The Arginine Finger Loop of Yeast and Human GAP Is a Determinant for the Specificity toward Ras GTPase†

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ABSTRACT: In this work, we have studied the role of the arginine finger region in determining the specificity of the GTPase activating proteins (GAPs) *Saccharomyces cerevisiae* Ira2p and human p120-GAP toward yeast Ras2p and human Ha-Ras p21. It is known that p120-GAP can enhance both Ras2p and Ha-Ras GTPase activities, whereas Ira2p is strictly specific for Ras2p and fails to activate Ha-Ras GTPase. Substitution in Ira2p of the arginine following the arginine finger with alanine, the residue found in the corresponding position of p120-GAP, or by glycine as found in neurofibromin, evokes a low but significant stimulation of Ha-Ras GTPase. The stimulatory activity of Ira2p on Ha-Ras increased by substituting segments of the finger loop region with p120-GAP residues, especially with the six residues forming the tip of the arginine loop. In p120-GAP, substitution of the entire finger loop with the corresponding region of Ira2p led to a construct completely inactive on Ha-Ras GTPase but active on yeast Ras2p GTPase. Analysis of these results and modeling of Ira2p·Ras complexes emphasize the importance of the finger loop region not only for the catalytic activity but also as a structural determinant involved in the specificity of GAPs toward Ras proteins from different organisms.

Ras proteins are ubiquitous GTPases with highly conserved structures, acting in the eukaryotic cell as switches of pathways regulating cell growth and differentiation (see *1*). They cycle between the active GTP-bound and the inactive GDP-bound form. The intrinsically very slow GTP hydrolysis and the GDP/GTP exchange control the level of the active conformation and are enhanced by GTPase activating proteins (GAPs)[1] and GDP/GTP exchange factors (GEFs), respectively. The strong sequence conservation of the catalytic domains within different families of GAPs and GEFs indicates that the molecular mechanisms regulating the activities of Ras proteins have been conserved during evolution. In line with this, the functions of Ras proteins and these regulators in mammals and the yeast *Saccharomyces cerevisiae* are interchangeable (see *2*). An exception is represented by the *S. cerevisiae* GAPs Ira1p and Ira2p, that as genetic and biochemical data indicate (*3*−*7*) are strictly specific for homologous yeast Ras1p and Ras2p GTPases and are completely inactive on Ha-Ras GTPase. In contrast, p120-GAP can act equally well on both homologous Ha-Ras p21 and heterologous yeast Ras2p GTPases. In previous work, we were able to identify the major structural determinant of Ras responsible for the differential specificity toward human and yeast GAP (*2*, *8*). This is located in the region of Ras2p comprising the N-terminal moiety of helix α3. Thus, we were able to construct by site-directed mutagenesis Ras2p variants responsive to human p120-GAP and, conversely, Ha-Ras p21 variants with a decreased response to Ira2p. The group of Wittinghofer has characterized the 3D structure of the complexes between the catalytic domain of p120-GAP (*9*) or neurofibromin (*10*) and Ha-Ras. An arginine situated in a loop of the catalytic domain of p120-GAP (L1c) hydrogen-bonds to AlF_3 that mimics the γ-phosphate in the hydrolysis transition state of Ha-Ras·GTP. This residue ("arginine finger") was proposed to stabilize the transition state of the p120-GAP·Ha-Ras·GTP complex, thus playing a crucial role in catalysis (*11*). A similar picture was found in the model of the complex between neurofibromin and AlF_3−GDP-bound Ha-Ras (*10*, *12*). The importance of the arginine finger in p120-GAP- or neurofibromin-dependent GTP hydrolysis of Ha-Ras was confirmed by site-directed mutagenesis analysis (*13*).

These findings have offered the possibility to study the mechanism(s) of the specificity of GAP proteins toward Ras, by adopting the reverse approach to that chosen by Parrini et al. (*2*), i.e., trying to identify possible structural determinants of GAP that are involved in the specificity toward the GTPase activity of different Ras proteins. For this purpose, we have mutated the finger loop region, introducing substitutions of single or multiple residues up to the whole finger loop, taking into account the sequence homologies of the corresponding regions of p120-GAP, neurofibromin, and Ira2p. The obtained results show that the finger loop region is not only essential for stimulating the catalytic reaction but is also important for determining the specificity of GAPs

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[1] Abbreviations: GAP, GTPase activating protein; Ira2p-383, catalytic domain of *Saccharomyces cerevisiae* GAP Ira2p comprising 383 residues; GAP-350, catalytic domain of human p120-GAP comprising the 350 C-terminal residues; GEF, guanine nucleotide exchange factor; NF-1, human GAP neurofibromin; GST, glutathione *S*-transferase; DTT, dithiothreitol; ME, 2-mercaptoethanol.

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toward the GTPase of Ras proteins from different organisms. Homology modeling of GAP·Ras complexes suggests a structural rationale for the difference in specificity of these GAP proteins.

EXPERIMENTAL PROCEDURES

Construction of Chimeras and Site-Directed Mutagenesis. The genes encoding GAP-350 and Ira2p-383 were cloned into the vector pGEX-2T. The Ira2p-383 and p120-GAP-350 encoding genes containing one point mutation were constructed using the Quick Change site-directed mutagenesis kit of Stratagene.

The genes encoding Ira2p-383 with multiple substitutions with residues from p120-GAP and neurofibromin finger loops were constructed by means of a cassette method. At the two sides of the Ira2p-383 finger loop, *Bgl*II and *Pac*I sites were introduced using the Quick Change site-directed mutagenesis kit. The mutated gene was obtained by ligating two synthesized complementary oligonucleotides containing the sequences encoding the substitutions into the vector pGEX-Ira2p-383 cut at the *Bgl*II and *Pac*I sites.

The gene encoding p120-GAP-350/Ira2p[1733−1746] was constructed by a PCR megaprimer method. In a first reaction, a ∼500 bp fragment of pGEX-GAP-350 was amplified using as mutagenic primer a 75 bp oligonucleotide comprised in the middle of 42 bp from the Ira2p finger loop containing the substitution(s) and a 20 base downstream second primer hybridizing to pGEX-2T (nucleotides 719−741). In a second PCR reaction, the obtained fragment was used as a primer together with a 22 base oligonucleotide hybridizing upstream to pGEX-2T (nucleotides 1261−1249) to amplify a ∼1700 bp fragment which was cloned *Bam*HI−*Eco*RI into pGEX-2T, yielding pGEX-p120-GAP-350/Ira2p[1733−1746].

Production and Purification of Biological Components. The catalytic domains of p120-GAP, GAP-350 (C-terminal 350 aa residues, ref *14*), and Ira2p-383 (aa 1644−2026, ref *15*) were purified as a GST-fusion protein. For expression of Ira2p-383 and its mutants in *Escherichia coli* HB101 or DH5α, a 3 h induction with 0.1 mM isopropyl thiogalactoside at 37 °C was used. The cells were broken by sonication (3 bouts × 30 s) and the overproduced proteins purified by affinity chromatography on glutathione−Sepharose. Proteins were concentrated by Aquacide II (Calbiochem) for 2 h, dialyzed overnight against 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 7 mM ME, and 50% glycerol, and stored at −20 °C at a concentration of 0.5−1 mg·mL^{-1}. If necessary, they were concentrated about 10 times just prior to use by ultrafiltration, using Vivaspin-500. Alternatively, preparations were first concentrated with Vivaspin-4, followed by a second concentration with Vivaspin-500 and then used directly in the GTPase assay. For expression of GAP-350 and its mutated forms in *E. coli*, a 15 h induction with 10 mM isopropyl thiogalactoside at 24 °C was used. The cell pellet was washed and solubilized in PBS containing 2 mM phenylmethylsulfonyl fluoride, and sonicated 5 times for 10 s. The GAP proteins were purified by affinity chromatography on glutathione−Sepharose. After concentration with Aquacide II for 2 h and dialysis overnight against 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 7 mM ME, and 50% glycerol, they were stored at −20 °C at a concentration of 0.5−1 mg·mL^{-1}. Ras2p and Ha-Ras p21 were expressed as GST-fusion proteins (*15*) and purified by affinity chromatography on glutathione−Sepharose, followed by thrombin-mediated cleavage of the fusions bound to the resin (*16*). The purity of the Ras proteins was > 90%.

Measurement of GAP Activity. Ha-Ras·[γ-^{32}P]GTP and Ras2p·[γ-^{32}P]GTP complexes were formed by incubating Ras·GDP at 25 °C for 10 and 5 min, respectively, with a 4-fold excess of [γ-^{32}P]GTP in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, and 0.2 mg·mL^{-1} bovine serum albumin. They were stabilized by the addition of 3 mM $MgCl_2$. The Ras·[γ-^{32}P]GTP complexes (∼0.25 μM) were then incubated at 25 °C for 15 min in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, and 1 mg·mL^{-1} bovine serum albumin in the presence of different amounts of Ira2p-383 or GAP-350 (final volume 30 μL). The stimulated GTP hydrolysis was determined by following the nitrocellulose-retained radioactivity that represented the Ras·[γ-^{32}P]GTP complexes. Twenty microliters of the incubation mixture was passed through nitrocellulose membranes (Sartorius, pore size 0.45 μm). The amount of Ras·[γ-^{32}P]GTP retained on the filters after incubation of all components in the absence of Ira2p-383, GAP-350, or its mutated forms was taken as 100% reference. Therefore, the values of GTPase activity reported represent the GAP-dependent stimulated activity. Sensitivity to Ha-Ras and Ras2p was measured by determining the concentration of GAP-334, Ira2p-383, or their mutants required to induce 50% GTP hydrolysis under the experimental conditions used (C_{50}).

Generation of Structural Models of Ras2p·Ira2p and Ha-Ras-p21·Ira2p by Homology Modeling. Structural models of the complexes were automatically generated by the SWISS-MODEL homology modeling server (http://www.expasy.ch/swissmod/) using the Ha-Ras-p21·GDP·AlF_3·GAP-350 model of Sheffzek et al. (PDB code 1wq1) as a template. First, models of the individual protein components were made; then the two were reassembled into an energy-minimized complex by the server as suggested in the SWISS-MODEL documentation (default parameter settings were used). The cofactors (GDP, Mg^{2+}, and AlF_3) could not be included in this step. As a control, the same procedure was done with Ha-Ras-p21 and RasGAP, thus effectively reconstructing a nucleotide-free energy-minimized protein complex. No dramatic differences were observed with the original template model. Even the final orientation of the "arginine finger" that seems strongly influenced by its interaction with the AlF_3 group proved not essentially different. For the illustrations of Figure 4, the coordinates of the missing GDP, Mg^{2+}, and AlF_3 were substituted from 1wq1 into the models.

RESULTS

Production and Purification of Ira2p-383, GAP-350, and Their Mutants. Wild-type Ira2p-383 and its mutants were purified under native conditions, as described under Experimental Procedures. The soluble Ira2p-383 product varied between 10 and 30% of the total amount in the cell extract. After glutathione−Sepharose chromatography, the resulting Ira2p preparations were virtually pure on the Coomassie Blue-stained gel except for the presence of an *E. coli* protein (3−12% of the Ira2p-383 amount depending on the preparation). This protein did not interfere in the reaction, as carefully checked. On storage at −20 °C, the activities of

A)	Ira2p	1730 E I E K S S R P T D I L R R N S C A T
	p120-GAP	777 E I S M E D E A T T L F R A T T L A S
	NF-1	1264 E V E L A D S M Q T L F R G N S L A S
B)	Ira2p-383/GAP-350[781-792]	1730 E I E K E D E A T T L F **R** A T T C A T
	Ira2p-383/GAP-350[787-792]	1730 E I E K S S R P T D L F **R** A T T C A T
	GAP-350/Ira2p-383[1733-1746]	777 E I S K S S R P T D I L **R** R N S C A S

FIGURE 1: Primary sequence of the finger loop region of wild-type GAPs and mutants. Finger loop regions of Ira2p, p120-GAP, and NF-1 as aligned by Scheffzek et al. (*9*) (A) and of the mutants of Ira2p and p120-GAP containing multiple substitutions (B). The arginine finger is shown in boldface type, and the substituted area is underlined.

GST-Ira2p-383, GST-Ira2p-383[R1743A], GST-Ira2p-383-[R1743G], and GST-Ira2p-383/NF-1[1274−1280] were stable for months. In comparison, the activity of GST-Ira2p-383 carrying the substitutions from the p120-GAP finger loop was less stable and was therefore determined after purification. The GAP proteins and their mutants were devoid of contaminating GTPase activities. The intrinsic GTPase activity of Ras proteins amounted to ∼5 mmol of GTP hydrolyzed·min^{-1}·(mol of Ras protein)$^{-1}$.

The catalytic domain of wild-type p120-GAP-350 was more soluble than that of wild-type Ira2p-383 (about 50% of the GAP amount in the cell extract). After glutathione−Sepharose chromatography, it was >90% pure. On storage at −20 °C, GST-GAP-350 was stable for at least several months. Solubility, purity, and stability at −20 °C of GAP-350[A790R] and p120-GAP-350/Ira2p-383[1733−1746] were comparable to those of wild-type p120-GAP-350.

Arg1743 of Ira2p Is a Determinant for Specificity toward Ras GTPase. The arginine finger region of the catalytic domain of p120-GAP has been considered to comprise 19 amino acid residues (residues 777−795) including in p120-GAP the arginine finger loop L1c, the C-terminal portion of α-helix 1c, and the N-terminal part of α-helix 2c (*10*). This region shares a significant degree of identity with the corresponding region of neurofibromin as well as a similar structural arrangement, as has been shown by their three-dimensional models (*12*). A homologous region can also be identified in the primary sequence of the catalytic domain of Ira2p, of which to date no experimental 3D structure has been published. As illustrated in Figure 1A, in this region p120-GAP and neurofibromin show a 47% identity, whereas Ira2p displays a lower identity to these two GAP proteins: 26% for p120-GAP and 31% for neurofibromin. At first, we investigated whether in Ira2p the substitution of the putative arginine finger (R1742) with alanine or lysine had the same negative effect on the activity as the substitution of the corresponding residues in p120-GAP (R789) and neurofibromin (R1276) (*13*). This was indeed the case, because in the presence of the substituted Ira2p proteins the Ras2p GTPase was extremely reduced (>99%, not illustrated) as compared to its activity in the presence of wild-type Ira2p.

Interestingly, the arginine finger of these three GAPs is flanked on the C-terminal side by alanine (A790) in p120-GAP and by glycine (G1277) in neurofibromin, both small, neutral residues. In contrast, Ira2p has in the corresponding position an arginine (R1743), a charged residue with a bulky side chain. This difference in the nature of the residue flanking the arginine finger suggested that the second arginine could be involved in the selectivity of these three

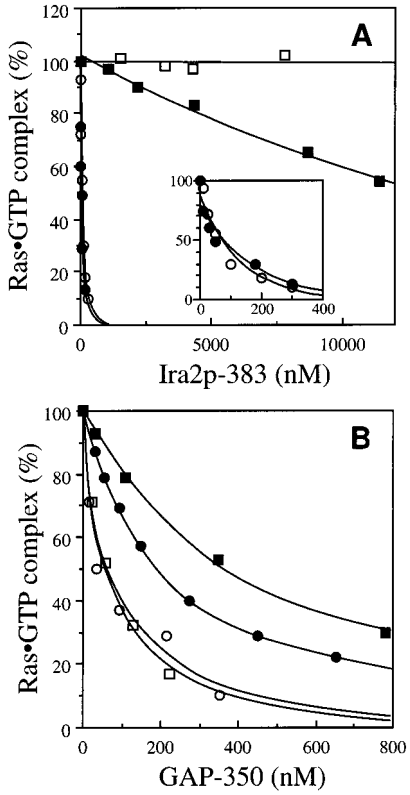

FIGURE 2: Stimulation of the GTPase activity of Ha-Ras and Ras2p as a function of the concentration of the various GAP proteins. Stimulation of the GTPase on Ras2p (○, ●) and Ha-Ras (□, ■), in panel A, by Ira2p-383 (open symbols) and Ira2p-383 R1743A (filled symbols) and, in panel B, by GAP-350 (open symbols) and GAP-350 A790R (filled symbols). The inset shows the effect of wt and mutant Ira2p-383 on Ras2p over a smaller concentration range. GTP hydrolysis was determined differentially by measuring, after 15 min incubation at 30 °C in the presence of the indicated concentration of GAP, the amount of Ras·[γ-^{32}P]GTP complexes retained by filtration on nitrocellulose membranes. As mentioned under Experimental Procedures, 100% represents the amount of Ras[γ-^{32}P]-GTP retained on the filters after incubation in the presence of all components except for Ira2p-383, GAP-350, or its mutated forms.

GAPs for yeast and human Ras proteins. Noteworthy, in the primary sequence of other GAPs from mammals or other organisms, the C-terminal flanking residue of the arginine finger is invariably a glycine (*10*, *12*). Thus, we started our analysis by substituting R1743 of Ira2p by an alanine and A790 of p120-GAP by an arginine. We found that the former substitution did not greatly affect the ability of the construct to enhance the GTPase activity of its homologous target Ras2p, as shown in a representative experiment (Figure 2A). However, this mutant displays a significant change in specificity, showing some stimulatory effect on Ha-Ras as

Table 1: Effect of Point and Multiple Mutations in the Finger Loop Region of Ira2p-383 and p120-GAP on the Stimulation of Ras2p and Ha-Ras GTPase Activities[a]

	Ras2p C_{50} (nM)	Ha-Ras C_{50} (nM)
(A) Ira2p-383 and Their Mutants		
Ira2p-383	52 ± 4.8 (6)	>20000 (6)
Ira2p-383[R1743A]	49 ± 3.3 (4)	12000 ± 248 (4)
Ira2p-383[R1743G]	47 ± 5.0 (4)	5400 ± 70 (4)
Ira2p-383/GAP-350[781−792]	5900 ± 278 (4)	5500 ± 49 (4)
Ira2p-383/GAP-350[787−792]	3500 ± 83 (3)	2500 ± 61 (3)
(B) GAP-350 and Their Mutants		
GAP-350	49 ± 3.3 (4)	53 ± 3.8 (4)
GAP-350[A790R]	158 ± 5.0 (5)	293 ± 9.2 (5)
GAP-350/Ira2p-383[1733−1746]	473 ± 30 (5)	>20000 (5)

[a] C_{50} represents the concentration (nM) of Ira2p-383, GAP-350, and mutated constructs inducing 50% hydrolysis of Ras-bound GTP at 30 °C in 15 min. The values are the averages of several experiments (*n*) ± standard deviations.

well. An even twice better stimulation was obtained by substituting this arginine with glycine, the corresponding residue found in neurofibromin (Table 1A). This novel activity of Ira2p[R1743A] on Ha-Ras was reproducible but low, approximately 240 (Ira2p[R1743A]) and 100 (Ira2p-[R1743G]) times lower than the activity of wild-type p120-GAP-350. Nevertheless, these results suggested some role of R1743 of Ira2p in achieving a productive interaction with Ha-Ras as well. An interesting result was obtained by replacing A790 of p120-GAP-350 with arginine (the reverse of the above mutation). This substitution decreased the stimulatory activity on Ha-Ras more than twice as strongly as that on Ras2p (Table 1B and for more details a representative experiment, Figure 2B).

One can conclude that the nature of the residue at this position already contributes to determining the specificity of Ira2p and Ras2p toward their target Ras proteins.

Changing Ira2p into p120-GAP and p120-GAP into Ira2p. The described results encouraged us to construct and analyze Ira2p mutants containing part of or nearly the complete L1c of p120-GAP and, conversely, a GAP-350 mutant containing the L1c of Ira2p. Figure 1B illustrates the multiple substitutions introduced into the primary sequence of the finger loop of Ira2p and p120-GAP. As shown in Table 1A, Ira2p-383/p120-GAP-350[781−792] was able to enhance the GTPase activity of Ha-Ras twice as strongly as Ira2p[R1743A]. On the other hand, its activity on Ras2p was decreased approximately 110 times compared to wild-type Ira2p-383, whereas that of Ira2p[R1743A] was not essentially changed. Limiting the substitution to the six residues constituting the tip of the p120-GAP finger loop (Ira2p-383/p120-GAP-350-[787−792]) further increased the stimulatory activity on Ha-Ras GTPase at least 2-fold (cf. Table 1A), whereas that on Ras2p was now decreased only about 65 times. Substituting the tip of the arginine finger loop of Ira2p by that of neurofibromin gave similar results (not illustrated).

Most interestingly, when we substituted in p120-GAP-350 14 residues corresponding to nearly the entire putative finger loop of Ira2p (Figure 1B), we obtained a catalytic domain of p120-GAP totally unable to stimulate Ha-Ras but still capable of enhancing to a substantial extent the GTPase activity of Ras2p, only some 10 times less than the wild-type catalytic domain (Table 1B). A more detailed analysis of these effects is given in Figure 3 that in representative

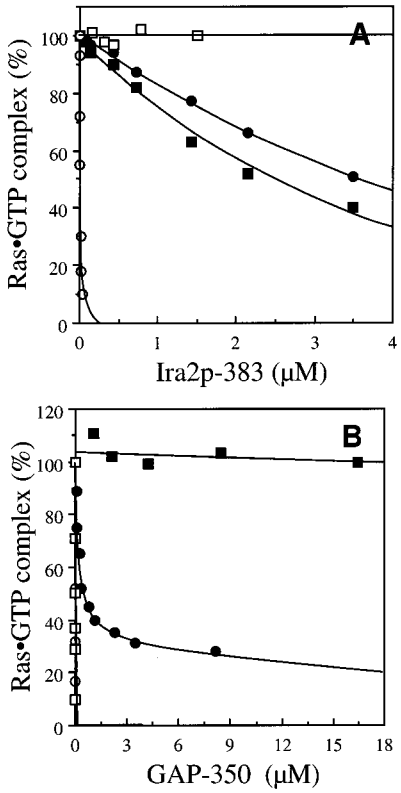

FIGURE 3: Stimulation of GTPase activities of Ha-Ras and Ras2p as a function of the concentration of the various GAPs. Stimulation of the GTPase on Ras2p (○, ●) and Ha-Ras (□, ■), in panel A, by Ira2p-383 (open symbols) and Ira2p-383/GAP-350[787−792] (filled symbols) and, in panel B, by GAP-350 (open symbols) and GAP-350/Ira2p-383[1733−1746] (filled symbols). GTP hydrolysis was determined as described in the legend to Figure 2.

experiments compares the activity of increasing concentrations of the two constructs Ira2p-383/p120-GAP-350[787−792] (panel 3A) and p120-GAP350/Ira2p[1733−1746] (panel 3B) on Ha-Ras and Ras2p with for reference the activity of the wild-type catalytic domains.

These results show that by modifying the respective L1c regions via mutual exchange of amino acid residues, it is possible to modify the selectivity of Ira2p and p120-GAP toward the GTPase activity of Ras proteins from human and yeast organisms, thus indicating that this region constitutes an important determinant for the specificity of the reaction.

DISCUSSION

The recent dramatic development of three-dimensional structure determination has been a decisive factor for improving our knowledge of function−structure relationships. Increasingly, experimentally determined structures of protein−protein or protein−ligand complexes contribute to our understanding of functional interactions of proteins and the regulation of their activities. Concerning the GTPases, several insights have resulted from these studies. In the case of Ras, Rho, or the Gα subunit of heterotrimeric G-proteins, the experimental observation of the interaction of an arginine residue from RasGAP (*10*, *12*), from RhoGAP (*17*), or from another domain of the Gα subunit (*18*, *19*) with an $AlF_{3/4}$ complex occupying the location of the γ-phosphate of Ras·GTP has led to the proposal of a mechanism in which this arginine makes the same interaction with the γ-phosphate

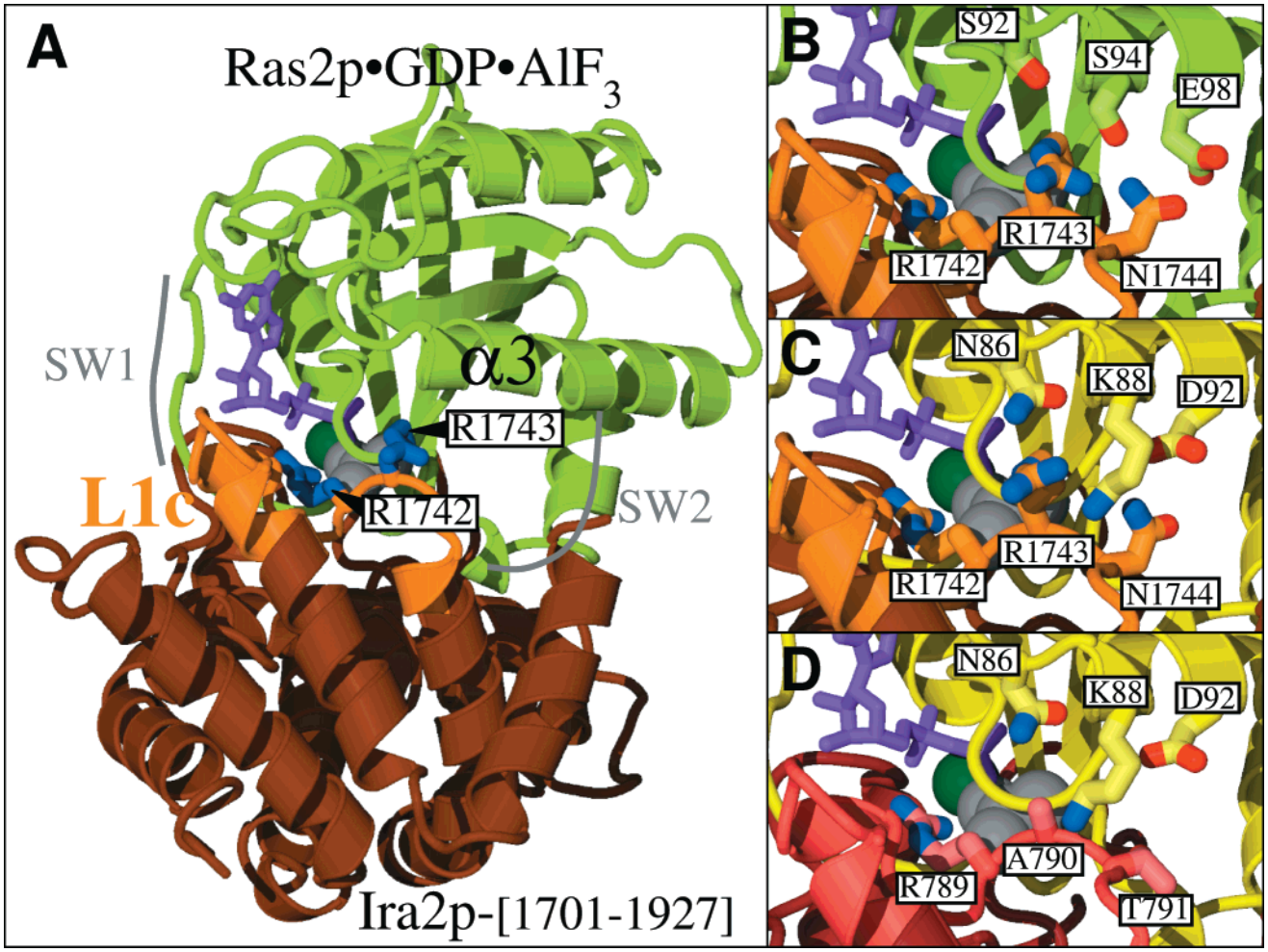

FIGURE 4: Theoretical structural models of the contacts between Ras2p or Ha-Ras and Ira2p or p120-GAP. Panel A: Putative structural model of an Ira2p-383·GDP·AlF_3·Ras2p complex generated automatically by the SWISS-MODEL homology modeling server (see Experimental Procedures). GDP is shown in dark blue, magnesium in dark green, and AlF_3 in dark gray. The L1c region into which substitutions were made in this work is highlighted in orange, and the "arginine finger" R1742 and the adjacent arginine R1743 are shown in blue. SW1 and SW2 indicate the "switch 1" and "switch 2" regions of Ras2p, respectively, while the indicated helix α3 was shown previously to be involved in specificity of the interaction between Ira2p and Ras2p GTPase (*2*, *8*). Panels B−D: Close-ups of different (theoretical) complexes illustrating putative contacts between the L1c region of Ira2p or p120-GAP and the L6-α3 region of Ras, that possibly contribute to specificity. The view orientation is the same as in panel A. Selected residues are shown as tube structures with nitrogen atoms in blue and oxygens in red. Panel B, Ira2p (brown/orange) with Ras2p (green); panel C, Ira2p (brown/orange) with Ha-Ras (yellow); panel D, p120-GAP (dark red/pink) with Ha-Ras (yellow). Note that for the combination of Ira2p and Ha-Ras, that does not interact productively, a clash occurs between side chains R1743−N1744 of Ira2p and N86/K88 of Ha-Ras-p21. Substitution of these residues by those of RasGAP (shown in panel D) enables activation of Ha-Ras by Ira2p-383, though not a complete switch of specificity (see Table 1A). The figure was prepared using Molscript (*24*) and Raster3D (*25*).

in the transition state of hydrolysis, thus providing catalytic action. Noteworthy, in other GTPases, the interaction with GAP proteins can take place differently from the above models. For instance, in the RabGAP·Rab complex, non-charged amino acids (two serines) directly interact with the γ-phosphate of GMPPNP (*20*). In the ArfGAP·Arf complex, the regulator interacts with a domain of the GTPase other than the substrate binding pocket, apparently acting via an allosteric long-range mechanism (*21*), while RGS4 also stimulates the Gα GTPase indirectly by changing the conformation of Gα (*22*). We show in this work that substitution in Ira2p of Arg1742, the putative Ira2p arginine finger (as based on sequence homologies with p120-GAP and neurofibromin), eliminates nearly any catalytic activity, similarly to p120-GAP and neurofibromin (*13*).

It is important to emphasize that the catalytic domains of the various GAP proteins are conserved within their respective families. Despite that, *S. cerevisiae* Ira1p and Ira2p display a strict specificity for the homologous yeast Ras GTPase and fail to activate the GTPase activity of Ha-Ras. In contrast, human p120-GAP, neurofibromin, and other GAPs, such as GAP1m from rat (*23*), are active on both mammalian Ha-Ras p21 and yeast Ras2p. On these two Ras proteins, the major determinant for the specific activation by GAP and Ira2p was found to be located in a Ras2p region of higher sequence variability than the neighboring regions (identities: ∼20% vs ∼45%), including residues 89−108 (82−101 in Ha-Ras) that comprise the C-terminal part of strand β4, loop L6, and the N-terminal moiety of helix α3. Of these, the N-terminal portion of helix α3 constitutes the most important element (*2*, *8*).

In this work we observed that the arginine finger regions of Ira2p and p120-GAP are involved in the ability of GAP to recognize Ras proteins. The results obtained by substituting the residue flanking C-terminally the arginine finger of Ira2p, and to a minor extent that of p120-GAP, already

suggested this possibility. Indeed, substitution of R1743 in Ira2p-383 with alanine, the homologous residue of p120-GAP, or glycine, the homologous residue in neurofibromin, influenced the specificity toward Ha-Ras and Ras2p GTPases. Both Ira2p-383 mutants acquire the novel property to enhance somewhat the GTPase activity of Ha-Ras. This result was supported by the effect of the reciprocal substitution in p120-GAP, where GAP-350[A790R] displayed a twice lower activity on Ha-Ras than on Ras2p, whereas the activity of wild-type GAP-350 on both Ras proteins is nearly the same. On the other hand, Ira2p-383[R1743A] stimulates the GTPase activity of Ras2p as efficiently as wild-type Ira2p-383, whereas in p120-GAP the reciprocal substitution reduces 6 times its activity on Ha-Ras. This indicates that the structural context of these residues has an influence on the stimulation of the GTPase activity of Ras.

More extensive substitutions show the importance of L1c in the recognition of Ras proteins. In Ira2p, substitution with the corresponding 12 residues of p120-GAP (Ira2p-383/GAP-350[781−792]) increased another 2 times the activity on Ha-Ras p21, compared to the single substitution R1743A as well as R1743G. Interestingly, introduction of only the tip of GAP-350 Lc1 comprising six residues (Ira2p-383/GAP-350-[787−792]) entails a better activation of Ha-Ras (2 times) than the substitution of a larger segment, while its action on Ras2p is also comparably less affected. That in both cases the effects are associated with a strong decrease in the stimulation of the GTPase activity of Ras (>100 times) shows that a correct conformation of the whole L1c region is essential for GAP activity on Ras. A similar effect also resulted from substitution of the Ira2p finger loop with the corresponding sequence of neurofibromin (experiment not illustrated).

Most interestingly, the introduction into p120-GAP of a L1c segment corresponding to nearly the entire finger loop (GAP-350/Ira2p-383[1733−1746]) caused abolition of the effect on the Ha-Ras GTPase, whereas that on the Ras2p GTPase remained considerable. This construct can be considered to represent an Ira2p-like p120-GAP with respect to its specificity toward Ras proteins. Taken together with the results of Parrini et al. (*2*), our observations strongly suggest that the arginine loop interacts directly with helix α3 of Ras, even though crystallographic studies could not precisely define the interaction of the helix α3 residue lysine 88 with the finger loop (*9*).

To rationalize our observations, we constructed theoretical models of various complexes by homology modeling, based on the structure of the p120-GAP·Ha-Ras complex (Figure 4A−D). Panels A and B show an overview and a close-up, respectively, of the putative structure of the native homologous complex Ira2p·Ras2p, while panel C shows that of the unproductive heterologous complex Ira2p·Ha-Ras. In this case, a clash between the bulky side chains of R1743−N1744 of Ira2p and N86/K88 of Ha-Ras-p21 occurs. In contrast, this collision can be avoided by the substitution of these two residues for those of RasGAP (A790 and T791, cf. panel D), a construct which enables activation of Ha-Ras-p21 by Ira2p-383, though not a complete switch of specificity, as shown in this work (cf. Table 1A). Even though at present these models are speculative, they suggest a structural rationale that can in part explain the strong specificity of Ira2p toward the homologous Ras protein.

Taken together, the results of this work point to an important role of the L1c region in determining the specificity of GAPs. From modeling data, this region was found to interact mainly with the Switch 1 and 2 regions of Ha-Ras (*9*). On the other hand, our experimental data clearly show that the full specificity involves more than the arginine loop residues and strongly suggest that other regions of GAP are contributing directly or indirectly to the recognition of Ras proteins.

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