In the budding yeast Saccharomyces cerevisiae, association with the 70-kDa cyclase-associated protein (CAP) is required for proper response of adenylyl cyclase to Ras proteins. We show here that a small segment comprising the N-terminal 36 amino acid residues of CAP is sufficient for association with adenylyl cyclase as well as for its function in the Ras-adenylyl cyclase pathway as assayed by the ability to confer RAS2Val-19-dependent heat shock sensitivity to yeast cells. The CAP-binding site of adenylyl cyclase was mapped to a segment of 119 amino acid residues near its C terminus. Both of these regions contained tandem repetitions of a heptad motif αXXαXXX (where α represents a hydrophobic amino acid and X represents any amino acid), suggesting a coiled-coil interaction. When mutants of CAP defective in associating with adenylyl cyclase were isolated by screening of a pool of randomly mutagenized CAP, they were found to carry substitution mutations in one of the key hydrophobic residues in the heptad repeats. Furthermore, mutations of the key hydrophobic residues in the heptad repeats of adenylyl cyclase also resulted in loss of association with CAP. These results indicate the coiled-coil mechanism as a basis of the CAP-adenylyl cyclase interaction.

The budding yeast Saccharomyces cerevisiae has two RAS genes, RAS1 and RAS2, whose protein products are structurally, functionally, and biochemically similar to mammalian Ras proto-oncoproteins (for reviews, see Refs. 1 and 2). The yeast Ras proteins are essential regulatory elements of adenylyl cyclase, which catalyzes the production of cAMP, a second messenger vital for cell growth (3, 4). The Ras-adenylyl cyclase pathway has been implicated in transduction of a signal triggered by glucose to an intracellular environment where a protein phosphorylation cascade is induced by cAMP. Yeast cells bearing the activated RAS2 gene, RAS2Val-19, exhibit an elevated level of intracellular cAMP and display abnormal phenotypes, including sensitivity to heat shock, sensitivity to nutritional starvation, and failure to sporulate (3, 5).

Yeast adenylyl cyclase, encoded by the CYR1 gene, consists of 2026-amino acid residues that comprise at least four domains: the N-terminal, the middle leucine-rich repeat, the C-terminal domains (6, 7). The leucine-rich repeat domain contains a binding site for Ras proteins (8, 9). Adenylyl cyclase forms a complex with 70-kDa CAP.1 CAP was identified biochemically as the only protein associated tightly with adenylyl cyclase and also by genetic screening of a gene whose mutation abolished the RAS2Val-19-dependent heat shock sensitivity (10, 11). Studies on the function of CAP revealed that CAP is a multifunctional protein. It was shown that the N-terminal region, mapped to residues 1–168, is required for acquisition of heat shock sensitivity in the RAS2Val-19 background while the C-terminal region, mapped to residues 369–526, is required for normal cell morphology and responsiveness to nutrient deprivation and excess (12). The C-terminal function appears to be related to regulation of the actin cytoskeleton as evidenced by complementation of its defect by overexpression of profilin or SNC1 (13, 14) and by demonstration of its direct association with actin monomer and of its actin-sequencing activity (15, 16). In addition, CAP possesses two proline-rich sequences in its middle region intervening between the two regions, with which associations of actin-binding protein 1, elongation factor 1α, and ribosomal protein L3 were recently shown (17, 18).

The N-terminal region of CAP binds to the C-terminal region of adenylyl cyclase (19), and this association appears to be required for the proper in vivo response of adenylyl cyclase to Ras, because its loss by mutation of either CAP or adenylyl cyclase resulted in disappearance of the RAS2Val-19-dependent heat shock sensitivity and in a reduced cAMP response to glucose stimulation (19). This function resides solely in the CAP N-terminal region and is separable from the functions of the other regions as reported (12). We have recently shown biochemically that the association of adenylyl cyclase with the CAP N-terminal region is responsible for efficient stimulation of adenylyl cyclase activity by the posttranslationally modified form of Ras, although the molecular mechanism underlying this process remains to be clarified (20). In this report, we have mapped a minimal region of CAP responsible for its N-terminal function and analyzed the molecular mechanism for its association with adenylyl cyclase.

**EXPERIMENTAL PROCEDURES**

**Cell Strains and Growth Media—**The S. cerevisiae strains used are listed in Table I. Replacement of the chromosomal CAP gene with its

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1. The abbreviations used are: CAP, adenylyl cyclase-associated protein; CYR1, adenylyl cyclase; GST, glutathione S-transferase; PCR, polymerase chain reaction; GBT, GAL4 DNA-binding domain; GAD, GAL4 transactivation domain; MES, 2-(N-morpholino)ethanesulfonic acid.
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**TABLE I**

**Strain**

| Yeast strains used in this study |
|-----------------------------|
| **Genotypes** |
| SPI | MATa his3 leu2 trp1 ura3 ade8 can1 |
| FS1 | MATa his3 leu2 trp1 ura3 ade8 can1 cap::pCAPAN1 |
| TK161-R2V | MATa his3 leu2 trp1 ura3 ade8 can1 RAS2val19 |
| TK161-R2V(CAPΔN) | MATa his3 leu2 trp1 ura3 ade8 can1 RAS2val19 cap::pCAPAN1 |
| YPB2 | MATa his3 leu2 trp1 ura3 ade2 can1 gal4 gal80 lys2 gal1-his3 ura3::gal41–17 mers3(3x)::cyc1-tata-lacZ cap::pCAPAN1 |
| YPB2(CAPΔN) | MATa his3 leu2 trp1 ura3 ade2 can1 gal4 gal80 lys2::gal1-his3 ura3::gal41–17 mers3(3x)::cyc1-tata-lacZ |

*Strains SPI, FS1, TK161-R2V, and YPB2 were described previously (3, 5, 20, 25).*

N-terminal deletion mutant CAPAN-1 was carried out as described previously (20). The resulting yeast strain expresses only the C-terminal segment of CAP corresponding to residues 369–526 under control of the yeast ADC1 promoter. Yeast cells were grown in YPD (2% Bactopeptone, 1% Bacto-yeast extract, 2% glucose) or yeast synthetic medium (0.67% yeast nitrogen base, 2% glucose) with appropriate auxotrophic supplements. Genetic manipulations of yeast cells were performed as described previously (21). Transformation into yeast cells was carried out with lithium acetate (22).

**Construction of Expression Plasmids and Oligonucleotide-directed Mutagenesis**—pAD-GST plasmid, pAD-GST-CAP (18), was used to express the full-length CAP in yeast as a fusion protein with GST under control of the ADC1 promoter. Various deletions were introduced into the CAP gene by cleavage of pAD-GST-CAP with suitable pairs of restriction endonucleases and resealing by T4 DNA ligase with a linker oligonucleotide, 5′-CTAGTCTAGACTAG-3′, bearing stop codons in all reading frames, between. The resulting plasmids were designated as pAD-GST-CAP-(x–y), where x–y represents the range of the expressed CAP polypeptides in amino acid positions. A 5′-terminal 107-base pair fragment corresponding to residues 1–36 of CAP, CAP-(1–36), was amplified by PCR (23) using suitable oligonucleotide primers and, after cleavage with BamHI and Smal in the primer sequences, cloned into pAD-GST to produce pAD-GST-CAP-(1–36). Similarly, DNA fragments encoding various C-terminal polypeptides of adenylyl cyclase were amplified by PCR using suitable primers and cloned into pAD-GST to produce pAD-GST-CYR1-(y–y), where y–y represented the range of the expressed adenylyl cyclase polypeptide in amino acid positions. Specific amino acid substitution mutations were introduced into adenylyl cyclase by the gapped duplex method using suitable mutagenic oligonucleotides (24). The mutant genes were used to replace the corresponding wild-type genes in the expression plasmids. YEP24-ADC1-CYR1-(1–40, 1769–2026) and YEP24-ADC1-CYR1-(1–40, 606–2026), which expressed adenylyl cyclase carrying internal deletions of residues 41–1768 and 41–605, were identical to YEP24-ADC1-CYR1-(1–40, 1769–2026) and YEP24-ADC1-CYR1-(1–40, 606–2026), which expressed adenylyl cyclase carrying internal deletions of residues 41–1768 and 41–605, respectively, as described previously (19). A rabbit polyclonal antiserum for GST (anti-GST) was used for detection of GST fusion proteins.

**RESULTS**

**Mapping of the Mutual Binding Sites of CAP and Adenylyl Cyclase**—Previous experiments had already mapped the N-terminal function of CAP to residues 1–168 (12) and the CAP-binding site of adenylyl cyclase to residues 1879–2026 (19). To further delineate the binding sites, we introduced various deletion mutations into CAP and adenylyl cyclase as described under “Experimental Procedures.” Interactions of the various CAP mutants with CYR1-(1879–2026) and of the various CYR1 C-terminal mutants with CAP were examined by employing the yeast two-hybrid system (Fig. 1A). As an indicator strain, we used YPB2(CAPΔN), whose chromosomal CAP gene was replaced by its N-terminal deletion mutant CAPAN-1 in order to exclude the possibility that endogenous CAP complexed with an otherwise negative GAD-fusion CAP mutant may serve as a bridge to yield a positive interaction with the GBT-fusion CYR1. Formation of such a CAP dimer had been reported before (16). To our surprise, the shortest CAP construct carrying only the N-terminal 36 residues, CAP-(1–36), as well as the longer CAP-(1–66), CAP-(1–77), and CAP-(1–88) exhibited a positive interaction with the C-terminal region of adenylyl cyclase (Fig. 1A). In contrast, CAP-(78–526) lacking the N-terminal region did not exhibit any interaction. On the other hand, the shortest fragment of CYR1 giving a positive interaction with CAP was 119 residues corresponding to positions 1898–2016 (Fig. 1A). An N-terminal deletion up to position 1935 destroyed the activity to interact with CAP.

Physical associations of the same sets of CAP and CYR1 mutants with their counterparts were also examined biochemically as shown in Fig. 1, B and C. The CAP deletion mutants were expressed as GST fusions from pAD-GST-CAP-(x–x) in yeast FS1 cells harboring YEP24-ADC1-CYR1-(1–40, 1769–2026). The GST-CAP fusion proteins were purified by glutathione-Sepharose chromatography and examined for the bound CYR1-(1–40, 1769–2026) by immunoblotting with anti-CYR1CT antibody (Fig. 1B). Similarly, proteins copurified with GST-CYR1-(y–y) from yeast cells harboring pAD-GST-CYR1-(y–y) were examined for anti-CAP antibody (Fig. 1C).
Western immunoblotting with anti-CAP antibody (by SDS-polyacrylamide gel electrophoresis (10% gel) and detected by copurified with GST-CYR1-(muscle triosephosphate isomerase (32 kDa), dehydrogenase (62 kDa), rabbit muscle aldolase (47 kDa), and rabbit tose-binding protein-fusion paramyosin (83 kDa), bovine liver glutamic resin were detected by immunoblotting with anti-GST antibody (CYR1-)). GST-CAP-(part resin were detected by immunoblotting with anti-GST antibody (part resin were detected by immunoblotting with anti-GST antibody (upper part). The molecular size markers indicated were Escherichia coli maltose-binding protein-fusion paramyosin (83 kDa), bovine liver glutamic dehydrogenase (62 kDa), rabbit muscle aldolase (47 kDa), and rabbit muscle triosephosphate isomerase (32 kDa). C, the endogenous CAP copurified with GST-CYR1-(y-y) proteins from SP1 cells was separated by SDS-polyacrylamide gel electrophoresis (10% gel) and detected by Western immunoblotting with anti-CYR1CT antibody as described under “Experimental Procedures” (lower part). GST-CAP-(x-x) proteins eluted from glutathione-Sepharose resin were detected by immunoblotting with anti-GST antibody (upper part). The results were in good agreement with those of the yeast two-hybrid analysis; the shortest fragment exhibiting this activity. An equivalent results were obtained in three independent experiments.

The results were in good agreement with those of the yeast two-hybrid analysis; the shortest CAP and CYR1 fragments that retained the activity to associate with their counterparts were CAP-(1–36) and CYR1-(1989–2016), respectively. These results indicated that residues 1–36 of CAP and residues 1898–2016 of adenyl cyclase are sufficient for their mutual association.

**CAP-(1–36) Is Sufficient for Its N-terminal Function in Vivo**—We examined the abilities of the CAP deletion mutants to confer heat shock sensitivity to TK161-R2V(CAPΔN) cells, which carried the CAPΔN-1 gene encoding the protein lacking its N-terminal function and thereby were made resistant to heat shock in the RAS2^{Val-19} background. As shown in Fig. 2A, the short fragment, CAP-(1–36), as well as other longer CAP N-terminal fragments was sufficient to restore the heat shock sensitivity in this yeast strain. As observed in the binding assays, both GST-CAP-(77–526) and GST only were found inactive. This result implied that the N-terminal 36 residues are functional in the Ras-adenyl cyclase pathway.

Heat shock sensitivity was also used for examining the CAP binding activity of various CYR1 fragments. It had been shown that overexpression of the CAP-binding region of adenyl cyclase suppressed the RAS2^{Val-19}-dependent heat shock sensitivity presumably by competitive sequestration of CAP from the endogenous adenyl cyclase (19). We examined the activity of the CYR1-deletion mutants overexpressed from pAD-GST-CYR1-(y-y) to suppress the heat shock sensitivity of TK161-R2V (Fig. 2B). Again, the result was in good agreement with that of the CAP-binding assays, i.e. CYR1-(1898–2016) was the shortest fragment exhibiting this activity.

**Identification of Mutations That Abrogate the CAP-Adenylyl Cyclase Interaction**—The mutually interacting regions, CAP-(1–36) and CYR1-(1989–2016), were searched for a peculiar sequence motif hinting at the mechanism of their interaction. The search identified tandem repetitions of a heptad motif αXXαXXαXXαXX (where α and X represent a hydrophobic amino acid and any amino acid, respectively; for reviews, see Refs. 30 and 31) in both residues 13–30 of CAP and residues 1916–1930 of adenyl cyclase (Fig. 3A). If the heptad repeat motif is taken as that of a leucine zipper LXXXLXXX (32), adenyl cyclase has one more repeat unit in residues 1931–1937. These heptad repeats enabled us to predict formation of α-helices that are wound around each other to form a superhelix, the coiled-coil structure (30, 31). This was also supported by calculation of the
Coiled-coil Mechanism for CAP-Cyclase Interaction

Amino acid substitutions found in CAP mutants

| Mutant CAP clones | Mutations in residues 1–36 |
|-------------------|---------------------------|
| HS10              | V30D                      |
| HS27              | L20P, D29G                |
| HS92              | Q91                        |
| HS113             | T24A                      |
| HS139             | L27S                      |
| HS143             | T31A                      |
| HS155             | Y6L, T7A                  |
| HS197             | L16R                      |
| HS205             | T31P, Q34L                |
| HS208             | L20P                      |

but those from five clones (HS92, HS113, HS143, HS155, and HS197) were found to have lost the ability to attach onto glutathione-Sepharose resin (data not shown), suggesting gross alterations in their conformations, and, therefore, were excluded from further analyses. The remaining five clones were examined for physical association with a deletion mutant CYR1 protein that retained the Ras-responsive adenyl cyclase activity (8) (Fig. 4A). The results clearly indicated that GST-CAP-(1–77) from the five clones HS10, HS27, HS139, HS205, and HS208 lost the ability to associate with CYR1-(1–40, 606–2026). Concurrently, the same CAP mutants lost the activity to confer heat shock sensitivity to TK161-R2V(CAPAN) yeast cells (Fig. 5A). Strikingly, four out of the five clones turned out to carry an amino acid substitution mutation at Leu-20, Leu-27, or Val-30, all of which corresponded to the key hydrophobic residues in the heptad repeats (Fig. 3A). Moreover, the mutations were introduced in such a way that the hydrophobic residues were converted to neutral or hydrophilic residues (Table II). The other clone HS205 carried two mutations, T31P and Q34L. Although Gln-34 is located at the position in the heptad repeats corresponding to the key hydrophobic residue, it is presently unclear which of the two mutations is responsible for the effect.

Next, we examined the importance of hydrophobic residues in the predicted heptad repeats of adenyl cyclase in association with CAP. The key hydrophobic residues Leu-1916 and Leu-1923 corresponding to the α-position (Fig. 3A) were converted to Ser and to either Pro or Arg, respectively, by oligonucleotide-directed mutagenesis. The CYR1 C-terminal fragments carrying L1916S, L1923P, and L1923R mutations all lost the ability to associate with CAP as assayed by the yeast two-hybrid method (Fig. 4B) or by the in vivo binding assay (Fig. 4C). Overexpression of CYR1-(1822–2026) bearing the same mutations could not suppress the RAS-G15V-dependent heat shock sensitivity (Fig. 5B). These results indicated that the hydrophobic residues of both CAP and adenyl cyclase are indeed critical not only for their mutual association but also for their proper function in the Ras-adenyl cyclase pathway and further supported the involvement of the coiled-coil mechanism for their interaction.

**DISCUSSION**

We have shown that the N-terminal 36 residues of CAP are sufficient for association with adenyl cyclase as well as for its in vivo function in the Ras-adenyl cyclase pathway. The CAP binding site of adenyl cyclase was mapped to a 119-residue segment near the C terminus. Close inspection of the primary sequences of the two mutual binding sites has identified typical heptad repeat motifs (αXXαXXX), indicative of a coiled-coil interaction (30, 31) (Fig. 3A). Furthermore, the presence of the coiled-coil in the CAP N terminus was predicted by the computer program COILS (33). Coiled-coils are composed of two, three, or four α-helices wound around each other to form a
Coiled-coil Mechanism for CAP-Cyclase Interaction

A further proof for the coiled-coil interaction came from the studies on mutations of CAP and adenylyl cyclase, which abrogated the interaction. Strikingly, the three residues of CAP (Leu-20, Leu-27, and Val-30) and two residues of adenylyl cyclase (Leu-1916 and Leu-1923) that were identified to be essential for the interaction based on these mutational studies are all hydrophobic and located at position a or d. These results strongly support the notion that the coiled-coil mechanism forms a molecular basis for the CAP-adenylyl cyclase interaction.

At present, it is impossible for us to predict from the amino acid sequences how many strands of CAP and adenylyl cyclase contribute to the formation of the coiled-coil superhelix. It is also impossible to predict the relative orientation, parallel or anti-parallel, of the strands of CAP and adenylyl cyclase and how individual pairs of the residues from each strand are formed, both of which are primarily determined by polar and ionic interactions between residues flanking the hydrophobic core (30, 31). Elucidation of these structural features awaits determination of the three-dimensional structure of the CAP-adenylyl cyclase complex.

The heptad repeat structure is well conserved in CAP homologues identified in other organisms including Schizosaccharomyces pombe and mammals, although the N-terminal function of the budding yeast CAP is not conserved among them (37–40). This suggests that in those organisms CAP may establish a coiled-coil interaction at its N-terminal short segment with a certain protein to exert a function that is presumably different from that of the Ras-adenylyl cyclase pathway. The identification of such a CAP-interacting protein may reveal a novel function of CAP in addition to its C-terminal cytoskeletal function, which is known to be conserved between yeasts and mammals (37–40).

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