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Adenylyl Cyclase in Yeast: Antibodies and Mutations Identify a Regulatory Domain

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The adenylyl cyclase system of the yeast *Saccharomyces cerevisiae* contains the *CYR1* polypeptide, responsible for catalyzing formation of cAMP from ATP, and two *RAS* polypeptides, responsible for stimulation of cAMP synthesis by guanine nucleotides. We have obtained rabbit antibodies that recognize the *CYR1* protein. Antibodies were raised against synthetic oligopeptides and against a recombinant β-galactosidase/*CYR1* fusion protein. These antibodies have allowed the identification of the *CYR1* gene product as a 205 kDa protein. Treatment with trypsin (2 μg/ml) reduced the size of the *CYR1* protein from 205 to 155 kDa and produced an activated enzyme which no longer responded to guanine nucleotides. This result is consistent with a model in which adenylyl cyclase activity is regulated by an inhibitory domain near the amino-terminus of the *CYR1* protein. This model is further supported by the finding that adenylyl cyclase activity is also markedly elevated and unresponsive to guanine nucleotides in mutant yeast strains that express only the carboxy-terminal half of the *CYR1* protein. Treatment with high trypsin concentrations (>10 μg/ml) caused release of adenylyl cyclase activity from the membrane. Comparison of immunoreactive *CYR1* fragments released by trypsin and membrane bound genetically altered proteins suggests that the *CYR1* protein is attached to the membrane via a separate trypsin sensitive anchoring protein rather than via a membrane anchoring domain.

Key words: cAMP, *RAS*, g-protein, signal transduction, inhibitory domain

Adenylyl cyclase in the yeast *Saccharomyces cerevisiae* resembles mammalian adenylyl cyclase in many ways. Both enzymes are tightly bound to the membrane, are stimulated by Mn^{2+}-ATP, and are regulated by homologous guanine nucleotide binding proteins—G_s in mammals and *RAS* proteins in yeast. Research on these enzymes has followed somewhat different pathways. Intense efforts in several laboratories have led to the purification of mammalian adenylyl cyclase from brain tissue, yielding a 120–150 kDa glycosylated protein [1–3]. The α-subunit of G_s can directly stimulate the enzyme [4]. *CYR1*, the gene encoding adenylyl cyclase in yeast [5], has been isolated [6], and

Abbreviations used: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate.

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sequenced [7,8], yielding a long open reading frame of 2026 amino acids. Deletion experiments [7] localize the catalytic portion of the enzyme to the carboxy-terminal 417 amino acids of the protein. The function of the remaining three quarters of the protein remains unknown. Northern blot analysis reveals a message corresponding to the entire open reading frame, as well as a shorter 3.5 kb mRNA that hybridizes to the 3′ end of the gene, but not to the 5′ end [7]. Thus, although the sequence of the *CYR1* gene is known, genetic analysis has not revealed how much of the open reading frame is actually translated into protein or proteins.

We are interested in the mechanism by which *RAS* proteins activate adenylyl cyclase. Experiments in this laboratory have shown that trypsin treatment activates adenylyl cyclase while abolishing guanine nucleotide sensitivity [9]. This result is consistent with a model in which *RAS* proteins activate adenylyl cyclase by releasing the enzyme from a tonic inhibition that may be exerted by an inhibitory domain of the *CYR1* protein itself.

In order to determine whether activation by trypsin involves cleavage of the *CYR1* protein, we have developed anti-*CYR1* antibodies. These antibodies recognize a 205 kDa protein that corresponds in length to translation of roughly the entire open reading frame. Trypsin cleavage experiments, in accord with our model, indicate that the amino-terminal quarter of the *CYR1* protein is an inhibitory domain mediating the effects of the *RAS* proteins. This model is further supported by experiments with genetically truncated *CYR1* proteins.

MATERIALS AND METHODS

Materials

α-[^{32}P]ATP was from Amersham, [^{3}H]cAMP was from New England Nuclear. [^{35}S]Methionine was from Irvine Chemical and Nuclear. Trypsin and soybean trypsin inhibitor were from Sigma.

Deletion of the *CYR1* Gene

The *CYR1* gene was disrupted in strain TC41 (a *ura3-52 leu2-3 leu2-118 his3 his4 trp1-1 cam CYR1*) by transformation with the plasmid pCYR1 Δ5. The resulting strain, TC41-1, had no adenylyl cyclase activity, and required exogenous cAMP for growth. Integration of the plasmid at *CYR1* was confirmed by Southern analysis. The plasmid pCYR1 Δ5 was a derivative of pUC18 [10], with a disrupted *CYR1* gene inserted at the polylinker. The insert was made by removing 5 kb of internal *Eco*RI fragments from the 8 kb *Bam*HI-*Hind* III fragment of pAC2 (YEp:CYR1-2 in ref. 6) and replacing them with the *URA3* gene. This deletion removed approximately 85% of the DNA in the open reading frame, corresponding to amino acids 135 through 1822.

Isolation of 102R-10

The strain 102R-10 was selected in the cyr1$^-$ strain AM18-5C, as a spontaneous pseudorevertant, by plating approximately 10^6 cells on YEPD (1% yeast extract, 2% bacto peptone and 2% glucose) media containing no cyclic AMP.

Expression of a *CYR1* Protein With an In-Frame Internal Deletion.

The plasmid pAC103 was constructed by removal of the 3.7 kb *Sac*I fragment from pAC2. This produced an in frame deletion of amino acids 27–1,233, leaving an

open reading frame of 821 amino acids, encoding a polypeptide of approximately 85 kDa. This plasmid and the wildtype pAC2 were used to transform the *cyr 1-1* yeast strain NW23-9C [6].

Preparation of Yeast Particulate Fractions

Yeast were grown to an optical density (O.D.) of approximately 2, at 660 nm. The cultures were centrifuged and cells were disrupted by vortexing with glass beads in YMB buffer (50 mM 2[N-morpholino]ethanesulfonic acid, pH 6.0; 0.1 mM EDTA; 0.1 mM $MgCl_2$; 1 mM phenylmethylsulfonylfluoride; and 50 μg/ml leupeptin) as previously described [11]. Membranes were stored in YMB at $-70°$ until needed.

[^{35}S]-labelling of Yeast Proteins

A typical 100 ml culture was grown in minimal medium lacking methionine to which 1 mCi of [^{35}S]methionine was added. The cultures were grown at 30°C overnight to a density of approximately 1 O.D.; a particulate fraction, prepared as described above, yielded a crude membrane preparation containing approximately 500×10^6 cpm.

β-galactosidase/adenylyl Cyclase Fusion Protein and Preparation of Antibodies

The 4.4 kb *Cla*I fragment of pAC2 [6] was isolated and filled in with Klenow [12], *Hin*dIII linkers were added, the fragment was digested with *Pst*I and *Hin*dIII, and the 2.1 kb fragment was isolated and inserted into pUR 292 [13]. This placed the carboxyl-terminal one-third of the *CYR1* gene (Fig. 1) in frame at the carboxy-terminal end of the β-galactosidase. The resulting plasmid, pYC101, was used to transform *E. coli* strain SR101. Induction of cultures with isopropylthio-β-D-galactoside resulted in large quantities of a 180 kDa protein in bacteria transformed with pYC 101. The 180 kDa protein

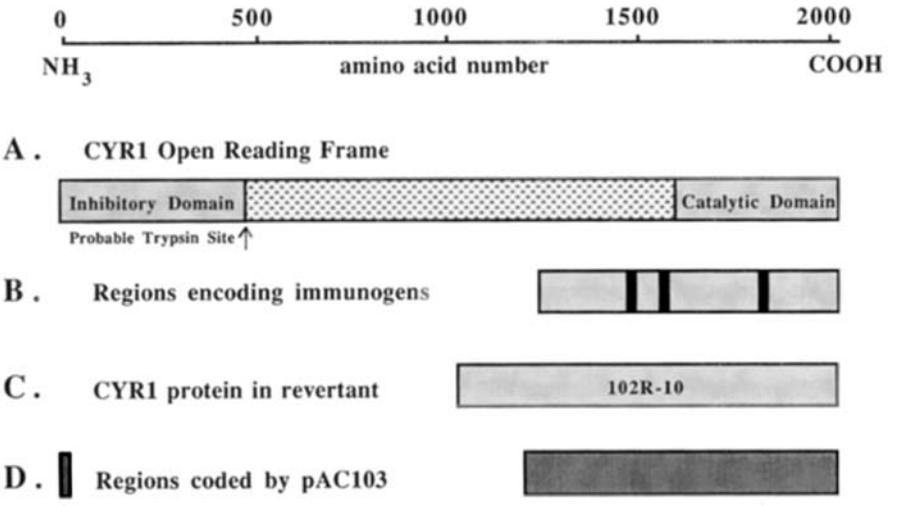

Fig. 1. Map of *CYR1* protein. **A:** Locations of catalytic domain and postulated inhibitory domain. Arrow indicates location of amino acids 470–484, a stretch rich in arginine and lysine residues. **B:** Portion of *CYR1* gene used to make fusion protein; solid bars indicate locations of oligopeptides used as immunogens. **C:** Proposed portion of *CYR1* protein expressed by 102R-10. **D:** Portion of *CYR1* encoded by pAC103.

was purified by SDS polyacrylamide gel electrophoresis, dialyzed into phosphate buffered saline, and used to immunize rabbits.

Antipeptide antibodies were generously provided by J. Ramachandran, Genentech, South San Francisco, CA. The antibodies were prepared against a mixture of three peptides corresponding to residues 1506–1520, 1591–1606, and 1828–1842 from the predicted sequence of Kataoka et al. [7]. These particular peptide sequences were chosen because they were hydrophilic and included proline residues. Peptides were individually coupled to bovine serum albumin according to the method of Kishida et al. [14], using glutaraldehyde at a peptide to carrier ratio of 1:5 (wt:wt). The peptide-carrier conjugates were dialyzed against phosphate buffered saline, combined, and used to immunize rabbits.

Antibodies were purified by adsorption onto affinity columns prepared by coupling the immunogens to agarose gel. The antibodies were eluted with 0.1 M glycine, pH 2.5, followed by immediate neutralization with 1 M Tris, pH 10.5.

Immunoprecipitation

Labelled membranes were extracted with 20 mM Tris, pH 7.5; 1% Nonidet® P-40; 0.4% deoxycholate; 66 mM EDTA; 0.3% SDS (NDET.3). Typically, samples containing between 100 and 500 μg protein and approximately 50×10^6 cpm were extracted in 200 μl NDET.3 for 30 min at room temperature, the mixture was centrifuged 15 min at 10,000*g*, and the supernatant was incubated overnight at 4°C with 500 μl pre-blocked antibody solution containing 10 μg antibody and approximately 10 mg unlabelled NDET.3 extract prepared from TC41-1 membranes. Antibodies were precipitated with 25 μl of a 10% suspension of fixed *Staphylococcus aureas*. The *S. aureas* cells were collected by centrifugation, resuspended in NDET.3, and washed twice by centrifugation through a cushion of 30% sucrose in 0.5 ×NDET.3. The pellet was then washed in water and immunoprecipitated proteins were released for SDS gel electrophoresis by boiling in sample buffer containing 2-mercaptoethanol. Proteins were electrophoresed on 6% polyacrylamide gels, and the gels were stained, treated with Enhance®, and dried for autoradiography.

Immunoblotting

Samples were concentrated by precipitation with 10% trichloroacetic acid; pellets were neutralized by the addition of 1 M Tris, pH 10.5, and run on a 6% polyacrylamide gel. Proteins were transferred to nitrocellulose, and the filter was blocked with a modified "Blotto" [15], containing 50 mM Tris (pH 7.5), 5% non-fat dry milk, 150 mM NaCl, 5 mM EDTA, 0.01% sodium azide, and 0.05% Tween®20, and developed with antibody solution containing 5 μg/ml primary antibody in Blotto containing paraformaldehyde fixed TC41-1 membranes at a concentration of 1 mg/ml. Immunoreactive proteins were visualized by the addition of [^{125}I]-labelled goat-anti-rabbit antibodies, followed by autoradiography of the washed filter.

Adenylyl Cyclase Assay

Adenylyl cyclase was assayed by a modification of the method of Salomon et al. [16] as described by Casperson et al. [6]. Protein was assayed by the method of Lowry et al. [17].

RESULTS

Antibody Probes

In order to identify the *CYR1* gene product, antibodies were raised to a β-galactosidase/adenylyl cyclase fusion protein containing the carboxy-terminal 775 amino acids of *CYR1*, and to synthetic oligopeptides corresponding to sequences located in the carboxy-terminal portion of the protein (see Fig. 1).

Identification of the *CYR1* gene product using antibodies posed problems of specificity and sensitivity. All of the preimmune sera reacted quite strongly with multiple yeast proteins. This problem was compounded by the fact that adenylyl cyclase is a minor component of the yeast membrane. Thus, we could expect the non-specific background signal to be higher than that produced by the authentic cyclase. Assuming that the turnover number of yeast adenylyl cyclase is similar to that of the enzyme purified from brain [3], yeast adenylyl cyclase may compose as little as 4 parts in 10^6 of the total membrane protein. The problem of low abundance was most acute with immunoblotting. We therefore used immunoprecipitation of [^{35}S]-labelled proteins for studies involving crude particulate fractions. In some preparations with relatively high specific activity, immunoblotting was effective.

Several steps were taken in order to identify the *CYR1* gene product, enhance the signal, and decrease the background. First, radiolabelled proteins immunoprecipitated from HR125::pAC2, a yeast strain carrying *CYR1* on the multi-copy plasmid YEp24, were compared with those from TC41-1, a strain in which the *CYR1* gene had been deleted. This allowed identification of immunoprecipitated proteins expressed only in the *CYR1*$^+$ strain, bands common to both strains being irrelevant. Yeast carrying the plasmid pAC2 express approximately fivefold more adenylyl cyclase activity than those carrying the parent plasmid, thus increasing the signal. All other characteristics of the adenylyl cyclase produced in the yeast with the multicopy plasmid (sensitivity to guanine nucleotide stimulation, hydrodynamic size, sensitivity to proteolytic activation) were indistinguishable from the normal enzyme. Antibodies were blocked 1 h prior to use by the addition of an excess of unlabelled extract prepared from the *cyr1*$^-$ strain. This treatment blocked antibodies which reacted nonspecifically to proteins carried in both strains, but did not block the antibodies to adenylyl cyclase, since TC41-1 has no gene for the protein.

The *CYR1* Gene Product is a 205 kDa Protein

Under these conditions, both the fusion protein antibodies (Fig. 2A) and the synthetic peptide antibodies (Fig. 2B) precipitated a 205 kDa band which was prominent in the HR125::pAC2 extract and was completely absent in the TC41-1 extract. The size of this protein was consistent with the 220 kDa predicted from the amino acid sequence of *CYR1* [7]. This band was also observed in wild-type strains at intensities which were roughly proportional to enzyme activity (not shown), however in these strains the signal was more difficult to detect.

Taken together, the data indicate that this 205 kDa protein is the *CYR1* gene product. The protein is present in overexpressing cells, as well as wild type, but not in the TC41-1. The same polypeptide is recognized by antibodies obtained by two different strategies. Furthermore the apparent size of the protein closely approximates the molecular weight predicted from the cDNA sequence.

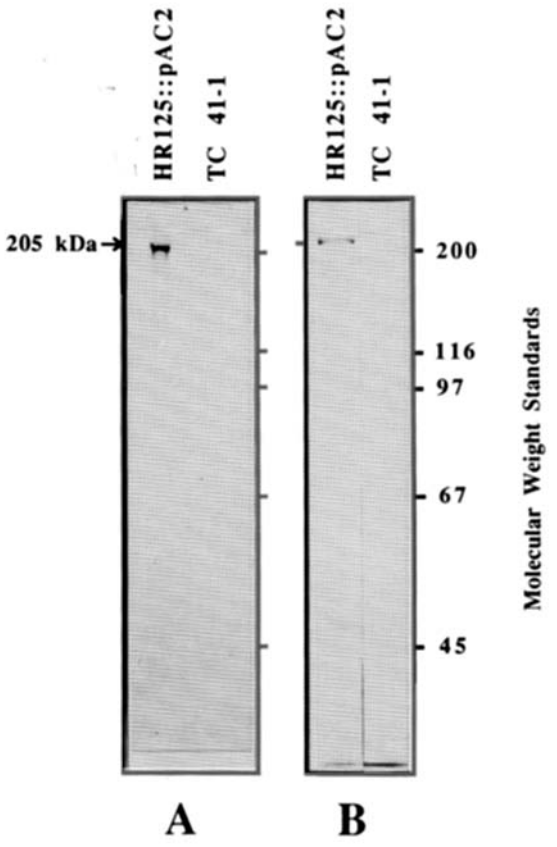

Fig. 2. Immunoprecipitation of yeast adenylyl cyclase. [^{35}S]methionine, labelled extracts from TC41-1, the *CYR1* deletant, or HR125::pAC2, the *CYR1* overexpressor, were prepared and immunoprecipitated as described in Methods. Each sample contained 61 × 10^6 cpm. **A:** Immunoprecipitations using anti-fusion protein antiserum. **B:** Immunoprecipitation with antipeptide antiserum. Autoradiograms were exposed for 16 h.

With overnight exposure as in Figure 2, only the 205 kDa protein can be seen; this was the case in all of the experiments shown. However, at longer exposure times multiple immunoreactive proteins can be identified between 150 and 205 kDa (Figs. 3B, 6). Several points can be made about these bands: They are specific to the *CYR1*$^+$ strain; they are present at much lower levels than the major band at 205 kDa, requiring approximately tenfold longer exposures to achieve comparable density; the relative amounts of these bands vary somewhat between membrane preparations; similar bands can be observed on immunoblots of yeast overexpressing *CYR1* with a Gal 10 promoter (not shown). Because these proteins are *CYR1* specific and yet present at much lower levels than the 205 kDa protein, they probably represent minor degradation products of the 205 kDa protein rather than non specific binding, or proteins associated with the enzyme at stoichiometric.

Proteolytic Activation of Adenylyl Cyclase is Associated With Cleavage of the *CYR1* Gene Product

Adenylyl cyclase activity in membranes treated with low concentrations of trypsin increases severalfold; as proteolysis progresses, the enzyme is released into the superna-

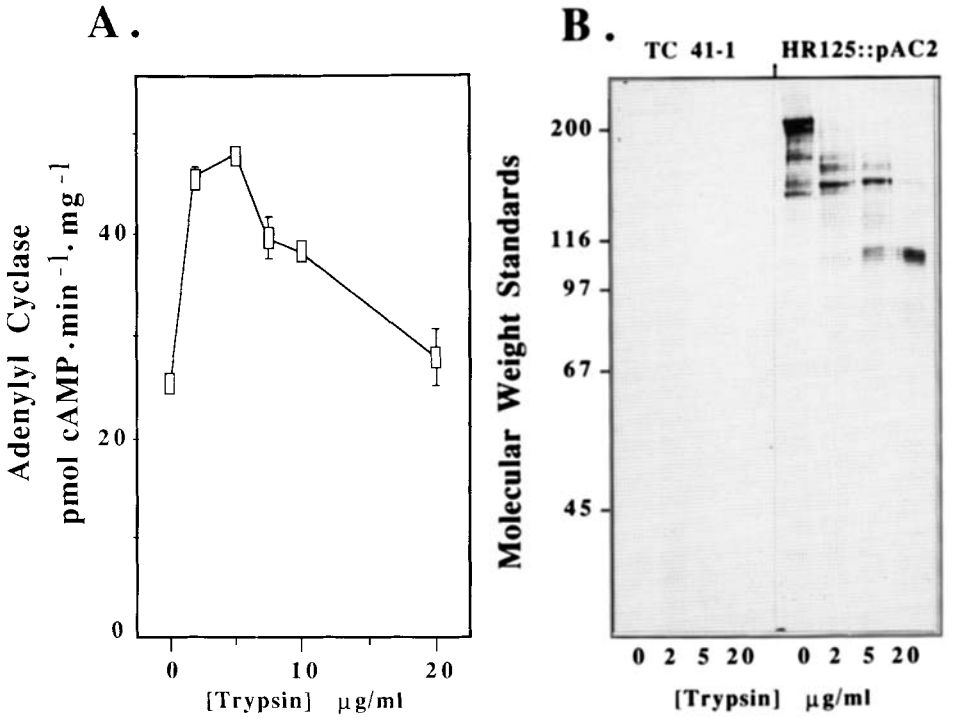

Fig. 3. Trypsin activation of adenylyl cyclase in yeast membranes. Yeast particulate fractions (0.5 mg/ml) from either TC41-1, the *CYR1* deletant, or HR125::pAC2, the *CYR1* overexpressor, were incubated with trypsin at the indicated concentration for 3 min at 30°C, the reaction was stopped with soybean trypsin inhibitor 0.5 mg/ml, and the mixture was centrifuged 15 min at 10,000*g*. **A:** Unlabelled pellets were resuspended and assayed for adenylyl cyclase activity. **B:** [^{35}S] pellets, 58 $\times 10^6$ cpm/point, were extracted and immunoprecipitated using anti-fusion protein antiserum as described in Methods. The autoradiogram was exposed for 180 h.

tant (ref. 9, Figs. 3, 4). Figure 3 correlates tryptic activation of membrane bound adenylyl cyclase with proteolysis of the *CYR1* protein. Low concentrations of trypsin activated membrane bound adenylyl cyclase, while at higher concentrations activity bound to the membrane falls as the enzyme is released from the membranes (Fig. 3A). A parallel immunoprecipitation experiment (Fig. 3B) demonstrated that activation was accompanied by cleavage of the *CYR1* protein; at 2 μg/ml trypsin the immunoreactive 205 kDa protein was almost completely destroyed. A number of proteolytic products were observed: At the range of trypsin concentrations producing maximal activity (2–5 μg/ml), several products in the 150–180 kDa range were observed, with the most prominent band at 155 kDa. These bands disappeared at higher trypsin concentrations (20 μg/ml), and were replaced by a product of approximately 110 kDa. The loss of the 205 kDa *CYR1* protein during trypsin activation is consistent with, but does not prove, our model in which adenylyl cyclase is regulated by an inhibitory domain of the enzyme.

Tryptic Release of Adenylyl Cyclase Activity From the Membrane is Associated With Release of a 145 kDa Immunoreactive Fragment

The observation that trypsin releases adenylyl cyclase activity from the membrane [9] indicated the possibility that a membrane anchoring domain had been cleaved from

the enzyme. Figure 4 shows that the release of activity from the membranes by trypsin is paralleled by the release of immunoreactive proteins. At relatively low trypsin concentrations (5–20 μg/ml) a 145 kDa fragment was released. At higher trypsin concentrations (10–50 μg/ml) a 100 kDa fragment was the predominant immunoreactive species released. Release of the enzyme from the membrane may be the result of the loss of a membrane anchoring domain. If so, this domain is not likely to lie in the middle of the protein, in that a 145 kDa fragment would retain this region of the protein. It is also possible, however, that release results from tryptic cleavage of an unidentified protein linking the adenylyl cyclase to the membrane.

Expression of *CYR1* Proteins Lacking Amino Terminal Sequence Produces Unregulated High Activity

Adenylyl cyclase activity is markedly elevated in two yeast strains which produce altered *CYR1* polypeptides. The first strain, 102R-10, is a mutant that produces adenylyl cyclase activity that is greatly elevated over that of the parental wild type AM3-4B (Table I). This mutant was isolated as a spontaneous cAMP independent pseudorevertant of a *cyr1* mutant, AM18-5C. Immunoprecipitation experiments show that this strain produces a truncated *CYR1* protein of approximately 100 kDa in place of the normal 205 kDa protein (Fig. 5). This altered *CYR1* protein has not been identified in any other strain examined. Interestingly, we were unable to detect any *CYR1* protein in

TABLE I. Adenylyl Cyclase Activity in Yeast Particulate Fractions*

Strain	Adenylyl cyclase activity (pmol cAMP · min^{-1} · mg^{-1})		
	GDPβS	GppNHp	Mn^{2+}
AM3-4B[a] (wild type) *αade 6 ade10 amp 1 cam 1 cam 2 cam 3*	0.3 ± 0.17	0.9 ± 0.04	2.6 ± 0.45
AM18-5C[a] (*cyr1*) *cyr1* mutant of AM3-4B	<0.1	<0.1	<0.1
102R-10[b] (*cyr1* pseudorevertant) cAMP independent revertant of AM18-5C	12.2 ± 0.76	12.2 ± 0.48	73.7 ± 6.51
HR125::pAC2[c] (Multi copy *cyr1*) a*leu 2-3 leu 2-112 ura 3-52 his 3-532 his 4 trp 1-1::YEp24: CYR1-2*	3.8 ± 0.30	26.1 ± 0.51	30.6 ± 0.58
TC41-1[c] (*cyr1* deleted) a*leu 2-3 leu 2-112 ura 3-52 his 3-532 his 4 trp 1-1::pCYR1 Δ5*	<0.1	<0.1	<0.1

*Yeast crude membrane fractions were assayed for adenylyl cyclase in the presence of 5 mM $MgCl_2$ and either 100 μM GDPβS, 100 μM GppNHp, or 5 mM $MnCl_2$ for 30 min at 30°C.

[a]Matsumoto *et al.* [5].

[b]Casperson *et al.* [6].

[c]Materials and Methods.

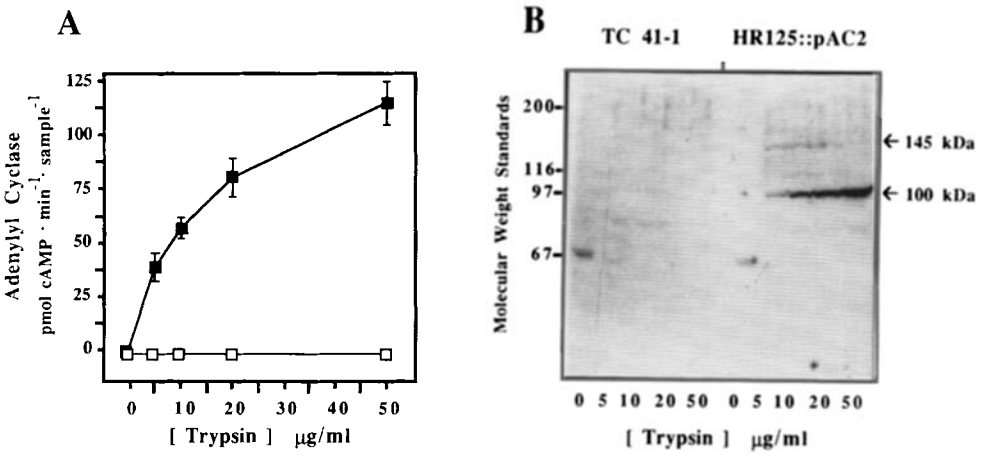

Fig. 4. Trypsin release of adenylyl cyclase from yeast membranes. Yeast particulate fractions, 15 mg per sample, were incubated with the indicated trypsin concentration for 6 min at 30°C in a volume of 1.5 ml. The reaction was stopped with soybean trypsin inhibitor 0.5 mg/ml, centrifuged 15 min at 10,000*g*, and the supernatant fractions were **A**) assayed for adenylyl cyclase activity (triplicate 75 μl aliquots) and **B**) precipitated with trichloroacetic acid (1 ml aliquots) for immunoblotting using anti-fusion protein antiserum. Closed symbols represent the HR125::pAC2 strain; open symbols, TC41-1.

the parent *cyr1*⁻ strain AM18-5C (not shown). We have identified neither the site of the original *cyr1* mutation, nor the nature of the mutation that caused the reversion. However, because the truncated *CYR1* protein in 102R-10 retains activity, and thus at least part of carboxy-terminal catalytic domain, most if not all of the protein lost must come from the amino terminus.

The second strain producing an altered enzyme was made by transforming a *cyr1*⁻ strain with pAC103, a plasmid containing an in-frame deletion in the *CYR1* gene. This plasmid encodes a *CYR1* protein lacking amino acids 27–1,233 (Fig. 1). Yeast carrying pAC103 express an immunoreactive protein of approximately 88 kDa, matching the size predicted for the remaining coding region. This result provides additional confirmation that the *CYR1* gene product is the 205 kDa immunoreactive protein. The shortened protein is produced at levels which are comparable to the level of expression of the full length protein by yeast carrying the control plasmid pAC2 (Fig. 6). However, the level of adenylyl cyclase activity produced by the strain carrying pAC103 is greatly elevated compared to yeast expressing the plasmid pAC2 carrying the wild-type gene (Table II).

Both strains expressing altered *CYR1* polypeptides produce enzyme activity which matches that of trypsin activated membranes: the activity is elevated, it cannot be further elevated by trypsin (not shown), and it is insensitive to guanine nucleotides. The fact that trypsin activation and stimulation by guanine nucleotides are not additive suggested that both agents shared a common pathway. An alternative explanation, that trypsin destroyed the *RAS* proteins while activating adenylyl cyclase, could not be ruled out. However, in strains carrying genetically shortened *CYR1* proteins, increased adenylyl cyclase activity was also associated with loss of guanine nucleotide sensitivity. This indicates that the loss of guanine nucleotide sensitivity is due to changes on the *CYR1* polypeptide itself rather than an effect on the *RAS* proteins.

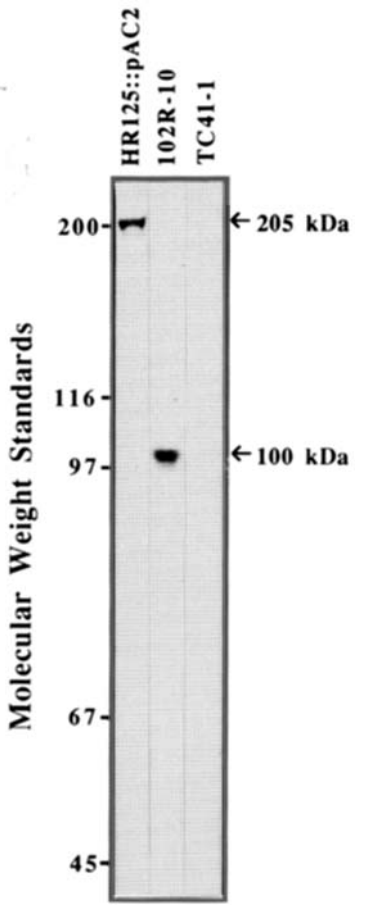

Fig. 5. Immunoprecipitation of 102R-10. Extracts from [^{35}S]-labelled cells of the indicated strains (61×10^6 cpm per sample) were immunoprecipitated using anti-fusion protein antiserum as described in Methods. The dried gel was autoradiographed for the indicated time.

DISCUSSION

Yeast adenylyl cyclase is a potentially useful genetic and biochemical model system for analyzing the molecular mechanism of GTP dependent regulation of enzyme activity. Biochemical evidence, combined with the genetic deletion experiments of Kataoka et al. [7], formed the basis of a working model of the regulation of adenylyl cyclase by *RAS* proteins [9]. The model divided the protein into three functional domains: 1) an amino-terminal inhibitory domain postulated to mediate *RAS* activation; 2) a proposed membrane binding domain located in the middle of the protein; and 3) a catalytic domain located near the carboxy-terminus. The present evidence confirms the first tenet of the model, regarding location of the proposed inhibitory domain. As we discuss below, the data contradict the membrane binding part of our model. How the enzyme is attached to the membrane remains unclear.

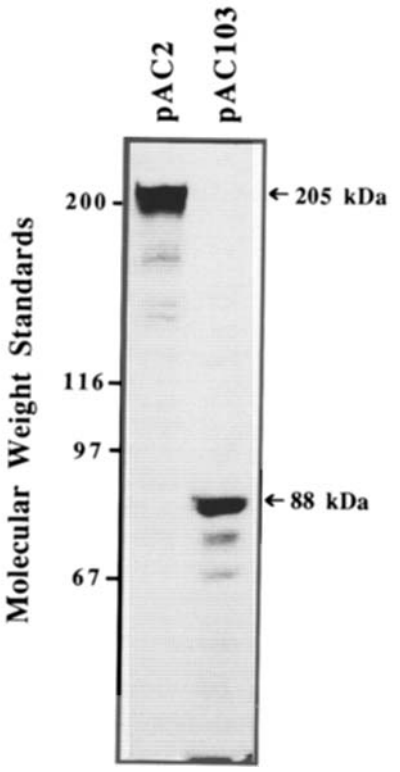

Fig. 6. Immunoprecipitation of NW23-9C::pAC103 adenylyl cyclase. Extracts from NW23-9C yeast, transformed with either wild-type plasmid, pAC2, or the plasmid carrying the in-frame deletion, pAC103, were labelled with [^{35}S]methionine and immunoprecipitated with fusion protein antibodies as described in Methods. Each sample contained 40 × 10^6 cpm. The autoradiogram was exposed for 120 h.

TABLE II. Adenylyl Cyclase Activity in Yeast Expressing a Truncated Adenylyl Cyclase*

	Adenylyl cyclase activity (pmol cAMP · min^{-1} · mg^{-1})		
Strain	GDPβS	GppNHp	Mn^{2+}
NW23-9C::pAC2[a] (wild type *CyR1-*) a*leu 2-3 leu 2-112 ura 3-52 his 3-532 his 4 trp 1-1 cyr1-1:: YEp24:CYR1-2*	1.4 ± 0.10	7.8 ± 0.58	27.0 ± 0.91
NW23-9C::pAC103[b] (truncated *cyr1*) a*leu 2-3 leu 2-112 ura 3-52 his 3-532 his 4 trp 1-1 cyr1-1:: pAC103*	6.9 ± 0.25	6.6 ± 0.32	128.0 ± 19.6

*Yeast crude membrane fractions were assayed for adenylyl cyclase in the presence of 5 mM $MgCl_2$ and either 100 μM GDPβS, 100 μM GppNHp, or 5 mM $MnCl_2$ for 30 min at 30°.
[a]Casperson *et al.* [6].
[b]Materials and Methods.

Antibodies Identify the *CYR1* Gene Product

We have identified the *CYR1* gene product by a number of criteria: 1) Abundance of the immunoreactive protein correlated with manipulations of *CYR1* gene expression. 2) The size of the protein corresponds to the length of the open reading frame in the cDNA sequence reported by Kataoka et al. [7]. 3) This size varies with alteration of the open reading frame. 4) The protein is recognized by different antisera obtained by differing strategies. Field et al. [18] have reported the immunopurification of a recombinant *CYR1* protein by using an epitope tagging method to add a short amino-terminal leader, creating an immunoreactive fusion protein. The size of the *CYR1* protein purified by this method is consistent with our results.

None of the antisera precipitated adenylyl cyclase activity under native conditions. This may have resulted from our use of SDS denatured protein as the immunogen. Indeed, we were unable to precipitate the [^{35}S]-labelled 205 kDa protein unless it was denatured by SDS.

Biochemical and Genetic Evidence for an Inhibitory Domain in the *CYR1* Protein

Our previous model [9] placed the inhibitory domain at the amino-terminal end of the *CYR1* protein. Without anti-*CYR1* antibodies, however, we were unable to determine whether trypsin acted directly on the enzyme. The present experiments show that proteolytic activation is associated with tryptic cleavage of the *CYR1* protein.

Because the effects of trypsin in a crude membrane system are not confined to adenylyl cyclase, we cannot rule out cleavage of other proteins as the cause of the activation that we observed. However, genetic truncation of the *CYR1* protein produced the same effect: enzyme activity was elevated; it was not sensitive to guanine nucleotides, and it was not further activated by trypsin. This indicates that a portion of the *CYR1* protein normally inhibits catalytic activity.

The inhibitory domain can be localized to the largest region removed in common between the trypsin activated enzyme and the genetically altered enzyme. Experiments with the genetically altered strains indicate that this domain lies in the amino terminal half of the protein. Enzyme encoded by pAC103 lacks amino acids 27–1,233. The 102R-10 pseudorevertant is presumably missing a smaller region, in that it produces a larger protein; the largest possible amino-terminal deletion in this strain would be from amino acids 1 to approximately 980.

Experiments with the genetically altered *CYR1* protein place the inhibitory domain somewhere within the amino-terminal 50% of the protein. Data from the trypsin activation experiment shown in Figure 3 suggest that it is sufficient to remove only the amino-terminal 25% of the *CYR1* protein to produce the activated phenotype. Activation by trypsin correlated well with the disappearance of the 205 kDa band and the production of several proteolytic products between 150 and 180 kDa. At increased concentrations of trypsin, these products disappeared and were replaced by a 110 kDa product. The finding that maximal activation is reached at trypsin concentrations producing *CYR1* cleavage products of 150–180 kDa suggests that loss of 25% or less of the *CYR1* protein is sufficient to activate the enzyme.

This model rests on the assumption that at least one of the proteolytic fragments observed is catalytically active. Although we have not demonstrated catalytic activity in these fragments, several points are in favor of this presumption: These fragments are the

only tryptic products observed at the trypsin concentration that produces activation. The most prominent fragment produced during activation is observed at approximately 155 kDa, consistent with cleavage within an unusual amino acid sequence (residues 470–484) that contains 6 lysines and 4 arginines in a space of only 15 amino acids. Cleavage at this site would produce an immunoreactive, catalytically active fragment of the observed molecular weight. Production of the 155 kDa fragment, and other fragments of similar size correlates well with enzyme activation by trypsin.

These estimates assume that the fragments have not been cleaved at the carboxy-terminus; the putative inhibitory region would be even smaller if these fragments have also lost a portion of the carboxy-terminus. It remains possible that the tryptic fragments observed are inactive, and that enzyme activity actually resides on a fragment not observed in the experiment. Such a result would be possible if trypsin cleaved the catalytic domain into fragments too small to be resolved by the gel, or if the epitopes recognized by the anti fusion protein antibody lie only outside of the catalytic domain.

Other Mutations That Increase Adenylyl Cyclase Activity

The postulated inhibitory domain of *CYR1* would be a likely target for other activating mutations. Several reported mutations, at loci mapped at or near the *CYR1* gene, increase adenylyl cyclase activity. *IAC* mutants, isolated by Uno et al. [18], produce elevated adenylyl cyclase activity. Cannon et al. [19] isolated *SRA4* as a *ras2* revertant that accumulates elevated cAMP levels. Although uncharacterized in terms of guanine nucleotide sensitivity and protein size, these mutations may be equivalent to the mutation that caused the 102R-10 reversion. Two point mutations close to the catalytic domain have also been described. De Vendittis et al. [20] have described a mutation at position 1651 that activates the enzyme and produces overstimulation by *RAS*, while a nearby mutation at position 1547 [21] changes the specificity for *RAS* interaction. Kataoka et al. [7] reported greatly elevated adenylyl cyclase activity in yeast expressing carboxy-terminal fragments of *CYR1*, but attributed this to increased expression of the enzyme.

Proteolytic Release of Activity

Trypsin treatment released both enzyme activity and immunoreactive protein fragments from the membrane. If adenylyl cyclase is anchored directly to the membrane, the anchoring domain must have been removed by the trypsin treatment. Such a domain must be absent from the 145 and 100 kDa immunoreactive proteins released by trypsin (Fig. 4), yet present in the fragments of 150–180 and 110 kDa that are not released by trypsin. If trypsin releases the 145 kDa fragment in Figure 4 by removal of a membrane binding domain, this domain must lie within 600 amino acids from either the amino- or carboxy-terminus; cleavage any closer to the middle of the protein would release a smaller fragment. This indicates that the middle region of the protein does not bind directly to the membrane as has been previously proposed [7,9]. The amino-terminal region is also unlikely to serve this function, in that adenylyl cyclase produced by the revertant, pAC103, and enzyme activated by trypsin all remain bound to the membrane, and are all probably missing at least some amino-terminal sequence. Although location of an anchoring domain at the extreme carboxy-terminus cannot be ruled out, this region contains no obviously hydrophobic stretches of amino acids.

The data is more readily explained by a model in which adenylyl cyclase is bound indirectly to the membrane via an unidentified protein. In this model, tryptic release could be due to cleavage of the protein linking the adenylyl cyclase to the membrane, rather than the enzyme itself. Release from this protein might in turn lead to the production of the 145 and 100 kDa fragments when cleavage sites on the *CYR1* that are not normally accessible to trypsin become exposed. Such a model is in agreement with the apparent molecular weight of 600,000 for the detergent solubilized enzyme as measured by hydrodynamic techniques [9]. This large particle size indicates a complex of polypeptides. Because such a complex would probably not survive the immunoprecipitation conditions that we used, subunits other than the catalytic polypeptide would not have been identified. Identification of the proteins that make up this complex will allow a greater understanding of adenylyl cyclase in yeast.

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