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IRA2, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein

(oncogene/GTP binding protein/*Saccharomyces cerevisiae*)

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ABSTRACT **The ras GTPase-activating protein (GAP), identified and characterized in mammalian cells, stimulates the intrinsic GTPase activity of ras proteins. We have previously proposed that the *IRA* genes, negative regulators of *RAS* genes in *Saccharomyces cerevisiae*, encode yeast homologs of the mammalian GAP. In this paper, we present the following evidence that a product of the *IRA2* gene exhibits GAP activity similar to that of the mammalian GAP protein. (*i*) Extracts of yeast cells overexpressing *IRA2* stimulated the GTPase activity of the yeast RAS2 protein. (*ii*) An epitope for a monoclonal antibody (12CA5) was added to the N terminus of the IRA2 protein. The GAP activity of extracts prepared from cells expressing this fusion protein was shown to be immunoprecipitable by 12CA5. (*iii*) An IRA2 protein fused to glutathione *S*-transferase (GST) was produced and partially purified from *Escherichia coli* cells. GAP activity was detected with this purified GST–IRA2 fusion protein. (*iv*) The GAP activity of IRA2 proteins described above did not stimulate the GTPase activity of the $RAS2^{Val19}$ protein, a protein having an amino acid alteration analogous to that found in mammalian oncogenic ras proteins. This result parallels studies showing that mammalian GAP is incapable of stimulating the GTPase activity of mammalian oncogenic proteins. The remarkable conservation between the GAP activity in mammalian and yeast cells supports the idea that the function of GAP is to negatively regulate ras proteins in mammalian cells.**

Accumulating evidence indicates that ras proteins play a key role in the control of growth and differentiation of eukaryotic cells. The ras proteins exist in either of two guanine nucleotide-binding states, a GTP-bound or a GDP-bound form, and only the GTP-bound form can transmit a signal to a downstream effector molecule. Consistent with this idea is the observation that the intrinsic GTPase activity of activated (oncogenic) ras mutants is impaired; these proteins exist predominantly in the GTP-bound state (reviewed in ref. 1).

The ras GTPase-activating protein (GAP), a cytoplasmic protein in vertebrate cells, was recently found to stimulate the intrinsic GTPase activity of normal ras, but not that of oncogenic ras (2). Bovine and human GAP proteins contain 1044 (3) and 1047 amino acids (4), respectively, and the C-terminal region of 343 amino acids was shown to be responsible for the GAP catalytic activity (5). Because GAP interacts with ras directly, GAP is proposed to be either an upstream regulator or a downstream effector of ras (reviewed in refs. 6 and 7).

Previously, we have reported on the identification of two *Saccharomyces cerevisiae* genes, *IRA1* and *IRA2*, which encode proteins with domains homologous to the mammalian GAP protein (8, 9). Genetic results suggest that these genes function as upstream negative regulators of yeast *RAS* genes (8, 9). A mutation in either *IRA1* or *IRA2* produces a set of phenotypes that are also observed in strains carrying an activated mutation of *RAS2*, such as $RAS2^{Val19}$. These phenotypes include heat shock sensitivity, nitrogen starvation sensitivity, and sporulation deficiency.

The following observations further support the idea that IRA proteins have functions similar to those of the mammalian GAP. First, expression of mammalian GAP suppresses the heat shock-sensitive phenotype of *ira* mutants (10, 11). Second, the fraction of RAS proteins in the GTP-bound form is increased in *ira* mutants (10).

We report here that IRA2 indeed possesses a GAP activity. This provides evidence that IRA proteins are yeast homologs of the mammalian GAP proteins. Since *IRA* genes are upstream regulators of *RAS* genes, the mammalian GAP may also have an upstream regulatory function.

MATERIALS AND METHODS

Strains and Medium. The yeast strain TK161-R2V (*MATα ura3 leu2 trp1 his3 ade8* $RAS2^{Val19}$) (12) was used as the host for transformation. This $RAS2^{Val19}$ strain was chosen because the putative GAP activity of IRA2 may be positively feedback-regulated by cAMP-dependent protein phosphorylation (9). Transformants were grown in SDA medium, which is 2% dextrose/0.5% ammonium sulfate/0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco)/0.5% Casamino acid (Difco)/0.63 mg of adenine sulfate per ml/0.25 mg of L-tryptophan per ml.

Plasmids. The 5183-base-pair (bp) *Pvu* II/*Pvu* II fragment of *IRA2* was placed into the plasmid pKT10 (9) under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter. The resulting plasmid, pKT16, encodes a protein derived from amino acids 528–2255 of IRA2, with N-terminal and C-terminal additions of MGTARA and VID, respectively. The DNA sequence of pKT16 encoding the N-terminal MGTARA peptide was replaced by oligonucleotides encoding the HA1 epitope, MYPYDVPDYASLDGPMST (13), to form a plasmid, pKT64. To construct a plasmid pGEX-1/IRA2 used for expression of the glutathione *S*-transferase (GST)–IRA2 fusion protein in *Escherichia coli*, a 1082-bp DNA fragment of *IRA2*, encoding amino acids 1665–2025 of the IRA2 protein, was placed into pGEX-1/RCT, in-frame with a gene encoding the 26-kDa C-terminal portion of Sj26, the GST of the parasitic helminth *Schistosoma japonicum* (14). pGEX-1/RCT (a gift of H. Maruta, Ludwig Cancer Institute, Australia), which is a derivative of pGEX-1 (14), contains 15 extra amino acid residues (DPKKLPKGGGCCVLS) added to the C terminus of GST. (This extra sequence is replaced by the IRA2 sequence in pGEX-1/IRA2.) The *E. coli* expression plasmid for bovine GAP,

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Abbreviations: GAP, GTPase-activating protein; GST, glutathione *S*-transferase.

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pUC8-GAP1 (5) was a gift from J. Gibbs (Merck Sharp & Dohme).

Purification of RAS Proteins. The full-length RAS2 protein expressed under the control of the *recA* promoter (15) in *E. coli* cells was purified essentially as described (16). The C-terminal 113 amino acid residues of RAS2 were replaced by nonrelated peptides, RAAVID, to make RAS2–CTT and $RAS2^{Val19}$–CTT, which were purified as follows. Crude supernatant in buffer D (20 mM Tris·HCl, pH 7.4/0.1 mM EDTA/0.2 mM dithiothreitol/1 mM $MgCl_2$) was applied to a DEAE-Sepharose CL-6B column equilibrated with buffer D. Bound protein was eluted by a linear NaCl gradient (0–0.4 M) in buffer D. Then, fractions were assayed for the RAS-specific [^{3}H]GDP binding activity by using monoclonal antibody Y13-259 essentially as described (16). Pooled peak fractions were concentrated and then applied onto a Sephadex G-75 column equilibrated in buffer D containing 50 mM NaCl. Purified proteins (>80% purity) were concentrated, mixed with glycerol to 50%, and stored at −20°C. Purified Ha-ras protein (17) was a gift from S. Nishimura (National Cancer Center, Tokyo).

Preparation of Cell Extracts and IRA2 Proteins. Yeast cells in the exponential growth phase were collected, disrupted with glass beads in a lysis buffer (100 mM Mes-NaOH, pH 6.5/0.1 mM $MgCl_2$/0.1 mM EGTA/1 mM 2-mercaptoethanol) containing 1% Lubrol-PX and 1 mM phenylmethylsulfonyl fluoride, and centrifuged for 5 min at 17,000 × *g*. Resulting supernatants with a protein concentration of ≈8 mg/ml were used for the GAP assay. HA–IRA2 was immunoprecipitated by adding purified 12CA5 (5 μg) to 100 μl of crude supernatant in lysis buffer containing 0.5 M NaCl. After incubation for 30 min on ice, 10 μl of 50% protein A-Sepharose (Pharmacia) was added to the incubation mixture and then rotated for 3 hr at 4°C. After washing three times with wash buffer (lysis buffer with 0.5 M NaCl/0.1% Lubrol-PX/1 mg of bovine serum albumin per ml), the immunoprecipitates were dissolved in 40 μl of wash buffer. *E. coli* extracts for pUC8-GAP1 were prepared as described (5).

Purification of the GST–IRA2 fusion protein was carried out as follows. Isopropyl β-D-thiogalactopyranoside was added to *E. coli* cells carrying pGEX-1/IRA2 or pGEX-1/RCT to express GST–IRA2 or GST–RCT protein. Cells were collected and lysed by sonication in MTPBS buffer (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, and the resulting supernatants were then rotated with 0.5 ml of glutathione/agarose beads (50% solution in MTPBS; Sigma) at 4°C for 30 min. The protein then eluted from the beads in a solution containing 5 mM reduced glutathione (Sigma) and 50 mM Tris·HCl (pH 7.5). On a SDS/polyacrylamide gel, 68- and 26-kDa proteins appeared as major bands in the GST–IRA2 and GST–RCT preparations, respectively, which corresponded to the expected size of the GST proteins.

GAP Assay. RAS protein (20 pmol) was incubated at 25°C for 15 min in 50 μl of a nucleotide exchange buffer containing 40 nM [α-^{32}P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) or [γ-^{32}P]GTP (5000 Ci/mmol; Amersham), 50 mM Mes-NaOH (pH 6.5), 1 mM EDTA, 2 mM dithiothreitol, and 300 μg of bovine serum albumin per ml. In the case of Ha-ras, 50 mM Mes·NaOH was replaced with 50 mM Tris·HCl (pH 7.5). The reaction was stopped on ice. This reaction mixture (22 μl) was then added to 180 μl of prewarmed GAP assay buffer (25 mM Mes·NaOH, pH 6.5/1.25 mM $MgCl_2$/1.25 mM dithiothreitol/1.25 mg of bovine serum albumin per ml), and the GAP assay was started with the addition of 22 μl of extracts or immunoprecipitates or partially purified GST fusion proteins. At each time point during the incubation at 25°C or 30°C, a 50-μl sample was taken out and added to 0.5 ml of ice-cold stop buffer (20 mM Mes·NaOH, pH 6.5/5 mM $MgCl_2$). This mixture was applied to nitrocellulose filters (GSWP 02500; Millipore) and then rinsed three times with the stop buffer. When [γ-^{32}P]GTP was used, the radioactivity retained on the filter was determined by Cerenkov counting (filter binding assay). When [α-^{32}P]GTP was used, guanine nucleotides bound to the RAS protein were eluted by immersing the filter in 25 mM Tris·HCl, pH 7.4/20 mM EDTA/2% SDS/1 mM GDP/1 mM GTP at 65°C for 5 min. More than 90% of the radioactivity on the filter was released. The released nucleotides were separated by polyethyleneimine-cellulose (Sigma) chromatography using 1 M LiCl as a developing solvent followed by autoradiography (guanine nucleotide analysis).

RESULTS

Identification of IRA2-Dependent GAP Activity in Yeast Extracts. The GAP activity of the IRA2 protein was demonstrated by using extracts of yeast cells overexpressing *IRA2*. A DNA fragment encoding amino acid residues 527–2255 of IRA2, which includes the region homologous to mammalian GAP, was placed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. This plasmid, pKT16, suppressed the heat shock-sensitive phenotype of *ira* mutants. Extracts prepared from cells transformed with either pKT16 or the control plasmid pKT10 were incubated with the RAS2 protein preloaded with [γ-^{32}P]GTP. The reaction mixtures were then passed through nitrocellulose filters to measure the remaining radioactivity bound to the RAS2 protein. As shown in Fig. 1*A*, ≈90% of the initial radioactivity was released after a 10-min incubation with extracts of pKT16 cells, while virtually no decrease of radioactivity was observed with the pKT10 extracts (Fig. 1*A*) or with the cell lysis buffer (data not shown). Essentially the same results were obtained with five other independent transformants each of pKT16 and pKT10. The activity of pKT16 extracts was dose dependent and even extracts diluted 1:5 showed significant activity (data not shown). The GAP activity was also observed at pH 7.5 (50 mM Tris·HCl) as well as at pH 6.5 (50 mM Mes-NaOH). Detergent in the extraction buffer (Lubrol-PX) was essential for the GAP activity, suggesting that IRA2 (residues 527–2255) is membrane bound.

The reduction in the radioactivity retained on the filter was not observed when [α-^{32}P]GTP was used in place of [γ-^{32}P]GTP. This indicates that IRA2 stimulated the release of γ-phosphate from the RAS2–GTP complex. To confirm this point, the assay was carried out using RAS2 protein preloaded with [α-^{32}P]GTP; then, the RAS-bound guanine nucleotides were released from RAS2 and analyzed by thin-layer chromatography. As shown in Fig. 1*B*, conversion of GTP to GDP was observed with the pKT16 extracts as well as with *E. coli* extracts containing bovine GAP, but not with the pKT10 extracts. The extent of the GTP to GDP conversion observed in Fig. 1*B* is consistent with the result of the filter binding assay shown in Fig. 1*A*.

Specificity of IRA2 GAP Activity on Mutant RAS Proteins. It has been shown that mammalian GAP is incapable of stimulating the GTPase activity of ras^{Val12} (2, 18). To see whether the GAP activity of IRA2 has a similar specificity, $RAS2^{Val19}$–CTT was expressed and purified from *E. coli* cells and used in the GAP assay. As shown in Fig. 2, extracts of pKT16 stimulated the GTPase activity of RAS2–CTT, but not that of $RAS2^{Val19}$–CTT. This result is in line with the observation that a high percentage of $RAS2^{Val19}$ protein contains bound GTP in wild-type cells as well as in *ira* mutant cells (10). It is worth pointing out that the RAS2–CTT protein used as a control in Fig. 2 is lacking a C-terminal 120-amino acid stretch of RAS2. Thus, the domain of RAS2 required for the interaction with IRA2 seems to reside in the conserved N-terminal region.

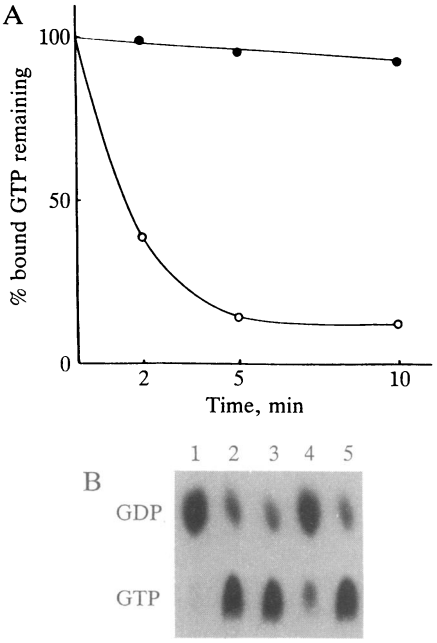

FIG. 1. Identification of IRA2-dependent GAP activity in yeast extracts. The GAP assay was carried out either by filter binding assay (*A*) or by guanine nucleotide analysis (*B*). (*A*) Extracts of TK161-R2V/pKT16 cells (180 μg of total protein; ○) or of TK161-R2V/pKT10 cells (170 μg of total protein; ●) were incubated at 25°C with RAS2–[γ-^{32}P]GTP and the reaction mixtures were passed through a nitrocellulose filter. Radioactivity retained on the filter was measured and the percentage of initial radioactivity at each time point was shown. A value of 100% corresponds to 126,000 cpm. (*B*) The GAP assay was carried out as in *A* for 10 min with RAS2–[α-^{32}P]GTP. Guanine nucleotides bound to RAS2 were then released and analyzed by polyethyleneimine-cellulose chromatography. Sources of extracts are TK161-R2V/pKT16 [175 μg of total protein (lane 1)], TK161-R2V/pKT10 [180 μg of total protein (lane 2)], cell lysis buffer (lane 3), JM103/pUC8-GAP1 [45 μg of total protein (lane 4)], and JM103/pUC18 [46 μg of total protein (lane 5)]. pUC8-GAP1 encodes bovine full-length GAP under the *lac* promoter (5). Positions of authentic GTP and GDP standards are shown on the left.

The above result points to the idea that yeast IRA2 acts in a manner similar to the mammalian GAP protein. However, IRA2 appears to have a strict specificity toward yeast RAS proteins. As shown in Fig. 2, IRA2 could not stimulate the GTPase activity of Ha-ras, while the GTPase activity of the Ha-ras protein was stimulated by *E. coli* extracts expressing bovine GAP (data not shown). The inability of IRA2 to stimulate the GTPase activity of Ha-ras is not due to the pH used (pH 6.5), since a similar result was obtained in experiments performed at pH 7.5 (the pH optimum for the intrinsic

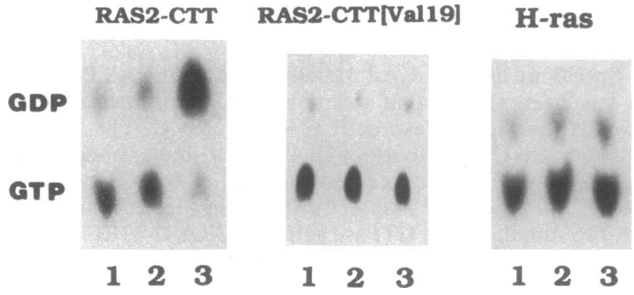

FIG. 2. IRA2 GAP activity on various RAS proteins. Twenty picomoles of each protein preloaded with [α-^{32}P]GTP was incubated with cell lysis buffer (lanes 1), TK161-R2V/pKT10 extracts [150 μg of total protein (lanes 2)], or TK161-R2V/pKT16 extracts [170 μg of total protein (lanes 3)] for 10 min at 25°C; then, guanine nucleotides bound to RAS proteins were analyzed.

GTPase activity of Ha-ras protein). This specificity of IRA2 toward yeast RAS proteins is in marked contrast to that of mammalian GAP, which stimulates the GTPase activity of both Ha-ras and yeast RAS2 proteins (Fig. 1*B*).

Immunoprecipitation of IRA2 Also Precipitates the GAP Activity. To demonstrate that the IRA2 protein is responsible for the GAP activity detected above, IRA2 was immunoprecipitated. For this purpose, the HA epitope, a peptide sequence that is recognized by the monoclonal antibody 12CA5, was added to the IRA2 protein. This method has been successfully used for isolation of the catalytic subunit of adenylyl cyclase (13) as well as STE18 protein (19). We constructed a plasmid, pKT64, which expressed the IRA2 protein with an HA epitope added to its N terminus. Addition of the epitope did not affect the function of IRA2, since pKT64 complemented the heat shock-sensitive phenotype of the *ira2* mutant. Extracts of pKT64 or pKT16 transformants were prepared and subjected to immunoprecipitation by the 12CA5 antibody. As shown in Fig. 3, the antibody precipitated a significant GAP activity from pKT64 extracts. With the pKT16 extracts, however, little GAP activity was precipitated. The immunoprecipitated GAP activity stimulated the GTPase activity of RAS2–CTT, but neither that of RAS2^{Val19}–CTT nor Ha-ras (data not shown), consistent with the GAP activity found in extracts. Immunoprecipitation of the GAP activity was blocked by competition with a peptide containing the HA epitope, YPYDVPDYA (kindly provided by J. Field, Cold Spring Harbor Laboratory) (data not shown). This result suggests that the immunoprecipitation is specific for the HA epitope. Thus, it seems that the IRA2 protein itself has a GAP activity.

GAP Activity of Bacterially Expressed IRA2 Fusion Protein. To further verify that IRA possesses GAP activity, we have expressed the *IRA2* gene in *E. coli*. This was accomplished by fusing a 1082-bp DNA fragment encoding amino acids 1665–2027 of IRA2 corresponding to the region homologous to mammalian GAP to the 3′ end of the GST gene to make a plasmid, pGEX-1/IRA2. *E. coli* cells carrying the pGEX-1/IRA2 or a control vector pGEX-1/RCT were grown and fusion proteins were partially purified by using glutathione/agarose as described. GAP assays were then performed on the partially purified proteins. As shown in Fig. 4*A*, GAP activity on the RAS2 protein was observed with GST–IRA2,

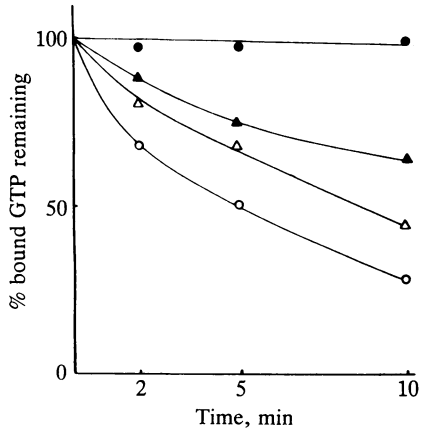

FIG. 3. Immunoprecipitation of GAP activity. Extracts of TK161-R2V/pKT16 expressing IRA2 (residues 528–2255) (700 μg of total protein; ○ and ●) or of TK161-R2V/pKT64 expressing HA–IRA2 (residues 528–2255) (680 μg of total protein; △ and ▲) were subjected to immunoprecipitation with monoclonal antibody 12CA5. Extracts before immunoprecipitation (154 μg and 150 μg of total protein for IRA2 and HA–IRA2, respectively; ○ and △) or immunoprecipitates (● and ▲) were assayed for GAP activity at 25°C using a filter binding method. The amount of extracts and immunoprecipitates was selected to give a linear dose-dependent activity.

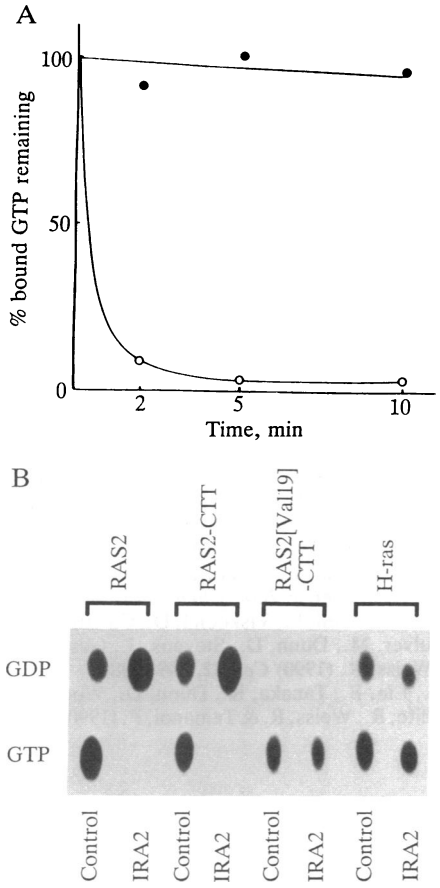

FIG. 4. Partially purified GST–IRA2 has GAP activity. GAP assays were carried out by either filter binding assay (*A*) or guanine nucleotide analysis (*B*) as in Fig. 1 except that the reaction was performed at 30°C. (*A*) Enzyme source is 30 μg of either partially purified GST–IRA2 (○) or GST–RCT (●). The RAS2–[γ-^{32}P]GTP was used as a substrate. (*B*) GAP assays were carried out for 10 min using the RAS protein indicated above each lane. The enzyme source was either partially purified GST–IRA2 protein (6 μg; IRA2) or GST–RCT protein (8 μg; Control).

but not with GST–RCT. Within 2 min after starting the incubation with GST–IRA2, 90% of the GTP bound to RAS2 was hydrolyzed to GDP. In contrast to this, stimulation of GTP hydrolysis was not observed with the control GST–RCT protein. The activity of the GST–IRA2 protein exhibited specificities for different RAS proteins that were identical to what was shown using crude extracts of yeast cells overexpressing *IRA2*. As shown in Fig. 4*B*, IRA2 could stimulate the GTPase activity of RAS–CTT, but not that of the RAS2^{Val19} mutant or the Ha-ras protein. Thus, the GAP activity of purified GST–IRA2 protein resembles that found both in yeast extracts overexpressing *IRA2* and in immunoprecipitated HA–IRA2 (compare Figs. 2 and 4*B*).

DISCUSSION

In this paper, we have shown that the IRA2 protein possesses GAP activity. This was shown by using crude extracts of yeast cells overexpressing *IRA2* as well as by using GST–IRA2 fusion protein affinity purified from *E. coli* extracts. This GAP activity stimulated the GTPase activity of RAS2 and RAS2–CTT, but not that of either RAS2^{Val19}–CTT or Ha-ras, consistent with the result of RAS-bound guanine nucleotide analysis in *ira* mutants and wild-type cells (10). Although we have not yet demonstrated GAP activity of the IRA1 protein, an extensive homology between IRA1 and IRA2 (>50% identity in the region homologous to GAP) suggests that IRA1 also has a GAP activity. Since, in yeast, GAP activity of IRA2 down-regulates the GTP-bound form of RAS (10), GAP activity in mammalian cells may also be used in the negative regulation of ras activity.

The experiments using GST–IRA2 fusion protein indicate that IRA2 (residues 1665–2027) (362 amino acids) is sufficient for GAP activity. This region of IRA2 is homologous to the catalytic domain of mammalian GAP. Recently, it was reported that a synthetic peptide corresponding to residues 888–910 of GAP (MRTRVVSGFVFLRLICPAILNPR) can inhibit GAP activity, suggesting that this region may be a site of interaction with ras proteins (20). Interestingly, this region of GAP shows the highest homology to IRA—39% identity between IRA2 and GAP and 43% identity between IRA1 and GAP (9). The corresponding stretch of IRA2, residues 1843–1863, is located in the middle of the 362-residue sequence. Thus, this stretch might be the site of interaction between IRA2 and RAS proteins.

In regard to a domain of RAS2 interacting with IRA2, the following points can be made. IRA2 can stimulate the GTPase activity of RAS2–CTT, a RAS2 protein lacking the C-terminal 120 amino acids. This indicates that IRA2 interacts with a domain of the N-terminal region of RAS2, a region that is conserved throughout the ras family. The specific site for interaction with IRA2 may be located in the effector domain corresponding to residues 37–47 of RAS2 (residues 30–40 of Ha-ras) (6, 21). Our preliminary results with one such effector mutant of RAS2, RAS2^{Asn45}, point to the importance of the effector domain. The intrinsic GTPase activity of RAS2^{Asn45} was similar to that of wild-type RAS2, while stimulation of GTPase activity by IRA2 was significantly impaired in this mutant (unpublished results). This mutation also affects downstream functions such as the ability to complement the growth defect of *ras2* cells on nonfermentable carbon sources (22). Therefore, the effector region appears to be a site of interaction between RAS and IRA as well as between RAS and a downstream effector. Further work using RAS proteins having various mutations within the effector domain should enable us to define these sites.

The GTPase activity of Ha-ras could not be stimulated by IRA2. In contrast, mammalian GAP stimulated the GTPase activity of yeast RAS proteins. Moreover, the mammalian GAP is incapable of stimulating the GTPase activity of smg21/K-rev1/rap1 (23). Thus, GTPase-activating proteins such as IRA and GAP have specificity toward substrate proteins. Since all three proteins—Ha-ras, yeast RAS, and smg21—have the identical effector domain, this may indicate that an additional domain plays a role in the stimulation of GTPase activity of ras-like proteins by their GAP. Such a domain may be located within amino acid sequence 17–32 of Ha-ras, because a peptide from this region was shown to compete with the binding of Ha-ras to GAP (20).

In summary, we have shown that IRA2 exhibits GAP activity similar to that of mammalian GAP. Recently, it has been discovered that a product of the neurofibromatosis type 1 (NF1) gene shares homology with GAP and IRA (24). Thus, IRA, GAP, and NF1 may comprise a family of GAP proteins that regulate ras action. Characterization of these proteins should contribute to the understanding of how ras activity is regulated.

Note Added in Proof. We have recently demonstrated that a segment of the NF1 gene product exhibits GAP activity (25). This supports our idea that IRA, GAP, and NF1 proteins comprise a family of GAP proteins.

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