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Leucine-rich repeats and carboxyl terminus are required for interaction of yeast adenylate cyclase with RAS proteins

(oncogene/cAMP/guanine nucleotide-binding regulatory protein)

NOBORU SUZUKI*, HYE-RYUN CHOE†, YOSHIMITSU NISHIDA*, YURIKO YAMAWAKI-KATAOKA†, SHIN OHNISHI†, TATSUYA TAMAOKI†, AND TOHRU KATAOKA*†

*Department of Physiology, Kobe University School of Medicine, Kobe 650, Japan; and †Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115

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ABSTRACT **A *Saccharomyces cerevisiae* gene encoding adenylate cyclase has been analyzed by deletion and insertion mutagenesis to localize regions required for activation by the *Sa. cerevisiae* RAS2 protein. The NH_2-terminal 657 amino acids were found to be dispensable for the activation. However, almost all 2-amino acid insertions in the middle 600 residues comprising leucine-rich repeats and deletions in the COOH-terminal 66 residues completely abolished activation by the RAS2 protein, whereas insertion mutations in the other regions generally had no effect. Chimeric adenylate cyclases were constructed by swapping the upstream and downstream portions surrounding the catalytic domains between the *Sa. cerevisiae* and *Schizosaccharomyces pombe* adenylate cyclases and examined for activation by the RAS2 protein. We found that the fusion containing both the NH_2-terminal 1600 residues and the COOH-terminal 66 residues of the *Sa. cerevisiae* cyclase rendered the catalytic domain of the *Sc. pombe* cyclase, which otherwise did not respond to RAS proteins, activatable by the RAS2 protein. Thus the leucine-rich repeats and the COOH terminus of the *Sa. cerevisiae* adenylate cyclase appear to be required for interaction with RAS proteins.**

The *ras* oncogenes were identified as the transforming genes of retroviruses and the physiological function of ras proteins still remains to be elucidated (for review, see ref. 1). However, in the yeast *Saccharomyces cerevisiae*, RAS proteins are essential regulatory elements of adenylate cyclase (2). The *Sa. cerevisiae* adenylate cyclase has been shown to consist of 2026 amino acids that comprise at least four domains: the NH_2-terminal, the middle-repetitive, the catalytic, and the COOH-terminal domains (3, 4). The catalytic domain, located between amino acids 1609 and 1890, is self-sufficient for the Mn^{2+}-dependent adenylate cyclase activity, but the remaining portion appears to be required for activation of the Mg^{2+}-dependent activity by RAS proteins and GTP (3–6). The middle-repetitive region is composed of a repeated 23-amino acid leucine-rich motif, PXXαXXLX XLXXLXLXXNXαXXα (where α is an aliphatic amino acid and X is any amino acid). Mammalian ras proteins can activate the *Sa. cerevisiae* adenylate cyclase but neither the mammalian ras nor the yeast RAS proteins can regulate mammalian adenylate cyclase (7–9). Even in a lower eukaryote, such as the fission yeast *Schizosaccharomyces pombe*, adenylate cyclase appears not to be regulated by RAS proteins (4, 10). Although the precise mode of interaction between RAS proteins and the *Sa. cerevisiae* adenylate cyclase is unknown, regulatory proteins of adenylate cyclase appear to have changed during the evolution of *Sa. cerevisiae* and *Sc. pombe* and so has the nature of an effector molecule of RAS proteins. Based on a sequence comparison of adenylate cyclase from the two yeast species, we suggested that the *Sc. pombe* adenylate cyclase might have lost the ability to interact with RAS proteins by the loss of sequences required for the interaction (4). In this report, by using a mutational analysis, we identify two sequence elements in *Sa. cerevisiae* adenylate cyclase required for its interaction with RAS proteins.

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MATERIALS AND METHODS

Cell Strains, Growth Media, and Transformation. *Sa. cerevisiae* strains, TK34-1 and TK35-1, were constructed by disruption of the chromosomal *RAS2* gene of T50-3A (*MAT*α, *leu2*, *his3*, *trp1*, *ura3*, *cyr1-2*) (3) using p*ras2*::*URA3* or p*ras2*::*LEU2* (11), respectively, as described (11, 12). Culture media for yeast cells and the method of yeast transformation were described (2–4, 7, 13).

Deletion and Insertion Mutagenesis. A plasmid, *YEP24–ADC1–CYR1*, which overexpressed the complete *Sa. cerevisiae* adenylate cyclase gene (*CYR1*) from the yeast alcohol dehydrogenase I (*ADC1*) promoter (14), was constructed by transferring a *Sal* I–*Nhe* I fragment bearing the *ADC1–CYR1* of *YRp7–ADC1–CYR1* (4, 15) to *YEP24* (16). The plasmid was subjected to deletion mutagenesis using BAL-31 exonuclease (17) after cleavage with suitable restriction endonucleases. The range of deletions in each mutant was examined by nucleotide sequencing, and mutants with an in-frame deletion were used for the subsequent study. A *Mlu* I–*Bst*EII fragment of the *CYR1* corresponding to the amino acids 605–1666 was cloned into a *Mlu* I–*Acc* I fragment of a plasmid, pUCMlu, after cohesive termini of the *Bst*EII and *Acc* I sites were filled-in by the large fragment of *Escherichia coli* DNA polymerase I. pUCMlu had been constructed by inserting a *Mlu* I linker into a *Sma* I site of pUC8 (18). The resulting plasmid, pUC-CYR1MB, was subjected to insertional mutagenesis by using 6-base-pair (bp) synthetic oligonucleotides (19) (TAB linkers obtained from Pharmacia) after digestion with suitable restriction endonucleases (see *Results* for details). The *Mlu* I–*Bst*EII fragments of the pUC-CYR1MBs bearing the mutations were subsequently transferred to *YEP24–ADC1–CYR1* for expression of the mutant cyclase proteins in yeast. The mutated *YEP24–ADC1–CYR1* plasmids were transformed into TK35-1, and the Ura^+ transformants were subjected to adenylate cyclase assays and Western (immuno) blot analysis as described below.

Construction of Chimeric Adenylate Cyclase Genes. A plasmid, pAD-spCYR1-2, which expressed the complete *Sc. pombe* adenylate cyclase gene (*spCYR1*) (4), was cleaved by suitable restriction endonucleases and the resulting fragments were used to construct chimeras with various fragments of the *Sa. cerevisiae CYR1* gene derived from *YEP24–ADC1–CYR1* plasmid (see *Results* and Fig. 3). The chimeric

Abbreviation: p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

plasmids were transformed into TK34-1 or TK35-1, and the transformants were subjected to adenylate cyclase assays.

Other Methods. The preparation of crude membrane fractions from yeast cells and the assay of adenylate cyclase activity in them were performed as described (2–4, 8, 15). Where indicated, 5 μg of the purified RAS2 protein was preincubated with 5 μl of 1 mM guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG) and added to the assay. Detection of the *Sa. cerevisiae* adenylate cyclase protein and its chimeras was carried out by Western blot analysis as described (4, 20).

RESULTS

Deletion Mutagenesis of the *Sa. cerevisiae* Adenylate Cyclase Gene. A preliminary deletion analysis of the *Sa. cerevisiae CYR1* gene was performed by introducing in-frame deletions by using restriction digestion, filling in the resulting cohesive termini by the large fragment of *Escherichia coli* DNA polymerase I, and resealing of the plasmid *YEP24–ADC1–CYR1* (Table 1). The resulting plasmids bearing the mutant genes were transformed into adenylate cyclase-deficient yeast cells whose *RAS2* gene had been disrupted (TK35-1) and the mutant proteins were overexpressed from the *ADC1* promoter. Crude membrane fractions of the transformants were assayed for the RAS2 protein-dependent activation of adenylate cyclase activity in the presence of Mg^{2+} and p[NH]ppG. As shown in Table 1, the NH_2-terminal 605 amino acids were found to be dispensable for the activation. Any smaller deletions within the NH_2-terminal 605 residues did not affect the activation (data not shown). However, deletion of a *Bgl* II fragment corresponding to amino acids 1116–1316 completely abolished the RAS2 protein-dependent activation, as did deletions of amino acids 1218–1338 and 1404–1528. For further analysis, we introduced stepwise deletions by using BAL-31 exonuclease starting from a cleavage site with *Hpa* I (at amino acid 335), *Mlu* I (at amino acid 605), *Xho* I (at amino acid 1067), or *Cla* I (at amino acid 1960). As shown in Table 1, deletions confined to the NH_2-terminal 657 amino acids had no discernible effect on the RAS2 protein-dependent activation, whereas deletions in a segment beyond amino acid 660 completely abolished it. In addition, all the adenylate cyclase mutants that lost their COOH-terminal 66 residues were rendered unactivatable by the RAS2 protein, reinforcing our report (4), which showed the requirement of the COOH-terminal 140 residues for the activation. Deletions of short sequences upstream of the COOH-terminal 66 residues were found to be permissive for the activation. The observed loss of activation did not appear to be explained by preferential degradation of mutant cyclase proteins, as shown in Fig. 1*A*, where the cyclase proteins produced from representative mutant genes were detected by Western blot analysis and found to be expressed without significant degradation. Their molecular sizes were similar to those expected from the extent of deletions.

Table 1. Adenylate cyclase activity of deletion mutant cyclases

Deleted residues	Method	Adenylate cyclase activity, units: Mn^{2+}	Mg^{2+}/p[NH]ppG	RAS2/Mg^{2+}/p[NH]ppG	Activation
None		202.6	3.3	59.1	+
Upstream of catalytic site					
1–42	A	204.1	6.3	98.2	+
42–605	A	107.3	0.98	72.2	+
335–630	B	97.0	3.1	27.9	+
605–655	B	249.3	15.3	167.9	+
605–657	B	269.0	6.2	46.5	+
605–660	B	138.2	6.8	7.2	−
605–771	B	193.2	2.8	2.3	−
736–839	C	543.4	5.2	7.4A	−
983–1066	B	203.3	5.3	8.0	−
1010–1052	C	523.7	8.8	10.5	−
1053–1066	C	198.0	2.1	2.1	−
1067–1141	C	285.3	5.8	7.8	−
1116–1316	A	158.2	8.3	9.1	−
1218–1338	A	313.4	5.7	9.4	−
1404–1528	A	557.1	22.8	16.9	−
1595–1646	C	415.7	13.8	21.8	−
Downstream of catalytic site					
1950–1959	B	215.8	2.5	125.1	+
1942–1959	B	151.5	3.5	35.3	+
1933–1959	B	236.2	2.9	71.6	+
1960–2026	B	39.5	1.04	1.24	−
1932–2026	B	178.8	8.8	11.7	−
1917–2026	B	70.4	0.65	0.18	−
1906–2026	B	428.9	9.3	5.2	−
1900–2026	B	12.4	0.0	0.0	−

Methods for preparation of the deletion mutants are classified into three categories: cleavage by restriction enzymes and resealing (method A), cleavage by restriction enzymes and BAL-31 digestion (method B), and cleavage twice by restriction enzymes and resealing with a 6-bp linker inserted (method C) (see *Results*). One unit of activity is defined as 1 pmol of cAMP formed in a 1-min incubation with 1 mg of membrane protein at 32°C under standard assay conditions. +, Activated; −, not activated.

Insertion Mutagenesis. To more precisely localize the segments that are indispensable for interaction with RAS proteins, we introduced 2-amino acid insertions into various locations between adenylate cyclase amino acids 605 and 1666. A 6-bp oligonucleotide pCGTCGA was inserted into every possible cleavage site by restriction endonucleases *Hpa* II, *Hin*PI, and *Taq* I after partial digestion of pUC-CYR1MB with the respective enzyme. Similarly, a 6-mer GGGCCC was used to make insertions into every possible cleavage site by *Bal* I, *Dra* I, *Eco*RV, *Hin*cII, *Pvu* II, *Sca* I, *Ssp* I, and *Xmn* I, all of which yielded flush ends upon digestion. The insertion of pCGTCGA resulted in the addition of three 2-amino acid blocks that, depending on the relative locations in the coding frame, did not alter the franking residues, whereas insertion of GGGCCC could change one residue immediately upstream of the 2-amino acid insertions. As shown in Table 2, a striking regional difference was observed in the effect of the insertions on the RAS2 protein-dependent activation. Almost all the insertions into a segment corresponding to amino acids 849–1296, which coincided with a region where the leucine-rich repeat motif was well retained (Fig. 2), completely abolished the activation. In contrast, almost all of the insertions into a segment (positions 1332–1648) between the repeats and the catalytic domain had no effect. The insertions between amino acids 700–839, where the leucine-rich repeats were not conspicuous but still identifiable, gave ambivalent results. There was only one location, at position 1126 in the middle of the leucine-rich repeats, where an insertion failed to abolish the activation. However, as shown in Fig. 2, this point conspicuously lacked major features of the repeat motif, and the insertion of Gly-Pro here actually created a sequence that better conformed to the motif. Insertions at positions 1588 and 1648 destroyed the activation, exceptions between amino acids 1332–1648 where insertions generally had no effect. Results of the adenylate cyclase assays showed a clear distinction between mutants activatable by the RAS2 protein and those that were not. The former was activated by a factor of at least 4, whereas the latter was activated by a factor of less than 1.7. The insertion mutant at position 1648, the only exception, was reproducibly activated by a factor of ≈2, and this extent

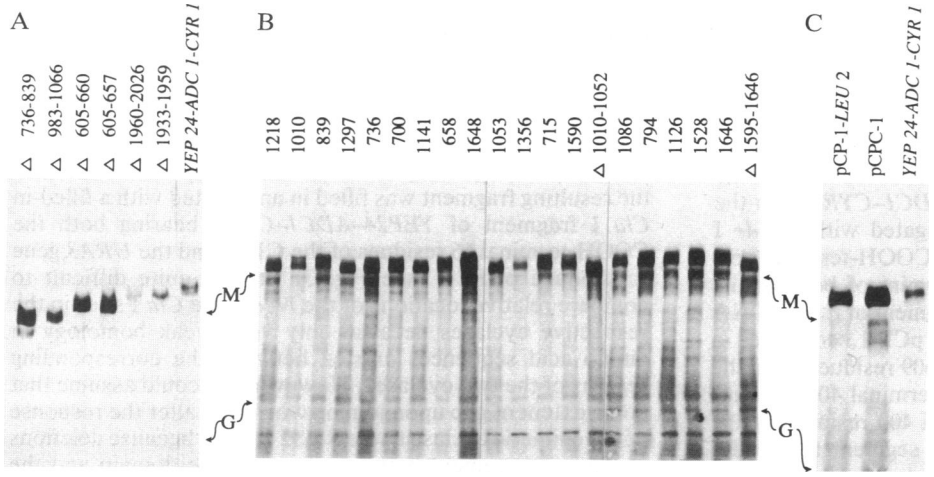

FIG. 1. Detection of the mutant adenylate cyclase proteins. Membrane proteins (50 μg) from yeast cells harboring the mutant *CYR1* genes derived from deletion mutagenesis (*A*), insertion mutagenesis (*B*), or chimeric gene construction (*C*) were fractionated in 5% polyacrylamide gels. *Sa. cerevisiae* adenylate cyclase protein was detected on Western blots of the gels as described (4). The lane labels represent the location (amino acid position) of a 2-amino acid insertion in the respective mutant cyclase. Numbers after Δ represent the residues deleted. The molecular mass markers were myosin heavy chain (M, 200 kDa) and *E. coli* β-galactosidase (G, 116 kDa).

of activation could be significant. During construction of the insertion mutants by partial digestion with restriction enzymes, several mutants with small in-frame deletions were obtained by twice cleaving and resealing with the 6-bp linker inserted. None of the mutants were activatable by the RAS2 protein (see Table 1). Deletion of a *Sca* I fragment corresponding to amino acids 1595–1646 did not destroy the Mn^{2+}-dependent catalytic activity, indicating that the catalytic domain could be localized between positions 1646 and 1890, a region 37 residues smaller than published (4). As shown in Fig. 1*B*, where representative mutant cyclases were detected by Western blot analysis, the loss of activation of the mutant cyclases could not to be explained by their preferential degradation.

Table 2. Adenylate cyclase activity of insertion mutant cyclases

Position of insertion	Inserted sequence	Adenylate cyclase activity, units: Mn^{2+}	Mg^{2+}/ p[NH]ppG	RAS2/ Mg^{2+}/ p[NH]ppG	Activation
658	-V-D-	107.4	2.1	79.4	+
700	-V-D-	121.5	2.4	2.8	−
715	-S-T-	83.9	0.98	84.0	+
736	-S-T-	287.3	3.9	4.5	−
794	(L)-G-P-	246.6	3.6	187.9	+
839	-S-T-	78.6	1.14	21.7	+
849	-G-P-	53.6	1.23	2.11	−
902	-S-T-	123.8	1.5	2.0	−
1010	-S-T-	153.1	1.9	2.3	−
1053	-S-T-	109.2	0.94	1.58	−
1066	-V-D-	52.6	1.03	1.41	−
1083	(M)-G-P-	56.1	0.9	1.6	−
1086	-G-P-	199.0	2.4	4.1	−
1126	-G-P-	186.3	6.5	38.4	+
1138	-G-P-	370.7	7.9	9.8	−
1141	-S-T-	146.9	2.5	3.4	−
1143	(G)-G-A-	527.8	6.8	11.8	−
1218	-V-D-	449.2	7.4	8.3	−
1236	-G-P-	265.2	5.2	4.7	−
1290	(W)-A-H-	395.4	5.9	7.4	−
1296	-R-R-	484.7	9.3	15.7	−
1332	-G-P-	88.1	6.7	43.5	+
1338	-S-T-	404.2	7.2	40.4	+
1356	-R-R-	74.1	1.21	53.8	+
1402	-G-P-	172.6	3.2	185.0	+
1484	(R)-A-L-	420.7	22.0	230.3	+
1528	-G-P-	308.9	15.1	175.8	+
1588	-G-P-	120.0	10.0	8.1	−
1590	-S-T-	225.6	2.3	32.1	+
1594	-G-P-	127.9	7.8	79.0	+
1602	(G)-A-P-	92.7	9.7	37.7	+
1616	-G-P-	150.7	2.4	76.8	+
1636	-G-P-	226.6	9.2	112.0	+
1646	-G-P-	143.1	18.4	91.7	+
1648	-V-D-	193.5	1.97	3.78	−/±
		180.4	2.44	4.67	−/±

Inserted amino acid sequences are shown by the one-letter code. Amino acids in parentheses indicate residues that are located adjacent to the insertion sites and altered. For definition of a unit, see Table 1.

Response of Chimeric Adenylate Cyclases to RAS Proteins. The plasmids, pAD-spCYR1-2 (4) and *YEP24–ADC1–CYR1*, which contained the *Sc. pombe* and *Sa. cerevisiae* adenylate cyclase genes, respectively, under the *ADC1* promoter, were

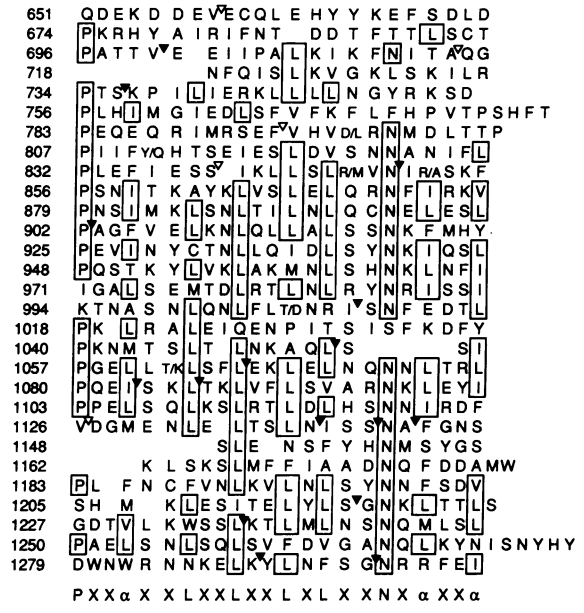

FIG. 2. Relationship between locations of mutations and their effects on the RAS–cyclase interaction. The sequence of amino acids 651–1301 of the *Sa. cerevisiae* adenylate cyclase (3) is indicated by the one-letter code. Numbers on the left are the amino acid coordinates. Slashes indicate areas where two positions have been compressed, and blanks indicate the placement of gaps that align the sequence to reflect the periodic structure (3). The bottom line indicates the consensus sequence of the repeats (where α is an aliphatic amino acid). Inverted open arrowheads represent locations where a 2-amino acid insertion did not destroy the RAS2 protein-dependent activation, and inverted closed arrowheads indicate those that abolished activation.

used to make chimeric genes. As depicted in Fig. 3*A*, pAD-spCYR1-2 had two *Nde* I cleavage sites, one at amino acid 1262 of the *spCYR1* and the other in the vector pAD-1. Also the *spCYR1* gene had two *Nco* I cleavage sites at amino acids 1332 and 1591. *YEP24–ADC1–CYR1* had two *Nco* I sites, one at amino acid 1608 of the *CYR1* and the other in the *URA3* gene, and two *Cla* I sites at position 1959 and in the vector. A *Nco* I fragment of *YEP24–ADC1–CYR1* with the NH_2-terminal half of the *CYR1* was ligated with a *Nde* I fragment of pAD-spCYR1-2 having the COOH-terminal portion of the *spCYR1* after cohesive termini of both of the fragments were filled in by the large fragment of *E. coli* DNA polymerase I. The resulting plasmid, pCP-1, contained a hybrid *CYR1* gene, the NH_2-terminal 1609 residues from the *Sa. cerevisiae CYR1*, and the COOH-terminal 400 residues from the *spCYR1*. The COOH-terminal 400 residues of the *Sc. pombe* cyclase contained a whole segment that had a striking homology with the catalytic domain of the *Sa. cerevisiae* cyclase (4) and might well encode the whole catalytic domain. Subsequently, a *Xho* I–*Sal* I fragment of the *LEU2* gene (21) was inserted into a *Sal* I site of pCP-1 to produce pCP-1-*LEU2* (see Fig. 3). Similarly, the remaining fragments derived from pAD-spCYR1-2 and *YEP24–ADC1–CYR1* were ligated to produce pPC-1. The amino acid sequences surrounding the *Nco* I site of the *CYR1* and the *Nde* I site of the *spCYR1* were very homologous (4), and the two sites were located at relatively identical locations in the respective cyclase protein. After filling in and joining at these sites, the resulting chimeric cyclase in pCP-1-*LEU2* had a

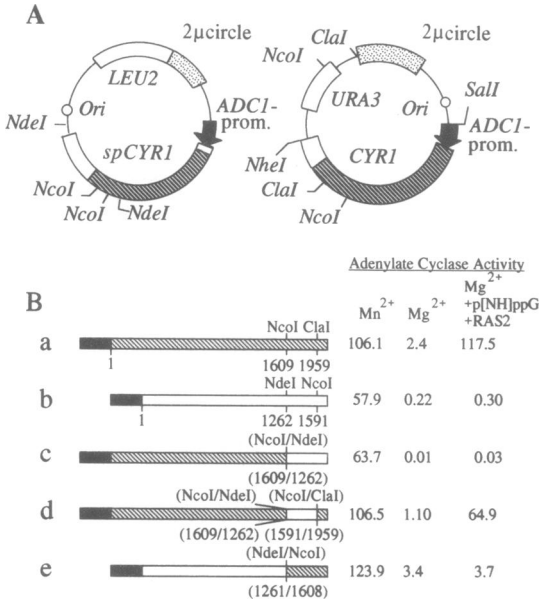

FIG. 3. Structures and properties of chimeric adenylate cyclase protein. (*A*) Structures of pAD1-spCYR1-2 (*Left*) and *YEP24–ADC1–CYR1* (*Right*), which express the *spCYR1* and *CYR1* genes, respectively, from the *ADC1* promoter, are shown. Restriction cleavage sites used for construction of the chimeric genes are indicated. (*B*) Structures of the chimeric adenylate cyclase genes in the plasmids pPC-1-LEU2 (diagram c), pCPC-1 (diagram d), and pPC-1 (diagram e) are depicted on the left along with those of the *CYR1* (diagram a) and *spCYR1* (diagram b) genes. Solid bars represent the *ADC1* promoter, cross-hatched bars are portions derived from the *Sa. cerevisiae CYR1* gene, and open bars are portions from the *spCYR1* gene. Numbers under the bars indicate amino acid positions at the junctions. A pair of restriction cleavage sites in the parentheses indicates that the two sites were filled in and ligated with each other. Adenylate cyclase activities of membranes from yeast cells harboring the respective genes are shown on the right. The activities are shown as 1 pmol of cAMP formed per min per mg of membrane protein at 32°C.

1-amino acid deletion at the junction and that in pPC-1 had a 3-amino acid insertion. We assumed that the deletion or insertion in the chimeric cyclases would not alter their response to the RAS2 protein because the 2-amino acid insertions around this region did not affect the RAS protein-dependent activation (see Table 2). Finally, pCP-1 was cleaved partially with *Nco* I and completely with *Cla* I, and the resulting fragment was filled in and ligated with a filled-in *Cla* I fragment of *YEP24–ADC1–CYR1* bearing both the COOH-terminal 66 residues of the *CYR1* and the *URA3* gene to produce pCPC-1 (see Fig. 3). It was quite difficult to compare relative locations of the *Nco* I and *Cla* I sites in the respective cyclases because only very weak homology in amino acid sequences existed between the corresponding regions of the two cyclases. However, we could assume that some extent of gap upon joining would not alter the response of the chimeric cyclase to the RAS2 protein because deletions involving a segment between the catalytic domain and the COOH-terminal 66 residues were found to have no effect as shown in Table 1. The chimeric plasmids were transformed into TK34-1 or TK35-1, depending on the genetic markers present, and the resulting transformants were examined for adenylate cyclase activity. The result, shown in Fig. 3*B*, clearly indicated that only the fusion with both the NH_2-terminal 1608 residues and the COOH-terminal 66 residues of the *Sa. cerevisiae* cyclase conferred an ability to be activated by the RAS2 protein to the putative catalytic domain of the *Sc. pombe* cyclase, whereas the fusion with either one of the two segments alone did not. As shown in Fig. 1*C*, chimeric proteins of the expected sizes were produced from pCP-1-LEU2 and pCPC-1 without significant degradation. Because we could not distinguish the chimeric proteins from the *Sa. cerevisiae* cyclase protein by the Western blot analysis due to their similar molecular sizes, there remained a possibility that a complete *Sa. cerevisiae* gene might be reconstructed by a recombination event that occurred during the transformation and propagation of the chimeric plasmids. To exclude the possibility, we recovered plasmid DNAs from the supernatant fractions obtained from a crude membrane preparation after transformation into *E. coli* cells and found that the chimeric plasmids were still retained without any structural change after the propagation of yeast cells (data not shown). The protein product from the plasmid pPC-1 was undetectable because the antibody used for the detection had been raised against a part of the NH_2-terminal sequence of the *Sa. cerevisiae* adenylate cyclase (4).

DISCUSSION

The *Sa. cerevisiae* gene encoding adenylate cyclase was subjected to mutational analysis to localize domains required for interaction with RAS proteins. As expected from the structural complexity of protein molecules, the analysis using deletion mutagenesis gave only limited information. The NH_2-terminal 657 amino acids and the segment between the catalytic domain and amino acid 1959 were found to be dispensable for the interaction. The COOH-terminal 66 residues were indispensable, reinforcing our report (4) that the COOH-terminal 140 residues were required for the RAS2 protein-dependent activation. Any deletions involving remaining parts upstream of the catalytic domain resulted in the loss of the activation. Therefore, we decided to perform detailed insertion mutagenesis in which every cleavage site of various restriction endonucleases was mutated by insertion of 6-bp linkers. The results clearly indicated that the leucine-rich repeats constituting the middle 600 residues were indispensable for the interaction with RAS proteins. The importance of both the leucine-rich repeats and the COOH-terminal 66 residues was further supported by the finding that fusion with both was required to render the *Sc. pombe* adenylate cyclase activatable by the RAS2 protein. Colicelli *et al.* (6)

has also shown the requirement of the leucine-rich repeats for the RAS2 protein-dependent activation of the *Sa. cerevisiae* adenylate cyclase, based on a similar deletion analysis and a much simpler version of insertion mutagenesis. However, they did not analyze a region on the COOH-terminal side of the catalytic domain. The importance of the leucine-rich repeats for interaction with RAS proteins has also been suggested by Field *et al.* (22), who showed that an overproduction of a segment of the *Sa. cerevisiae* adenylate cyclase (positions 605–1301) bearing the whole region of leucine-rich repeats could suppress abnormal yeast phenotypes brought about by introduction of an activating mutation into the *RAS2* gene to give the [Val^{19}]RAS2 protein. Although mechanisms underlying the suppression were unknown, they might be easily explained by competitive sequestration of the RAS2 protein or of a hypothetical protein mediating the interaction between the RAS2 protein and adenylate cyclase. We have confirmed their data and, in addition, found that overproduction of a peptide containing the COOH-terminal 260 residues (a segment of the *CYR1* on the COOH-terminal side of a *Stu* I cleavage site at amino acid 1769) could also suppress the heat-shock sensitivity of the [Val^{19}]RAS2 mutant (H.-R.C. and T.K., unpublished data). As pointed out (3, 4, 22), sequences homologous to the leucine-rich repeats have been identified in several proteins from diverse organisms including the human leucine-rich α_2-glycoprotein (23), α (24, 25) and β (26) chains of the human platelet glycoprotein Ib, the porcine ribonuclease inhibitor (27), the lutropin-choriogonadotropin receptor (28), chaeoptin (29), and the *Toll* gene product (30) of *Drosophila*. Our mutagenesis data suggest that the 600-amino acid leucine-rich repeats may form a rigid stereo structure that is vulnerable to minor structural perturbations at any location in the repeats and may, like the leucine-zipper sequence (31), provide a site for interaction with other proteins. Because the consensus sequence is not rigorously retained throughout the repeats (see Fig. 2), it is impossible to deduce any structural feature essential for the interaction with RAS proteins from our present data.

It is still not clear whether the interaction between RAS proteins and the *Sa. cerevisiae* adenylate cyclase is direct or indirect. A *Sa. cerevisiae* protein called CAP (cyclase-associated protein) was found to be tightly bound to the adenylate cyclase molecule (32). Disruption of the gene encoding CAP abolished the activation of adenylate cyclase by RAS proteins. In the CAP-deficient mutant, however, the adenylate cyclase protein was found to be undetectable presumably due to degradation. Therefore, it is possible that the function of CAP may be to stabilize the adenylate cyclase protein and the observed loss of the activation upon its absence may be ascribed to degradation of the cyclase protein. Further studies will be needed to give a clearer picture on the mode of protein–protein interaction associated with the RAS protein-dependent activation of the *Sa. cerevisiae* adenylate cyclase.

We have proposed (4) that the loss of the ability of the *Sc. pombe* adenylate cyclase to interact with RAS proteins might be brought about by the loss of an interaction site, based on the structural homology with the *Sa. cerevisiae* adenylate cyclase. A leucine-rich repeat-like structure does exist in the *Sc. pombe* cyclase, but it appears much more irregular and has a much lower homology with its counterpart in the *Sa. cerevisiae* cyclase than that between the catalytic domains of the two cyclases (4). Considering the extreme structural vulnerability of the *Sa. cerevisiae* leucine-rich repeats, the observed extent of homology appears too low to assure the conservation of the ability to interact with RAS proteins in the *Sc. pombe* cyclase. Moreover, very little homology exists in the COOH-terminal 66 residues between adenylate cyclases of the two yeast species. These results further support our hypothesis that the *Sc. pombe* cyclase may have lost the interaction sites with RAS proteins. In addition, we have shown that the fusion with the two putative interaction sites rendered the *Sc. pombe* cyclase activatable by the RAS2 protein. The result suggests that the interaction sites with RAS proteins may function as a separate entity from the catalytic domain and, when accidentally fused to a different protein, may confer RAS protein-dependent regulation to the protein. This possibility may explain the reason why the effector molecule of RAS proteins has changed during the evolution of *Sa. cerevisiae*, *Sc. pombe*, and mammals.

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