchange factors, which are also known as guanine nucleotide dissociation stimulators (GDSs) and which accelerate the release of the bound GDP. Thus, the quantity of GTPase in the active, GTP-bound state can be increased by increasing the GDS activity or by decreasing the GAP activity.

We previously cloned cDNAs that encode a mammalian GDS protein, ralGDS, that actively catalyzes guanine nucleotide release from the RalA and RalB gene products but not from the H-*ras*, N-*ras*, K-*ras*, R-*ras*, Rap1a, Rap2, RhoA, RhoB, CDC42Hs, Rab3a, or YPT1 gene products (Albright et al., 1993). Other groups have shown that the mammalian Ras exchange factors Ras-GRF and mSos-1 are similarly specific for Ras proteins but do not act on closely related, low molecular mass GTPases (Shou et al., 1992; Buday and Downward, 1993).

The Ras and Ral proteins are about 50% identical at the amino acid sequence level (Chardin and Tavitian, 1986). What does ralGDS "see" that allows it to distinguish between its target, Ral, and a close relative such as

Ras? To answer this question, we constructed chimeric Ral/Ras molecules and tested whether ralGDS or a catalytic fragment of a Ras exchange factor (GTCD-1, the catalytic domain of the *S. cerevisiae* protein CDC25; Lai et al., 1993) could catalyze guanine nucleotide exchange on these chimeric GTPases.

RESULTS

Synthesis and characterization of *ral*B/H-*ras* chimeric gene products

We constructed chimeric Ral/Ras GTPases using the polymerase chain reaction (PCR; Maruta et al., 1991). These chimeric proteins were expressed in bacteria and purified. We were able to construct a series of soluble Ral/Ras chimeras containing progressively greater lengths of RalB at their N-termini (Figure 1B), but all of a series of Ras/Ral chimeras carrying any of the N-terminus of H-Ras were insoluble in our expression system (data not shown). Four of these chimeric GTPases (BHλ2, BHλ3, BHλ4, and BHλ5) have intrinsic nucleotide off-rates similar to Ras and Ral (Fig. 2). We assume that interactions with the guanine nucleotide and the ability to release nucleotide are not significantly altered in chimeras with nucleotide off-rates similar to the parental GPTases. One chimera, BHλ1, has a nucleotide off-rate that is approximately three times faster than that of Ras (Fig. 2C).

We tested the Ral/Ras chimeras to determine whether ralGDS or a catalytically active fragment of the Ras exchange factor, CDC25 (GTCD-1; Lai et al., 1993) could act on them. These GDS proteins are specific for their target GTPases: ralGDS does not catalyze nucleotide release from Ras (Albright et al., 1993 and data not shown) and GTCD-1 does not catalyze nucleotide release from Ral (data not shown).

ralGDS was as active (38-fold increase) in stimulating release of guanine nucleotide from BHλ5, which carries the N-terminal 87 amino acids of Ral, as it was on the Ral GTPase (30-fold increase; Figure 2). Elimination of only eleven amino acids, however, decreased the ability of ralGDS to stimulate guanine nucleotide release approximately four-fold; ralGDS stimulated guanine

nucleotide release from pBHλ4 about 10-fold (Fig. 2F). Replacement of Ral residues 60 - 76 with amino acids 49 - 65 of Ras (chimera BHλ3) created a GTPase that was barely responsive to ralGDS (Fig. 2E).

None of the chimeras respond to GTCD-1 under conditions in which this GDS increases the rate of nucleotide release from Ras 24-fold (Fig. 2). BHλ1, which releases nucleotide faster than Ras, may fail to respond because of an altered conformation of the nucleotide binding pocket. BHλ2, however, which only has 47 amino acids of Ral, and BHλ3, which has only 59 amino acids of Ral, also failed to respond to GTCD. These chimeric proteins have nucleotide binding half-lives similar to Ras and do not respond to ralGDS, consistent with the notion that BHλ2 and BHλ3 lack key specificity-determining residues for recognition by the Ral exchange factor. We speculate that the amino terminal 59 residues of Ral, which is eleven amino acids longer than the Ras N-terminus, may somehow interfere with the interaction of these chimeras and the Ras exchange factor. Alternatively, some residues among the amino-terminal 36 amino acids of Ras may be required for the interaction with the Ras exchange factor.

These data suggest that Ral residues 60 - 87, corresponding to residues 49 - 76 in Ras, are necessary for specific interaction with the appropriate exchange factor. This region includes the residues comprising the switch II region (amino acids 60 - 76 in Ras); the amino acids in this region of Ras are known to assume substantially different conformations depending upon whether Ras is GDP- or GTP-bound (Milburn et al., 1990; Stouten et al., 1993).

Synthesis and characterization of H-*ras* switch II region mutants

The Ras and Ral proteins have similar switch II region residues, differing only at positions 63, 65, 67, 70, 72, and 74 (amino acid numbers are for Ras). Residues 63, 65, 67, and 70 are well exposed (Milburn et al., 1990) and form part of the epitope for the Y13-259 anti-Ras monoclonal antibody (Sigal et al., 1986). Having shown that residues in this switch II region are necessary for a GTPase to interact with its proper exchange factor, we wanted to see whether this region was sufficient to allow exchange factor interaction and to determine which residues in the switch II region affect the specificity of this interaction. We therefore constructed a Ras vector (pRas/SW2) in which the nucleotides coding for amino acids 59 - 73 were replaced with the corresponding Ral sequences (five amino acid changes). We also constructed mutant Ras vectors carrying Ral residues at positions 63 (glutamic acid to aspartic acid), 65 (serine to alanine), 67 (methionine to isoleucine), 70 (glutamine to asparagine), and 72 (methionine to phenylalanine).

All of the Ras switch II region point mutants have nucleotide binding half-lives similar to Ras (Table I). The Ras/SW2 protein has a decreased nucleotide binding half-life, releasing nucleotide about two-fold faster than Ras. We tested the ability of GDS proteins to stimulate guanine nucleotide release from these mutants. GTCD-1 stimulated nucleotide release from all of the Ras switch II region mutants (Table I), although the Ras/SW2 and RasM67I gene products were significantly less responsive than wild-type Ras. Indeed, Mosteller *et al.* (1994) have also recently shown that the M67I mutant is defective in its ability to interact with CDC25. Thus, position 67 appears important in the interaction between exchange factor and GTPase.

GTCD-1 greatly stimulated nucleotide release from the E63D mutant (118-fold) but the nucleotide off-rate in the presence of GTCD-1 was a third slower than that of Ras or the S65A, Q70N, or M72F mutants. Thus, position 63 may also be important in the interaction between exchange factor and GTPase.

To determine whether ralGDS was active on the Ras/SW2 mutant, we conducted a time-course analysis of nucleotide dissociation. In the experiment shown, ralGDS stimulated the release of GTP from Ras/SW2 six-fold compared to a twelve-fold stimulation for Ral itself (Fig. 3). Thus, changing only five amino acids in Ras created a GTPase at least partially responsive to ralGDS. These data suggest that residues in the switch II region play a central role in mediating the specific interaction between a GTPase and its exchange factor.

We also tested the activity of ralGDS toward the switch II region point mutants. ralGDS failed to stimulate guanine nucleotide exchange from any of the point mutants (data not shown). Presumably, several of these positions are important for the specific recognition of GTPase by exchange factor.

DISCUSSION

Our experiments demonstrate the importance of residues in the switch II region for recognition of a GTPase by its exchange factor. ralGDS is as active on chimeric Ral/Ras molecules as it is on wild-type Ral if the chimeras contain amino acids 60 - 87 of Ral. Furthermore, a mutant Ras protein carrying Ral residues from the switch II region (residues 59 - 73 of Ral) responded to ralGDS. Thus, residues in the switch II region impart at least partial sensitivity to the corresponding GDS protein.

These results are consistent with a growing body of evidence implicating the switch II region in the interaction between a GTPase and its exchange factor. Milburn *et al.* (1990) first suggested that this region might interact with upstream regulators. They speculated that the two regions whose structures differed most between the GDP- and GTP-bound forms of Ras (switch I and switch II) could interact with the upstream and downstream components of the signaling machinery; switch I had already been identified as the effector domain (Sigal et al., 1986; Willumsen et al., 1986). The switch II region had been shown to be dispensable for transformation by constitutively active Ras (Willumsen et al., 1986), consistent with this region interacting with an upstream activator. Furthermore, several amino acids in the switch II region had been shown to interact with the neutralizing antibody Y13-259 (Sigal et al., 1986), which inhibits nucleotide exchange (Hattori et al., 1987).

Studies of *ras* mutants have also implicated the switch II region in exchange factor interaction. Mistou *et al.* (1992) reported that substitutions in switch region I (positions 35 and 38) or II (positions 61, 62, or 63) inhibited the activity of the GDS but did not decrease the affinity of the Ras·GDS

interaction. Mutations at these positions did not significantly affect the intrinsic nucleotide dissociation.

Verrotti *et al.* (1992) reported that amino acid substitutions at positions 80 and 81 of the yeast *RAS2* gene product (positions 73 and 74 in mammalian Ras) impaired SDC25-catalyzed GDP to GTP exchange. Similarly, mutations at positions 75, 76, and 78 have been identified that abolish the growth inhibitory effects of the Asn-17 dominant negative Ras (Quilliam et al., 1994). The Asn-17 mutant protein interferes with Ras signaling by binding and sequestering the exchange factor (Munder and Furst, 1992; Powers et al., 1989). Thus, an Asn-17 protein unable to interact with an exchange factor would no longer interfere with signaling through the wild-type protein.

Beitel *et al.* (1990), working on the *let-60 ras* gene of *C. elegans*, identified recessive mutations affecting codons 37, 66, 75, 103 - 108, 131 - 132, and 136. With the exception of position 37, alteration of these residues does not affect the transforming ability of v-Ha-*ras* (Willumsen et al., 1986; Sigal et al., 1986). Positions 131, 132, and 136 are not highly conserved and are thus less likely to interact with activating proteins. Positions 66, 75, and 103 - 108, however, are highly conserved, not involved in guanine nucleotide binding, and located on the surface (Willumsen et al., 1986); these properties are consistent with residues that interact with the signaling machinery. Amino acids in the 103 - 108 region have been implicated in exchange factor interaction by other workers: replacement of amino acids 101 - 103 has little effect on the ability of activated v-Ha-*ras* to transform cells but cripples the ability of c Ha-*ras* to cooperate with the E5 product of bovine papillomavirus in transformation of NIH 3T3 cells (Willumsen et al., 1991) and Ras products bearing a deletion of amino acids 102 - 108 (but not 101 - 103) could not be activated *in vivo* by

Cdc25 (Segal et al., 1993). These investigators postulated that some of the mutations might affect the interaction with activating proteins.

To determine whether any of the recessive *let-60 ras* mutations affected exchange factor interaction, Howe and Marshall (1993) introduced these changes into wild-type, oncogenic (Asp-12), and dominant negative (Asn-17) *ras*. All of the *let-60 ras* mutations alleviated the growth inhibitory properties of the Asn-17 *ras*, consistent with the idea that some of these positions might be essential for exchange factor interaction. Howe and Marshall (1993) demonstrated that the proteins mutated at position 66 or 75 are stable but cannot be activated by exchange factor. The position 75 mutation, however, affects a hinge region involved in switching conformations, increasing the GTP off-rate. Furthermore, a comparable yeast mutant (RAS2-S82) can be stimulated by SDC25 GDS (Verrotti et al., 1992). The mutation of amino acid 89 or deletion of amino acids 103 - 108 destabilizes the GDP-bound form of the Ras protein. This destabilization is sufficient to explain why position 102 - 103 mutants have a greater effect on the biological activity of cellular Ras. Thus, of the recessive *let-60 ras* mutations, only the alteration of position 66 appears to affect exchange factor interaction.

Recently, Mosteller *et al.* (1994) identified Ras mutations at positions 62, 63, 67, and 69 as intragenic suppressors of the dominant negative Ala-15 mutation; like Asn-17 Ras, Ala-15 Ras is thought to inhibit the activation of normal Ras by tying up all of the exchange factor. The position 62, 63, 67, and 69 mutants do not bind CDC25. Mosteller *et al.* (1994) also demonstrated that mSos1 and CDC25 GDS proteins block binding of the anti-Ras monoclonal antibody Y13-259, consistent with the hypothesis that the Ras exchange factors interact with the Y13-259 epitope. These antibody binding studies

appear to contradict the work of Gross et al. (1992), who demonstrated that Y13-259 co-immunoprecipitates Cdc25 protein from unstimulated yeast cells. It may be significant that in their experiments Gross and co-workers co-immunoprecipitated full-length Cdc25 protein, whose binding is not blocked by Y13-259, whereas the antibody binding experiments of Mosteller *et al.* (1994) used only the smaller catalytic domains of Cdc25 and mSos1, which block binding by Y13-259. These observations could, therefore, suggest that regions outside of the catalytic domain of Cdc25 interact with the GTPase target, possibly increasing the strength of interaction between the molecules, and that this binding may involve parts of the GTPase away from the switch II region.

The growing evidence for exchange factor interaction with the switch II region suggests an attractive model for exchange factor-mediated activation (Milburn et al., 1990; Mosteller et al., 1994). This region, which lies near the γ-phosphate of bound GTP (Fig. 1A), is one of two areas of the Ras molecule that undergoes a conformational change upon the exchange of GTP for GDP. Conceivably, exchange factor interaction with this region could destabilize the interaction between the GTPase and GDP, perhaps by stabilizing the nucleotide-free intermediate (Lai et al., 1993; Haney and Broach, 1994). GTP would then bind, disrupting the complex of the GTPase and exchange factor ((Lai et al., 1993; Munder and Furst, 1992; Mosteller et al., 1994).

MATERIALS AND METHODS

Generation of chimeric GTPases

The chimeric GTPase BHλ4 was generated using two-step PCR as described in Maruta *et al.* (1991). The first step consisted of two PCR reactions to separately amplify the *ral* and *ras* pieces of the chimera. For the *ral* fragment, PCR was performed on pRalB/pET plasmid DNA (Albright et al., 1993) using an oligonucleotide corresponding to amino acids 1 - 6 of RalB and a downstream oligonucleotide. For the *ras* fragment, PCR was performed on pSVEcHrasA plasmid DNA (kindly provided by Dr. Sean Egan, unpublished work) an oligonucleotide corresponding to the last six codons of the *ras* gene and an upstream chimeric oligonucleotide. For the second step, the products of these two reactions were mixed and PCR was performed using an upstream *ral* and a downstream *ras* oligonucleotide. These oligonucleotides contained *Nde*I and *Bam*HI sites to facilitate cloning into pET-5c (Novagen). The authenticity of this chimeric gene was verified by DNA sequencing.

The chimeric GTPases BHλ1, BHλ2, BHλ3, BHλ5 were generated using a similar two-step PCR strategy. The *ral* and *ras* PCR reactions were conducted as for BHλ4 except that the downstream *ras* oligonucleotide corresponded to codons 111 - 116, which are immediately downstream from a unique *Nco*I site. The vector pET5/Ras contains the c-H-*ras* insert from pSVEcHrasA; the insert was isolated by PCR using the oligonucleotides containing NdeI and BamHI sites to facilitate subcloning into pET-5cRI$^-$, a derivative of pET-5c in which the *Eco*RI site has been eliminated by filling in the site with Klenow. The PCR products for the chimeras BHλ1, BHλ2, BHλ3, BHλ5 were digested with *Nde*I and *Nco*I and subcloned into pET5/Ras, which had been digested with

*Nde*I and *Nco*I. The authenticity of these chimeric genes was verified by DNA sequencing.

Generation of Ras mutants

The vector pET5/RasSA was constructed carrying a *Sac*II site after nucleotide 176 of H-*ras* and an *Age*I site after nucleotide 220 using two-step PCR. This vector allows us to replace nucleotides 177 to 220 of the H-*ras* gene, corresponding to amino acids 59 to 74. pET5/RasSA was digested with *Sac*II and *Age*I and phosphorylated oligonucleotides corresponding to the appropriate mutant sequences were ligated into the doubly cleaved vector. To generate pRas/SW2, we used oligonucleotides with the sequences GGGGCAAGAGGACTACGCAGCCATTCGAGATAACTACTTTCGGA (sense) and CCGGTCCGAAAGTAGTTATCTCGAATGGCTGCGTAGTCCTCTTGCCCCG C (antisense) in which the underlined sequences correspond to nucleotides 211 to 253 of the *ral*B gene. The sequences of these mutants were verified by DNA sequencing.

Preparation of low molecular mass GTPases

Ras, Ral, chimeric GTPases, and Ras switch II region mutant gene products were purified from *E. coli* that overexpress these proteins as previously described (Settleman et al., 1992)

Purification of exchange factors

ralGDS was purified as described in Albright *et al.* (1993). pGTCD-1 was

provided by Scott Powers and was expressed as described in Lai *et al.* (1993) and purified as described in Smith and Johnson (1988).

Assays for GTPase Nucleotide Dissociation

Guanine nucleotide dissociation assays were carried out as described in Albright *et al.* (1993) using [α-^{32}]PGTP (3000 Ci/mmol). The rate constants are calculated for best-fit curves assuming first-order kinetics for nucleotide dissociation. These assays are linear with respect to the amount of GDS protein until approximately 50% of the bound nucleotide is removed (Albright et al., 1993; Lai et al., 1993). In experiments to test the effects of the ras exchange factor, GTCD-1, purified GST protein was used in the negative control (no GDS) assays.

FIGURE LEGENDS

Fig. 1. (A) Representation of the three-dimensional structure of Ras. Regions of α-helix (α), β-sheets (β), and loops (λ) are indicated. Adapted from Krengel et al. (1990). (B) Ral/Ras chimeras. Amino acid numbers for each protein segment are given above the representation of each chimera. The diagram of secondary structure, based on Pai *et al.* (1989), depicts the linear arrangement of regions of α-helix , β-sheets , and loops.

Fig. 2. Effect of GDS proteins on Ral/Ras chimeras. Chimeric GTPases were loaded with and incubated in the absence (open circles) or presence of the GDS proteins GTCD-1 (7 nM final concentration; closed circles) or ralGDS (200 nM final concentration; closed squares). The products were separated by filtration and the filter-bound radioactivity was counted. Background filter-bound nucleotide levels were subtracted from the total filter-bound radioactivity and these values were normalized to the initial amount of filter-bound nucleotide. Data points represent the average of 2 - 4 assays and are representative of several assays. (A) Effect of GDS proteins on Ras. The k_{off} values are 0.006 min^{-1} without any GDS and 0.144 min^{-1} with GTCD-1 (24-fold increase in the rate of release). (B) Effect of GDS proteins on Ral. The k_{off} values are 0.008 min^{-1} without any GDS and 0.243 min^{-1} with ralGDS (30-fold increase in the rate of release). (C) Effect of GDS proteins on the BHλ1 chimeric GTPase. The k_{off} values are 0.019 min^{-1} without any GDS, 0.026 min^{-1} with GTCD-1, and 0.026 min^{-1} with ralGDS. (D) Effect of GDS proteins on the BHλ2 chimeric GTPase. The k_{off} values are 0.005 min^{-1} without any GDS, 0.005 min^{-1} with GTCD-1, and 0.007 min^{-1} with ralGDS. (E) Effect of GDS proteins

on the BHλ3 chimeric GTPase. The k_{off} values are 0.008 min^{-1} without any GDS, 0.009 min^{-1} with GTCD-1, and 0.014 min^{-1} with ralGDS. (F) Effect of GDS proteins on the BHλ4 chimeric GTPase. The k_{off} values are 0.006 min^{-1} without any GDS, 0.006 min^{-1} with GTCD-1, and 0.060 min^{-1} with ralGDS (10-fold increase in the rate of release). (G) Effect of GDS proteins on the BHλ5 chimeric GTPase. The k_{off} values are 0.008 min^{-1} without any GDS, 0.009 min^{-1} with GTCD-1, and 0.303 min^{-1} with ralGDS (38-fold increase in the rate of release).

Figure 3. ralGDS stimulates guanine nucleotide release from the Ras/SW2 mutant. The k_{off} values for Ral (upper panel) are 0.010 min^{-1} without any GDS and 0.120 min^{-1} with ralGDS (a 12-fold increase in the rate of release). The k_{off} values for Ras/SW2 mutant (lower panel) are 0.015 min^{-1} without any GDS and 0.084 min^{-1} with ralGDS (a 6-fold increase in the rate of release). Assays were performed as in Fig 2 using approximately 50 nM ralGDS (final concentration). Data represent the average of duplicate assays.

Fig. 1A

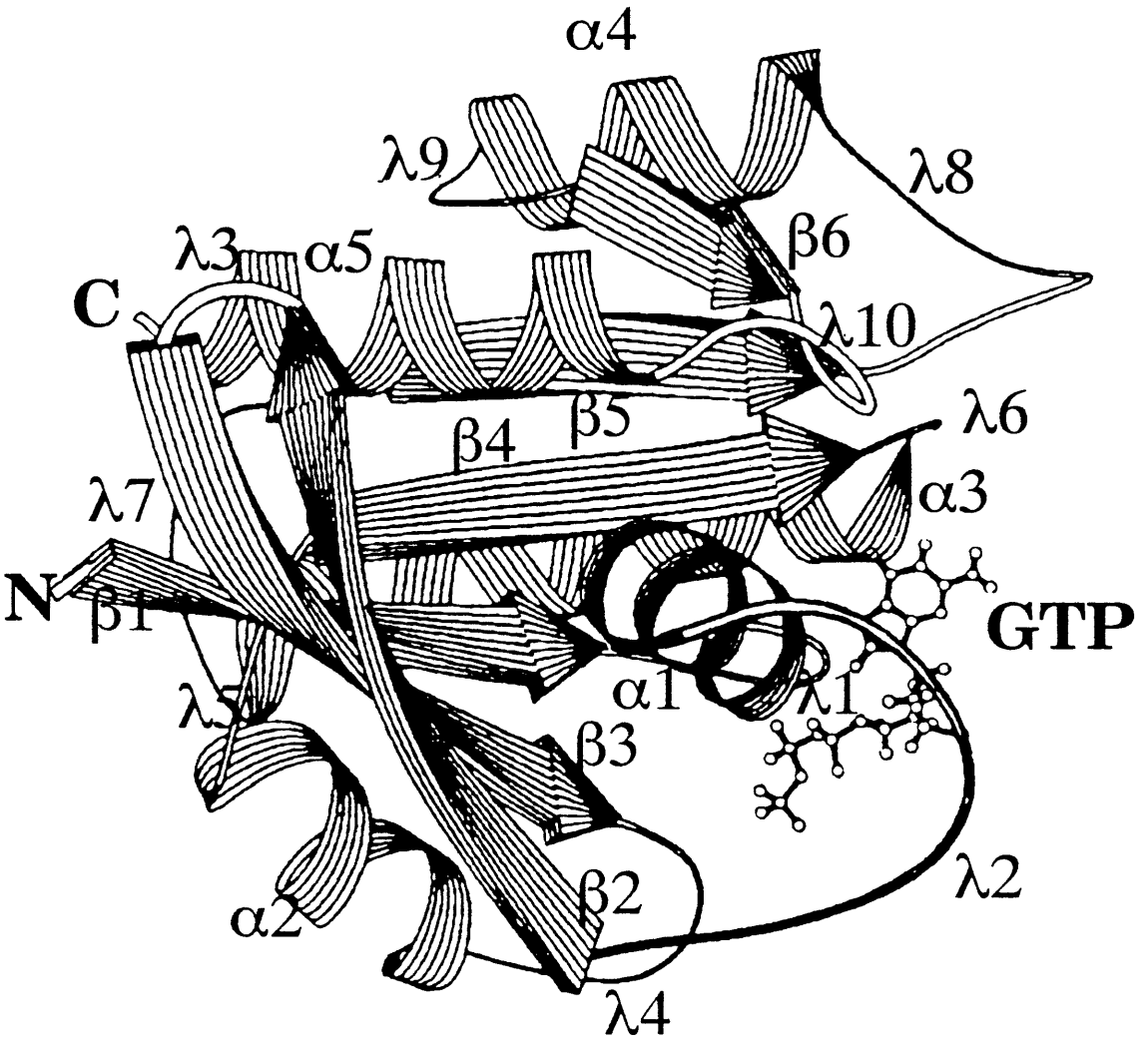
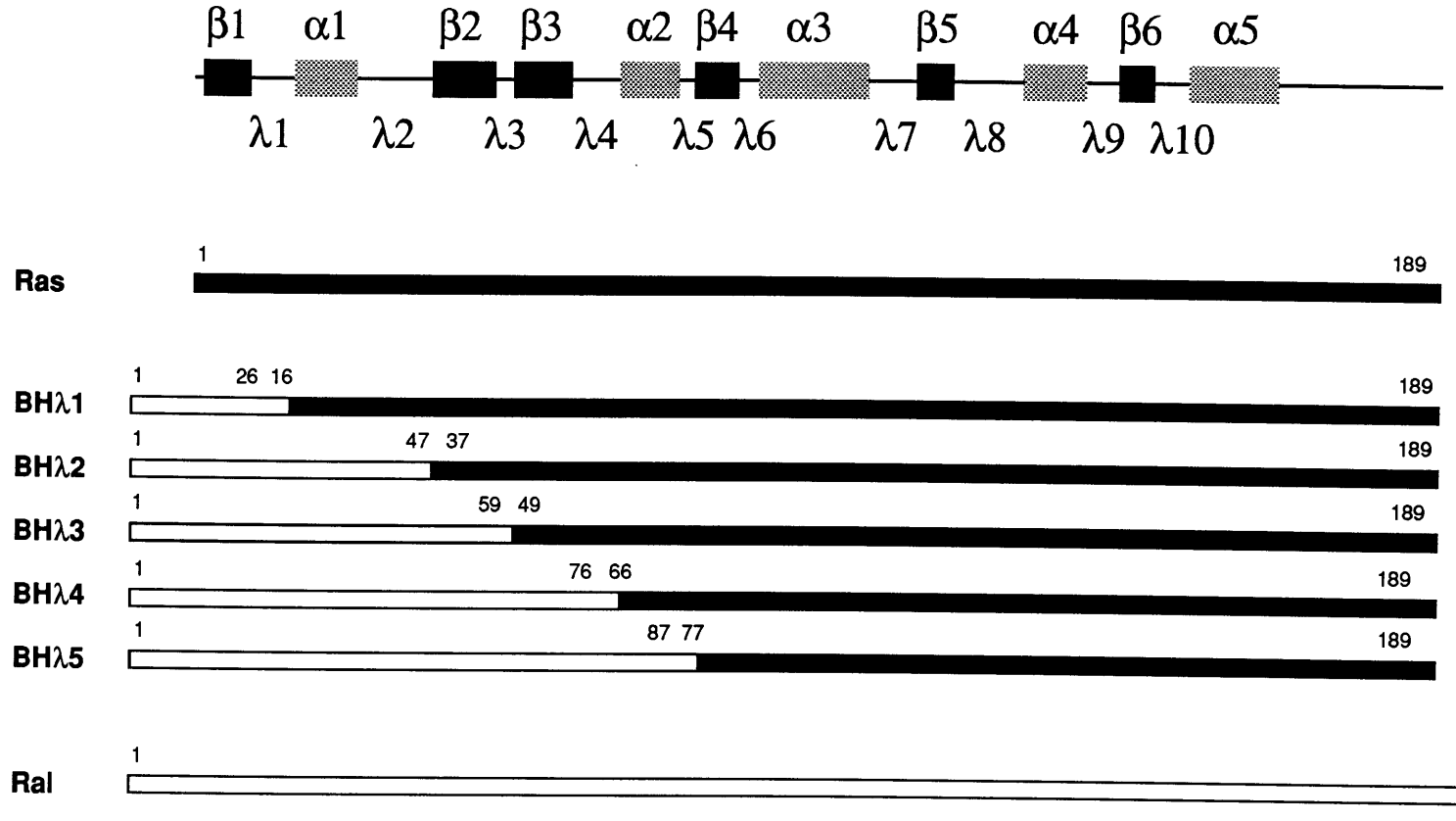


Fig. 1B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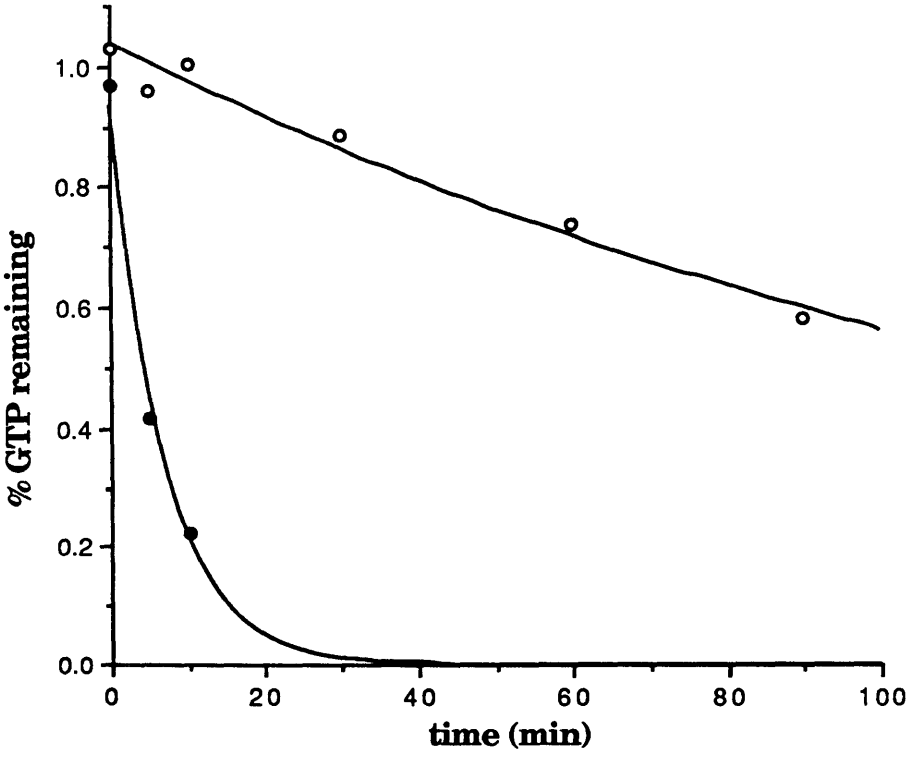
λ1 λ2 λ3 λ4 λ5 λ6 λ7 λ8 λ9 λ10
Ras
1
189
BHλ1
1 26 16
189
BHλ2
1 47 37
189
BHλ3
1 59 49
189
BHλ4
1 76 66
189
BHλ5
1 87 77
189
Ral
1
206

Fig. 2A



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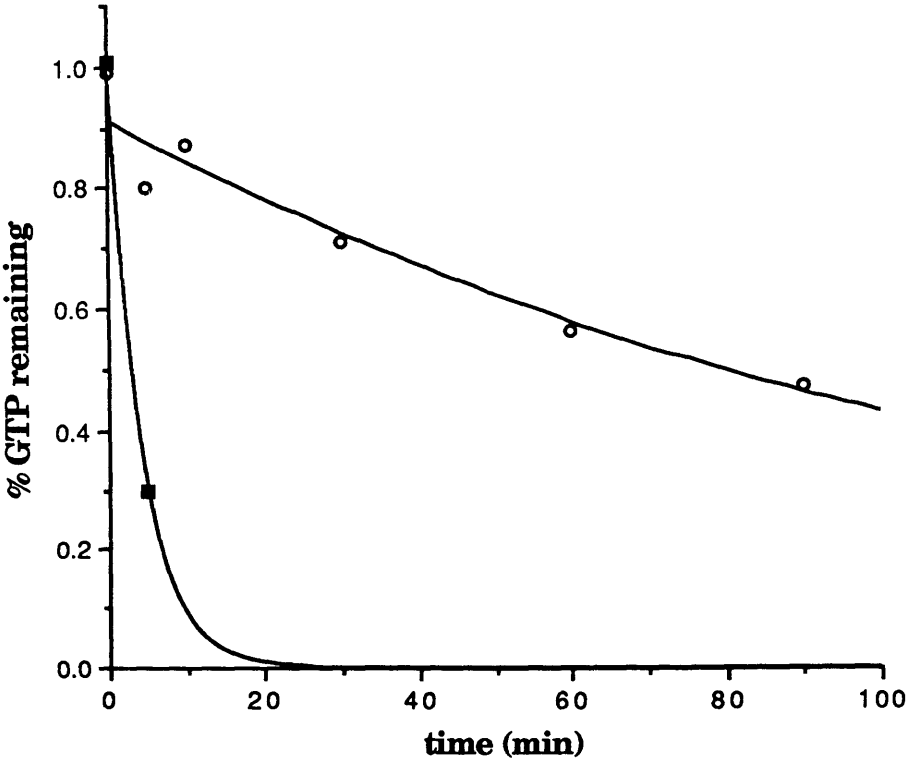

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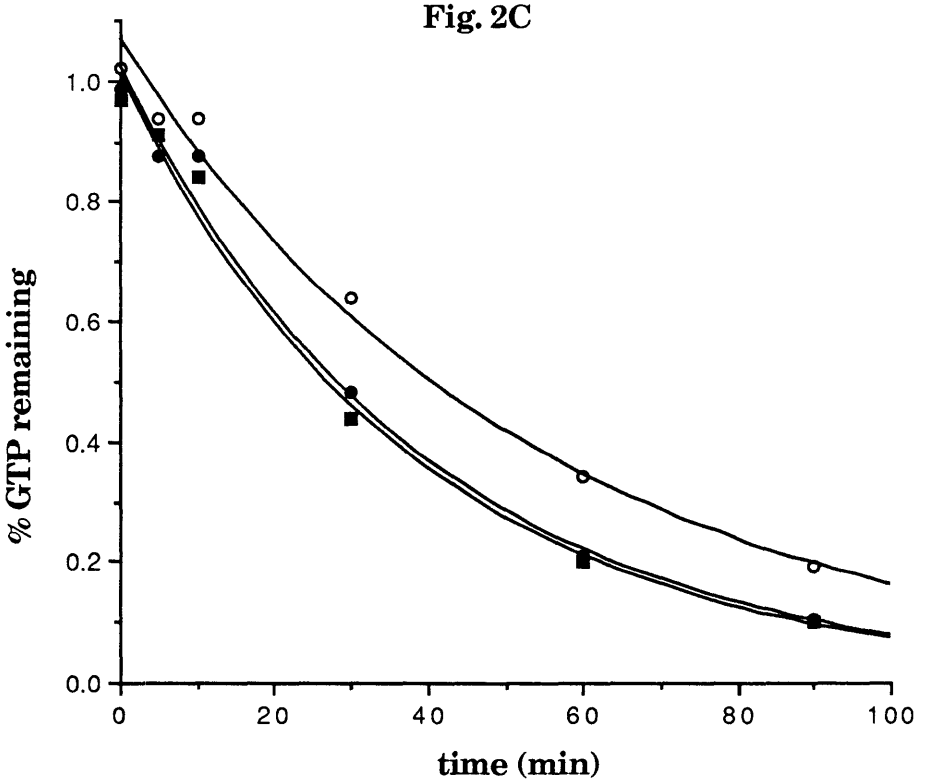


Fig. 2D

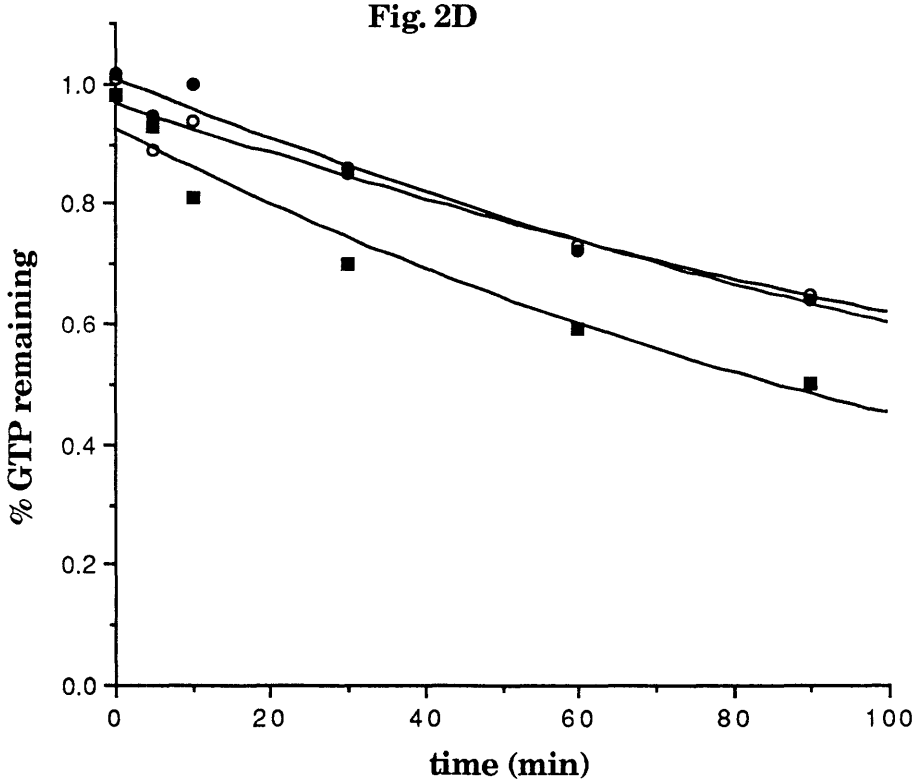


Fig. 2E

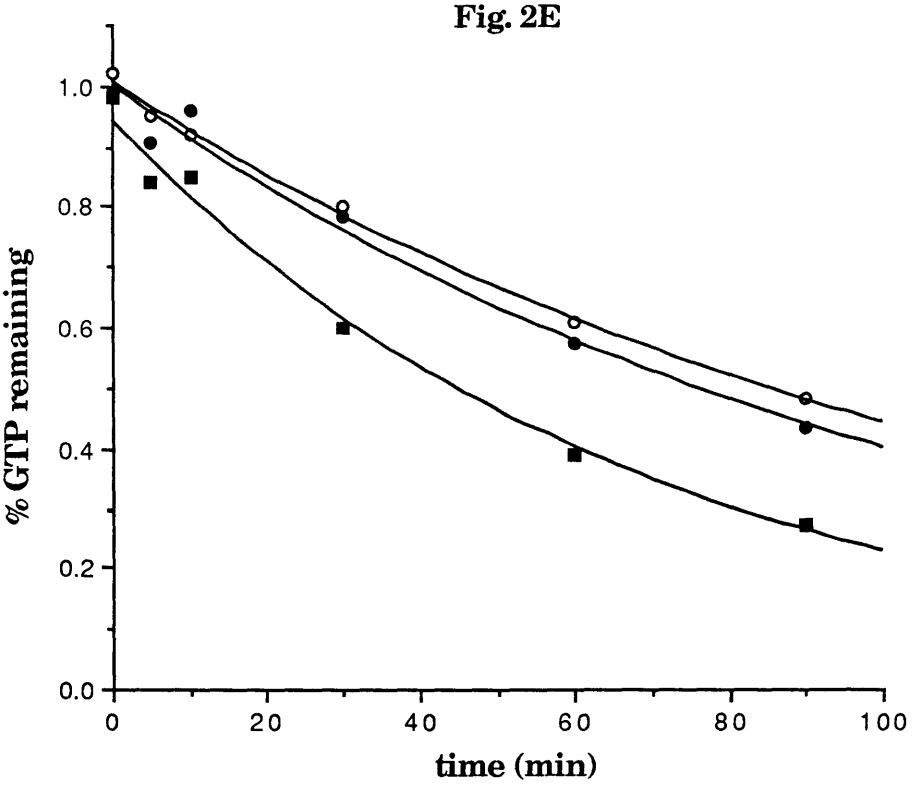


Fig. 2F

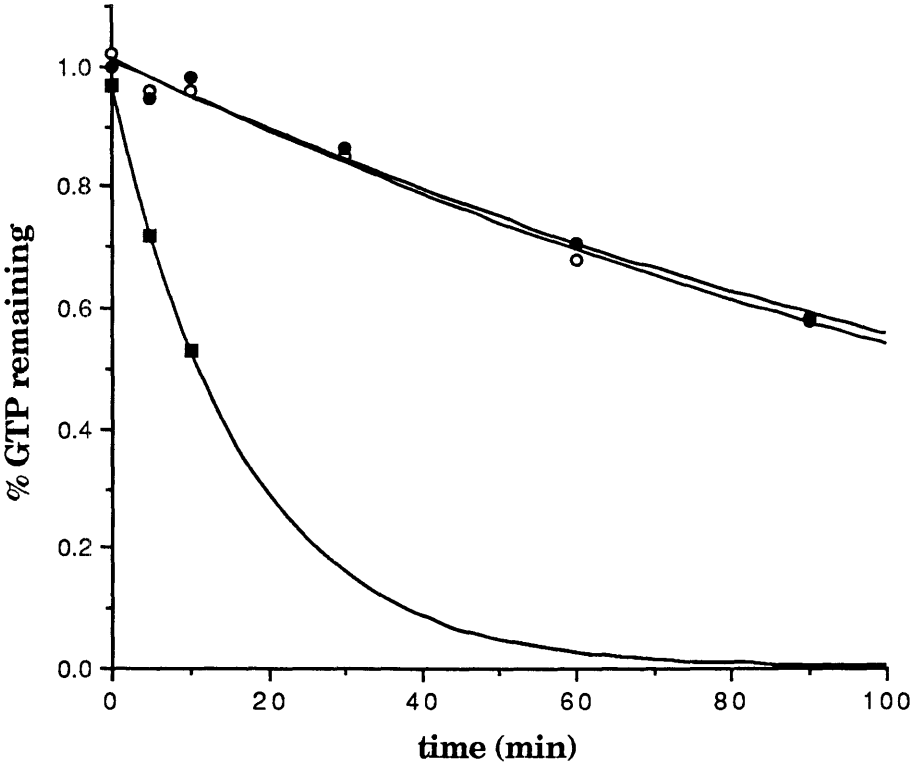


Fig. 2G

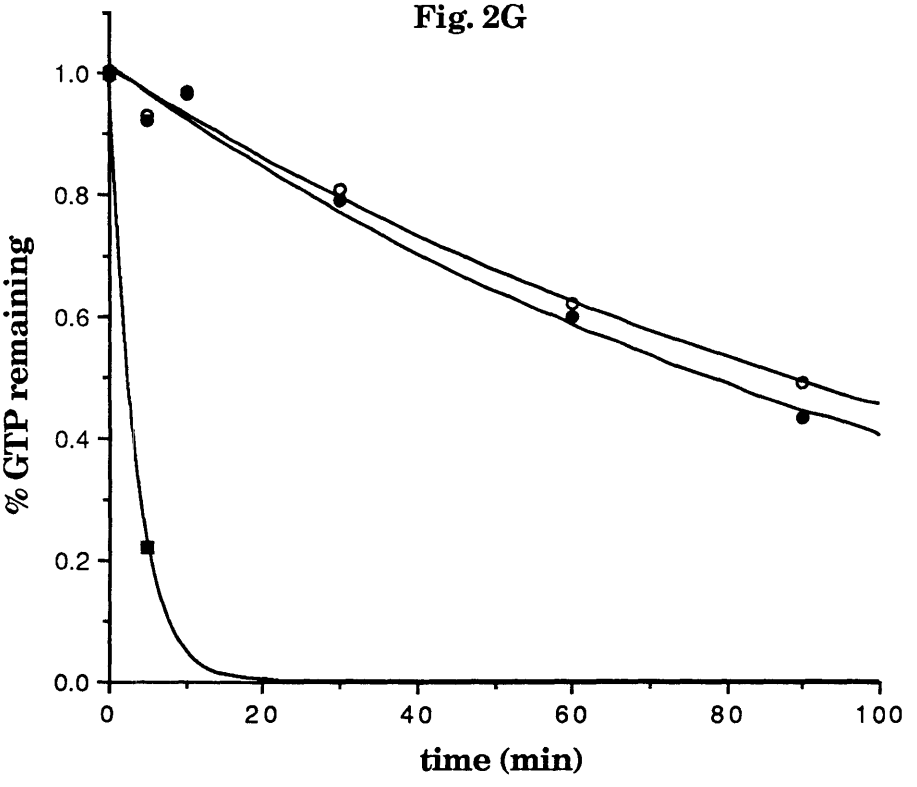
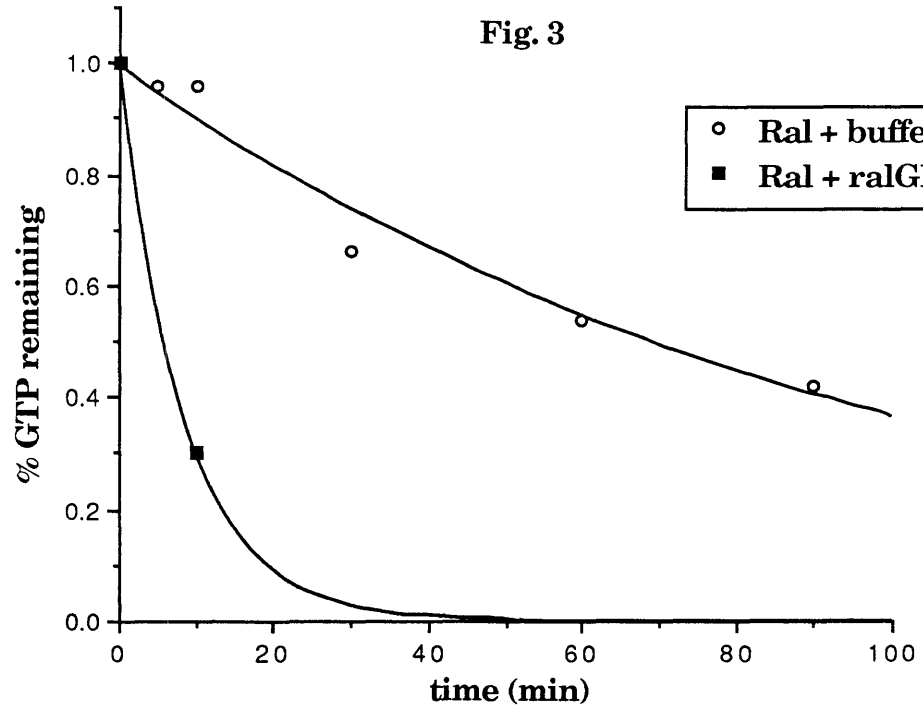


Fig. 3


1.0
0.8
0.6
0.4
0.2
0.0
% GTP remaining
0
20
40
60
80
100
time (min)
Ral + buffer
Ral + ralGDS

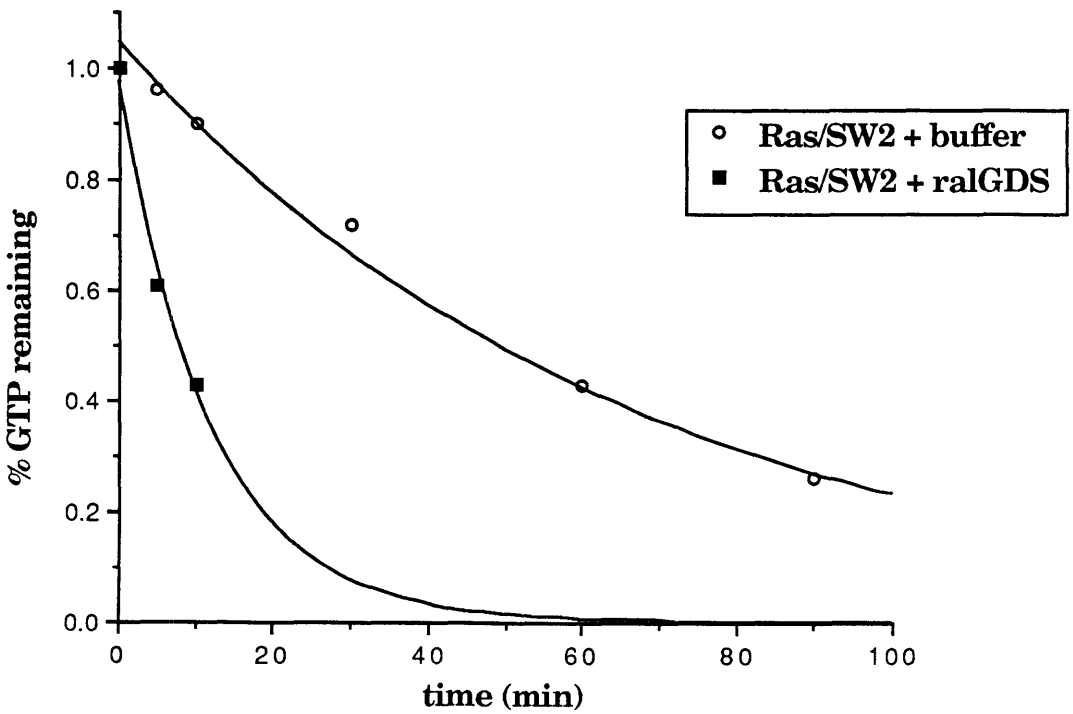

1.0
0.8
0.6
0.4
0.2
0.0
% GTP remaining
0
20
40
60
80
100
time (min)
Ras/SW2 + buffer
Ras/SW2 + ralGDS

Table I Effect of GTCD-1 on Ras Switch II Region Mutants

Mutant	k_{off} (no GDS)	k_{off} (GTCD-1)[a]	Stimulation
wild-type Ras	0.007 min^{-1}	0.912 min^{-1}	130 x
pRas/SW2	0.012 min^{-1}	0.254 min^{-1}	21 x
pRasE63D	0.005 min^{-1}	0.589 min^{-1}	118 x
pRasS65A	0.009 min^{-1}	0.877 min^{-1}	97 x
pRasM67I	0.007 min^{-1}	0.230 min^{-1}	33 x
pRasQ70N	0.005 min^{-1}	0.886 min^{-1}	177 x
pRasM72F	0.009 min^{-1}	0.933 min^{-1}	104 x

[a]The final concentration of GTCD-1 protein used in these experiments was approximately 70 nM.

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CHAPTER 4

Discussion:

Exchange factors in signal transduction.

Exchange factors in signal transduction.

Numerous reports have documented the involvement of GDS proteins in Ras signal transduction (for review, see Downward, 1992). These factors were first identified genetically as activators of Ras in *S. cerevisiae* (Camonis et al., 1986; Broek et al., 1987; Robinson et al., 1987; Boy-Marcotte et al., 1989), in *S. pombe* (Hughes et al., 1990), and in *Drosophila* (Simon et al., 1991; Bonfini et al., 1992), and as biochemical activities in mammalian cells (West et al., 1990; Wolfman and Macara, 1990; Downward et al., 1990b; Kaibuchi et al., 1991; Mizuno et al., 1991; Hiraoka et al., 1992). Then, several groups cloned mammalian genes related to Cdc25, a rasGDS from *S. cerevisiae*. The mammalian members of the Cdc25 family of exchange factors include rasGRF (also known as $CDC25^{Mm}$; Martegani et al., 1992; Shou et al., 1992; Wei et al., 1992; Cen et al., 1992); the murine and human homologs of the *Drosophila* Ras exchange factor, Sos (Bowtell et al., 1992; Chardin et al., 1993); and ralGDS (Albright et al., 1993). In Chapter 2, I present work describing the cloning and characterization of ralGDS, a mammalian exchange factor for a Ras-related GTPase. This study was one of several demonstrating the existence of Cdc25 homologues in mammalian cells and describing their biochemistry (see Boguski and McCormick, 1993; and Chapter 1).

One of the unresolved issues from the biochemical analysis of Cdc25-like proteins is the low GDS activity observed *in vitro*. My coworkers and I (Albright et al., 1993; Egan et al., 1993; Chapter 3 of this thesis) and others (Shou et al., 1992; Liu et al., 1993) observed only relatively low basal activities for purified GDS proteins and protein fragments. Relatively large amounts

(several micrograms per assay) of ralGDS, for example, stimulate guanine nucleotide release from Ral only 30-fold (Albright et al., 1993; Chapter 3 of this thesis). Results for Ras are similar: the specific activities of Ras exchange factors are relatively low (Shou et al., 1992; Egan and Weinberg, 1993; Chardin et al., 1993). rasGAP, on the other hand, can stimulate GTP hydrolysis 100,000-fold *in vitro* (Gideon et al., 1992). Since Ral and other GTPases coexist with both GDS and GAP proteins in the cell, the relatively potent GAP activity would immediately neutralize the attempts of GDS proteins to load GTP onto the GTPase. *In vivo* measurements, however, can detect significant GDS activity. Downward et al. (1990a), for example, have demonstrated significant exchange factor activity in T cells. Thus, one or more ways to increase the activity of the GDS proteins (post-translational modification, interaction with cofactors, specific localization) should exist.

Mechanisms that affect the activity of exchange factors could regulate signaling through these molecules. One model of GDS regulation invokes phosphorylation of the exchange factor by some component of the upstream signaling machinery. In this model, phosphorylation would increase the activity of the exchange factor. At least two reports support this model. First, Wolfman and Macara (1990) observed that phophatase inhibitors were required to maintain Ras exchange factor activity in lysates. Second, the exchange activity of the Vav oncoprotein (which catalyzes nucleotide release from Ras) is stimulated by phosphorylation by the tyrosine kinase, $p56^{lck}$ (Gulbins et al., 1993; Gulbins et al., 1994). However, since Vav is not related to Cdc25, it is not clear whether these observations are relevant to the regulation of Cdc25-like exchange factors.

In our examination of the Ral exchange factor, we were unable to demonstrate regulation of ralGDS by phosphorylation. ralGDS is phosphorylated on serine residues in COS cells (Albright et al., 1993), leading us to speculate that phosphorylation might increase or decrease exchange activity. We tested whether there were differences in the specific activity of phosphorylated and unphosphorylated forms of ralGDS protein from a baculovirus expression system but observed similar activities for both forms (Albright et al., 1993). Conceivably, residues critical to ralGDS function are not phosphorylated in this system.

At least one Cdc25 family member is regulated by phosphorylation. In *S. cerevisiae*, phosphorylation negatively regulates Cdc25 (Gross et al., 1992). Glucose treatment of yeast cells induces phosphorylation of Cdc25, which moves from the membrane fraction to the cytoplasm, where it no longer interacts with Ras.

A second model of GDS activation, the localization model, invokes regulatory molecules ferrying GDS proteins to the proximity of their GTPase targets. This relocation increases the localized concentration of GDS proteins in some cellular microenvironment. Support for this model comes from work on the rasGDS, Sos. Mammalian Sos proteins activate Ras in response to epidermal growth factor (EGF) stimulation (reviewed in Boguski and McCormick, 1993). Tyrosine kinase activation of Sos is, in turn, mediated by an adaptor protein, Grb2, which is the mammalian equivalent of Sem-5 from *Caenorhabditis elegans* and Drk from *Drosophila*. The Grb2 protein consists entirely of a Src-homology 2 domain (SH2) flanked by two Src-homology-3 (SH3) domains. These Src-homology domains mediate protein-protein

interactions: SH2 domains bind phosphorylated tyrosine residues, while SH3 domains bind short proline-rich sequences.

In the Appendix to this thesis, I present work done with Sean Egan and others demonstrating that the SH3 domains of Grb2 bind proline-rich regions on mammalian Sos. Furthermore, the SH2 domain of Grb2 binds phosphorylated tyrosines on activated EGF receptors. Thus, Grb2 recruits Sos into a multi-protein complex on the activated receptor. Similar observations have been reported by a number of other groups (Buday and Downward, 1993; Li et al., 1993; Rozakis-Adcock et al., 1993; Gale et al., 1993; Chardin et al., 1993; Matuoka et al., 1993).

How does Grb2 regulate Sos function? EGF treatment of cells does not appear to cause tyrosine phosphorylation of Sos (Egan et al., 1993; Buday and Downward, 1993), suggesting that some mechanism other than direct phosphorylation of Sos is responsible for activation. Buday and Downward (1993) did not observe any increase in the specific activity of Sos immunoprecipitates upon epidermal growth factor (EGF) treatment of cells. They did, however, report that upon EGF stimulation, Grb2 ferries Sos from the cytoplasm to the particulate (membrane) fraction where, presumably, it can intereact with and activate its target, Ras.

As yet, there is no evidence to implicate adaptor proteins in the regulation of other Cdc25 family members. In fact, the proline-rich SH3 binding domain of Sos is not present in other Cdc25-like GDS proteins (McCormick, 1993; Boguski and McCormick, 1993). Other sequence motifs may mediate signal-dependent activation (or localization). For example, Cdc25, Sdc25, and Ste6 contain each contain one SH3 domain. rasGRF contains two plekstrin-homology (PH) regions, while murine and fly Sos

proteins each contain one. The function of the SH3 and PH regions of Cdc25 family members is unknown. ralGDS contains no known signaling motifs.

A third model of GDS activation envisions regulatory proteins interacting with and overcoming the effects of inhibitory regions in the GDS proteins. Consistent with this inhibitory region model, several studies suggest that exchange factors contain regions that decrease their GDS activities: deletions and point mutations in *CDC25* (Broek et al., 1987; Kim and Powers, 1991), deletions in *SDC25* (Damak et al., 1991), deletions in *dbl* (Eva et al., 1988; Ron et al., 1988), deletions in *vav* (Katzav et al., 1991; Coppola et al., 1991), and mutations in *Sos* (Rogge et al., 1991; Bonfini et al., 1992; S. Egan, personal communication) all display phenotypes or biochemical properties consistent with increased GDS activity.

Once activated or relocated, how do exchange factors convey signals to their target GTPases? The mechanism by which GDS proteins stimulate guanine nucleotide release is not known, but studies of the interaction of these proteins and their targets provide some clues. In Chapter 3, I examine the molecular basis of specific recognition of a GTPase by its exchange factor. I constructed chimeric Ral/Ras GTPases and tested the ability of Ral and Ras exchange factors to catalyze guanine nucleotide release from these chimeras. Ral residues 60 - 87 (corresponding to residues 49 - 76 in Ras) are necessary for ralGDS-stimulated release of nucleotide. These residues include the switch II region (amino acids 60 - 76 in Ras), which is one of two regions that undergoes a significant conformational change upon the exchange of GTP for GDP.

Having shown that residues in the switch II region are necessary for the GTPase·GDS interaction, I wanted to test whether this region is sufficient to allow exchange factor interaction. I constructed a Ras mutant (Ras/SW2) in which the switch II region was replaced with the corresponding Ral sequences. The Ras/SW2 protein responded at least partially to ralGDS. These data suggest that residues in the switch II region play a central role in mediating the specific interaction between a GTPase and its GDS protein.

This study contributes to a growing body of evidence pointing to exchange factor interaction with the GTPase's switch II region (reviewed in Chapter 3 of this thesis). An obvious model for exchange factor-mediated activation proposes that the GDS protein's interaction with this region can destabilize the interaction between the GTPase and GDP. GTP would then bind, disrupting the nucleotide-free GTPase·GDS complex and activating the GTPase.

In this discussion, I note that several of the mysteries of signal transduction have been solved, at least partially, within the last few years. Since 1991, several mammalian exchange factors have been cloned and characterized biochemically. Furthermore, we now understand (at least in outline) how one GDS protein, Sos, links upstream signals to its target, Ras. We also have some insight into the mechanism by which exchange factors relay their signals to their GTPase targets.

Acknowledgements

I would like to thank Sean Egan for sharing his unpublished observations on Sos mutants.

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APPENDIX

Association of Sos Ras exchange factor protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation

Preface

This chapter represents work I did with Dr. Sean E. Egan, Mary Brooks, and Dr. Andrew M. Sizeland in the laboratory of Professor Robert A. Weinberg and Dr. Lázló Buday in the laboratory of Professor Julian Downward. I was involved in several aspects of this project. My contributions include assisting Dr. Egan in *Sos* transformation studies and in preparing some of the many expression constructs used in this study. I was also involved in critically interpreting data and assisted in the preparation of the manuscript.

This manuscript was previously published as an article in the 6 May 1993 issue of Nature, vol. 363, pp.45-51.

Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation

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The proteins Grb2–Sem-5, Shc and Sos have been implicated in the signalling pathway from tyrosine kinase receptors to Ras. Grb2–Sem-5 binds directly to murine Sos1, a Ras exchange factor, through two SH3 domains. Sos is also associated with ligand-activated tyrosine kinase receptors which bind Grb2–Sem-5, and with the Grb2–Sem-5 binding protein, Shc. Ectopic expression of *Drosophila* Sos stimulates morphological transformation of rodent fibroblasts. These data define a pathway by which tyrosine kinases act through Ras to control cell growth and differentiation.

MANY oncogenes encode protein tyrosine kinases (PTKs) which transmit signals regulating cellular growth and differentiation from the cell surface[1,2]. Another class of oncoprotein implicated in the process is the Ras family of low-M_r GTPases[3–7]. Inhibition of Ras function by microinjection of anti-Ras antibodies blocks the growth and transformed phenotype of tyrosine-kinase-transformed fibroblasts, indicating that Ras functions downstream from activated tyrosine kinases[8], and many tyrosine kinase receptors when activated, also activate Ras[9].

Genetic analysis of eye development in *Drosophila melanogaster* has shown that the Sevenless tyrosine kinase receptor is critical for induction of R7 photoreceptor neurons[10], making it possible to define other elements in the signalling pathway triggered by sevenless[11]. The *Son of Sevenless* gene (*Sos*) has thus been shown to function downstream from *Sevenless* and downstream from a second tyrosine kinase receptor, the *Drosophila* epidermal growth factor receptor homologue (DER)[12,13]. Sos is homologous to exchange proteins that activate Ras by inducing loading of GTP[13,14]. A second downstream gene encodes the *Drosophila* Ras protein DRas1 (ref. 13) providing strong evidence that in insect cells, sevenless and DER use the Ras pathway to transduce growth and differentiation signals.

The situation is similar in the system governing vulval development in *Caenorhabditis elegans*[15,16], where the locus *let*-23 encodes the *C. elegans* homologue of the EGF receptor[17], and a second gene (*let*-60) specifying a downstream function encodes a Ras protein[18,19]. Yet another gene in this pathway, termed *sem*-5, encodes a small protein consisting entirely of two src-homology-3 (SH3) domains flanking a src-homology-2 (SH2)[20] domain. Mutations in *sem*-5 can be rescued by activated *ras* alleles indicating that this protein functions upstream of the Let-60 Ras protein (ref. 20 and S. G. Clark and H. R. Horvitz, personal communication).

Sem-5 is thought to function as an adapter that assembles other proteins into multi-protein complexes[20,21] by means of its SH3 and SH2 domains, which bind short proline-rich sequence motifs and phosphotyrosine-containing peptides, respectively. SH2 domains, such as that in Sem-5, are found in certain effector proteins and have been implicated in the recruitment of these effectors into complexes with autophosphorylated tyrosine kinase receptors[22].

In mammals, Ras guanine nucleotide exchange activity is stimulated by tyrosine kinase receptors, including the *trk*-encoded nerve growth factor receptor, the EGF receptor (EGF-R), and the insulin receptor[23–25]. A mammalian homologue of Sem-5, designated Grb2, has been cloned in screens both for proteins which bind to the autophosphorylated EGF-R cytoplasmic tail[26] and for SH2-containing proteins[27]; mammalian homologues of the *Drosophila Sos* gene (termed m*Sos*1 and m*Sos*2) are also known[28]. The interactions between mammalian tyrosine kinases, Grb2, the mammalian Sos proteins and Ras can now be analysed, together with their involvement in signal transduction. Here we describe physical complexes between several of these signalling proteins strongly supporting the hypothesis that tyrosine kinase receptors introduce mitogen signals into the Ras signalling pathway by forming multiprotein complexes with the Grb2 and mSos proteins. One implication of these interactions has been confirmed through study of the

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effects of ectopically expressed *Drosophila* Sos protein in rodent fibroblasts.

Association of mSos with Grb2

Genetic analyses suggest that the *Drosophila* Sos and *C. elegans* Sem-5 proteins function near one another in their respective signalling cascades, downstream from receptor tyrosine kinases but upstream from Ras. We therefore investigated whether Grb2 and mSos formed physical complexes *in vivo* by tagging the Grb2 protein at its C terminus with the 9E10 Myc epitope to give the Grb2myc chimaera[29]. This gene was subcloned into the pBabepuro retroviral vector and packaged to produce retroviral stocks[30]. Rat1 cells were then infected with this virus, with control virus encoding untagged Grb2 or with control pBabepuro virus and puromycin-resistant populations from each infection were selected. Cell clones infected with the Grb2myc virus were also isolated. Western blot analysis of cell lysates using the 9E10 antibody revealed the expected 26K Grb2myc protein in cells infected with Grb2myc (Fig. 1*a*). The cloned cell line dR1Grb2myc1, which expresses tagged Grb2, and the control-infected cell population (dR1Babemix) were used for further analysis.

The mouse Sos protein (mSos1) was provided with a distinct antigenic tag by fusing the mSos1 reading frame at its N terminus to three copies of the influenza virus haemagglutinin (HA) epitope[31]. This peptide is efficiently recognized by monoclonal antibody 12CA5. The resulting chimaeric gene (3HAmSos1) was introduced into the pBabehygro retrovirus transducing vector, which carries a hygromycin resistance marker instead of the puromycin marker present in the Grb2 vector[30]. Retroviral stocks were generated from the pBabehygro3HAmSos1 and control pBabehygro vectors, which were used to infect dR1Grb2myc1 or dR1Babemix cells, and infected populations were selected in the presence of hygromycin.

Lysates of the resulting double drug-resistant cell populations were then immunoprecipitated with the anti-HA antibody 12CA5, and the precipitates analysed by western blot analysis using anti-HA (Fig. 1*b*). The 165K HA-tagged 3HAmSos1 pro-

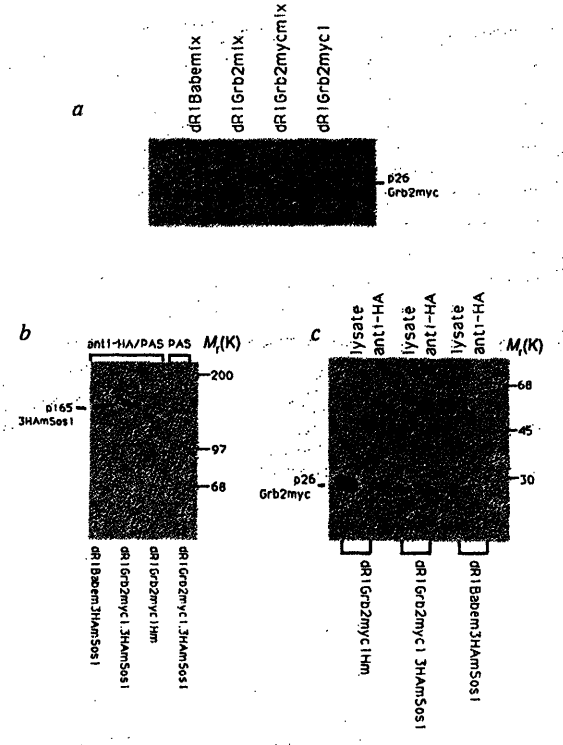

FIG. 1 *In vivo* association of Grb2 and mSos1. *a*, Western blot analysis of Rat 1 cells infected with Grb2myc virus. *b*, Western blot of anti-HA immunoprecipitates probed with 12CA5 anti-HA antibody. *c*, Western blot of whole-cell lysates and anti-HA immunoprecipitates probed with 9E10 anti-myc antibody.

METHODS. *a*, The human Grb2 gene was cloned from HT1080 complementary DNA by PCR using the 5′ primer GCTAAAGGATCCCTCAGAATGGAAGCCATCGCC (where the natural ATG start codon is underlined) and the 3′ primer GGATTCTCTAGAGTCGACTCAGACGTTCCGGTTCACGGGGGT. The PCR product was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI–*Xba*I-cut pBSK+. After sequencing clones and subcloning of non-mutant fragments, the vector pBSKGrb2A1 was generated which encoded full length Grb2. The *Bam*HI–*Sal*I Grb2 fragment was then subcloned into *Bam*HI–*Sal*I-digested pBabepuro to generate the retroviral vector pBabeGrb2A. The myc-tagged Grb2 gene was generated in the same way except PCR used the 3′ primer GGATTCTCTAGAGTCGACTCACAAGTCTTCTTCAGAAATAAGCTTTTGTTCGACGTTCCGGTTCACGGGGGT, where the underlined segment encodes the myc epitope. The vector pBSKGrb2mycAAA was generated which encoded full-length Grb2myc. The authenticity of this gene was also confirmed by sequence analysis. The *Bam*HI–*Sal*I Grb2myc fragment from pBSKGrb2mycAAA was also subcloned into *Bam*HI–*Sal*I-digested pBabepuro to generate pBabeGrb2mycA. pBabepuro, pBabeGrb2A and pBabeGrb2mycA were transiently transfected into the retroviral packaging line GP+E (ref. 40) and viral supernatants collected 48 h later. Rat1 cells were infected with these viral stocks and selected in 5 μg ml^{-1} puromycin. The cell cultures dR1Babemix, dR1Grb2mix and dR1Grb2mycmix are polyclonal mixtures of puro-resistant cells. dR1Grb2myc1 was isolated as a cloned line. Confluent monolayers of these lines were collected in 1 ml of lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl, 10 mM NaF, 10 μg ml^{-1} aprotinin, 1 mM PMSF, 1 mM Na_3VO_4) per 9-cm dish and clarified by centrifugation. Each lysate (20 μl) was boiled in SDS–PAGE sample buffer and resolved on a 12% SDS-PAGE gel. The gel was electroblotted onto nitrocellulose. This filter was then blocked in 5% dry milk powder, 0.05% Tween 20, PBS, washed in 1% dry milk powder, 0.05% Tween 20, PBS, probed with 1 μg ml^{-1} of 9E10 monoclonal antibody in wash buffer, washed, incubated in 1/5,000 dilution of Pierce Goat anti-mouse Fc horseradish peroxidase (HRP)-conjugated antiserum, washed and developed by ECL (Amersham). *b* and *c*, The triple HA-tagged mSos1 gene was constructed from its fragments. The 5′ end of the gene was replaced by the sequence AAGCTTAGATCTACCATGGGCGGCGCTTGTCTCCCACCTCATC by PCR-mediated mutagenesis, where the underlined ATG is the start codon. This altered 5′ end contained a *Not*I restriction site into which the triple HA epitope was cloned[31]. A retroviral expression vector (pBabeH3HAmSos1A) was generated by cloning fragments of the gene from the *Bgl*II site present in the 5′ PCR primer to a *Sal*1 site downstream of the gene (originally in the pBSIlm-Sos1.385 partial cDNA-containing plasmid[28]) into pBabehygro digested with *Bam*HI and *Sal*I. pBabeH3HAmSos1A or pBabehygro were transiently transfected into GP+E packaging cells and viral supernatants collected. dR1Babemix cells were infected with the H3HAmSos1 viral stock and the polyclonal population dR1Babem3HAmSos1 selected in 100 μg ml^{-1} hygromycin. dR1Grb2myc1 cells were infected with either H3HAmSos1 viral stocks or hygro control viral stocks. These cells were then selected in 100 μg ml^{-1} hygromycin and the polyclonal populations dR1Grb2myc1.3HAmSos1 and dR1Grb2myc1Hm isolated separately. Four confluent 9-cm dishes of each line were serum starved overnight in DME, 0.2% FCS, treated with 100 μM Na_3VO_4 for 30 min followed by 100 ng ml^{-1} EGF for 5 min. The cells were then washed with ice-cold PBS and lysed in 1 ml of 30 mM HEPES pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mg ml^{-1} BSA, 1 mM Na_3VO_4, 2 mM para-nitrophenylphosphate, 10 mM NaF, 25 μM phenyl arsine oxide, 25 μg ml^{-1} leupeptin, 25 μg ml^{-1} trypsin inhibitor, 25 μg ml^{-1} pepstatin A, 25 μg ml^{-1} aprotinin, 10 mM benzamidine, 1 mM PMSF. Lysates were clarified and incubated with 2 μg of anti-HA antibody covalently coupled to protein A–Sepharose[41] for 2 h at 4 °C. Immunoprecipitates were washed four times in 1 ml of PBS, 0.1% Triton X-100, 0.005% SDS, 0.25 M NaCl, 1 mM Na_3VO_4, 1 mM PNPP. Samples were run on 7 or 12% gels and immunoblotted with anti-HA (1 μg ml^{-1}) or 9E10 anti-myc (1 μg ml^{-1}), followed by anti-mouse IgG Fc-HRP (Pierce) with visualization by ECL (Amersham). The anti-HA precipitate from cell line dR1Grb2myc1.3HAmSos1 in *c* was from 450 μl of extract, whereas 30 μl of extract was loaded in the total lysate track.

tein was easily observed in cells infected with the 3HAmSos1 virus but not in cells infected with control virus. As expected, the 26K-tagged Grb2 protein was also present in hygromycin-resistant derivatives of the dR1Grb2myc1 cell line (Fig. 1*c*). When anti-HA immunoprecipitates from these lysates were western blotted with the anti-myc antibody, however, the Grb2myc protein was only precipitated from cells that expressed the 3HAmSos1 protein, indicating that Grb2 and mSos1 do associate *in vivo* (Fig. 1*c*).

SH3 binding

To understand the interaction between Grb2 and mSos1 in more detail, we made glutathione-*S*-transferase (GST) fusion proteins with Grb2myc, the mSos1 N-terminal domain, the mSos1 central domain (homologous to the yeast CDC25 Ras exchanger), a longer version of the mSos1 central domain, and the mSos1 C-terminal domain. Expression of the mSos1 fusions or a non-chimaeric GST control protein was then induced in bacteria and the lysates analysed by far-western blotting using a purified GSTGrb2myc fusion protein. The Grb2 protein was detected using the 9E10 anti-myc antibody.

Grb2 bound specifically to the C-terminal domain of mSos1 (amino-acids 1,135–1,336), indicating that no additional partners are required to couple these two proteins (Fig. 2*A*, *a*). This binding was specific to Grb2, because a nonfunctional myc-tagged Grb2 mutant described below did not bind to mSos1 fragments (Fig. 2*A*, *d*). Grb2 also bound to an overlapping fusion protein containing residues 613–1,166 of mSos1, but not to a smaller protein containing residues 613–1,135, indicating that a binding site for Grb2 is located between residues 1,135 and 1,166 of mSos1. As these 31 amino acids contain a proline-rich stretch which is similar in sequence to recently described SH3-binding motifs[32], we speculated that Grb2 uses one or both of its SH3 domains to bind to this proline-rich sequence in mSos1.

Nonfunctional alleles of *sem*-5 have been found to contain

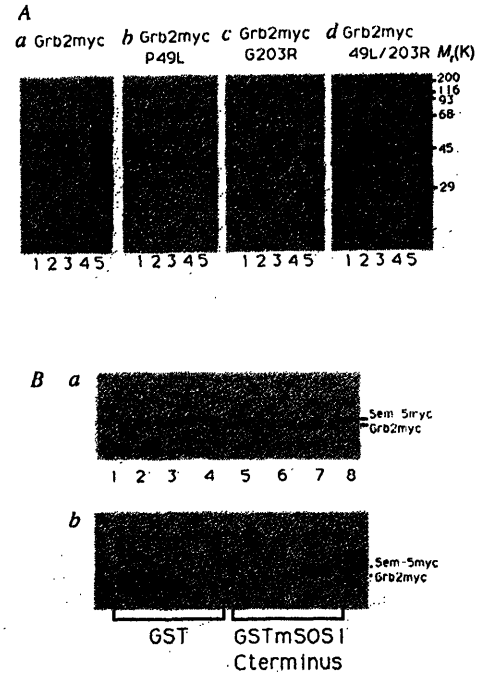

FIG. 2 *A*, Binding of GSTGrb2myc fusion proteins to mSos1 fusion proteins immobilized on nitrocellulose filters. Replicate filters with bacterial lysates containing: lanes 1, GST protein; 2, GSTmSos1Nterminus; 3, GSTmSos1-central; 4, GSTmSos1central+31; 5, GSTmSos1Cterminus. Filters were probed with *a*, GSTGrb2myc; *b*, GSTGrb2myc49L; *c*, GSTGrb2myc203R; *d*, GSTGrb2myc49L/203R. *B*, Analysis of solution binding between Grb2/Sem-5-myc proteins and GSTmSosCterminus beads. *a*, Western blot of total cell lysates for myc-tagged proteins produced in Cos1 cells transfected with; lanes 1 and 5, pSVE+; lanes 2 and 6, pSVEGrb2B; lanes 3 and 7, pSVEGrb2mycA.6A; lanes 4 and 8, pSVESem-5mycB. *b*, Western blot of protein samples 1 to 8 from *a* precipitated with either GST beads (lane 1–4) or GSTmSos1Cterminus beads (lanes 5–8).
METHODS. *A*, Fragments of mSos1 were cloned into derivative pGEX vectors as follows. GSTmSos1Nterminus: the N terminus of mSos1 was constructed from three partial clones[28] and the starting sequence replaced by PCR-mediated mutagenesis using the 5′ oligonucleotide ACCGCCAAGCTTAGA-TCTTAGCCTAAGGCAATGCTT, where the underlined ATG is the natural start codon. PCR-derived fragments were sequenced. The N-terminal sequences beginning at the *Bgl*II site in the above primer and ending at the unique *Bam*HI site was cloned into *Bam*H1-digested pGex20T. This fusion protein encodes the first 615 amino acids of mSos1. GSTmSos1central: this vector was made by subcloning the *Bam*H1–*Bgl*II (nucleotide (nt) 3,437) fragment of mSos1 into pGex30X. It encodes amino acids 615–1,135. GSTmSos1central+31: the central region of mSos1 from the unique *Bam*H1 site to the *Pvu*II site at nt 3,534 representing amino acids 613–1,166 was cloned into modified pGex30X, starting at the *Bam*HI site. GSTmSos1Cterminus: the C terminus of mSos1 from amino acid 1,135 to 1,336 was subcloned on a *Bgl*II–*Xho*I fragment into *Bam*HI–*Xho*I-digested pGex20T. The GSTGrb2myc-encoding vector was made by subcloning the *Bam*HI–*Xba*I fragment containing Grb2myc from pSVEGrb2mycA.6A into *Bam*HI–*Xba*I-digested pGex20T. The resulting plasmid codes for full-length Grb2myc fused to GST. The pSVEGrb2mycA.6A plasmid was itself made by inserting the *Bam*HI–*Sac*I fragment from pBSKGrb2mycAAA (described in Fig. 1) into *Bam*HI–*Sac*I-cut pSVE+ (ref. 42). All mutants were made by phagemid rescue of pSVEGrb2mycA.6A using the BioRad mutagenesis kit, following manufacturer's instructions. Mutants were sequenced and were subcloned into pGex20T. Induced bacterial cultures expressing GSTmSos1 fusion proteins or pGex30X-encoded GST were centrifuged, resuspended in protein sample buffer, boiled, separated on 12% SDS–PAGE gels and transferred to nitrocellulose using standard techniques[41]. Filters were blocked with 5% dry milk powder, 0.05% Tween 20 in PBS for 1 h and then incubated with 1 μg ml^{-1} purified Grb2myc fusion proteins in 1% dry milk powder, 0.05% Tween 20, PBS; the myc epitope was detected with 9E10 anti-myc antibody as in Fig. 1*a*. A western blot of these samples using anti-GST antiserum confirmed that fusion proteins of the expected sizes were present in all tracks (data not shown). The full-length C-terminal fusion protein is insoluble in bacteria and despite direct lysis of induced cultures in SDS–PAGE sample buffer with immediate boiling, degradation of this protein was reproducibly observed. Grb2myc fusion proteins were purified by binding to glutathione agarose and eluted with glutathione[43]. *B*, *Cos*1 cells were transfected with 20 μg of plasmid DNA encoding tagged Grb2–sem-5 genes or control vectors by calcium phosphate coprecipitation[44]. About 48 h later, cells were washed in cold PBS and collected in lysis buffer (Fig. 1*a*) and centrifuged. Clarified lysates were either directly run on 12% SDS–PAGE gels, transferred to nitrocellulose and western blotted for the myc-tagged protein (*a*) as described in Fig. 1 (1/50th of lysate) or precipitated with beads (GST or GSTmSosCterminus) for 1 h (*b*). The precipitated proteins were then washed (4×1 ml 20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), separated on a 12% SDS–PAGE gel, transferred and western blotted for the myc epitope (*a*) (49/50ths of lysate). The pSVEGrb2mycA.6A plasmid used to direct synthesis of myc-tagged Grb2 was made as described in methods from *A*. The pSVEGrb2B plasmid was made by subcloning the *Bam*HI–*Sac*I fragment from pBSKGrb2A1 (described in Fig. 1) containing full length Grb2 into *Bam*HI–*Sac*I-digested pSVE+. The myc-tagged *sem*-5 gene was synthesized by PCR with the 5′ primer GCTAAAGGATCCGAATTCCG-AAGTTTGAGG and the 3′ primer GGATTCTCTAGAGTCGACTCACAAGTCT-TCTTCAGAAATAAGCTTTTGTTCATTTCCAAAGTTGAAGCC (where the underlined sequence is the antisense sequence coding for a C-terminal 9E10 myc epitope) using pBSK-sem-5 as a template. The PCR product was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI–*Xba*I-digested pBSK+ to yield pBSKsem-5mycA. The majority of the sem-5 coding sequence was then replaced by subcloning the *Eco*RI to *Sna*BI fragment of wildtype sem-5 from pBSK-sem-5 into *Eco*RI (partially digested) to *Sna*BI-digested pBSKsem-5mycA which generated pBSKsem-5mycA1. The *Sna*BI to stop codon including the myc epitope tag region were checked by sequencing. Finally, the tagged gene was cut out from pBSKsem-5mycA1 on a *Bam*HI–*Sac*I fragment and ligated into *Bam*HI–*Sac*I-digested PSVE+ plasmid.

TABLE 1 Transforming potential of D*Sos*

Hygro	
Hygro 3 viral stock	< 0.00035 foci per hygro-resistant colony
Neu	
Neu4 viral stock	0.39 foci per hygro-resistant colony
SOS	
H.*Sos*.B viral stock	0.034 foci per hygro-resistant colony
H.*Sos*.J viral stock	0.025 foci per hygro-resistant colony

Rat 1 cells were infected with retroviral supernatants from cloned producer cell lines shedding either the pBabehygro virus or pBabehygro derivatives that encode the activated *neu* gene or D*Sos*. After infection, cells were either grown to confluence to score for the production of transformed foci or cultured in the presence of 100 μg ml^{-1} hygromycinB to determine infection frequency. Hygromycin-selected plates were stained on day 14 and drug-resistant colonies counted. Focus-forming assays were stained on day 21 after infection. Transformed foci were counted from the stained plates. Foci resulting from infection by the *neu* virus were typically larger than D*Sos*-induced foci and were apparent earlier. Producer lines were obtained by transfection of pBabe-vectors into the ΩE packaging cell line[30], followed by selection of hygromycin-resistant transfectant clones which were screened for production of hygromycin resistance-encoding viral supernatants. Highest titre cloned producer lines were used as a source of supernatants for transformation assays. The pBabeHNeuNT retroviral vector was made by subcloning the blunted *Hin*dIII-*Sal*1 Neu-fragment originally from pSV2NeuNT (ref. 46) into *Sna*BI-digested pBabehygro[30]. The pBabeH*Sos*A vector is also a derivative of pBabehygro. The natural 5' end of D*Sos* was replaced by the sequence CATTCGGATCCAGAACCATGTTCTCGGGGCCCAGCGGCCAT through polymerase chain reaction (PCR)-mediated mutagenesis. The PCR-derived 5' fragment was digested with *Bam*HI and *Pst*I. This small fragment containing the first 53 codons was cloned together with the *Pst*I (partially digested) to *Dra*I fragment containing the remainder of the gene into *Bam*HI-*Sna*BI-digested pBabehygro. The PCR-derived fragment was confirmed by DNA sequence analysis

mutations in the N-terminal SH3 domain, the SH2 domain or the C-terminal SH3 domain[20], and analogous SH3 mutations in human Grb2 also impair its function[26]. We therefore used site-directed mutagenesis to introduce these mutations in the SH3 domains of Grb2myc and analysed their effect on mSos1 binding. A proline to leucine substitution at residue 49 (P49L) in the N-terminal SH3 domain of Grb2 decreased its overall binding to mSos1. The mutant bound efficiently to the intact GSTmSos1 Cterminus protein, but binding to the fusion protein containing only residues 613–1,166 was greatly reduced, as was binding to degradation products of the mSos1 C-terminal fusion protein (Fig. 2*A*, *b*). The N-terminal SH3 domain of Grb2 thus appears to be responsible for binding to the 31 amino-acid domain of mSos1 described above.

This 31-residue domain of mSos1 contains the sequence VPVPPPVPP (single-letter amino-acid code), which differs only slightly from the SH3-binding binding consensus of XPXXPPPΨXP (where X is any amino acid and Ψ is hydrophobic)[32]. In contrast to the P49L mutation, a glycine to arginine mutation at codon 203 (G203R) in the C-terminal SH3 domain also decreases overall binding to the mSos1 C terminus, but leaves binding to the 613–1,166 domain intact (Fig. 2*A*, *c*). The binding site for the C-terminal SH3 domain must therefore be distinct from that for the N-terminal SH3, and must be located near the C terminus of mSos1. There are several proline-rich stretches in this region that are candidate binding sites. No specific binding by a 49L/203R double mutant of Grb2myc in which both Grb2 SH3 sites have been altered was observed (Fig. 2*A*, *d*), indicating that Grb2:mSos1 binding depends upon the cooperative actions of the two Grb2 SH3 domains, either of which can mediate loose binding to mSos1 on its own.

These associations were further confirmed by expressing myc-tagged Grb2 or Sem-5 in Cos1 cells and measuring the ability of the mSos1 C-terminal region immobilized on glutathione agarose beads to absorb these proteins from cell lysates. As seen in Fig. 2*B*, these beads were indeed able to bind both Grb2 and Sem-5, while control GST beads showed no affinity for either.

In other experiments, a Grb2 mutant carrying a glutamic acid to lysine substitution at residue 89 in the SH2 domain (again analogous to a loss-of-function *sem*-5 allele) also bound to the mSos1 beads efficiently (results not shown). Experiments with Cos cells transiently transfected with SH3 mutants were also consistent with the results described above (data not shown). These data indicate that the proteins Grb2 and Sem-5 share a high affinity for the C-terminal region of mSos1 despite more than 600 million years of separate evolution. High-affinity binding depends on the cooperative binding of the Grb2 N-terminal and C-terminal SH3 domains to a proline-rich motif between mSos1 residues 1,135 and 1,166 and a second site mapping closer to the C terminus of mSos1, respectively.

mSos linked to tyrosine kinase signalling

Grb2 has a second, distinct binding activity that allows it to associate with autophosphorylated tyrosine kinase receptors[26].

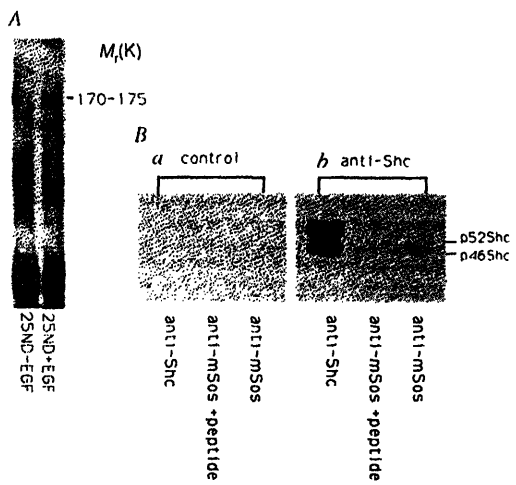

FIG. 3 Association of mSos with activated tyrosine kinase growth factor receptors and the kinase substrate Shc. *A*, Lysates from NIH/EGFR-25ND cells were either untreated or treated with 100 ng ml^{-1} EGF, immunoprecipitated with anti-mSos antiserum and western blotted with anti-phosphotyrosine monoclonal antibody 4G10. *B*, Lysates from v-*src*-transformed Rat2 cells were immunoprecipitated with either anti-Shc serum, anti-mSos peptide serum in the presence of competing peptide, or anti-mSos peptide serum in the absence of competing peptide. The resulting precipitates were probed with either control rabbit serum (*a*) or with anti-Shc serum (*b*).
METHODS. *A*, Two confluent dishes of NIH/EGFR-25ND cells were serum starved for 18 h in 0.2% fetal calf serum. One culture was treated with 100 ng ml^{-1} EGF for 5 min. Cells were washed, lysed as in Fig. 1*b*, clarified by centrifugation, precleared with protein A-Sepharose, then incubated with anti-mSos peptide serum. (Anti-mSos serum was generated against residues 100–120 of mSos1 and affinity purified, as described in detail elsewhere[45]). Immunoprecipitates were washed as in Fig. 1*b*, separated on a 7.5% SDS–PAGE gel, transferred and probed with 4G10 anti-phosphotyrosine monoclonal. *B*, High-density Ratsrc2 cultures were collected in lysis buffer as described in Fig. 1*a* and clarified by centrifugation. Lysates were precleared with protein A-Sepharose beads and then incubated for 1 h with either precoupled anti-Shc protein A-Sepharose beads[41] (anti-Shc antibodies from UBI, Lake Placid, NY), anti-mSos peptide serum precoupled to protein A-Sepharose beads[41], or anti-mSos peptide serum precoupled to protein A-Sepharose beads that has been preincubated with the immunizing peptide. Beads were washed as in Fig. 2*B*, eluted in sample buffer for 15 min at 70 °C, and then resolved on a 10% SDS-PAGE gel. This gel was then transferred to nitrocellulose, blocked and probed as in Fig. 1*a* except that the primary antiserum used was either control rabbit serum or anti-Shc antiserum and the secondary antibody used was HRP-conjugated goat anti-rabbit Fc antiserum (Pierce).

This binding is mediated by specific phosphotyrosine-containing peptides on the receptor and the SH2 domain of Grb2 (ref. 26). Taken together with the Grb2:mSos1 association found here, these earlier data indicate that Grb2 may function to recruit mSos1 into a multiprotein complex with an autophosphorylated receptor containing tyrosine kinase by acting as a bridge between the receptor and mSos1. We therefore used an affinity-purified anti-peptide antiserum raised against residues 100–120 of mSos1 and mSos2 (the two are identical in this region) to search for complexes between the endogenous mSos proteins and epidermal growth factor (EGF) receptors overexpressed on the surface of NIH/EGFR-25ND cells[33]. This cell line is a derivative of NIH 3T3 transfected with a construct expressing the human EGF receptor[33], and is analogous to the NIH 3T3 derivative HER14 in which the association of Grb2 with activated EGF receptors was demonstrated[26]. mSos precipitates from EGF-treated cells contained a diffuse 170–175K phosphotyrosine-containing band with the mobility of the EGF receptor which was absent in precipitates from cells that had not been treated with EGF before cell lysis (Fig. 3*A*). EGF treatment of cells thus results in the formation of multi-protein complexes containing both the EGF receptor and the mSos protein(s).

Another phosphotyrosine-containing protein that can associate with Grb2 is the recently described Shc oncogene product[34,35], which is heavily phosphorylated on tyrosine residues in cells transformed by the non-receptor tyrosine kinases v-Src and v-Fps[36]. Shc can transform fibroblasts and induce PC12 pheochromocytoma cells to differentiate[35]. The induction of PC12 differentiation is blocked by the N17 dominant inhibitory mutant of Ras[35], however, indicating that Shc, like Grb2, functions upstream from Ras. We therefore considered whether Shc acts analogously to autophosphorylated receptors like the EGF-R in binding to mSos by its phosphotyrosine residues.

To explore this possibility, we tested whether Shc is bound to mSos proteins in the Src-transformed Ratsrc2 line[37]. mSos precipitates from these cells were resolved by gel electrophoresis and analysed by western blotting with either a polyclonal anti-Shc antiserum (Fig. 3*B*, *b*) or with a control rabbit serum (Fig. 3*B*, *a*). Both p46Shc and p52Shc were specifically detected in the mSos immunoprecipitates unless the antiserum was preabsorbed with the mSos-derived immunizing peptide (Fig. 3*B*, *b*). Shc precipitation by the mSos antiserum must thus have been due to interactions between Shc and mSos in the immunoprecipitates and not to nonspecific crossreactivity of the antiserum with Shc.

The mSos proteins therefore occur in complexes containing a tyrosine kinase receptor (the EGF receptor) or a tyrosine kinase substrate (the Shc protein), both of which also bind Grb2 through its SH2 domain. As the mSos1 protein associates with the SH3 domains of Grb2, Grb2 appears to act as a bridging protein coupling tyrosine kinase receptors or tyrosine kinase substrates like Shc to mSos proteins. The formation of such complexes suggested, in turn, that these represent a means by which tyrosine kinases convey signals to mSos proteins.

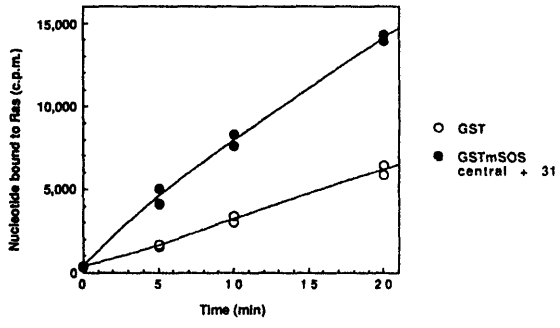

FIG. 4 Guanine-nucleotide-exchange activity of mSos1 towards Ras. [α-^{32}P]GTP binding to H-Ras was determined in the presence of GST–Sos fusion protein or GST alone.
METHODS. Assays were done in 20 μl 50 mM HEPES pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 0.1 mg ml^{-1} BSA, 0.2 mM PNPP, 0.05% Triton X-100 and 1.5 μM [α-^{32}P]GTP (2.5 μCi) with 1 μg fusion protein. This GSTmSos1 fusion protein was isolated from soluble lysates of induced bacteria expressing the GSTmSos1central + 31 fusion protein as described in Fig. 2. About 2% of the fusion protein represented full-length product. The remaining 98% of the soluble fusion protein is a C-terminal breakdown product of about 28K which encodes little more than the GST peptide. Reactions therefore included about 20 ng of the GSTmSos fusion protein. The reaction was started by addition of 10 ng, per assay point, of Ras. After the indicated time, samples were put on ice and binding stopped by addition of 1 ml 50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 μl ml^{-1} BSA, 0.1 mM unlabelled GTP. Supernatant (900 μl) was then spotted onto nitrocellulose filters, washed three times with 5 ml cold PBS, 5 mM $MgCl_2$, and the radioactivity bound to the filters was Cerenkov-counted.

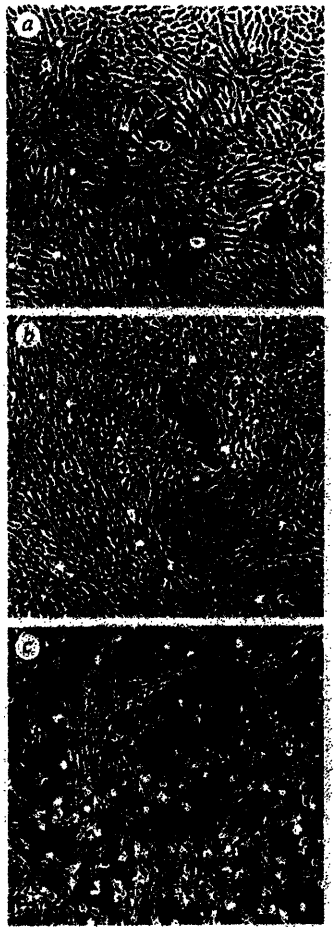

FIG. 5 Morphology of parental Rat1 fibroblasts (*a*) and DSos-infected derivative cell lines Rat1*Sos*F7C (*b*) and Rat1*Sos*F10C (*c*).
METHODS. Rat1 cells were infected with viral supernatants from a cloned ΩE cell line transfected with pBabeH*Sos* as described above. Transformed foci were isolated by micromanipulation and grown into stable cell lines.

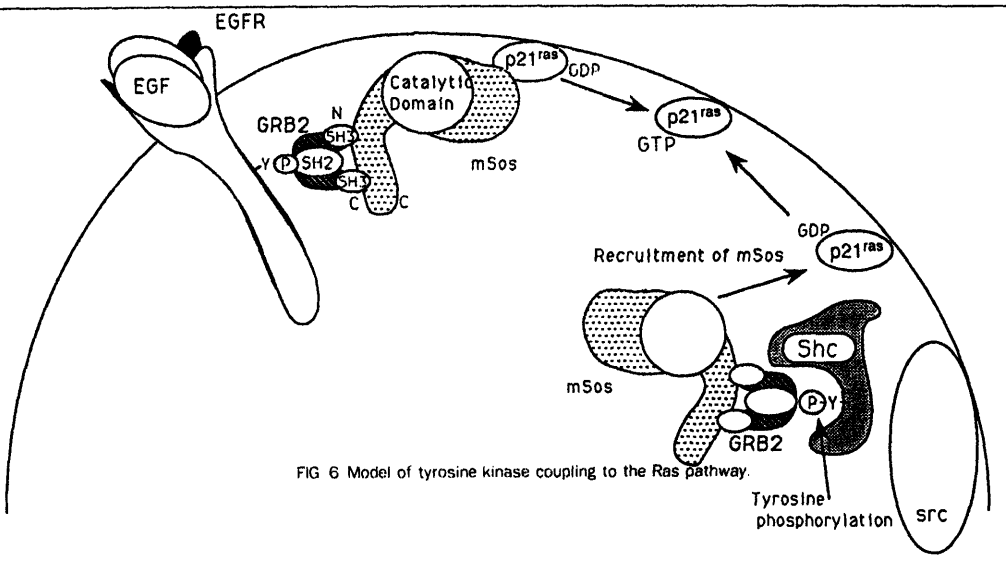

FIG 6 Model of tyrosine kinase coupling to the Ras pathway.

mSos1 is a Ras nucleotide-exchange protein

Sos proteins are homologous to several yeast nucleotide exchange factors that induce guanine nucleotide exchange by yeast Ras proteins[13,14,28]. mSos1 has not, however, been shown to induce nucleotide exchange by mammalian Ras.

Bacterially expressed GST-Sos fusion protein containing residues 613-1,166 of mSos1, was therefore purified and tested for its ability to increase the rate at which guanine nucleotide binds to H-Ras. The rate at which [α-^{32}P]GTP was loaded on H-Ras was three times higher in the presence of the Sos fusion protein than in the presence of GST control showing that mSos can act as a guanine nucleotide exchange factor for Ras *in vitro* (Fig. 4). Additional experiments revealed that the mSos1 catalytic-domain fusion protein also stimulated release of guanine nucleotides from the Ras protein (data not shown). This Sos fusion protein had no exchange activity towards the closely related protein R-Ras (data not shown), indicating that the Sos-Ras interaction was highly specific. By promoting physical complexes between tyrosine kinases (or their substrates) and the mSos proteins, Grb2 thus couples such kinases to a protein (mSos) capable of activating the Ras signalling pathway.

Morphological transformation by *Drosophila* Sos

Mutation or overexpression of the components of this signal transduction pathway, including growth factors and their tyrosine kinase receptors, nonreceptor tyrosine kinases and their substrate Shc, Grb2 and Ras, results in the induction of cell growth or transformation. The only components not so far shown to do so are the Sos proteins. We therefore devised a test for Sos function to strengthen the conclusion that the Sos proteins participate in this cascade.

As this pathway has apparently been strongly conserved in evolution, we reasoned that the *Drosophila* Sos protein might couple tyrosine kinases to Ras in mammalian cells and that its overexpression there should stimulate the Ras pathway. As an assay for biological function, we scored the appearance of morphologically transformed cell foci in monolayers of rat cells exposed to a retrovirus transducing the *Drosophila Sos* gene (D*Sos*). As a positive control, we used a retrovirus transducing an oncogenic *neu* allele. Helper-free retroviral stocks were generated from cloned producer lines expressing D*Sos*/hygro virus, Neu/hygro virus or hygro virus, and transforming potential was measured as the ratio of transformed foci to hygromycin-resistant colonies for each viral stock. Rat1 fibroblast cells were used for these assays, as they have a low rate of spontaneous transformation and, unlike NIH 3T3 cells, are not sensitive to weakly oncogenic stimuli like those resulting from overexpression of normal Ras[38].

The D*Sos* virus induced focal transformation of these cells, but with less than one-tenth the efficiency of the *neu* virus (Table 1). In addition, the *neu*-induced foci typically appeared earlier than D*Sos* foci. Weak transformation of NIH 3T3 cells by the catalytic domain of a yeast protein that can stimulate the exchange of guanine nucleotides on Ras has been previously reported[39].

Foci were picked to establish stable D*Sos*-infected cell lines. These lines were found to express D*Sos* RNA on northern blots (data not shown), and were refractile, growing to a high saturation density (Fig. 5). The *Drosophila Sos* protein, when overexpressed in mammalian cells, is thus able to trigger growth-stimulating pathways, such as the Ras pathway, so as to produce growth deregulation and transformation. This strengthens the idea that Sos proteins, initially discovered in the context of differentiating fly eye tissue, are so well conserved in evolution that they can participate in activating a mammalian mitogenic pathway.

Conclusions

An extensive body of data indicates that receptor and nonreceptor tyrosine kinases lie upstream of Ras in a signalling cascade that is widely conserved among metazoa. This signalling cascade is used to transduce both mitogenic and differentiation signals and seems to operate in some form in virtually all cell types studied to date. In spite of this intensive study, the biochemical links in this cascade have until recently remained unclear.

We have shown here that several of the components of this cascade are able to form physical complexes with one another. The central protein in this interaction is Grb2. On the one hand, it can bind tightly to mSos1 through its two SH3 groups; on the other, it can bind to tyrosine kinase substrates[26,35] (in this case either the autophosphorylated tail of the EGF receptor, or Shc, a substrate of the Src kinase) through its SH2 group. This bifunctionality allows it to serve as an adapter molecule that brings together the upstream signallers (the kinases) and the element two steps further down the cascade (mSos).

The mSos proteins are homologous to several yeast factors that stimulate GTP loading onto Ras. Such loading activates

these Ras proteins, allowing them to transmit signals to subsequent steps in the pathway. Here we have shown that mSos1 too can increase GTP loading on mammalian Ras, and that overexpression of the *Drosophila* mSos homologue, *DSos*, can transform rat cells.

Together, these experiments solidify thinking about the organization of the tyrosine kinase to Ras signalling cascade, which can now be schematized as depicted in Fig. 6.

Nonetheless, vital pieces of evidence supporting this scheme are still lacking. Although Grb2 has all of the attributes of the adaptor that links an autophosphorylated receptor to mSos, and such complexes can indeed be observed in the living cell, we have not yet succeeded in showing that the formation of the observed (EGF) receptor:mSos complexes depends on Grb2 rather than another bridging molecule with an analogous function.

The functional significance of the observed, multi-protein complexes also needs further clarification. Although there is strong evidence that these complexes assemble in the presence of mitogens, we have not shown directly that their formation is tightly linked with the transduction of signals between tyrosine kinases, their substrates and mSos1. But loss-of-function Grb2 mutants with altered SH3 domains are unable to induce DNA synthesis in cooperation with Ras^{26}, and have altered mSos1 binding profiles, indicating that mSos1:Grb2 complex formation and induction of DNA synthesis by Grb2 are indeed functionally linked. The signal transduction cascade characterized in this report is likely to represent only one of several ways in which tyrosine kinases affect Ras function and thereby trigger cell growth and differentiation. □

Received 13 April; accepted 23 April 1993.

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ACKNOWLEDGEMENTS. We thank J. Downward for help and encouragement; C. Albright, J. Settleman, P. Rodriguez-Viciana, L. Fisher, D. Smith, G. Hicks, C. Wilson, V. Tybulewicz, A. Gifford, T. Basu, P. Warne, S. Rayter, Y. Geng, A. Fazeli, J. Liu, M. Hatakeyama, P. Sicinski, D. Shaywitz, V. Episkopou, D. Templeton, S. Mittnacht, J. Morgenstern, B. Strauss, A. Greenberg, coworkers in the Weinberg laboratory, R. Mulligan and his laboratory, H. Land and B. Amati, D. Cantrell and laboratory and P. Parker and laboratory for valuable discussion, help, encouragement and reagents. We also thank D. Bowtell, T. Pawson, B. Futcher, S. Ponnambalam, S. Clark, D. Lowy, D. Pellman, M. Simon and G. Rubin for reagents and S. G. Clark and H. R. Horvitz for communication of unpublished data. S.E.E. has been funded by NCI of Canada and is presently funded by an MRC of Canada Centennial Fellowship; B.W.G. is supported by an NIH Training Grant; L.B. is a Fellow of the Federation of European Biochemical Societies and R.A.W. is an American Cancer Society Professor. Much of this work was supported by a grant from the US National Cancer Institute to R.A.W.

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