MOLECULAR AND CELLULAR BIOLOGY, Nov. 1994, p. 7483–7491
0270-7306/94/$04.00+0

Vol. 14, No. 11

ralGDS Family Members Interact with the Effector Loop of *ras* p21

AKIRA KIKUCHI, SUSAN D. DEMO, ZHI-HAI YE, YEN-WEN CHEN, AND LEWIS T. WILLIAMS*

Cardiovascular Research Institute, University of California San Francisco, San Francisco, California 94143-0130

Received 26 May 1994/Returned for modification 19 July 1994/Accepted 9 August 1994

Using a yeast two-hybrid system, we identified a novel protein which interacts with *ras* p21. This protein shares 69% amino acid homology with *ral* guanine nucleotide dissociation stimulator (ralGDS), a GDP/GTP exchange protein for *ral* p24. We designated this protein RGL, for ralGDS-like. Using the yeast two-hybrid system, we found that an effector loop mutant of *ras* p21 was defective in interacting with the *ras* p21-interacting domain of RGL, suggesting that this domain binds to *ras* p21 through the effector loop of *ras* p21. Since ralGDS contained a region highly homologous with the *ras* p21-interacting domain of RGL, we examined whether ralGDS could interact with *ras* p21. In the yeast two-hybrid system, ralGDS failed to interact with an effector loop mutant of *ras* p21. In insect cells, ralGDS made a complex with v-*ras* p21 but not with a dominant negative mutant of *ras* p21. ralGDS interacted with the GTP-bound form of *ras* p21 but not with the GDP-bound form in vitro. ralGDS inhibited both the GTPase-activating activity of the neurofibromatosis gene product (NF1) for *ras* p21 and the interaction of Raf with *ras* p21 in vitro. These results demonstrate that ralGDS specifically interacts with the active form of *ras* p21 and that ralGDS can compete with NF1 and Raf for binding to the effector loop of *ras* p21. Therefore, ralGDS family members may be effector proteins of *ras* p21 or may inhibit interactions between *ras* p21 and its effectors.

ras p21 is a member of the small GTP-binding protein (G-protein) superfamily and plays an important role in cell growth and differentiation. The molecular mechanisms by which *ras* p21 regulates cell growth and differentiation are not understood (2, 17, 24, 32). *ras* p21 has GDP/GTP-binding and GTPase activities and cycles between the GDP-bound inactive and GTP-bound active forms. The GDP-bound inactive form can be activated by guanine nucleotide exchange proteins (24, 32) which promote the exchange of GDP for GTP, thereby converting *ras* p21 to the GTP-bound active form. Presumably the GTP-bound active form of *ras* p21 interacts with effector proteins that can mediate *ras* p21-dependent processes such as growth factor-stimulated cell proliferation.

Identification of effector proteins of the active form of *ras* p21 has been difficult. One candidate effector protein of *ras* p21 is Raf, a cytoplasmic serine/threonine protein kinase that has been previously shown to act downstream of *ras* p21 (8, 15, 21). It has been recently demonstrated that Raf interacts with the GTP-bound but not with the GDP-bound form of *ras* p21 (18, 20, 29, 33–35, 41), that Raf binds to the effector loop of *ras* p21 (20, 33–35, 41), and that Raf inhibits the GTPase-activating activity of GTPase-activating protein (GAP), which is known to interact with the effector loop of *ras* p21 (35, 41). These results have indicated that Raf is an effector protein of *ras* p21, consistent with previous observations that Raf acts downstream of *ras* p21 in signaling pathways that mediate both the differentiation and mitogenic responses to receptor tyrosine kinases (8, 15, 17, 21, 24, 30, 36). However, it is possible that *ras* p21 has effector proteins other than Raf, since *ras* p21 has multiple functions (2, 17, 24).

We used a yeast two-hybrid system, originally developed by Fields and Song (12), to identify other possible effector proteins of *ras* p21. This system has been very useful in detecting the physical interaction between two fusion proteins expressed in *Saccharomyces cerevisiae*. With the yeast two-hybrid system, it has been found that SIP1 is a substrate of *S. cerevisiae* SNF1 (40), that Raf interacts with *ras* p21 (33, 34, 41), and that Bcl-2 binds to R-*ras* p23 (11). We report here the identification of a novel protein that interacts with *ras* p21 in the yeast two-hybrid system. This protein, termed RGL (ralGDS-like), is highly homologous with *ral* guanine nucleotide dissociation stimulator (ralGDS), a GDP/GTP exchange protein for *ral* p24, a member of small G-protein superfamily (1). Our results indicate that the *ras* p21-binding domain of RGL binds to *ras* p21 through the effector loop of *ras* p21 and that this domain is highly conserved in ralGDS. These results prompted us to examine whether this new family of proteins (RGL and ralGDS) could be effector proteins of *ras* p21. Since ralGDS has been well characterized (1), we used ralGDS to examine this possibility. We tested three characteristics that could be considered to be criteria for identification of an effector protein of *ras* p21: (i) the protein must interact with the GTP-bound active form of *ras* p21 but not with the GDP-bound inactive form, (ii) it must interact with *ras* p21 through the effector loop of *ras* p21, and (iii) ideally the protein should inhibit the interaction of *ras* p21 with other effector proteins such as Raf. By both in vitro and in vivo studies, we demonstrate that ralGDS fulfills these three characteristics.

MATERIALS AND METHODS

Materials and chemicals. PC62 and the PC51/mouse embryonic cDNA library were provided by P. M. Chevray and D. Nathans (Johns Hopkins University, Baltimore, Md.) (6). PC62 contains an *ADH* promoter expressing the GAL4 DNA-binding domain (amino acids 1 to 147), and PC51 contains the *ADH* promoter expressing the GAL4 transactivation domain (amino acids 768 to 881). The ralGDSb and *ralB* p24 cDNAs and the anti-ralGDS antibody were provided by B. W. Giddings, C. F. Albright, and R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, Mass.) (1). The c-H-*ras* p21 cDNA, dominant negative *ras* p21 cDNA (*ras* $p21^{S17N}$ [a form of *ras* p21 in which Ser-17 is changed to Asn]), and the

* Corresponding author. Mailing address: University of California, San Francisco, Parnassus Ave., CVRI, Box 0130, San Francisco, CA 94143-0130. Phone: (415) 476-4402. Fax: (415) 476-0429.

hybridoma cells producing anti-*ras* p21 antibody (Y13-259) were provided by J. Downward (Imperial Cancer Research Institute, London, England). pGBT9, pGBT/*ras* p21, and pGBT/*rap1* p21 were provided by L. Van Aelst and M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (33). pGBT9 contains an *ADH* promoter expressing the GAL4 DNA-binding domain (amino acids 1 to 147). *S. cerevisiae* YPB2 and pGAD were provided by G. Hannon and D. Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). pGAD contains the *ADH* promoter expressing the GAL4 transactivation domain (amino acids 768 to 881). *S. cerevisiae* Y153 was provided by S. Field (University of California, Berkeley) (9). *rac1* $p21^{G12V}$ (a form of *rac1* p21 in which Gly-12 is changed to Val) was provided by Alan Hall (Institute of Cancer Research, London, England). The neurofibromatosis 1 (NF1) cDNA was provided by G. Xu (University of Utah, Salt Lake City) (39). *Spodoptera frugiperda* (Sf9) cells, pVL1393, and BaculoGold linearized baculovirus DNA were purchased from Pharmingen (San Diego, Calif.). High-five cells were from Invitrogen (San Diego, Calif.). The anti-*ras* p21 antibodies (Y13-238 for immunoprecipitation assay and F235 for immunoblot analysis) were from Oncogene Science Inc. (New York, N.Y.). [γ-^{32}P]GTP and [α-^{32}P]GTP were from DuPont NEN Research Product (Boston, Mass.). All procedures of passage, infection, and transfection of Sf9 cells and the isolation of recombinant baculoviruses were carried out as described previously (31). c-*ras* p21 and ralGDS were purified from the cytosolic fraction of Sf9 cells and High-five cells, respectively, as described previously (1, 27). Glutathione *S*-transferase (GST) fused to the N-terminal region of Raf (amino acids 1 to 322) (GST–N-Raf) and GST fused to the NF1 catalytic domain (GST-NF1) were purified from Sf9 cells expressing GST–N-Raf and *Escherichia coli* expressing GST-NF1, respectively, as described previously (18, 39).

Plasmid constructions. To construct PC62 encoding c-H-*ras* p21, the 0.6-kb fragment containing c-H-*ras* p21 with the *Sal*I site upstream from the initiator methionine codon and the *Bam*HI site downstream from the termination codon was synthesized by PCR. This fragment was digested with *Sal*I and *Bam*HI and inserted into *Sal*I- and *Bam*HI-cut PC62 to generate PC62/*ras* p21. To make pGAD encoding the *ras* p21-interacting domain (RID) of RGL (amino acids 605 to 768), a 0.5-kb fragment containing RID with *Bam*HI and *Sal*I sites was synthesized by PCR. This fragment was digested with *Bam*HI and *Sal*I and inserted into *Bam*HI- and *Sal*I-cut pGAD to generate pGAD/RID. To construct pGAD containing ralGDS, pBluescript KS/*ralGDS* was digested with *Nco*I. The 1.8-kb fragment which represents the N-terminal two-thirds of ralGDS (N-ralGDS) was blunted with Klenow enzyme and inserted into pGAD which was digested with *Sma*I to generate pGAD/N-*ralGDS*. Then pEV55/*ralGDS* was digested with *Avr*II and *Eco*RI, and the 1.1-kb fragment, which represents the C terminus of ralGDS, was inserted into *Avr*II- and *Eco*RI-cut pGAD/N-*ralGDS* to generate pGAD/*ralGDS*. *ras* $p21^{C186S}$ (a form of *ras* p21 in which Cys-186 is changed to Ser) and *ras* $p21^{T35A}$ (a form of *ras* p21 in which Thr-35 is changed to Ala) were made by PCR. To construct pGBT9 encoding *ras* $p21^{C186S}$ and *ras* $p21^{T35A}$, the 0.6-kb fragments containing *ras* $p21^{C186S}$ and *ras* $p21^{T35A}$ with *Sma*I and *Bam*HI sites were synthesized by PCR. These fragments were digested with *Sma*I and *Bam*HI and inserted into *Sma*I- and *Bam*HI-cut pGBT9 to generate pGBT/*ras* $p21^{C186S}$ and pGBT/*ras* $p21^{T35A}$. To construct pGBT9 encoding *ralB* p24 and *rac1* $p21^{G12V}$, the 0.6-kb fragments containing *ralB* p24 and *rac1* $p21^{G12V}$ with *Bam*HI and *Sal*I sites were synthesized by PCR. These fragments were digested with *Bam*HI and *Sal*I and inserted into *Bam*HI- and *Sal*I-cut pGBT9 to generate pGBT/*ral*B p24 and pGBT/*rac*1 $p21^{G12V}$. To construct pVL1393 encoding c-*ras* p21 and *ras* $p21^{S17N}$, the 0.6-kb fragments containing c-*ras* p21 and *ras* $p21^{S17N}$ with *Bam*HI and *Pst*I sites were synthesized by PCR. These fragments were digested with *Bam*HI and *Pst*I and inserted into *Bam*HI- and *Pst*I-cut pVL1393. Constructions of v-*ras* p21 (a form of *ras* p21 in which Gly-12 is changed to Val) and the N-terminal region of Raf in pVL1393 and pV-IKS were carried out as described previously (18).

Two-hybrid screening. The yeast reporter strain YPB2 was cotransformed with PC62/*ras* p21 and the PC51/mouse embryonic cDNA library and plated at a density of 2.5×10^4 colonies per plate on synthetic minimal media lacking histidine, leucine, and tryptophan and supplemented with 30 mM 3-aminotriazole. The plates were incubated for 4 days at 30°C. Of the 0.5 million colonies that were plated, approximately 200 grew in the absence of histidine. These colonies were patched to selective plates and assayed for β-galactosidase activity by a filter assay (4). Eight colonies were positive. Of these, five were specific for PC62/*ras* p21 when tested with PC62/SH3 domain of mouse p85 (amino acids number 1 to 100). Library inserts from these five colonies were sequenced by using a Promega Sequenase kit (Promega Corp., Madison, Wis.) after subcloning the inserts as *Sal*I-*Not*I fragments into pBluescript KS. cDNAs from five colonies had the same orientation, and both strands of these cDNAs were determined. To identify the full-length cDNA of RGL, a probe was made by using the *Sal*I-*Not*I fragment with a Quick Prime kit (Pharmacia Biotechnology, Piscataway, N.J.) and used to screen a BALB/c3T3 fibroblast cDNA library as described previously (19).

Interaction assay of RID or ralGDS with *ras* p21 and other small G proteins in the yeast two-hybrid system. The yeast reporter strain Y153 (9) was cotransformed with pGAD/RID or pGAD/*ralGDS* and the indicated plasmids and plated on synthetic minimal medium lacking leucine and tryptophan. Plates were incubated for 3 days at 30°C and assayed for β-galatosidase activity by filter assay (4).

Interaction assay of ralGDS and *ras* p21 in vivo. Monolayers of Sf9 cells (2×10^7 cells) were infected singly or doubly with high-titer recombinant baculoviruses (10^8 PFU/ml) at a multiplicity of infection of 5 per cell. At 72 h postinfection, the cells were washed with cold phosphate-buffered saline and lysed in 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 10 μg of leupeptin per ml) at 4°C for 1 h. Insoluble material was removed by centrifugation at 4°C for 30 min at 13,000 $\times$ *g*, and 0.2 ml of lysate (0.24 mg of protein) was used for each assay. The lysates expressing ralGDS and v-*ras* p21 or *ras* $p21^{S17N}$ were prepared, and the proteins of the lysates were immunoprecipitated with the anti-*ras* p21 antibody. Y13-238 was used in the immunoprecipitation experiments except that Y13-259 was used for Fig. 2D. The immunoprecipitates were washed once with lysis buffer, twice with 100 mM Tris-HCl (pH 7.5) and 0.5 M LiCl, and once with 10 mM Tris-HCl (pH 7.5). The precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gel) (23), transferred to nitrocellulose filters, and probed with the anti-ralGDS or anti-*ras* p21 antibody.

Interaction assay of ralGDS and *ras* p21 in vitro. To make the guanosine 5′-(3-*O*-thio)triphosphate (GTPγS)- or GDP-bound form of *ras* p21, c-*ras* p21 (20 pmol) was incubated for 10 min at 30°C with 25 μM GTPγS or GDP in 40 μl of reaction mixture (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT]). After the incubation, 600 mM $MgCl_2$ was added at a final concentration of 15 mM. The

GTPγS- or GDP-bound form of *ras* p21 was incubated for 30 min at 4°C with ralGDS (20 pmol) in 80 μl of reaction mixture (20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 10 mM $MgCl_2$, 0.5 mM DTT, 25 μM GTPγS or GDP). Then, the anti-*ras* p21 antibody (Y13-238) was added to this mixture, and the mixture was subjected to immunoprecipitation. The precipitate was subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed with the anti-ralGDS antibody.

GAP assay of NF1. The GAP assay for c-*ras* p21 was performed as described previously (14). Briefly, c-H-*ras* p21 (2.5 pmol) was preincubated for 5 min at 30°C in 5 μl of preincubation mixture (100 mM sodium phosphate [pH 6.8], 0.5 mM EDTA, 0.5 mg of bovine serum albumin per ml, 0.5 mM DTT, 0.5 μM [γ-^{32}P]GTP [20,000 to 30,000 cpm/pmol]). To this preincubation mixture, 45 μl of reaction mixture (500 μM GTP, 22.2 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 1.1 mM $MgCl_2$, 1.1 mg of bovine serum albumin per ml, 0.11 mM DTT, 2.2 mM Tris-HCl [pH 7.5]) containing 10 nM GST-NF1 and the indicated amounts of ralGDS or GST-N-Raf was added, and a second incubation was performed at 24°C. Assays were quantified by rapid filtration on nitrocellulose filters. GAP activity was calculated from the decrease of the radioactivity of [γ-^{32}P]GTP compared with a reaction performed in the absence of GST-NF1, and GAP inhibition activity was expressed as percent decrease of GAP activity of GST-NF1.

Interaction assay of Raf and *ras* p21. To make the [α-^{32}P]GTP-bound form of *ras* p21, c-H-*ras* p21 (2.5 pmol) was preincubated for 15 min at 30°C in 5 μl of the preincubation mixture described above except that [α-^{32}P]GTP was used instead of [γ-^{32}P]GTP. To the preincubation mixture, 45 μl of the reaction mixture described above containing 20 nM GST–N-Raf and the indicated amounts of ralGDS was added, and a second incubation was performed for 30 min at 4°C. GST–N-Raf was precipitated with glutathione-Sepharose 4B, the precipitates were washed, and the remaining radioactivity was counted.

Other assays. Protein concentrations were determined with bovine serum albumin as a standard (3).

Nucleotide sequence accession number. The GenBank accession number for the mouse *RGL* cDNA sequence is U14103.

RESULTS

Isolation of a protein which interacts with *ras* p21 in the yeast two-hybrid system. To identify proteins that physically interact with H-*ras* p21, a fusion protein of *ras* p21 and the GAL4 DNA-binding domain was cotransformed with a mouse embryonic cDNA library expressed as a fusion with the GAL4 transactivation domain into a yeast reporter strain carrying the GAL4 binding sites upstream of both the *S. cerevisiae HIS3* gene and the *E. coli lacZ* gene. Of the 0.5 million yeast transformants, five grew in the absence of histidine and expressed β-galactosidase activity in a *ras* p21-dependent manner. The cDNA inserts from these five all encoded a single sequence containing an open reading frame of 164 amino acids and the consensus sequence for a stop codon and polyadenylation. We designated this *ras* p21-interacting domain RID.

To characterize RID, we examined the association of RID with *ras* p21 mutants and other small G proteins in the yeast two-hybrid system (Table 1). As assessed by filter assays of β-galactosidase, RID interacted with *ras* p21. To examine the effect of posttranslational modification of *ras* p21 on its interaction with RID, *ras* p21^{C186S} was used. It is known that this mutant is not posttranslationally modified (16). Coexpression of RID with *ras* p21^{C186S} reconstituted β-galactosidase activity. However, RID did not interact with the effector loop mutant of *ras* p21, *ras* p21^{T35A}. These findings indicate that the posttranslational modification of *ras* p21 is not necessary for its binding to RID and that the effector loop of *ras* p21 is required. We also examined the specificity of small G proteins which interact with RID. RID interacted with *rap1* p21 as well as with *ras* p21 but not with *ralB* p24 or with *rac1* p21^{G12V}. *rap1* p21 is known to have the same effector loop as *ras* p21 and to associate with the same effector-loop binding protein as *ras* p21, but how rap1 interacts with actual signaling effectors is not yet clear (24, 32).

TABLE 1. Interaction of RID with *ras* p21 in the yeast two-hybrid system[a]

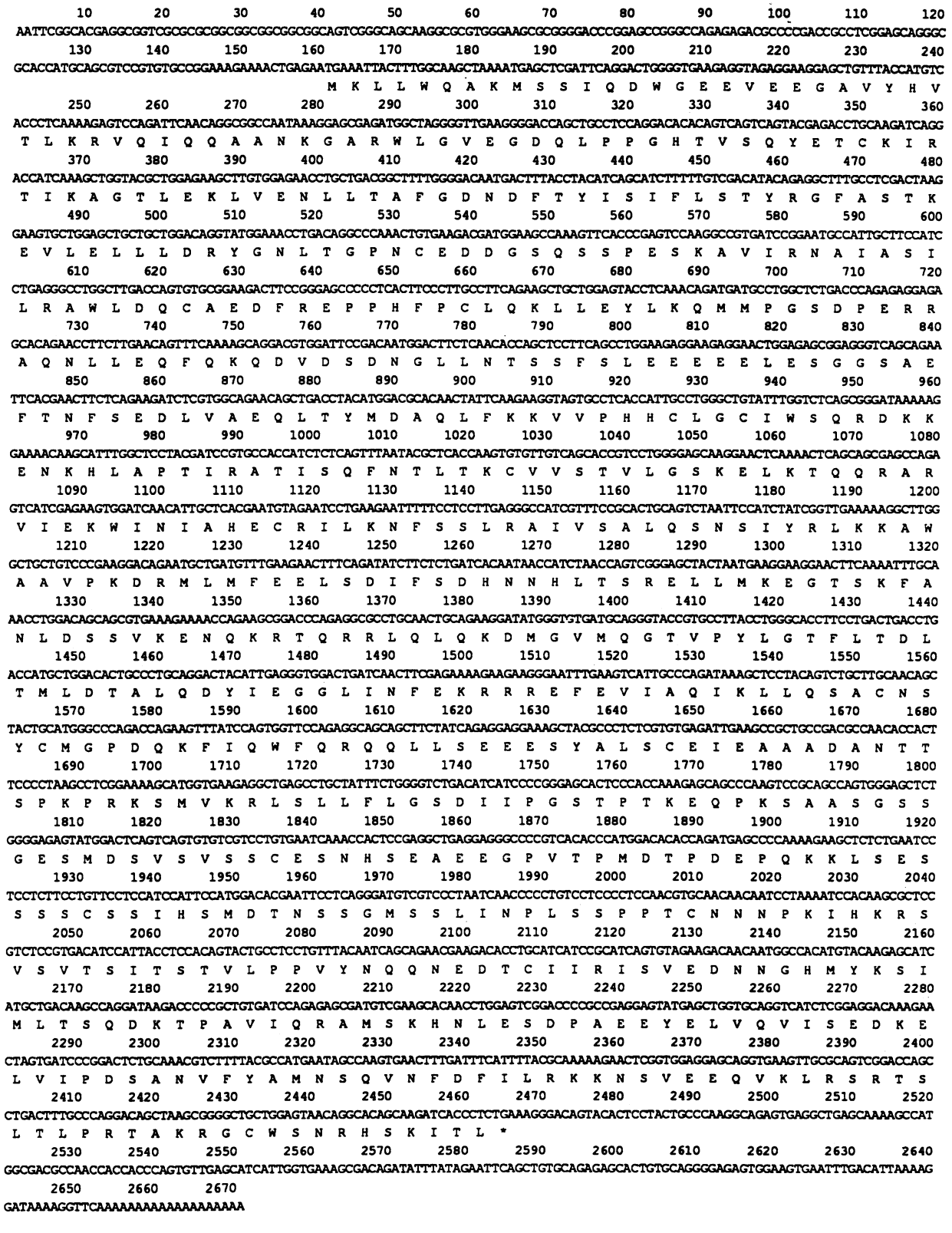
GAL4 DNA-binding domain fusion	GAL4 transactivation domain fusion	β-Galactosidase activity
Vector	RID	−
ras p21	Vector	−
ras p21	RID	+
ras p21^{C186S}	RID	+
ras p21^{T35A}	RID	−
rap1 p21	RID	+
ralB p24	RID	−
rac1 p21^{G12V}	RID	−

[a] Y153 was cotransformed with RID and *ras* p21 mutants or other small G proteins and assayed for β-galactosidase activity. A blue signal, representing β-galatosidase activity, is indicated by a +, and a white signal, indicating a lack of β-galactosidase activity, is shown as a −. In each case, β-galactosidase expression was not detected when cells were transformed with the DNA-binding domain (amino acids 1 to 147) or the transactivation domain (amino acids 768 to 881) fusion alone.

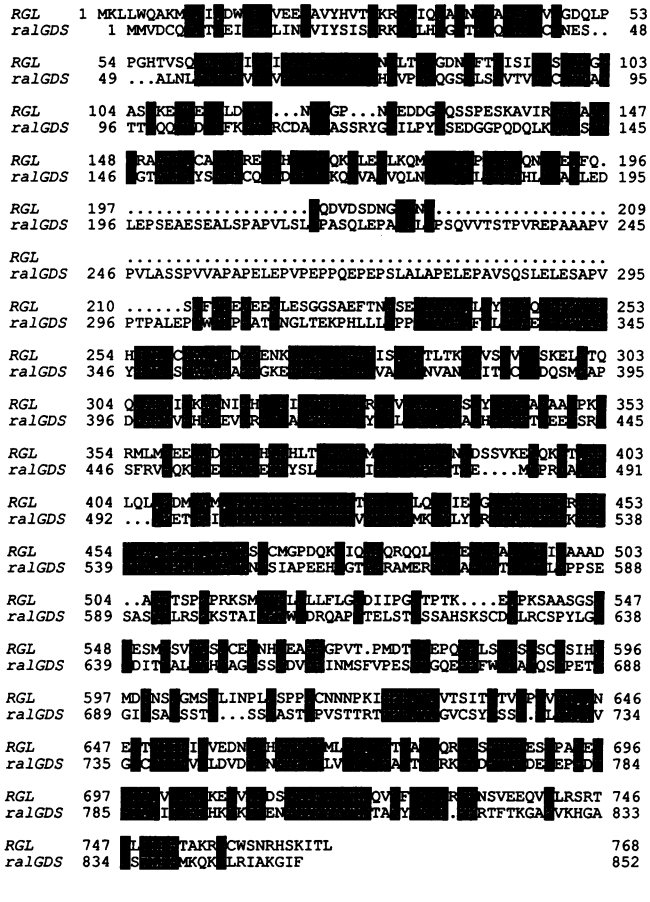
Molecular cloning of *RGL*. Using RID to probe a BALB/c3T3 fibroblast cDNA library, we identified a 2.7-kb cDNA containing an open reading frame of 768 amino acids (Fig. 1A). The predicted protein sequence had 69% amino acid homology with ralGDS, which is a GDP/GTP exchange protein for *ral* p24, a member of small G-protein superfamily (Fig. 1B) (1). We designated this protein RGL. In the *RGL* cDNA, the 5′ noncoding region was long and had a high percentage of G · C base pairs, which is typical in the 5′ noncoding region. The neighboring sequence of the first ATG was consistent with the translation initiation start proposed by Kozak (22).

The sequence of 290 amino acids (amino acids 210 to 499) of RGL was similar to a comparable region of CDC25, which is a GDP/GTP exchange protein for *ras* p21 (24, 25) (Fig. 1C). It is known that this region of CDC25 is important for GDS activity (1, 24, 25). RGL and ralGDS had an additional extensive region C terminal to the CDC25-like domain (1). RID was located in this region of RGL. There was a region exhibiting 66% amino acid homology with RID in the C terminus of ralGDS. This strong homology in the overall sequence suggests that ralGDS and RGL constitute a family. Furthermore, this structural analysis suggests that ralGDS and RGL may interact with the effector loop of *ras* p21. Since ralGDS has been well characterized (1), we used ralGDS in an attempt to examine whether ralGDS could be an effector protein of *ras* p21.

Interaction of ralGDS with *ras* p21 in the yeast two-hybrid system. We examined whether ralGDS interacts with *ras* p21 in the yeast two-hybrid system (Table 2). As assessed by filter assays of β-galactosidase, ralGDS interacted with *ras* p21. Consistent with the data shown in Table 1, ralGDS interacted with *ras* p21^{C186S} but not with *ras* p21^{T35A}. Furthermore, ralGDS interacted with *rap1* p21 as well as with *ras* p21 but not with *ralB* p24 or with *rac1* p21^{G12V}.


A
10 20 30 40 50 60 70 80 90 100 110 120
AATTCGGCACGAGGCGGTCGCGCGCGGCGGCGGCGGCGGCAGTCGGGCAGCAAGGCGCGTGGGAAGCGCGGGGACCCGGAGCCGGGCCAGAGAGACGCCCCGACCGCCTCGGAGCAGGGC
130 140 150 160 170 180 190 200 210 220 230 240
GCACCATGCAGCGTCCGTGTGCCGGAAAGAAAACTGAGAATGAAATTACTTTGGCAAGCTAAAATGAGCTCGATTCAGGACTGGGGTGAAGAGGTAGAGGAAGGAGCTGTTTACCATGTC
M K L L W Q A K M S S I Q D W G E E V E E G A V Y H V
250 260 270 280 290 300 310 320 330 340 350 360
ACCCTCAAAAGAGTCCAGATTCAACAGGCGGCCAATAAAGGAGCGAGATGGCTAGGGGTTGAAGGGGACCAGCTGCCTCCAGGACACACAGTCAGTCAGTACGAGACCTGCAAGATCAGG
T L K R V Q I Q Q A A N K G A R W L G V E G D Q L P P G H T V S Q Y E T C K I R
370 380 390 400 410 420 430 440 450 460 470 480
ACCATCAAAGCTGGTACGCTGGAGAAGCTTGTGGAGAACCTGCTGACGGCTTTTGGGGACAATGACTTTACCTACATCAGCATCTTTTTGTCGACATACAGAGGCTTTGCCTCGACTAAG
T I K A G T L E K L V E N L L T A F G D N D F T Y I S I F L S T Y R G F A S T K
490 500 510 520 530 540 550 560 570 580 590 600
GAAGTGCTGGAGCTGCTGCTGGACAGGTATGGAAACCTGACAGGCCCAAACTGTGAAGACGATGGAAGCCAAAGTTCACCCGAGTCCAAGGCCGTGATCCGGAATGCCATTGCTTCCATC
E V L E L L L D R Y G N L T G P N C E D D G S Q S S P E S K A V I R N A I A S I
610 620 630 640 650 660 670 680 690 700 710 720
CTGAGGGCTGGCTTGACCAGTGTGCGGAAGACTTCCGGGAGCCCCCTCACTTCCCTTGCCTTCAGAAGCTGCTGGAGTACCTCAAACAGATGATGCCTGGCTCTGACCCAGAGAGGAGA
L R A W L D Q C A E D F R E P P H F P C L Q K L L E Y L K Q M M P G S D P E R R
730 740 750 760 770 780 790 800 810 820 830 840
GCACAGAACCTTCTTGAACAGTTTCAAAAGCAGGACGTGGATTCCGACAATGGACTTCTCAACACCAGCTCCTTCAGCCTGGAAGAGGAAGAGGAACTGGAGAGCGGAGGGTCAGCAGAA
A Q N L L E Q F Q K Q D V D S D N G L L N T S S F S L E E E E E L E S G G S A E
850 860 870 880 890 900 910 920 930 940 950 960
TTCACGAACTTCTCAGAAGATCTCGTGGCAGAACAGCTGACCTACATGGACGCACAACTATTCAAGAAGGTAGTGCCTCACCATTGCCTGGGCTGTATTTGGTCTCAGCGGGATAAAAAG
F T N F S E D L V A E Q L T Y M D A Q L F K K V V P H H C L G C I W S Q R D K K
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
GAAAACAAGCATTTGGCTCCTACGATCCGTGCCACCATCTCTCAGTTTAATACGCTCACCAAGTGTGTTGTCAGCACCGTCCTGGGGAGCAAGGAACTCAAAACTCAGCAGCGAGCCAGA
E N K H L A P T I R A T I S Q F N T L T K C V V S T V L G S K E L K T Q Q R A R
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
GTCATCGAGAAGTGGATCAACATTGCTCACGAATGTAGAATCCTGAAGAATTTTTCCTCCTTGAGGGCCATCGTTTCCGCACTGCAGTCTAATTCCATCTATCGGTTGAAAAAGGCTTGG
V I E K W I N I A H E C R I L K N F S S L R A I V S A L Q S N S I Y R L K K A W
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
GCTGCTGTCCCGAAGGACAGAATGCTGATGTTTGAAGAACTTTCAGATATCTTCTCTGATCACAATAACCATCTAACCAGTCGGGAGCTACTAATGAAGGAAGGAACTTCAAAATTTGCA
A A V P K D R M L M F E E L S D I F S D H N N H L T S R E L L M K E G T S K F A
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
AACCTGGACAGCAGCGTGAAAGAAAACCAGAAGCGGACCCAGAGGCGCCTGCAACTGCAGAAGGATATGGGTGTGATGCAGGGTACCGTGCCTTACCTGGGCACCTTCCTGACTGACCTG
N L D S S V K E N Q K R T Q R R L Q L Q K D M G V M Q G T V P Y L G T F L T D L
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
ACCATGCTGGACACTGCCCTGCAGGACTACATTGAGGGTGGACTGATCAACTTCGAGAAAAGAAGAAGGGAATTTGAAGTCATTGCCCAGATAAAGCTCCTACAGTCTGCTTGCAACAGC
T M L D T A L Q D Y I E G G L I N F E K R R R E F E V I A Q I K L L Q S A C N S
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
TACTGCATGGGCCCAGACCAGAAGTTTATCCAGTGGTTCCAGAGGCAGCAGCTTCTATCAGAGGAGGAAAGCTACGCCCTCTCGTGTGAGATTGAAGCCGCTGCCGACGCCAACACCACT
Y C M G P D Q K F I Q W F Q R Q Q L L S E E E S Y A L S C E I E A A A D A N T T
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
TCCCCTAAGCCTCGGAAAAGCATGGTGAAGAGGCTGAGCCTGCTATTTCTGGGGTCTGACATCATCCCCGGGAGCACTCCCACCAAAGAGCAGCCCAAGTCCGCAGCCAGTGGGAGCTCT
S P K P R K S M V K R L S L L F L G S D I I P G S T P T K E Q P K S A A S G S S
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
GGGGAGAGTATGGACTCAGTCAGTGTGTCGTCCTGTGAATCAAACCACTCCGAGGCTGAGGAGGGCCCCGTCACACCCATGGACACACCAGATGAGCCCCAAAAGAAGCTCTCTGAATCC
G E S M D S V S V S S C E S N H S E A E E G P V T P M D T P D E P Q K K L S E S
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
TCCTCTTCCTGTTCCTCCATCCATTCCATGGACACGAATTCCTCAGGGATGTCGTCCCTAATCAACCCCCTGTCCTCCCCTCCAACGTGCAACAACAATCCTAAAATCCACAAGCGCTCC
S S S C S S I H S M D T N S S G M S S L I N P L S S P P T C N N N P K I H K R S
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
GTCTCCGTGACATCCATTACCTCCACAGTACTGCCTCCTGTTTACAATCAGCAGAACGAAGACACCTGCATCATCCGCATCAGTGTAGAAGACAACAATGGCCACATGTACAAGAGCATC
V S V T S I T S T V L P P V Y N Q Q N E D T C I I R I S V E D N N G H M Y K S I
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280
ATGCTGACAAGCCAGGATAAGACCCCCGCTGTGATCCAGAGAGCGATGTCGAAGCACAACCTGGAGTCGGACCCCGCCGAGGAGTATGAGCTGGTGCAGGTCATCTCGGAGGACAAAGAA
M L T S Q D K T P A V I Q R A M S K H N L E S D P A E E Y E L V Q V I S E D K E
2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
CTAGTGATCCCGGACTCTGCAAACGTCTTTTACGCCATGAATAGCCAAGTGAACTTTGATTTCATTTTACGCAAAAAGAACTCGGTGGAGGAGCAGGTGAAGTTGCGCAGTCGGACCAGC
L V I P D S A N V F Y A M N S Q V N F D F I L R K K N S V E E Q V K L R S R T S
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520
CTGACTTTGCCCAGGACAGCTAAGCGGGGCTGCTGGAGTAACAGGCACAGCAAGATCACCCTCTGAAAGGGACAGTACACTCCTACTGCCCAAGGCAGAGTGAGGCTGAGCAAAAGCCAT
L T L P R T A K R G C W S N R H S K I T L *
2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640
GGCGACGCCAACCACCACCCAGTGTTGAGCATCATTGGTGAAAGCGACAGATATTTATAGAATTCAGCTGTGCAGAGAGCACTGTGCAGGGGAGAGTGGAAGTGAATTTGACATTAAAAG
2650 2660 2670
GATAAAAGGTTCAAAAAAAAAAAAAAAAAAA

B



C

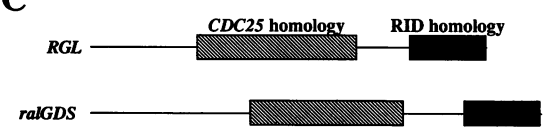


FIG. 1. Homology between RGL and ralGDS. (A) Nucleotide and predicted amino acid sequences of RGL. The single-letter amino acid code is shown below the DNA sequence. (B) Alignment of the amino acid sequences of RGL and ralGDS. Sequences were aligned by using the Best Fit program. Amino acid identity is denoted by a black background. Dots indicate gaps. *ralGDS* refers to mouse ralGDSa. (C) Schematic representation of RGL and ralGDS.

Interaction of ralGDS with *ras* p21 in intact cells. To examine whether ralGDS interacts with *ras* p21 in intact cells, we coexpressed ralGDS with v-*ras* p21 in insect cells. The expression level of transfected ralGDS in Sf9 cells expressing ralGDS alone was similar to that in the cells coexpressing ralGDS with v-*ras* p21, as assessed by immunoblotting (Fig. 2A, lanes 1 to 3). The lower band which is seen under ralGDS might be a degradation product of ralGDS. When the lysates coexpressing ralGDS with v-*ras* p21 were immunoprecipitated with the anti-*ras* p21 antibody, both ralGDS and *ras* p21 were detected in the *ras* p21 immune complex (Fig. 2B, lane 1). When the lysates expressing ralGDS alone or v-*ras* p21 alone were immunoprecipitated with the anti-*ras* p21 antibody, ralGDS was not detected (Fig. 2B, lane 2; Fig. 2D, lane 1). Neither ralGDS nor *ras* p21 was immunoprecipitated with nonimmune immunoglobulin in lysates expressing both proteins (Fig. 2B, lane 3).

TABLE 2. Interaction of ralGDS with *ras* p21 in the yeast two-hybrid system[a]

GAL4 DNA-binding domain fusion	GAL4 transactivation domain fusion	β-Galactosidase activity
Vector	ralGDS	−
ras p21	Vector	−
ras p21	ralGDS	+
ras $p21^{C186S}$	ralGDS	+
ras $p21^{T35A}$	ralGDS	−
rap1 p21	ralGDS	+
ralB p24	ralGDS	−
rac1 $p21^{G12V}$	ralGDS	−

[a] Y153 was cotransformed with ralGDS and *ras* p21 mutants or other small G proteins and assayed for β-galactosidase activity. A blue signal, representing β-galactosidase activity, is indicated by a +, and a white signal, indicating a lack of β-galactosidase activity, is shown as a −. In each case, β-galactosidase expression was not detected when cells were transformed with the DNA-binding domain (amino acids 1 to 147) or the transactivation domain (amino acids 768 to 881) fusion alone.

To characterize the interaction of ralGDS and *ras* p21 further, we examined the ability of ralGDS to interact with a *ras* p21 mutant, *ras* $p21^{S17N}$. *ras* $p21^{S17N}$ is well known as a dominant negative mutant that has higher affinity for GDP than GTP and strongly interacts with upstream molecules but not with downstream molecules (2, 10, 24). The expression level of *ras* $p21^{S17N}$ was similar to that of v-*ras* p21 (Fig. 2A, lanes 3 and 4). When the lysates coexpressing ralGDS with *ras* $p21^{S17N}$ were immunoprecipitated with the anti-*ras* p21 antibody, ralGDS was not coprecipitated with *ras* $p21^{S17N}$ under the same conditions in which ralGDS was coprecipitated with v-*ras* p21 (Fig. 2C, lanes 1 and 2). These results indicate that ralGDS makes a complex with v-*ras* p21 but not with *ras* $p21^{S17N}$ in intact cells.

We used Y13-238 as the anti-*ras* p21 antibody to immunoprecipitate *ras* p21 for these experiments. Another antibody, Y13-259, were tested for its ability to immunoprecipitate a *ras* p21-ralGDS complex. Y13-259 is known to be a neutralizing antibody (28). In contrast to Y13-238, Y13-259 could not immunoprecipitate the *ras* p21-ralGDS complex from the lysate coexpressing ralGDS with v-*ras* p21 under the same conditions (Fig. 2D, lanes 3 and 4). Y13-259 and Y13-238 immunoprecipitated similar amounts of *ras* p21 from the lysates expressing v-*ras* p21 alone (Fig. 2D, lanes 1 and 2).

Interaction of ralGDS with *ras* p21 in vitro. To examine whether the interaction of ralGDS with *ras* p21 is direct, we purified ralGDS and *ras* p21 from the cytosolic fraction of insect cells. The purity of both proteins was more than 95% by Coomassie brilliant blue staining (Fig. 3A). The GTPγS- or GDP-bound form of *ras* p21 was incubated with ralGDS, and this mixture was immunoprecipitated with the anti-*ras* p21 antibody. ralGDS was coprecipitated with the GTPγS-bound form of *ras* p21 in a dose-dependent manner but not with the GDP-bound form (Fig. 3B).

Effect of ralGDS on the GAP activity of NF1 for *ras* p21. It has been reported that ralGDS does not affect the dissociation of GDP and GTP from *ras* p21 (1). We examined the effect of ralGDS on the GTPase activity of *ras* p21. ralGDS did not alter the intrinsic GTPase activity of *ras* p21 (Fig. 4A). In contrast, ralGDS inhibited the GAP activity of GST-NF1 for *ras* p21 at a 50% inhibitory dose (IC_{50}) of about 300 nM (Fig. 4). GST–N-Raf also inhibited the GAP activity of GST-NF1 at an IC_{50} of about 50 nM under the same conditions (Fig. 4B). The

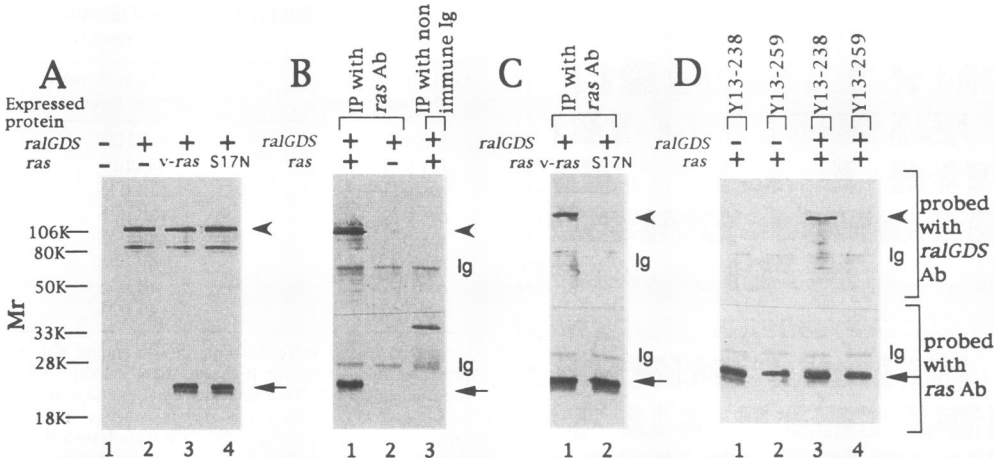


FIG. 2. Interaction of ralGDS with *ras* p21 in intact cells. (A) Coexpression of ralGDS with *ras* p21 in Sf9 cells. Aliquots (5 μl each) of lysates expressing no protein (lane 1), ralGDS alone (lane 2), both ralGDS and v-*ras* p21 (lane 3), or both ralGDS and *ras* $p21^{S17N}$ (lane 4) were probed with the anti-ralGDS and *ras* p21 antibodies. (B) Interaction of ralGDS with *ras* p21 in Sf9 cells. Sf9 cells expressing both ralGDS and v-*ras* p21 (lanes 1 and 3) and ralGDS alone (lane 2) were lysed, and the proteins of the lysates were immunoprecipitated (IP) with the anti-*ras* p21 antibody (Ab; lanes 1 and 2) or nonimmune rat immunoglobulin (Ig; lane 3). The precipitates were probed with the anti-ralGDS and *ras* p21 antibodies. (C) Inability of ralGDS to interact with *ras* $p21^{S17N}$. Sf9 cells coexpressing ralGDS with v-*ras* p21 (lane 1) or ralGDS with *ras* $p21^{S17N}$ (lane 2) were lysed, and the proteins of the lysates were immunoprecipitated with the anti-*ras* p21 antibody. The precipitates were probed with the anti-ralGDS and *ras* p21 antibodies. (D) Inability of Y13-259 to immunoprecipitate a *ras* p21-ralGDS complex. Sf9 cells expressing v-*ras* p21 alone (lanes 1 and 2) or both ralGDS and v-*ras* p21 (lanes 3 and 4) were lysed, and the proteins of the lysates were immunoprecipitated with Y13-238 (lanes 1 and 3) or Y13-259 (lanes 2 and 4). The precipitates were probed with the anti-ralGDS and *ras* p21 antibodies. An arrowhead and an arrow indicate the positions of ralGDS and *ras* p21, respectively. The results shown are representative of three independent experiments.

IC_{50} value of GST–N-Raf to inhibit the GAP activity of GST-NF1 was similar to previous observations (35). ralGDS did not interact with GST-NF1 (data not shown).

Effect of ralGDS on the interaction of Raf with *ras* p21. Since Raf is an effector protein of *ras* p21, we examined whether ralGDS inhibits the interaction of Raf with *ras* p21. GST–N-Raf interacted with *ras* p21 as described previously (18, 34, 35, 41). ralGDS inhibited this interaction in a dose-dependent manner (Fig. 5). The IC_{50} value of ralGDS to inhibit the interaction of Raf-1 with *ras* p21 was about 250 nM.

DISCUSSION

In this study, using the yeast two-hybrid system, we have identified a clone encoding a 164-amino-acid domain (RID) which interacts with H-*ras* p21. However, RID has no primary homology sequence with Raf and GAP, which interact with *ras* p21. Using this clone as a probe, we have isolated cDNA of RGL and sequenced it. We have found that RGL shares 69% amino acid homology with ralGDS. We have also found that the RIDs of RGL and ralGDS are located on the C-terminal side of the CDC25-like domains of these molecules. The high degree of homology between the RIDs of RGL and ralGDS suggests that these domains are functionally important. We are not aware of other proteins that have similar RIDs. Our results suggest that ralGDS and RGL are in a family of proteins that contain a domain that binds to *ras* p21.

ralGDS was originally isolated by PCR using regions conserved between CDC25 and Ste6 proteins, two guanine nucleotide exchange proteins known to regulate *ras* p21 in *S. cerevisiae* and *Schizosaccharomyces pombe*, respectively (1). ralGDS is a 115-kDa protein that has high homology with the region of CDC25 which is important for stimulating the GDP/GTP exchange of *ras* p21. However, ralGDS does not affect the GDP/GTP exchange of *ras* p21. Among 13 different small G proteins, ralGDS stimulates GDP/GTP exchange only of *ralA* p24 and *ralB* p24. From these observations, ralGDS has been implicated in the regulation of the GTP state of *ral* p24 (1). However, it is not known whether ralGDS has additional activities. The function of *ral* p24 is not yet understood (13). The *ral* p24 cDNA was originally isolated by probing with an oligonucleotide corresponding to one of the GTP-binding domains of *ras* p21 (5). There have been few published studies

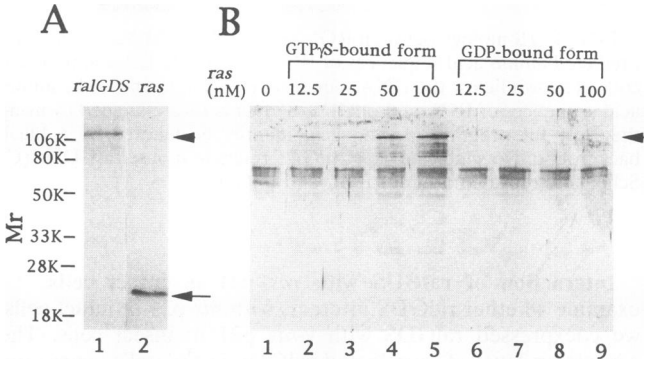


FIG. 3. Interaction of ralGDS with *ras* p21 in vitro. (A) Protein staining of ralGDS and c-*ras* p21. The purified ralGDS and c-*ras* p21 (0.5 μg of protein each) were subjected to SDS-PAGE (12% polyacrylamide gel) and stained with Coomassie brilliant blue. (B) Interaction of ralGDS with the GTP-bound form of *ras* p21. ralGDS (20 pmol) was incubated without (lane 1) or with the indicated amounts of the GTPγS-bound form (lanes 2 to 5) or GDP-bound form (lanes 6 to 9) of *ras* p21, and the mixtures were immunoprecipitated with the anti-*ras* p21 antibody. The precipitates were probed with the anti-ralGDS antibody. An arrowhead and an arrow indicate the positions of ralGDS and *ras* p21, respectively. The results shown are representative of three independent experiments.

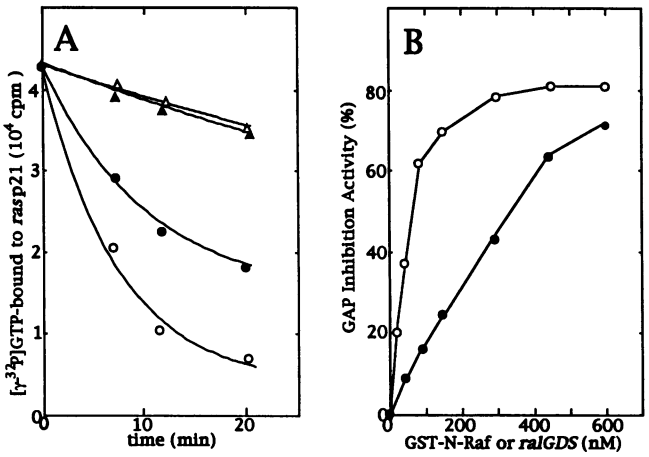


FIG. 4. Inhibition of GAP activity of NF1 by ralGDS. (A) Time course for the GAP inhibition activity of ralGDS. The [γ-^{32}P]GTP-bound form of *ras* p21 was incubated for the indicated periods of time with or without 300 nM ralGDS in the presence or absence of 10 nM GST-NF1. The mixtures were then collected on filters, washed, and counted. △, without ralGDS or GST-NF1; ▲, with ralGDS; ○, with GST-NF1, ●, with ralGDS and GST-NF1. (B) Dose-dependent effect of ralGDS and Raf on GAP inhibition activity. The [γ-^{32}P]GTP-bound form of *ras* p21 was incubated for 6 min with the indicated amounts of ralGDS or GST–N-Raf in the presence or absence of 10 nM GST-NF1. ●, with ralGDS; ○, with GST-N-Raf. The results shown are representative of three independent experiments.

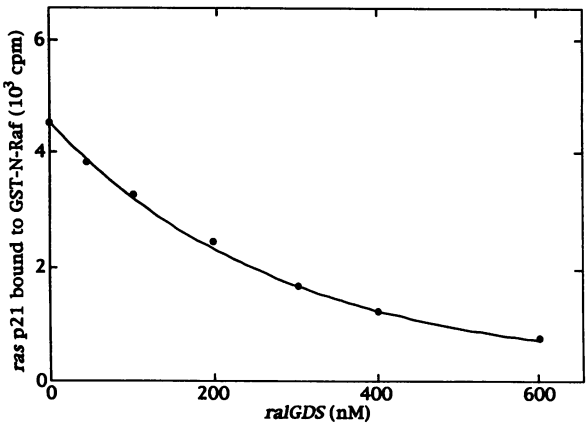


FIG. 5. Inhibition of the interaction of Raf-1 with *ras* p21 by ralGDS. The [α-^{32}P]GTP-bound form of *ras* p21 was incubated for 30 min with 20 nM GST–N-Raf in the presence of the indicated amounts of ralGDS. GST–N-Raf was precipitated by using glutathione-Sepharose 4B, the precipitates were washed, and the remaining radioactivity was counted. The results shown are representative of three independent experiments.

of the role of *ral* p24 in signal transduction. We speculate that ralGDS provides a potential link between *ras* p21 and *ral* p24.

We have shown that ralGDS interacts with v-*ras* p21 but not with *ras* p21^{S17N} in insect cells. It has been known that v-*ras* p21 is mainly in the GTP-bound form and that *ras* p21^{S17N} is mainly in the GDP-bound form in intact cells (2, 10, 24). Our in vitro results have indicated that the purified ralGDS interacts with the GTP-bound form of purified *ras* p21 but not with the GDP-bound form. These results indicate that ralGDS prefers the GTP-bound form to the GDP-bound form and that the interaction of ralGDS with *ras* p21 is direct.

We have demonstrated that ralGDS does not interact with an effector loop mutant of *ras* p21, *ras* p21^{T35A}, and that ralGDS interacts with *rap1* p21 but not with other small G proteins in the yeast two-hybrid system. It has been shown that mutations in the effector loop of *ras* p21 impair the ability of *ras* p21 to transform cells and that the mutants do not interact with Raf or GAP (2, 17, 20, 24, 32, 33, 41). *rap1* p21 is known to have the same effector loop as *ras* p21 and to associate with the same effector-loop-binding proteins as *ras* p21 (24, 32). We have also shown that one *ras* p21 antibody, Y13-238, immunoprecipitates the *ras* p21-ralGDS complex from the lysates expressing both ralGDS and v-*ras* p21, but that another antibody, Y13-259, does not. Y13-259 has been shown to be a neutralizing antibody for *ras* p21 and to recognize the region behind the effector loop of *ras* p21 (28). Y13-238 recognizes a region apart from the effector loop of *ras* p21 and does not inhibit *ras* p21 function (37). Therefore, it has been suggested that Y13-259 cannot recognize *ras* p21 after *ras* p21 interacts with an effector protein. In fact, Y13-238 immunoprecipitates a *ras* p21-Raf complex, but Y13-259 does not (20, 35). These results indicate that the effector loop of *ras* p21 is required for the interaction with ralGDS.

Although it has not yet been determined whether NF1 is an upstream molecule or a downstream molecule of *ras* p21, it has been shown that NF1 interacts with the effector loop of *ras* p21 and stimulates the GTPase activity of *ras* p21 (24, 32, 39). Raf also binds to the effector loop of *ras* p21 and is biologically, genetically, and biochemically an effector protein (8, 15, 18, 20, 21, 29, 33–35, 41). Raf inhibits the GAP activity of NF1 presumably by competing with NF1 for binding to *ras* p21 (35, 41). We have shown that ralGDS inhibits the GAP activity of NF1 for *ras* p21. Further, another experiment has demonstrated that ralGDS can compete with Raf for binding to *ras* p21. Our results indicate that ralGDS can compete with NF1 and Raf for binding to the *ras* p21 effector loop. Taken together, these results strongly suggest that ralGDS may be an effector protein of *ras* p21. The proof that ralGDS is the effector protein of *ras* p21 awaits demonstration that *ras* p21 regulates the ralGDS function or that ralGDS mediates *ras* p21-dependent processes.

We have measured the affinity of ralGDS for *ras* p21 and found that ralGDS inhibits the GAP activity of GST-NF1 for *ras* p21 with an IC_{50} of about 300 nM. Under the same conditions, GST–N-Raf inhibits the GAP activity of GST-NF1 for *ras* p21 with an IC_{50} of about 50 nM. ralGDS inhibits the association of GST–N-Raf with *ras* p21 with an IC_{50} of about 250 nM. It has been reported that the affinity of Raf for *ras* p21 is about 50 nM (35). Therefore, we roughly estimate the affinity of ralGDS for *ras* p21 to be five- to sixfold lower than the affinity of Raf for *ras* p21. A previous report has shown that the affinities of NF1 and GAP for *ras* p21 are about 250 nM and about 5 μM, respectively (26). How does *ras* p21 interact with ralGDS in the presence of Raf? It has been reported that cyclic AMP-dependent protein kinase (A-kinase) phosphorylates Raf and that the phosphorylated Raf does not interact with *ras* p21 (7, 38). It is intriguing to speculate that A-kinase regulates the interaction of *ras* p21 with Raf-1 or ralGDS.

We have also shown that ralGDS interacts with *ras* p21^{C186S} in the yeast two-hybrid system and that ralGDS interacts with *ras* p21 purified from the cytosol fraction of insect cells in vitro. Since these *ras* p21s are not posttranslationally modified (16, 27), these results suggest that the posttranslational modification of *ras* p21 is not essential for the interaction of ralGDS with *ras* p21. It is known that the posttranslational modification

of *ras* p21 is not essential for the interaction of *ras* p21 with Raf (18, 20, 29, 33–35, 41). However, we have found that the posttranslational modification of *ras* p21 is necessary for Raf activation in intact cells (18). Furthermore, it has been demonstrated that the posttranslational modification of *ras* p21 is important for its ability to induce transformation (2, 24, 32). Therefore, it is possible that the posttranslational modification of *ras* p21 is important for *ras* p21-dependent ralGDS action.

Taken together, these results suggest that members of the ralGDS family may be effector proteins of *ras* p21. It is possible that ralGDS mediates some *ras* p21-dependent processes. Alternatively, the primary function of ralGDS family members might be to modulate *ras* p21/Raf-mediated responses. In any case, a deeper understanding of the roles of ralGDS family members in *ras* p21-dependent processes is needed.

ACKNOWLEDGMENTS

We thank B. W. Giddings, C. F. Albright, and R. A. Weinberg for the ralGDSb and *ralB* p24 cDNAs, the anti-ralGDS antibody, and helpful advice; P. M. Chevray and D. Nathans for yeast strains; G. Hannon and D. Beach for yeast expression vectors; L. Van Aelst and M. Wigler for the *ras* p21 and *rap1* p21 cDNAs and helpful advice; J. Downward for the *ras* p21 mutant cDNA and the anti-*ras* p21 antibody; S. Fields and G. S. Martin for helpful advice and communication of results before publication; A. Hall, G. Xu, A. Klippel, M. Hirano, D. Pot, and Q. Hu for plasmids and helpful advice; and M. Kavanaugh and J. Escobedo for reading the manuscript.

This work was supported by NIH grant R01 HL32898 to L. T. Williams, by a grant from Daiichi Pharmaceuticals, and by a Cardiovascular Research Institute Comroe fellowship.

REFERENCES

1. **Albright, C. F., B. W. Giddings, J. Liu, M. Vito, and R. A. Weinberg.** 1993. Characterization of a guanine nucleotide dissociation stimulator for a *ras*-related GTPase. EMBO J. **12:**339–347.
2. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56:**779–827.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Breeden, L., and K. Nasmyth.** 1985. Regulation of the yeast HO gene. Cold Spring Harbor Symp. Quant. Biol. **50:**643–650.
5. **Chardin, P., and A. Tavitian.** 1986. The *ral* gene: a new *ras* related gene isolated by the use of a synthetic probe. EMBO J. **5:**2203–2208.
6. **Chevray, P. M., and D. Nathans.** 1992. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc. Natl. Acad. Sci. USA **89:**5789–5793.
7. **Cook, S. J., and F. McCormick.** 1993. Inhibition by cAMP of Ras-dependent activation of Raf. Science **262:**1069–1072.
8. **Dickson, B., F. Sprenger, D. Morrison, and E. Hafen.** 1992. Raf functions downstream of Ras1 in the sevenless signal transduction pathway. Nature (London) **360:**600–603.
9. **Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. E. Kilburn, W.-H. Lee, and S. J. Elledge.** 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:**555–569.
10. **Farnsworth, C. L., and L. A. Feig.** 1991. Dominant inhibitory mutations in the Mg^{2+}-binding site of Ras^{H} prevent its activation by GTP. Mol. Cell. Biol. **11:**4822–4829.
11. **Fernandez-Sarabia, M. J., and J. R. Bischoff.** 1993. Bcl-2 associates with the *ras*-related protein with R-*ras* p23. Nature (London) **366:**274–275.
12. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
13. **Frech, M., I. Schlichting, A. Wittinghofer, and P. Chardin.** 1990. Guanine nucleotide binding properties of the mammalian ralA protein produced in *Escherichia coli*. J. Biol. Chem. **265:**6353–6359.
14. **Gibbs, J. B., M. D. Schaber, W. J. Allard, I. S. Sigal, and E. M. Scolnick.** 1988. Purification of ras GTPase activating protein from bovine brain. Proc. Natl. Acad. Sci. USA **85:**5026–5030.
15. **Han, M., A. Golden, Y. Han, and P. W. Sternberg.** 1993. *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. Nature (London) **363:**133–140.
16. **Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall.** 1989. All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell **57:**1167–1177.
17. **Kaziro, Y., H. Itoh, T. Kozasa, M. Nakafuku, and T. Satoh.** 1991. Structure and function of signal-transducing GTP-binding proteins. Annu. Rev. Biochem. **60:**349–400.
18. **Kikuchi, A., and L. T. Williams.** 1994. The post-translational modification of *ras* p21 is important for Raf-1 activation. J. Biol. Chem. **269:**20054–20059.
19. **Klippel, A., J. A. Escobedo, M. Hirano, and L. T. Williams.** 1994. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. Mol. Cell. Biol. **14:**2675–2685.
20. **Koide, H., T. Satoh, M. Nakafuku, and Y. Kaziro.** 1993. GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. Proc. Natl. Acad. Sci. USA **90:**8683–8686.
21. **Kolch, W., G. Heidecker, P. Lloyd, and U. R. Rapp.** 1991. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature (London) **349:**426–428.
22. **Kozak, M.** 1987. An analysis of 5′-noncoding sequence from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15:**8125–8148.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
24. **Lowy, D. R., and B. M. Willumsen.** 1993. Function and regulation of *ras*. Annu. Rev. Biochem. **62:**851–891.
25. **Martegani, E., M. Vanoni, R. Zippel, P. Coccetti, R. Brambilla, C. Ferrari, E. Sturani, and L. Alberghina.** 1992. Cloning by functional complementation of a mouse cDNA encoding a homologue of *CDC25*, a *Saccharomyces cerevisiae* RAS activator. EMBO J. **11:**2151–2157.
26. **Martin, G. A., D. Viskochil, G. Bollag, P. C. McCabe, W. J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, R. M. Cawthon, M. A. Innis, and F. McCormick.** 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell **63:**843–849.
27. **Mizuno, T., K. Kaibuchi, T. Yamamoto, M. Kawamura, T. Sakoda, H. Fujioka, Y. Matsuura, and Y. Takai.** 1991. A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21. Proc. Natl. Acad. Sci. USA **88:**6442–6446.
28. **Mulcahy, L. S., M. R. Smith, and D. W. Stacy.** 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH3T3 cells. Nature (London) **313:**241–243.
29. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of ras GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658–1661.
30. **Schlessinger, J., and A. Ullrich.** 1992. Growth factor signaling by receptor tyrosine kinases. Neuron **9:**383–391.
31. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station, College Station.
32. **Takai, Y., K. Kaibuchi, A. Kikuchi, and M. Kawata.** 1992. Small GTP-binding proteins. Int. Rev. Cytol. **133:**187–230.
33. **Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between RAS and RAF and other protein kinases. Proc. Natl. Acad. Sci. USA **90:**6213–6217.
34. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
35. **Warne, P. H., P. R. Viciana, and J. Downward.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. Nature (London) **364:**352–355.
36. **Wendy, F. J., D. E. Johnson, and L. T. Williams.** 1993. Signaling by receptor tyrosine kinases. Annu. Rev. Biochem. **62:**453–481.
37. **Williumsen, B. M., A. G. Papageorge, H.-F. Kung, E. Bekesi,**

T. Robins, M. Johnsen, W. C. Vass, and D. R. Lowy. 1986. Mutational analysis of a *ras* catalytic domain. Mol. Cell. Biol. **6:** 2646–2654.

38. **Wu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill.** 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. Science **262:**1065–1069.
39. **Xu, G., B. Lin, K. Tanaka, D. Dunn, D. Wood, R. Gesteland, R. White, R. Weiss, and F. Tamanoi.** 1990. The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of S. cerevisiae. Cell **63:** 835–841.
40. **Yang, X., E. J. A. Hubbard, and M. Carlson.** 1992. A protein kinase substrate identified by the two-hybrid system. Science **257:** 680–682.
41. **Zhang, X.-F., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London) **364:**308–313.