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Residues crucial for Ras interaction with GDP–GTP exchangers

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ABSTRACT **Cdc25 is essential for Ras-mediated activation of adenylyl cyclase in the yeast *Saccharomyces cerevisiae*. This protein acts by catalyzing GDP–GTP exchange on yeast Ras. Harvey (Ha) *ras* expressed in *S. cerevisiae* is also recognized by both Cdc25 and Sdc25, a yeast homolog of Cdc25. Thus it is feasible to examine molecular aspects of mammalian Ras modulation by Cdc25 using the RAS/cAMP pathway in yeast as a model system. Here, we describe mutational analysis of Ha-*ras* for the identification of residues critical for the ability of Ras to interact with Cdc25 and related guanine nucleotide-release proteins. Mutations within codons 97–108 impaired Ras-mediated activation of adenylyl cyclase in the presence but not in the absence of mammalian GTPase-activating protein. Such mutations, therefore, affected the ability of Ras to undergo GDP–GTP exchange catalyzed by the guanine nucleotide exchanger without preventing Ras activation of the effector. Similar mutations were previously shown to impair the ability of c-*ras* to transform mammalian cells while having a less drastic effect on v-*ras*.**

Mammalian *ras* protooncogenes (c-*ras*) (see refs. 1 and 2) encode 21-kDa proteins ($p21^{ras}$) which bind guanine nucleotides and exhibit weak GTPase activity. Ras cycles between an active, GTP-bound and an inactive, GDP-bound form. The ratio of active to inactive forms *in vivo* is modulated by the combined action of guanine nucleotide release proteins (GNRPs), which catalyze GDP–GTP exchange generating the active GTP-bound form, and GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase and thus promote generation of the inactive GDP-bound form (3).

The yeast *Saccharomyces cerevisiae* provides a unique setup for studying Ras modulation. Two homologs of mammalian $p21^{ras}$, Ras1 and Ras2, have been identified in this organism (4). Yeast Ras is essential for guanine nucleotide-dependent activation of adenylyl cyclase and for cell viability (5, 6). So far, this is the only case in which an effector of Ras, the yeast adenylyl cyclase, has been assigned. Yeast Ras is modulated by Cdc25, which is a GNRP, and Ira1 and Ira2, which act as GAPs (7).

Biochemical assessment of Ras function is possible in yeast by measuring adenylyl cyclase activity. Yeast Ras can be replaced by mammalian Ras as the cyclase activator (8, 9). Further, a quantitative correlation between the transforming potential of $p21^{ras}$ mutants and their ability to activate yeast cyclase has been found (10). Thus, the RAS/cAMP pathway in *S. cerevisiae* serves as a model for studying $p21^{ras}$ function and interaction with its modulators.

In vitro nucleotide exchange assays showed that the yeast protein Sdc25, structurally and functionally related to Cdc25, promotes GDP–GTP exchange on $p21^{ras}$ when expressed as a truncated protein (11). Mutational analysis was applied to assign residues in Ras required for the protein to undergo Sdc25-catalyzed guanine nucleotide exchange *in vitro* (12, 13). Mutations within the switch I (12) and II (12, 13) regions strongly impaired GNRP-dependent exchange. This effect was not due to a decrease in the affinity of the Sdc25 C terminus toward the mutated p21 (12).

GNRPs, which exhibit significant homology within their catalytic domains, may promote GDP–GTP exchange by interacting with $p21^{ras}$ at a distal site and causing dissociation of the guanine nucleotide bound to Ras *in vivo*. The substrate for this reaction is GDP-bound Ras (3).

Willumsen *et al.* (18) reported mutations which impair the ability of c-*ras* to cause transformation but have less effect on the biological function of the oncogenic version, v-*ras*. It was suggested that such mutations might affect the interaction between $p21^{ras}$ and a GNRP, a function required in c-*ras*- but not in v-*ras*-encoded proteins for activity *in vivo*.

We have shown (19) that Cdc25 interacts with $p21^{Ha\text{-}ras}$ expressed in yeast along with mammalian GAP, further supporting the relevance of the yeast system in studying Ras modulation. We have continued to study this system to characterize mutations which might impair $p21^{ras}$ interaction with Cdc25 or related GNRPs without affecting the ability of $p21^{ras}$ to activate adenylyl cyclase. In agreement with Willumsen *et al.* (18), our results point to amino acids 97–108 as crucial for $p21^{Ha\text{-}ras}$ interaction with GNRPs.

MATERIALS AND METHODS

Yeast Strains. Strain MDS-1T (*MATα cdc25-2 ura3-52 trp1 leu2-3,112 ade2 his3Δ-200 ras1::URA3 ras2::LEU2* [pTPK1]) was derived from strain MDS-1 (19). The temperature-sensitive cdc25 (*cdc25^{ts}*) strain was MS-LL1 (*MATa cdc25-2 ura3-52 lys2 leu2-3,112 trp1 his3Δ-200 ade2*). Strains MS14-4T (*MATa cdc25Δ-1000 ura3-52 trp1 leu2-3,112 ade2 his3Δ-200 ras1::URA3 ras2::LEU2* [pTPK1]) and MS14-4HT (*MATa cdc25Δ-1000 ura3-52 trp1 leu2-3,112 ade2 his3Δ-200 ras1::URA3 ras2::HIS3* [pTPK1-2]) are isogenic derivatives of strain MS14-4 (19). For construction of isogenic *cdc25^{ts} ras1$^-$ ras2$^-$* strains expressing each of the c-Ha-*ras* mutants, strain MDS-1T was transformed with each c-Ha-*ras*-containing plasmid. The resulting Trp^+ transformants were grown without selection to saturation and plated on SD plates without tryptophan. Colonies were replica-plated onto SD plates without histidine and YPD plates. Trp^+ His^- colonies were picked. A similar procedure was followed with strains MS14-4T and MS14-4HT.

Yeast Techniques. Standard techniques were used (20). SD is a synthetic minimal medium (0.67% yeast nitrogen base without amino acids, with 2% glucose) supplemented with auxotrophic requirements. YPD (1% yeast extract/2% peptone/2% glucose) is a rich medium for nonselective growth.

Plasmids. pGAP is a multicopy plasmid expressing the catalytic domain of bovine GAP, and pM1 is the control vector resulting from deletion of GAP sequences in pGAP (19). pCDC25-2μ, pSDC25-2μ, and pTPK1 have been described (19). pCDC25-2 carries the 5.4-kb *Sal* I–*Pvu* II

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Abbreviations: GNRP, guanine nucleotide-release protein; GAP, GTPase-activating protein; GTP[β,γ-NH], guanosine 5′-[β,γ-imido]-triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate.

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fragment of *CDC25* in YEp21 (21). pCDC25^{Mm} was constructed by cloning the 1.3-kb *Xho* I fragment from plasmid pCDC25/12 (14), encoding a truncated *Mus musculus* Cdc25 protein (Cdc25^{Mm}), into the *Sal* I site of pAD$^4\Delta$ (22). pCDC25^{Mm}-TPK1 contains, in addition, the 3-kb *Bam*HI–*Sph* I fragment of *TPK1*. pTPK1-2 contains the *Bam*HI–*Sph* I fragment of *TPK1* in YEp21. Plasmid pCDC25-C was constructed by cloning the 2.6-kb *Bgl* II–*Pvu* II fragment encoding the C terminus of Cdc25 in pRS413 (23). pBW2396 was used for expression of wild-type and mutant c-Ha-*ras* cDNAs. This vector was derived from pYGA-Ras (24) by replacing Ha-*ras* sequences by a short oligonucleotide which introduced *Xho* I and *Eco*RI sites for cloning of the various c-Ha-*ras* mutants. The resulting plasmid expressed each cDNA under control of the yeast *PGK1* promoter followed by the *TRP1* terminator. The wild-type c-Ha-*ras* and in-frame deletion mutants were derived as described (18). Mutations are referred to in the text by the amino acid residues that have been deleted.

Membrane Preparation and Adenylyl Cyclase Assay. Membrane fractions were prepared and adenylyl cyclase (19) and protein (25) were assayed. When indicated, membranes were preincubated with 500 μM guanosine 5′-[β,γ-imido]-triphosphate (GTP[$\beta\gamma$-NH]) in the absence of $MgCl_2$ for 10 min at 4°C followed by 30 min at 25°C before dilution with the assay components. These conditions facilitate noncatalyzed (Cdc25-independent) guanine nucleotide exchange on Ras (26).

RESULTS

Genetic Analysis. *Interaction between p21^{ras} mutants and Cdc25.* Activation of adenylyl cyclase by wild-type p21$^{Ha\text{-}ras}$ expressed in yeast is independent of Cdc25 (19), since p21$^{Ha\text{-}ras}$ is not recognized by Ira1 and Ira2 (27) and, therefore, remains bound to GTP. However, upon transformation with pGAP, p21^{ras} is converted to its GDP-bound form and activation becomes strictly dependent on Cdc25 (19).

Therefore, mutations which impair the ability of p21$^{Ha\text{-}ras}$ to activate adenylyl cyclase in the presence, but not in the absence, of mammalian GAP in a *ras1*$^-$ *ras2*$^-$ strain are expected to reveal residues important for the ability of c-Ha-*ras* product to undergo Cdc25-dependent GDP–GTP exchange. Such mutations should not prevent rescue of a *cdc25^{ts}* mutation in an otherwise wild-type strain since the effector function of Ras—namely, activation of adenylyl cyclase—is not directly affected. For this reason, c-Ha-*ras* mutants were first tested for their ability to suppress a *cdc25^{ts}* mutation. Mutants which met this condition were further tested for rescue of a *cdc25^{ts} ras1*$^-$ *ras2*$^-$ strain in the presence of mammalian GAP.

Deletion/insertion mutants of c-Ha-*ras* expressed under control of the *PGK1* promoter were constructed and their ability to suppress a *cdc25^{ts}* mutation was determined (Table 1). pBW2419, encoding p21^{ras} mutated at positions 64–70, did not suppress the *cdc25-2^{ts}* mutation. pBW2430 (75–76 mutant) barely rescued the *cdc25^{ts}* mutation: only after prolonged incubation at 37°C (4 days) did patches grow uniformly above the level of the negative control (data not shown). In addition, two mutants in the variable region, encoded by pBW2424 and pBW2417 (165–180 and 165–184 mutants, respectively), failed to rescue the *cdc25^{ts}* mutation. These two mutant proteins were probably unstable, since they could not be detected by Western blot analysis using Y13-259 monoclonal antibody (data not shown).

c-Ha-*ras* mutants able to suppress the *cdc25^{ts}* mutation were further checked for their ability to rescue a *cdc25^{ts} ras1*$^-$ *ras2*$^-$ strain in the presence of mammalian GAP.

A series of isogenic strains carrying the *cdc25-2* allele, deleted of yeast *RAS* genes, and carrying each of the c-Ha-*ras* mutants was constructed and used for transformation with either pGAP or the control vector pM1 (Table 2).

Table 1. Ability of c-Ha-*ras* deletion mutants to suppress *cdc25^{ts}*

Plasmid	Structure*	Deletion	Rescue of *cdc25^{ts}*†
pBW2414	Full length, c-*ras*	None	+
pBW2427	Full length, v-*ras*‡	None	+
pBW2419	63SDQ71	64–70	−
pBW2430	74LIR77	75–76	±
pBW2421	92LIR96	93–95	+
pBW2444	96LIR104	97–103	+
pBW2418	100LIR104	101–103	+
pBW2431	101PDQ109	102–108	+
pBW2428	106ADQ109	107–108	+
pBW2423	123LIR130	124–129	+
pBW2420	128PDQ139	129–138	+
pBW2424	165PDQ180	166–179	−
pBW2417	165PDQ184	166–183	−

*Gene deletions are named according to the codons flanking the deletion site; letters indicate the three amino acids encoded by the oligonucleotide linker.

†Strain MS-LL1 was transformed with each c-*ras*-containing plasmid. One hundred independent transformants were patched onto selective plates, incubated at 23°C for 2 days, and then replica plated onto two YPD plates. Growth at 37°C or 23°C was scored after 2 days. +, >95 patches grew at 37°C; ±, patches grew uniformly after 4 days at 37°C; −, no patches grew at 37°C.

‡v-*ras* contains $Gly^{12} \rightarrow Arg$ and $Ala^{59} \rightarrow Thr$ mutations.

Strains expressing p21^{ras} mutants still able to interact with Cdc25 would undergo transformation efficiently with pGAP and become temperature sensitive. Temperature sensitivity confirms that, in the presence of GAP, p21$^{Ha\text{-}ras}$ activation of adenylyl cyclase is strictly dependent on Cdc25 (19).

The v-Ha-*ras*-encoded protein exhibits a reduced intrinsic GTPase which is insensitive to stimulation by GAP (28). Consistent with this fact, strain MS-BW2427 underwent transformation with pGAP efficiently but did not yield temperature-sensitive transformants. Strain MS-BW2431 (102–108 mutant) did not give transformants with pGAP unless pTPK1 was also present. The latter is a multicopy plasmid encoding the catalytic subunit of the cAMP-dependent protein kinase and, therefore, overrides the requirement of cAMP production for viability. In the absence of pTPK1, cells underwent a few divisions and were observed as microcolonies under a binocular microscope. Strain MS-BW2444 (97–103 mutant) underwent transformation with

Table 2. Ability of c-Ha-*ras* mutants to rescue *cdc25-2 ras1*$^-$ *ras2*$^-$ strains expressing GAP

Strain	Deletion	Transformation with pGAP*	Temperature sensitive†
MS-BW2414	None	+	Yes
MS-BW2427	None	+	No
MS-BW2421	93–95	+	Yes
MS-BW2444	97–103	+‡	Yes
MS-BW2418	101–103	+	Yes
MS-BW2431	102–108	−	—
MS-BW2428	107–108	+	Yes
MS-BW2423	124–129	+	Yes
MS-BW2420	129–138	+	Yes

*LiOAc-treated cells were transformed in parallel with either pGAP or pM1. +, Transformants were obtained with both plasmids with similar efficiencies (≈1000 colonies/μg of DNA); −, no transformants were obtained with pGAP whereas efficient transformation was observed with pM1.

†Fifty independent transformants were patched onto selective plates, incubated for 2 days at 23°C, and then replica plated in duplicate onto similar plates for incubation at either 37°C or 23°C. Growth was scored after 2 days. Transformants with pM1 were not temperature sensitive for all strains tested.

‡Small colonies were obtained upon transformation with pGAP.

high efficiency; however, tiny colonies were obtained (data not shown) which were temperature sensitive. This mutant was previously shown to exhibit reduced sensitivity to GAP *in vitro* (28). However, the temperature-sensitive phenotype observed implied that this mutant was responsive to GAP when expressed in yeast and that activation of adenylyl cyclase was still dependent on Cdc25.

The data pointed to residues 102–108 as critical for the interaction between Cdc25 and $p21^{ras}$.

Interaction of $p21^{ras}$ mutants with Sdc25 and $Cdc25^{Mm}$. To check whether mutations affecting Ras–Cdc25 interaction also impaired activation by other Ras GNRPs, a set of isogenic strains with a deletion in the *CDC25* locus, lacking *RAS* genes, and expressing each of the mutant c-Ha-*ras* genes was constructed and transformed with either pSDC25-2μ or $pCDC25^{Mm}$. The former encodes the C terminus of the yeast Sdc25 and the latter, the C terminus of the mouse homolog $Cdc25^{Mm}$. Both truncations were previously shown to suppress a $cdc25^{ts}$ mutation (14, 29). Since both plasmids encoded truncated proteins including the catalytic domain of the GNRP, plasmid pCDC25-C was also used to test a truncated Cdc25 under similar conditions. The resulting strains were then transformed with either pGAP or pM1 (Table 3).

Cells carrying pCDC25-2μ failed to undergo transformation with pGAP when carrying the 102–108 mutant. Although *CDC25* was present on a multicopy plasmid, results were similar to those in the background of the $cdc25^{ts}$ allele (Table 2). The truncated *CDC25* rendered comparable results.

In the case of yeast carrying pSDC25-2μ, transformation with pGAP was not obtained in the presence of the 102–108 mutant and, in addition, transformation with reduced efficiency occurred in the case of the 101–103 mutant. Strains carrying $CDC25^{Mm}$ in the presence of the 102–108 and 101–103 mutants did not give rise to colonies when transformed with pGAP. Yet the same strains underwent efficient transformation with pGAP when *TPK1* was also present.

These results suggested that the region around 101–103 might be important for the interaction of GNRPs and $p21^{ras}$. Yet differential ability of Ras proteins with mutations in this region to interact with each GNRP was observed.

Biochemical Analysis. Adenylyl cyclase was measured in membranes from strain MDS-1T ($cdc25^{ts}$ $ras1^-$ $ras2^-$ [pTPK1]) carrying the various c-Ha-*ras* mutants and, in addition, either pGAP or the control pM1. The presence of *TPK1* in high dosage ensured viability of strains expressing Ras mutants unable to interact with Cdc25.

The profiles of adenylyl cyclase activity in membranes from MS-BW2414[pTPK1] (c-Ha-*ras*) and MS-BW2431-[pTPK1] (102–108 mutant) are shown in Fig. 1.

Strains carrying the control vector pM1 exhibited similar levels of Mg^{2+}-, Mg^{2+}·GTP[$\beta\gamma$-NH]-, and Mg^{2+}·GDP[βS]-dependent activities. Under these conditions, even inhibition of cyclase activity by Mg^{2+}·GDP[βS] does not occur (19) since it is likely that GTP-bound Ras cannot be a substrate for the catalyzed exchange reaction. The profiles obtained correlated with the ability of pBW2414 and pBW2431 to suppress the $cdc25\text{-}2^{ts}$ mutation (Table 1).

In membranes from MS-BW2414[pTPK1], cyclase activity was stimulated by Mg^{2+}·GTP[$\beta\gamma$-NH] and inhibited by Mg^{2+}·GDP[βS], confirming sensitivity to GAP and responsiveness to Cdc25 (Fig. 1). In membranes from MS-BW2431[pTPK1] carrying pGAP, guanine nucleotide response was not detected, and the Mg^{2+}·GTP[$\beta\gamma$-NH]-dependent activity was very low (Fig. 1). This pattern resembled the one obtained with a $cdc25\Delta$ $ras1^-$ $ras2^-$ [pGAP pTPK1] strain expressing wild-type c-Ha-*ras* (19).

To further confirm that coupling between this $p21^{ras}$ mutant and adenylyl cyclase was not affected by GAP, cyclase activity was measured following preincubation of the membranes with GTP[$\beta\gamma$-NH] in the absence of Mg^{2+}, to favor Cdc25-independent guanine nucleotide exchange (26). A 10-fold stimulation was observed over the activity measured without preincubation (Fig. 2), indicating that the ability of this $p21^{ras}$ mutant to interact with and activate adenylyl cyclase in the presence of GAP was retained.

Adenylyl cyclase activity was also measured in membranes from strains MS-BW2444[pTPK1] and MS-BW2418[pTPK1] (97–103 and 101–103 mutants, respectively). Consistent with the genetic analysis, the mutants carried by these strains were less impaired in their interaction with Cdc25 than the 102–108 mutant, as reflected by the ratio of Mg^{2+}·GTP[$\beta\gamma$-NH]- and Mg^{2+}·GDP[βS]-dependent activities (Fig. 3).

The $p21^{ras}$ mutants were also evaluated biochemically in strain MS14-4TC ($cdc25\Delta$ *ras1 ras2* [$pCDC25^{Mm}$-TPK1]) expressing GAP. In agreement with the genetic analysis, impaired guanine nucleotide response was observed with the 102–108 and 101–103 mutants, as shown by the ratio of

Table 3. Ability of Ha-Ras mutants to interact with GNRPs

Mutant		Transformation with pGAP			
			C terminus		
Plasmid	Deletion	Cdc25	Cdc25	Sdc25	$Cdc25^{Mm}$
pBW2414	None (c-*ras*)	+	+	+	+
pBW2427	None (v-*ras*)	+	+	+	+
pBW2421	93–95	+	+	+	+
pBW2444*	97–103	+	+	+	+
pBW2418	101–103	+	+	±†	−
pBW2431	102–108	−	−	−	−
pBW2428	107–108	+	+	+	+
pBW2423	124–129	+	+	+	+
pBW2420	129–138	+	+	+	+

LiOAc-treated cells of strain $cdc25\Delta$ *ras1 ras2*::*LEU2* carrying pCDC25-2μ (Cdc25), pSDC25-2μ (C terminus of Sdc25), or pCDC25-C (C terminus of Cdc25) and of strain $cdc25\Delta$ *ras1 ras2*::*HIS3* carrying either pCDC25-2 (Cdc25) or $pCDC25^{Mm}$ (C terminus of mouse Cdc25) were transformed in parallel with pGAP or pM1. Transformation was scored as in Table 2.

*Small colonies were obtained in the presence of this mutant.

†Efficiency of transformation with pGAP was 30% of that with pM1.

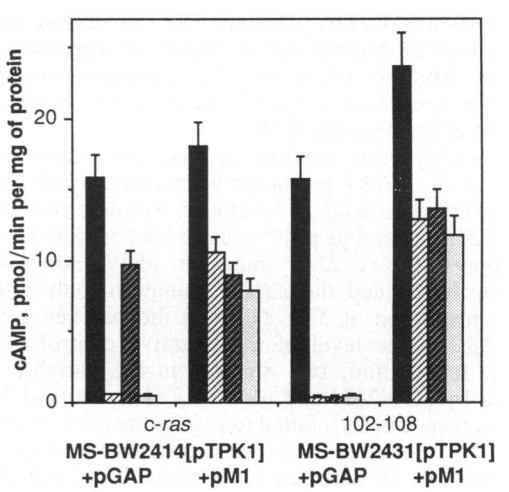

FIG. 1. Adenylyl cyclase activity in cells carrying c-*ras* or 102–108 mutant. Activity was measured in membranes from MS-BW2414[pTPK1] or MS-BW2431[pTPK1] cells carrying either pGAP or the control pM1. Divalent cations and guanine nucleotide analogs were as follows: 2.5 mM Mn^{2+} (■), 10 mM Mg^{2+} (▨), 100 μM GTP[$\beta\gamma$-NH] (▨), or 100 μM guanosine 5′-[β-thio]triphosphate (GDP[βS]) (□). Results are the mean of at least three independent measurements from two preparations.

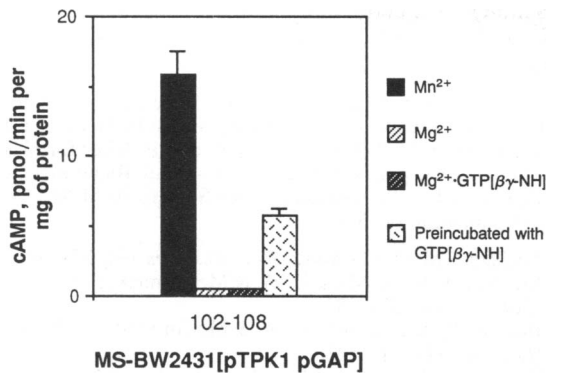

FIG. 2. Coupling of p21^{ras} 102–108 mutant and adenylyl cyclase in the presence of GAP. Cyclase activity was measured in membranes from MS-BW2431[pTPK1] carrying pGAP, as in Fig. 1. Membranes were preincubated with 500 μM GTP[βγ-NH].

Mg^{2+}·GTP[βγ-NH] to Mg^{2+}·GDP[βS] activities (Fig. 4). These two mutants mediated high constitutive activation of adenylyl cyclase in the absence of GAP (data not shown), indicating unimpaired ability to activate the effector.

DISCUSSION

Catalyzed GDP–GTP exchange on Ras might occur by the GNRP interacting with regions distal to the guanine nucleotide binding site, bringing about a decrease in the affinity toward the bound nucleotide. The use of the yeast system aided in the identification of residues of p21^{ras} which might be critical for such function. Substitutions in these residues did not affect drastically the ability of p21^{ras} to bind GTP *in vivo* and adopt the conformation essential to activate Ras effector.

Our data pointed to amino acids 97–108 as crucial for the ability of p21$^{Ha\text{-}ras}$ to undergo Cdc25-dependent guanine nucleotide exchange.

First, the various c-Ha-*ras* mutants were checked for their ability to suppress a *cdc25-2^{ts}* mutation in an otherwise wild-type strain (Table 1). This step enabled us to rule out mutants unable to activate adenylyl cyclase when expressed in yeast. Yet it remains possible that such mutations might affect the ability of Ras to interact with the GNRP as well.

Mutants which suppressed the *cdc25-2* allele were then tested for their ability to rescue a *cdc25^{ts} ras1^{-} ras2^{-}* strain when pGAP was present and, in addition, to confer a temperature-sensitive phenotype in such a background (Table 2). c-Ha-*ras* mutated at positions 102–108 did not suppress the *ras1^{-} ras2^{-}* double mutant in the presence of GAP. Guanine nucleotide-dependent activation of adenylyl cyclase was not observed in membranes from a *cdc25^{ts} ras1^{-} ras2^{-}* [pTPK1] strain carrying this mutant c-Ha-*ras* and pGAP (Fig. 1). However, under conditions which allowed Cdc25-independent GDP–GTP exchange *in vitro*, coupling of this mutant Ras and adenylyl cyclase was confirmed (Fig. 2). Two other mutants, 101–103 and 97–103, were partially impaired in their interaction with Cdc25 (Table 2 and Fig. 3).

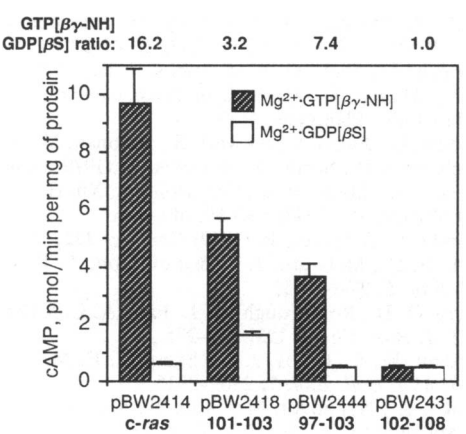

FIG. 3. Guanine nucleotide modulation of adenylyl cyclase mediated by yeast Cdc25 and p21^{ras} mutants. Cyclase was measured in membranes from MS-BW2414, MS-BW2418, MS-BW2444, and MS-BW2431 carrying pTPK1 and pGAP. Mn^{2+}-dependent activity (pmol of cAMP/min per mg of protein) was 16.5 in cells carrying c-*ras*, 17.2 in cells with the 101–103 mutant, 17.0 in cells with the 97–103 mutant, and 18.1 in cells with the 102–108 mutant.

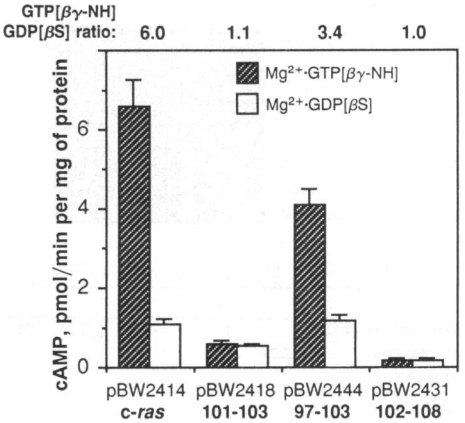

FIG. 4. Cdc25^{Mm}-dependent modulation of the various p21^{ras} mutants. Adenylyl cyclase activity was measured in MS14-4HT cells in which pTPK1-2 was replaced by pCDC25^{Mm}-TPK1, carrying pGAP and one of the following plasmids: pBW2414, pBW2418, pBW2444, or pBW2431. Mn^{2+}-dependent activity (pmol of cAMP/min per mg of protein) was 21.2 in cells carrying c-*ras*, 19.0 in the presence of the 101–103 mutant, 18.8 in cells carrying the 97–103 mutant and 19.2 in cells having the 102–108 mutant.

The behavior of the various Ras mutants toward the yeast Sdc25 and the mouse Cdc25^{Mm} protein was also tested in a strain having, in addition, a deleted *CDC25* locus. Both GNRPs proved unable to interact with the 102–108 mutant. The 101–103 mutant also failed to interact with Cdc25^{Mm} but still interacted with Sdc25 (Table 3 and Fig. 4). A truncated Cdc25 acted essentially like the full-length protein, indicating that the differential behavior of Sdc25 and Cdc25^{Mm} could not be attributed solely to their expression as truncated proteins.

Verrotti *et al.* (13) suggested that residues 80–83 of Ras2 (corresponding to 73–76 in p21$^{Ha\text{-}ras}$) may play a critical role in the response of Ras to a GNRP. Although mutations at these positions impaired Sdc25-stimulated GDP release *in vitro*, it was also reported that they lowered the affinity toward GTP[βγ-NH] (31), suggesting a more generalized conformational impairment. In contrast to their findings, we observed that deletion/insertions at positions 63–71 and 74–77 impaired the ability of the resulting c-Ha-*ras*-encoded mutants to activate adenylyl cyclase and rescue a *cdc25^{ts}* mutation (Table 1). Furthermore, analogous mutations in v-Ha-*ras* resulted in lower transformation efficiency of mammalian cells (unpublished results). These mutations lie within one of the two regions known to undergo major conformational changes in wild-type Ras upon binding of GTP (32). Most likely, these mutations affect the conformational switch of Ras and, indirectly, its ability to undergo catalyzed exchange and to efficiently activate the effector. However, Sigal *et al.* (10) reported other mutations within this region which did not impair v-Ha-*ras* biological activity. Gross *et al.* (33) have shown that Cdc25 and Ras proteins can be coimmunoprecipitated with Y13-259 antibodies. Since these antibodies recognize the 60–76 region of Ras, it is unlikely that this region interacts with Cdc25.

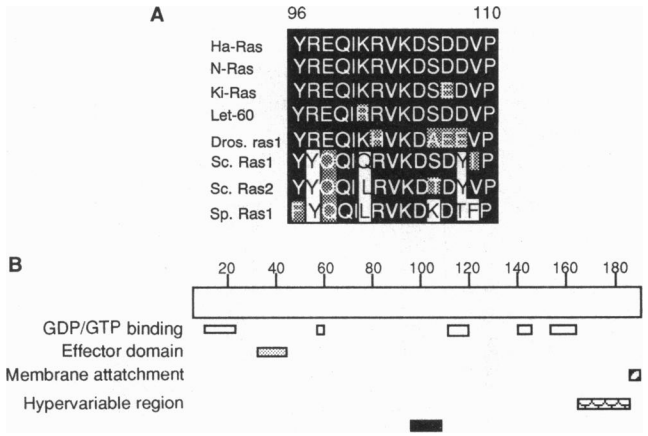

FIG. 5. (*A*) Comparison of Ras proteins at positions 96–110. Ha-Ras, Ki-Ras, and N-Ras are mammalian; Let-60 is from *Caenorhabditis elegans*; Dros. ras1 is from *D. melanogaster*; Sc. Ras1 and Sc. Ras2 are from *S. cerevisiae*; Sp. Ras1 is from *Schizosaccharomyces pombe*. (*B*) Domains of mammalian p21^{ras} proteins. Black box, region important for interaction with GNRP.

That the 102–108 mutant was equally impaired in Cdc25-, Sdc25-, or Cdc25^{Mm}-dependent GDP–GTP exchange indicates that proteins of the Cdc25 family interact with p21$^{Ha\text{-}ras}$ by a similar mechanism. However, less-drastic mutants displayed a differential behavior toward various GNRPs (Table 3), suggesting that Ras–GNRP interactions are specific.

Downward *et al.* (34) reported the effect of mutations on the ability of p21^{ras} to respond to a nucleotide-exchange activity isolated from human placenta. In agreement with our results, the 102–108 mutant was drastically impaired in stimulated exchange, though the same mutant also exhibited a reduced sensitivity to GAP *in vitro*. In our system, GAP downregulation of this mutant was comparable to that of wild-type p21^{ras} (Fig. 1). In ref. 34, however, the 101–103 and 97–103 mutants were not significantly impaired in the interaction with the putative exchanger.

Willumsen *et al.* (18) applied mutational analysis to identify residues required in c-Ha-*ras*-encoded proteins but dispensable in v-Ha-*ras*-encoded proteins for biological activity. Such residues would play a role in the interaction with a GNRP. In these studies, both the 101–103 and the 102–108 mutant showed reduced transformation efficiencies in the c-Ha-*ras* form. Although the 101–103 mutant proved mildly impaired in its interaction with Cdc25 in our system (Table 2 and Fig. 3), the substitution in this mutant (KRV to LIR at 101–103) was more related to yeast Ras (1LRV) than to mammalian Ras. For this reason, it is striking that this mutant was more impaired in its interaction with Cdc25^{Mm} (Table 3 and Figs. 3 and 4). This same mutant was slightly less affected than the 102–108 mutant when tested for transformation of mammalian cells (18).

Mistou *et al.* (12) reported that mutations within the switch I and II regions (32) impaired the ability of p21$^{Ha\text{-}ras}$ to undergo Sdc25-stimulated GDP dissociation without affecting binding of the GNRP, suggesting that interaction between the two proteins occurs through other sequences of p21^{ras}. We suggest that the region 100–110, which is highly conserved among Ras proteins (Fig. 5), could be involved in the interaction with GNRPs. In addition, this region is exposed to solvent, according to the proposed structure for Ras (32).

The mutants analyzed in this study could be impaired in catalyzed GDP–GTP exchange by lack of interaction with the GNRP, either due to substitutions at the actual site of interaction or to a conformational change preventing interaction with the exchanger. However, if such a conformational change was induced, it did not affect other properties of Ras, including activation of adenylyl cyclase, response to GAP, and *in vitro* noncatalyzed guanine nucleotide exchange.

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