# Properties and Regulation of the Catalytic Domain of Ira2p, a *Saccharomyces cerevisiae* GTPase-Activating Protein of Ras2p†

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ABSTRACT: This work describes the biochemical characterization of the catalytic domain of Ira2p, a *Saccharomyces cerevisiae* GTPase-activating protein (GAP) regulating the *RAS* gene products. A fragment of 383 residues (amino acids 1644−2026) was produced in *Escherichia coli* as glutathione *S*-transferase fusion protein (GST-Ira2p-383) and highly purified (>90%) by affinity chromatography. The affinity of Ras2p for the GST-fused Ira2p-383 was 18 $\mu$M and the maximal stimulation of the Ras2p GTPase activity 6 000 times. The Ira2p activity was confirmed to be strictly specific for Ras2p, no stimulatory effect on human c-H-ras p21 GTPase being detectable. Comparison with the GAP-like domain of mammalian p120-GAP and neurofibromin using yeast Ras2p as substrate showed that Ira2p-383 has an affinity and turnover intermediary between GAP-334 and NF1-414. The activity of Ira2p-383 was strongly inhibited by monovalent and divalent salts. The simultaneous presence of the catalytic domains of Ira2p and the yeast GDP/GTP exchange factor Cdc25p induced on Ras2p a multiple-round reaction of GTP hydrolysis and GDP/GTP exchange, showing that it is possible to reconstitute *in vitro* a *S. cerevisiae* system suitable for the study of the regulation of the Ras2p GDP/GTP cycle. The tubulin partially inhibited (25%) the GAP activity of the Ira2p-383. A larger Ira2p catalytic fragment, Ira2p-505 (amino acids 1549−2053), that showed the same $K_m$ for Ras2p as Ira2p-383, was also inhibited by tubulin to the same extent but with a higher affinity than Ira2p-383. This indicates that the conserved catalytic domain contains a binding site for tubulin that is extended to its N-terminal flanking region. These results show that the inhibition of neurofibromin by tubulin [Bollag, G., McCormick, F., & Clark, R. (1993) *EMBO J. 12,* 1923−1927] is a property shared with Ira2p.

In *Saccharomyces cerevisiae*, the two GTP-binding proteins Ras1p and Ras2p act as molecular switches of adenylylcyclase pathway by cycling between the GTP-bound "on" form activating the adenylylcyclase and the inactive GDP-bound "off" form (Broek et al., 1985; Toda et al., 1985). As for the other members of the ras protein family, the hydrolysis of the ras-bound GTP and the exchange of the resulting GDP with free GTP are controlled by two kinds of effectors: the GTPase-activating protein (GAP)[1] and the guanine nucleotide exchange factor (GEF). The *S. cerevisiae* Ira1p and Ira2p contain a domain, located in the intermediary portion of the molecule, that shares similarities with the catalytic core of mammalian p120-GAP and neurofibromin (Tanaka et al., 1990a; Buchberg et al., 1990). The ability of these two proteins to perform some of the Ira1p and Ira2p functions in the yeast cell led to the conclusion that Ira1p and Ira2p act as GAPs (Ballester et al., 1989, 1990; Tanaka et al., 1990b, 1993; Xu et al., 1990a; Martin et al., 1990). This was confirmed for Ira2p [3079 amino acids (aa)] by experiments showing that total extracts of *S. cerevisiae* cells overexpressing a Ira2p fragment (aa 527−2255) or a partially purified GST-fused Ira2p catalytic region (aa 1665−2025) produced in *Escherichia coli* were able to enhance the intrinsic GTPase activity of Ras2p *in vitro* (Tanaka et al., 1991). In *S. cerevisiae*, two GDP/GTP exchange factors were identified, Cdc25p and Sdc25p, which are members of the conserved CDC25-like family [for references, see Parmeggiani et al. (1993)]. Their catalytic domains were shown to have a GEF activity *in vitro* (Créchet et al., 1990; Jones et al., 1991; Lai et al., 1993; Chevallier-Multon et al., 1993; Jacquet et al., 1994).

In the past years, whereas the characterization of p120-GAP and neurofibromin made considerable progress (Eccleston et al., 1993) [for other references, see Boguski and McCormick (1993)], little appeared concerning the functions

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; ME, 2-mercaptoethanol; DTT, dithiothreitol; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; GST, glutathione *S*-transferase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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*in vivo* and the biochemical properties *in vitro* of Ira2p. The *in vitro* approach was hindered by the difficulties connected with the purification of soluble active *IRA2* products. In view of this situation, in the present work, we have undertaken an effort to analyze several biochemical parameters susceptible to the influence of the solubility and activity of Ira2p *in vitro*. We describe the isolation and characterization of the Ira2p catalytic domain, whose activity, specificity, and affinity were compared with those of the corresponding domain of the mammalian p120-GAP and neurofibromin. Like neurofibromin (Bollag et al., 1993), Ira2p was found to be inhibited by tubulin. We were able to achieve the reconstitution of a multiround turnover of the Ras2p GDP/GTP cycle *in vitro* by using only *S. cerevisiae* components.

## MATERIALS AND METHODS

*Production and Purification of the GST-Fused Ira2p and Neurofibromin Catalytic Domain.* Two *IRA2* fragments encoding Ira2p-383 (aa 1644−2026) and Ira2p-505 (aa 1549−2053), containing the GAP-like catalytic domain, were cloned into pGEX2T (Smith & Johnson, 1988) and expressed in *E. coli*. For Ira2p-383, a ClaI linker of 10 bp was inserted into the (1) filled MluI site of pCD1 carrying the entire *IRA2* gene (Bussereau et al., 1992) and (2) SmaI site of pGEX2T. The TaqI-TaqI (former MluI) fragment from pCD1 was cloned in the ClaI site of this modified pGEX2T-ClaI. For Ira2p-505, a Hind3 linker of 8 bp was inserted in the HpaI site of pCD1, and the Hind3-Hind3 (former HpaI) fragment from pCD1 was cloned in the Hind3 site of pGEX2TH (a gift of Dr. H. Maruta). The *E. coli* strain HB101 was used for the expression of the GST-fused products. For the GST-Ira2p-383, a 5 L culture at a cell concentration of 0.6−0.8 $A_{600}$ units was induced with 0.1 mM IPTG for 3 h at 37 °C. For the GST-Ira2p-505, the induction was started at a cell concentration of 0.2 $A_{600}$ units with 0.2 mM IPTG for 44 h at 24 °C. The cell pellets were frozen at −20 °C.

Two procedures were used to prepare the protein extract to be purified via affinity chromatography on glutathione sepharose, implying either native conditions or the use of the detergent sarkosyl. With the former procedure, the centrifuged cell pellet was resuspended in 50 mL of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 7 mM ME, and 1 mM PMSF and treated first with 0.4 mg/mL lysozyme for 30 min at 4 °C and then with 0.08 mg/mL DNAseI plus 15 mM $MgCl_2$ for 10 min at 4 °C. This was followed by three sonication bouts of 10 s each and then by a centrifugation at 12 000*g* for 15 min. In the second procedure, carried out after a modification of the method of Frangioni and Neel (1993), the cell pellet was washed and resuspended in 75 mL of STE buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA) and 2 mM PMSF. After treatment with 0.7 mg/mL lysozyme for 30 min at 4 °C, 6 mM DTT and 0.8% sarkosyl (*N*-lauroylsarcosine, sodium salt, 10% stock solution in STE) were added. After a vigorous mixing, the viscous extract was sonicated five times for 15 s and centrifugated at 12 000*g* for 15 min. Triton X-100 (final concentration, 1%) was then added to the supernatant to sequestrate the sarkosyl. Glutathione sepharose beads (10 mL) (Pharmacia) were mixed with the protein extract, gently shaken for 30 min at 4 °C, and washed with PBS (16 mM $Na_2HPO_4$/4 mM $NaH_2PO_4$, (pH 7.3) and 150 mM NaCl), and the fusion protein was eluted three times with 10 mL of 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. After concentration for 1−2 h on Aquacide II, the protein solution was dialyzed against 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 14 mM ME, and 50% glycerol and stored at −20 °C at a concentration of 0.5−1 mg/mL. For the GST-Ira2p-505 after the affinity chromatography, the preparation was immediately passed through a 5 mL Qsepharose Fast-flow column and eluted with a linear gradient of KCl (60 mL of 20−500 mM KCl in 50 mM Tris-HCl (pH 7.5) and 1 mM $MgCl_2$, at a flow rate of 1 mL/min). The GST-Ira2p-505 protein emerged at around 400 mM KCl.

The RsaI-RsaI *NF1* fragment encoding for a 414-amino acid catalytic domain (aa 1145−1558) of the neurofibromin (Xu et al., 1990b) was inserted into the SmaI site of a pGEX2T derivative. The production and purification of the GST-NF1-414 protein was performed essentially following the procedure with sarkosyl as described for the GST-fused Ira2p catalytic domain; the yield was approximately 1 mg per liter of culture and the purity around 90%.

*Measurement of GTPase Activity.* The GTPase activity of Ras2p was determined at 25 °C in buffer A (50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, and 1 mg/mL BSA) by measuring the release of $[^{32}P]P_i$ from the Ras2p·[$\gamma$-$^{32}$P]-GTP complex using the charcoal method (the reaction was stopped by addition of 400 $\mu$L of a 4% suspension of activated charcoal in 20 mM $H_3PO_4$, and after centrifugation, the radioactivity of an aliquot of the supernatant was counted). Alternatively, the GTP hydrolysis was determined by following the disappearance of the Ras2p-bound radioactivity from nitrocellulose filters (Sartorius, 0.45 $\mu$m pore size). When indicated, the Ras2p·[$\gamma$-$^{32}$P]GTP complex was preformed by incubating the Ras2p·GDP with a 2−4-fold excess of [$\gamma$-$^{32}$P]GTP in 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, and 0.2 mg/mL BSA at 30 °C for 5 min; then $MgCl_2$ (3 mM) was added to stabilize the complex. Control reactions were systematically performed to reveal the presence of contaminating nucleotidases in the protein preparations. Since a variable concentration of glycerol (0.1−10%) was carried over with the Ira2p storage buffer, it was verified that glycerol up to 20% had no effect on the Ira2p activity.

*Preparation of the Cdc25p Catalytic Domain.* Cloning, production, and partial purification of the GST-fused Cdc25p fragment containing the last 509 C-terminal residues (GST-Cdc25p-509) were performed as described (Jacquet et al., 1994). To eliminate nearly completely the contaminating nucleotidase activities, the GST-Cdc25p-509 preparation was passed through a small (1 mL) Qsepharose Fast-flow column and eluted with a linear gradient of NaCl (20 mL of 0−400 mM NaCl in 50 mM Tris-HCl (pH 7.5) and 1 mM $MgCl_2$, at a flow rate of 0.5 mL/min). The fractions corresponding to the peak of the GEF activity, emerging around 200 mM NaCl, were concentrated in Aquacide II, dialyzed against 50 mM Tris-HCl (pH 7.5) and 50% glycerol, and stored at −20 °C.

*Preparation of RAS Proteins.* Ras2p, as a 30 or 42/37 kDa form, was purified and stored as described (Jacquet et al., 1994). For the purification of c-H-ras p21, the filled NdeI-SalI fragment from pSKc-H-ras (Gross et al., 1985) was inserted into the SmaI site of pGEX2T. The induction with 0.1 mM IPTG at 30 °C of the transformed SCS1 was started at a cell concentration of 0.4 $A_{600}$ units and continued until 2 $A_{600}$ units. The resuspended cell pellet from a 2 L culture was sonicated five times for 30 s in 16 mL of buffer

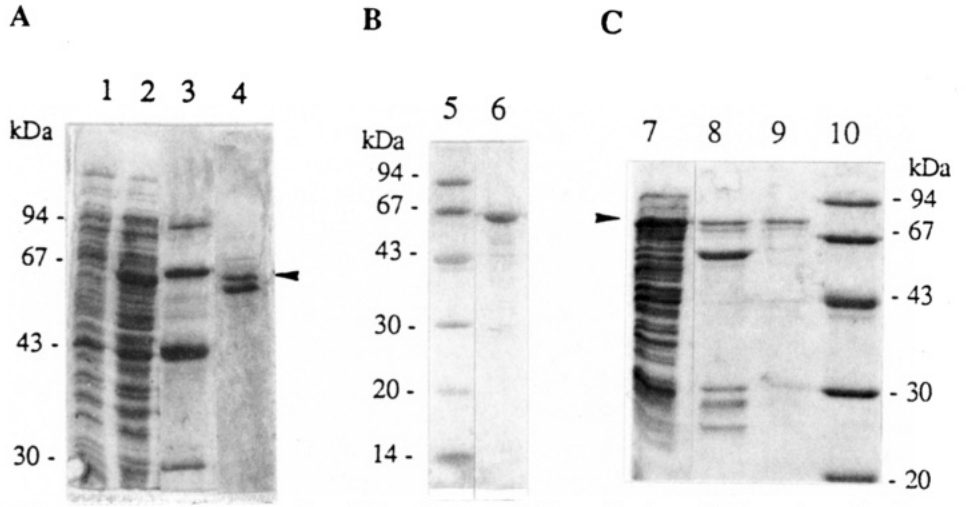


FIGURE 1: SDS−PAGE of GST-fused Ira2p-383 (A and B) and Ira2p-505 (C) purifications. (A) Lane 1, total cell extract before induction. Lane 2, total cell extract after GST-Ira2p-383 induction. Lane 4, preparation of GST-Ira2p-383 purified under native conditions. (B) Lane 6, preparation of GST-Ira2p-383 purified with the use of sarkosyl. (C) Lane 7, total cell extract after GST-Ira2p-505 induction. Lane 8, preparation of GST-Ira2p-505 after affinity chromatography. Lane 9, preparation of GST-Ira2p-505 after Qsepharose chromatography. Lanes 3, 5, and 10, molecular weight markers. The gels were stained with Coomassie Blue.

B (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 $\mu$M GDP, 7 mM ME, and 10% glycerol) and 0.5 mM PMSF. After centrifugation for 20 min at 17 000*g*, the supernatant was mixed with 4 mL of glutathione sepharose and gently shaken for 30 min at 4 °C. The resin-bound GST-p21 fusion was cleaved with 70 NIH of human thrombin (Sigma) for 45 min at 30 °C in 5 mL of buffer B plus 2.5 mM $CaCl_2$. The final step on monoQ (FPLC system, Pharmacia) yielded pure p21 (0.5 mg per liter of cell culture) that was stored as described for Ras2p.

*Other Materials and Methods.* The pure catalytic domain of human p120-GAP (GAP-334, aa 714−1047) was a gift of Dr. A. Wittinghofer. Pure dimer tubulin was a gift of Drs. M. F. Carlier and D. Pantaloni. The *in vitro* polymerization of tubulin was obtained by incubation for 30 min at 37 °C in 50 mM MES (pH 6.8), 1 mM EGTA, 6.5 mM $MgCl_2$, 0.6 mM GTP, 15% glycerol, and 20 $\mu$M taxol, a drug that irreversibly stabilizes the polymerization form. The microtubules were purified from bovine brain in the presence of taxol (Vallee, 1986) and carefully washed to eliminate the free tubulin. SDS−PAGE was carried out using 12% acrylamide separating gel. Polyclonal antibodies anti-GST and anti-Ira2p-383 were produced in rabbit. Protein concentration was determined by the Bio-Rad protein assay using BSA as a standard. To determine the percentage of a specific product in a protein mixture, the Coomassie Blue-stained gels were scanned with the Apple Scanner system and the results analyzed with the Scan Analysis software.

## RESULTS

*Purification of the Ira2p Catalytic Domain.* The Ira2p-383 (aa 1644−2026) closely corresponds to the GAP-like catalytic domain, as defined by comparison of the primary structure of the diverse members of the GAP family [376 residues (aa 1650−2025), Xu et al., 1990a; 347 residues (aa 1634−1980), Imai et al., 1991]. Despite considerable difficulties due to the low solubility of the protein and the instability of its activity, we were able to determine the conditions for a reproducible purification. The procedure under native conditions (see Materials and Methods) only gave a small percentage of soluble product (less than 5%), and after glutathione sepharose chromatography, the GST-Ira2p-383 fusion was 20−40% pure. On Coomassie Blue-stained gel, the contamination mainly consisted of only one *E. coli* protein (Figure 1, lane 4). The yield was at most 0.05 mg per liter of culture. The solubility was not significantly increased by the presence of 1.5 M NaCl or of 1−2% detergents, such as Triton X-100, *n*-octyl glucoside, deoxycholate, nonidet P40, CHAPSO, or lubrol. Nor was the solubility improved by inducing the cells at lower temperatures (25−30 °C). Only denaturating conditions such as 8 M urea or pH 12 induced a partial solubility. A breakthrough was obtained by the use of sarkosyl. The presence of 0.8% of this detergent allowed the complete solubilization of the GST-fused Ira2p-383 and the isolation of an active >90% pure protein (Figure 1, lane 6) in higher yield, at least 0.8 mg per liter of cell culture. With this method, the preparation of Ira2p was moreover free of contaminating nucleotidases that interfere in the experiments measuring the Ras2p GTPase activity.

To obtain stable Ira2p-383 preparations, it is important to perform the whole purification within 24 h. On storage at −20 °C (see Materials and Methods for conditions), GST-Ira2p-383 was stable for months. The precise amount of Ira2p in the various preparations was evaluated by combining total protein concentration and scanner analysis. It is important to mention that the specific activity of the fused Ira2p-383 product purified in the presence of sarkosyl, as measured at fixed concentrations of enzyme (32 nM GST-Ira2p-383) and substrate (0.3 $\mu$M Ras2p·GTP), was equal to that obtained in the absence of the detergent ($1 \times 10^{-3}$ $s^{-1}$ GTP molecules hydrolyzed per molecule of Ras2p, corresponding to a 10-fold stimulation of the intrinsic Ras2p GTPase). This indicates that the treatment with sarkosyl does not affect the Ira2p-383 activity. In Figure 1, the minor bands with a molecular mass between those of GST-Ira2p-383 (64 kDa) and GST (27 kDa) corresponded to fragments of GST-Ira2p-383 truncated at the C-terminal end, as confirmed by western blot carried out with both anti-GST and anti-Ira2p antibodies (not shown). This suggests the

existence of weak proteolytic phenomena during the extraction procedure or, alternatively, phenomena of premature termination of the GST-Ira2p-383 polypeptide chain. These C-terminal-truncated GST-Ira2p-383 products (<10%) were not taken into account in determining the specific activity of GST-Ira2p-383. In fact, genetic analysis indicates that the C-terminal boundary of the active domain of Ira2p is between positions 1991 and 1909 (Tanaka et al., 1990a); therefore, most of these fragments lost the Ira2p activity. The GST-Ira2p-383 could be efficiently digested with thrombin, yielding a preparation of cleaved GST and Ira2p-383 that was as active as the GST-fused Ira2p-383 in stimulating the Ras2p GTPase (not shown). A low digestion temperature (15 °C) and the presence of BSA (0.5 mg/mL) were critical to the conservation of the full Ira2p activity. Most experiments described in this article were carried out with GST-fused preparations obtained with the procedure using sarkosyl.

In contrast to Ira2p-383, sarkosyl induced a pronounced instability of the activity of Ira2p-505. Therefore, the purification of this Ira2p fragment was performed in the absence of this detergent. In this case, the best compromise between expression and solubility was obtained by carrying out an IPTG induction for a longer time (44 h) and at a lower temperature (24 instead of 37 °C). For the purification, extraction under native conditions and affinity chromatography were followed by Qsepharose chromatography, which yielded a 65% pure GST-Ira2p-505 protein (Figure 1, lanes 8 and 9). GST-Ira2p-505 was able to stimulate the Ras2p GTPase. Whereas, in some preparations, the specific activity reached the level of GST-Ira2p-383, in other cases, it was 2−4-fold lower. This suggests a major instability of the activity of the larger fragment.

*Activity of Ira2p Catalytic Domain.* In standard buffer A, the addition of GST-Ira2p-383 induced one burst of Ras2p GTPase (Figure 2A). In the presence of 3 mM EDTA and an excess of free [$\gamma$-$^{32}$P]GTP, the concentration of the Ira2p substrate (the Ras2p·[$\gamma$-$^{32}$P]GTP complex) remained virtually constant due to the rapid nucleotide exchange on Ras2p induced by the magnesium-chelating action of EDTA. This allowed us to obtain linear multicycles of Ira2p-stimulated Ras2p GTPase, whose rate was dependent on the concentration of GST-Ira2p-383 (Figure 2B). Noteworthy is the fact that the rates of both the intrinsic and the Ira2p-stimulated GTPases of Ras2p were not significantly influenced by the presence of 3 mM EDTA. These experiments were carried out with the 30 kDa form of Ras2p. The GTPase activities of both 30 and 42/37 kDa forms of Ras2p were stimulated by the GST-fused Ira2p-383 to a similar extent (not illustrated). Therefore, the Ras2p C-terminal domain of 112 residues, which is absent in the 30 kDa form, is not required for the Ras2p-Ira2p interaction, as suggested by the observations of other authors (Tanaka et al., 1991).

The Ira2p-dependent Ras2p GTPase activity (*i.e.* its linear initial rate minus the intrinsic rate) was measured in buffer A at 25 °C in the presence of a fixed amount of GST-Ira2p-383 (30 nM) and increasing amounts of the Ras2p·GTP complex (up to 44 $\mu$M). The double reciprocal plot allowed the determination of the $K_m$ of Ras2p·GTP for the GST-Ira2p-383, which was 18 $\mu$M. By comparison of the observed $k_{cat}$ value of the Ira2p-stimulated GTPase (0.65 $s^{-1}$) with that of the intrinsic GTPase of Ras2p ($1.08 \times 10^{-4} s^{-1}$), it turns out that the catalytic domain of Ira2p can stimulate

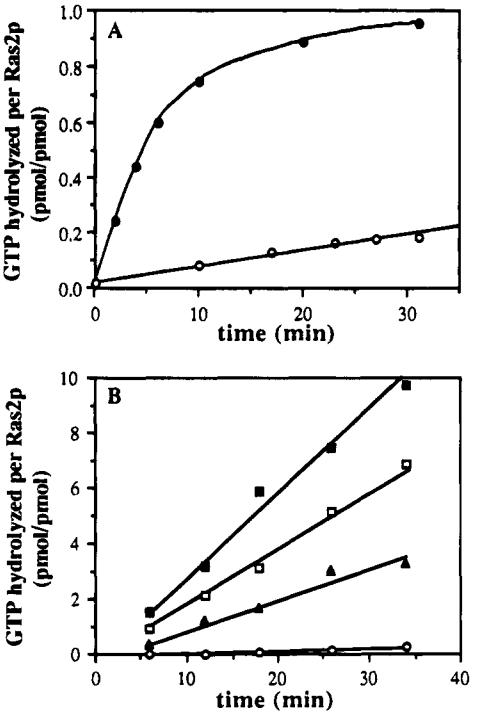


FIGURE 2: Stimulation of Ras2p GTPase activity by Ira2p-383. (A) The reaction mixture (100 $\mu$L) containing 0.22 $\mu$M [$\gamma$-$^{32}$P]GTP-bound Ras2p (specific activity, 204 Bq·pmol$^{-1}$) in buffer A was incubated at 25 °C in the absence (○) or in the presence (●) of 49 nM GST-Ira2p-383. At the indicated times, 15 $\mu$L aliquots were withdrawn and the liberated [$^{32}$P]$P_i$ was measured using the charcoal method. (B) The reaction mixture (20 $\mu$L) containing in buffer A 0.75 $\mu$M GDP-bound Ras2p, 3 mM EDTA, and 50 $\mu$M [$\gamma$-$^{32}$P]-GTP (51 Bq·pmol$^{-1}$) was incubated at 25 °C in the absence (○) or in the presence of 49 nM (▲), 122 nM (□), 245 nM (■) GST-Ira2p-383. At the indicated times, the liberated [$^{32}$P]$P_i$ was measured in 2 $\mu$L aliquots.

Table 1: Comparison of the $K_m$ and $k_{cat}$ of Ras2p·GTP and H-ras·GTP for Different GAP-like Domains[a]

| | Ras2p·GTP | | H-ras·GTP | |
|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
| Ira2p-383 | 18 | 0.65 | – | – |
| Ira2p-505 | 16 | ~0.2 | – | – |
| GAP-334 | >50 | >5 | 19[b] | 4.2[b] |
| NF1-414 | 0.11 | 0.15 | 0.4 | 0.9 |
| NF1-333 | ND | ND | 0.3[c] | 1.4[c] |

[a] The final concentrations in the tests were 30 nM Ira2p-383, 40 nM Ira2p-505, 30 nM GAP-334, and 2 nM NF1-414. [b] Gideon et al. (1992). [c] Wiesmüller and Wittinghofer (1992). ND, not determined.

the GTPase of Ras2p 6000 times (Table 1). The $K_m$ of Ras2p·GTP for the larger product GST-Ira2p-505 was 16 $\mu$M, *i.e.* very similar to that of the small one, whereas the $k_{cat}$ value was lower (~0.2 $s^{-1}$).

*Effect of the Temperature, Salts, and pH.* The stability of the GST-Ira2p-383 activity was very sensitive to the temperature, and it was significantly increased by the presence of 0.5−1 mg/mL BSA. In buffer A, at 25 °C or below, the activity was virtually stable for at least 1 h, whereas at 30 °C, it lost half its activity in 20 min (not shown). The presence of the substrate Ras2p·GTP partially protected the GST-Ira2p-383 against inactivation at 30 °C. The finding that the stability of the activity of the Ira2p catalytic domain strongly increased when the temperature was decreased to 25 °C was important for obtaining quantitatively reproducible experiments. It is likely that the

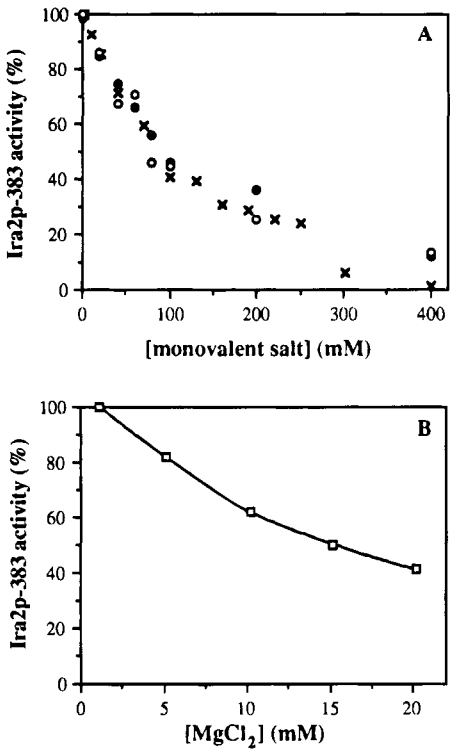


FIGURE 3: Influence of the salts on the Ira2p-383 activity. The Ras2p•[$\gamma$-$^{32}$P]GTP complex (0.13−0.76 $\mu$M, depending on the test) was incubated at 25 °C in the absence and in the presence of 55 nM GST-Ira2p-383 in buffer A (30 $\mu$L) with the indicated concentrations of NaCl (×), KCl (●), $NH_4Cl$ (○) (A), and $MgCl_2$ (□) (B) . After 5 min, 20 $\mu$L aliquots were withdrawn and filtered on nitrocellulose disks. The Ira2p-dependent GTPase was calculated as the difference between the radioactivity retained in the absence of GST-Ira2p-383 and the radioactivity retained in the presence of GST-Ira2p-383. The Ira2p-dependent Ras2p GTPase in buffer A (containing 1 mM $MgCl_2$) was taken as 100% activity.

folding of the catalytic domain of Ira2p is not very stable *in vitro* due to the absence of the other Ira2p domains and/or its cellular localization in a hydrophobic environment. The addition of different salts to buffer A strongly inhibited the Ras2p GTPase stimulation by GST-Ira2p-383 without significantly affecting the intrinsic Ras2p GTPase. The calculated $IC_{50}$ was 85−95 mM for the monovalent salts NaCl, KCl, and $NH_4Cl$ and 15 mM for $MgCl_2$ (Figure 3). The pH dependence was determined using a three-component buffer (Mistou et al., 1992). The activity of Ira2p-383 and Ira2p-505 displayed small variations in the range of pH between 6.4 and 8.5 with an optimum around pH 8 (not shown).

*A Multiround Ras2p•GDP/GTP Cycle Is Induced by the Simultaneous Presence of Ira2p and Cdc25p Catalytic Domains.* The extremely slow rate of the intrinsic GDP/GTP exchange on Ras2p under physiological concentration of $MgCl_2$ (1 mM) only allowed the investigation of the properties of the one-round GTPase reaction stimulated by Ira2p-383. Therefore, we increased the velocity of the GDP/GTP exchange by adding a GEF of Ras proteins, such as the catalytic domain of Cdc25p (Cdc25p-509, Jacquet et al., 1994), and we investigated the effect on the Ira2p-stimulated Ras2p GTPase. As shown in Figure 4, in the presence of only Ira2p-383, the preformed [$\gamma$-$^{32}$P]GTP-bound Ras2p showed a single round cleavage. Addition of the Cdc25p-509 induced a multiround turnover Ras2p GTPase, whose rate was linear. In these experiments, the substrate Ras2p•[$\gamma$-$^{32}$P]GTP remained constant due to the saturating concentrations of free [$\gamma$-$^{32}$P]GTP and Cdc25p, Ira2p being the rate-limiting component of the reaction. In fact, the rate of the multiround reaction corresponded to the initial velocity of the one-round GTP hydrolysis induced by Ira2p-383 in the absence of Cdc25p-509. Moreover, in other experiments not illustrated, this rate was found to be dependent on the concentration of the added Ira2p.

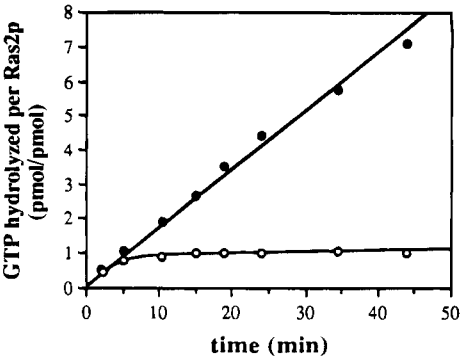


FIGURE 4: Induction by Cdc25p-509 of a multiround activity of the Ras2p GTPase stimulated by Ira2p-383. The [$\gamma$-$^{32}$P]GTP-bound Ras2p (1.4 $\mu$M) was added to 20 $\mu$L of buffer A containing 34 $\mu$M [$\gamma$-$^{32}$P]GTP (27 Bq•pmol$^{-1}$) and 100 nM GST-Ira2p-383 with (●) or without (○) 4 nM GST-Cdc25p-509. The mixture was incubated at 25 °C and at the given times, aliquots (2 $\mu$L) were withdrawn and the liberated [$^{32}$P]$P_i$ was measured.

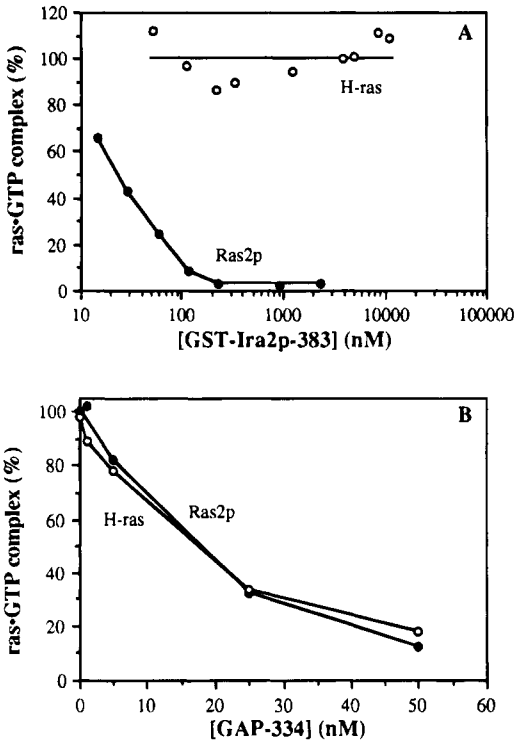


FIGURE 5: Comparison of the effects of Ira2p-383 and GAP-334 on the Ras2p and H-ras p21 GTPase activities. The Ras2p•[$\gamma$-$^{32}$P]-GTP (●) and H-ras•[$\gamma$-$^{32}$P]GTP (○) complexes (0.2 $\mu$M) were incubated at 25 °C in buffer A (20 $\mu$L) in the presence of increasing concentrations of GST-Ira2p-383 (A) or GAP-334 (B). After 15 min, the percentage of ras protein bound to GTP was measured by filtering 15 $\mu$L aliquots on nitrocellulose disks. The amount of ras•[$\gamma$-$^{32}$P]GTP after incubation without GST-Ira2p-383 or GAP-334 was taken as 100% concentration.

*Specificity of Ira2p.* The GTP hydrolysis rates of yeast Ras2p and human c-H-ras p21 were measured in the presence of various concentrations of GST-Ira2p-383 and GAP-334 (Figure 5). Nanomolar concentrations of GAP-334 stimu-

lated both Ras2p and H-ras GTPase to the same extent, and similarly, nanomolar concentrations of GST-Ira2p-383 were able to stimulate Ras2p; whereas even a 100-fold higher amount of GST-Ira2p-383 (11 $\mu$M) had no effect on the H-ras GTPase. Nor was the larger fragment Ira2p-505 able to stimulate the H-ras GTPase (not shown). Previous observations from other authors had indicated a strict specificity of Ira2p for *S. cerevisiae* Ras proteins (Xu et al., 1990a; Tanaka et al., 1991). Our results obtained in a highly purified system confirmed this peculiarity of Ira2p.

In experiments not illustrated, we observed that the H-ras·GTP complex, added at a concentration up to 20 $\mu$M, did not compete with the GTPase stimulation of Ras2p by Ira2p-383, showing that the insensitivity of the GTPase of H-ras to Ira2p is likely due to a lack of binding rather than to a nonproductive interaction.

*Comparison of the Activity of the Catalytic Domains of Ira2p, p120-GAP, and Neurofibromin on the Ras2p GTPase.* We compared the activity of the Ira2p catalytic domain with that of the homologous domain of mammalian p120-GAP and neurofibromin using the yeast Ras2p protein as substrate. The affinities ($K_m$ ) of Ras2p·GTP for GAP-334 and GST-NF1-414 and the corresponding maximal stimulated GTPase activities ($k_{cat}$ ) were measured as described for GST-Ira2p-383 (Table 1). The Ras2p·GTP affinity for GAP-334 was lower than that for Ira2p-383, but due to the difficulty in using a higher concentration of Ras2p·GTP, we could only estimate a minimal value for its $K_m$. Differently, the Ras2p·GTP affinity for NF1-414 was 150-fold higher. However, the $k_{cat}$ of GAP-334 (>5 $s^{-1}$) turned out to be higher than that of Ira2p-383 (0.65 $s^{-1}$) and NF1-414 (0.15 $s^{-1}$). Noteworthy is the fact that the $K_m$ and $k_{cat}$ values we obtained for the NF1-414 action on H-ras·GTP GTPase are in good agreement with the values obtained by Wiesmüller and Wittinghofer (1992) for NF1-333. These and other results, summarized in Table 1, point to the existence of highly specific differences between interactions of Ira2p, p120-GAP, and neurofibromin with Ras2p and H-ras.

*Inhibition of the Ira2p Activity by Tubulin.* It has been demonstrated that tubulin partially inhibits the activity of full-length neurofibromin and less efficiently that of its catalytic domain but not that of full-length p120-GAP (Bollag et al., 1993). We tested the effects of increasing concentrations of tubulin as a dimer form on the Ras2p GTPase activation by GST-Ira2p-383, GST-Ira2p-505, and GAP-334 (Figure 6). Whereas GAP-334-dependent GTP hydrolysis was essentially uninfluenced by tubulin, Ira2p-383- and Ira2p-505-dependent GTP hydrolysis were inhibited. The maximal inhibition was 25% for both fragments. Interestingly, Ira2p-383 showed a slight but reproducible stimulation at tubulin concentrations lower than 5 $\mu$M. To obtain the same percentage of inhibition, Ira2p-505 needed around 10 times less tubulin than Ira2p-383. This indicates that the affinity of Ira2p-505 for tubulin is 1 order of magnitude higher than that of Ira2p-383, suggesting that the stretch of the additional 95 N-terminal residues (aa 1549−1643) flanking the Ira2p catalytic domain contributes to the binding of tubulin. In good agreement with this, a homologous segment of 80 amino acids N-terminal to the GAP-like domain of neurofibromin was shown to be important for the interaction with tubulin (Bollag et al., 1993). However, a role of the additional 27 C-terminal residues (aa 2027−2053) of Ira2p-505 cannot be excluded. In experiments not illustrated, the tubulin after *in vitro* polymerization and also the microtubules purified from bovine brain (see Materials and Methods) did not inhibit the Ira2p-505 activity, suggesting that the inhibitory action on Ira2p is a property of the dimer form of tubulin.

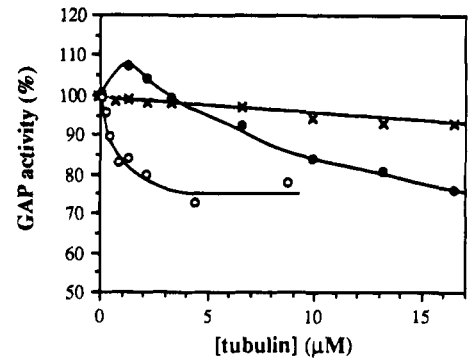


FIGURE 6: Inhibition of Ira2p-383 and Ira2p-505 by tubulin. The Ras2p·[$\gamma$-$^{32}$P]GTP complex (0.3 $\mu$M in 4 $\mu$L) was added to a 26 $\mu$L mixture containing fixed amounts of GAP-334 (final concentration, 24 nM) (×), GST-Ira2p-383 (30 nM) (●), or GST-Ira2p-505 (100 nM) (○) and the indicated increasing concentrations of tubulin in 50 mM MES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM GTP, 0.2 mg/mL BSA, and 7.5% glycerol. After incubation for 15 min at 25 °C, 20 $\mu$L aliquots were filtered on nitrocellulose disks. For each tubulin concentration, the GAP activity of GAP-334, GST-Ira2p-383, or GST-Ira2p-505 was measured as the difference between the radioactivity retained in the absence and in the presence of the various GAP products. The GAP activity in the absence of tubulin was taken as 100%. The presence of tubulin had no effect on the retention of Ras2p·[$\gamma^{32}$P]GTP on nitrocellulose.

## DISCUSSION

From this work, the stimulation of the Ras2p GTPase by the catalytic domain of Ira2p can reach factors of 3 orders of magnitude (around 6000 times), but it is as yet unknown whether the presence of other Ira2p domains may further increase the stimulatory activity on the Ras2p GTPase. In the case of p120-GAP, the full-length protein has been reported to induce a maximum acceleration of the p21 GTPase by 5 orders of magnitude, the maximal stimulation by GAP-334 being 4.5-fold lower (Gideon et al., 1992). Noteworthy is the fact that, in our experimental conditions, GAP-334 exerted on Ras2p a stimulatory effect higher than 45 000 times, whereas NF1-414 stimulated the Ras2p GTPase 1400 times. It has been reported that the catalytic domain of neurofibromin has a maximal stimulation on p21 GTPase of 11 000 times (Martin et al., 1990; Wiesmüller & Wittinghofer, 1992). The effect of Ira2p-383 on the Ras2p GTPase displayed a $K_m$ value in the micromolar range (18 $\mu$M). Moreover, we found that Ras2p had a much higher affinity for NF1-414 than for GAP-334 (0.11 *vs* >50 $\mu$M), similar to the situation found for the stimulation of the p21 GTPase activity: 0.3 $\mu$M for NF1-333 (Wiesmüller & Wittinghofer, 1992), 9.7 $\mu$M for the full-length p120-GAP, and 19 $\mu$M for GAP-334 (Gideon et al., 1992). Thus, the comparison of these three GAP-like domains with respect to Ras2p emphasizes the existence of specific differences in the enzymatic properties of the components of the GAP family, Ira2p-383 showing an affinity and a turnover activity intermediary between GAP-334 and NF1-414 (Table 1). The lower maximal stimulation obtained with the larger Ira2p-505 catalytic fragment could be due to intrinsic properties of the regions flanking the catalytic domain or, more likely, to a major instability of this fragment independent of

proteolytic phenomena, as suggested by the variations in the specific activity of the various preparations. The inhibitory action observed with diverse monovalent salts is a property shared by Ira2p, p120-GAP, and neurofibromin (McCormick et al., 1988; Gibbs et al., 1988; Wiesmüller & Wittinghofer, 1992). This effect indicates that the ionic conditions may constrain the activity of GAP proteins in the cell.

The ability of a very short fragment to express an efficient catalytic activity strongly suggests that the other domains of the Ira2p protein may be involved in complex regulation mechanims, in which cellular elements from other signal pathways could also participate. Interaction with other components has already been shown for mammalian p120-GAP (Kaplan et al., 1990; Wong et al., 1992; Settleman et al., 1992), whose dimension is less than half that of Ira2p. Recently, it has been reported that neurofibromin binds to and is partially inhibited by tubulin, whereas such an effect was not observed for p120-GAP (Bollag et al., 1993). We also found that the Ira2p activity is inhibited by tubulin and that the region of 95 amino acids N-terminal to the Ira2p catalytic domain contains an important determinant for the tubulin action. Similarly, the corresponding segment N-terminal to the catalytic domain of neurofibromin was found to contribute to the binding site for tubulin (Bollag et al., 1993). In Ira2p, the two regions flanking the catalytic domain (350 aa at the N-terminal site and 850 at the C-terminal site) display significant homologies with those of neurofibromin but not with those of p120-GAP (Tanaka et al., 1993), suggesting that there are some specific functions and/or interactions shared only by Ira2p and neurofibromin. These results indicate that one of these functions is the binding to tubulin. Bollag et al. (1993) reported that polymerized tubulin was able to induce on neurofibromin an inhibition comparable to that of the dimer form. On the contrary, in our heterologous system, the polymerized form was unable to inhibit Ira2p. The inhibition induced by tubulin on the Ira2p catalytic domain was only partial (25%). The effect could be much stronger with the full-length Ira2p, as has been shown for the intact neurofibromin, whose activity was inhibited by tubulin up to 70%. This action of tubulin suggests interesting biological implications, since it could increase the level of the active form of Ras in specific phases of the cell cycle.

Ira2p's strict specificity toward the yeast Ras proteins remains an intriguing and fully unexplained characteristic. The reason for this may have been the requirement of multicellular organisms to develop more complex regulatory mechanisms, imposing a higher evolutionary pressure on the ras pathway. Indeed, three GAP products acting on ras have been identified in mammals so far: p120-GAP, neurofibromin, and GAP1$^m$ (Boguski & McCormick, 1993; Maekawa et al., 1994). The great specialization of H-ras p21 for this multiple GAP system could be the reason for its insensitivity to Ira2p. In this context, an interesting and as yet unanswered question is the difference between GAP and GEF families in which the GDP/GTP exchange factors have remained fully interchangeable in the mammalian and yeast organisms. This difference may be interpreted as the result of a greater complexity in the function of GAP proteins *vs* that of the exchange factors, for example, in connecting multiple signaling pathways, a possibility that nevertheless remains to be proven.

As a relevant result, this work shows that it is possible to reconstitute a homologous *S. cerevisiae* system *in vitro* of the Ras2p GDP/GTP cycle, which is regulated by the catalytic domains of Ira2p and Cdc25p. The Ira2p-383 or Cdc25p-509 alone can perform their specific activity on Ras2p, that is, the stimulation of the intrinsic GTPase and the nucleotide dissociation, respectively. The simultaneous presence of these two regulators induces on Ras2p a multicycle reaction of GTP hydrolysis and GDP to GTP exchange. The multiround cycle is obtained in the presence of a large molar excess of Ras2p, showing that the action of both regulators is of catalytic type. Since Cdc25p and Ira2p should alternate their productive interaction with Ras2p during the GDP/GTP cycle, it is probable that their binding on Ras2p is also alternate, as in the case of the corresponding ligands of the elongation factor Tu, the elongation factor Ts, and the ribosome [for references, see Miller and Weissbach (1977)]. The binding of Cdc25p and Ira2p is likely dictated by the specific conformational changes of Ras2p induced by either GDP or GTP. It is known that Cdc25p or CDC25-like proteins show a higher activity with and a higher affinity for the GDP complex than they do with the GTP complex of ras proteins (Créchet et al., 1990; Jacquet et al., 1992, 1995; Haney & Broach, 1994). On its turn, p120-GAP has a much lower affinity for H-ras·GDP than for H-ras·GTP (Schaber et al., 1989). The *in vitro* system with these three components (Ras2p, Ira2p, and Cdc25p) may be a usefull tool for the analysis of the regulation of Ras2p activities by ligands and the search of as yet unknown elements of this pathway.

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