THE CHARACTERIZATION OF THE *CYR1* AND *IRA1* POLYPEPTIDES OF THE ADENYLATE CYCLASE SYSTEM OF THE YEAST *SACCHAROMYCES CEREVISIAE*

by

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List of Abbreviations

ATP- adenosine triphosphate

BSA- bovine serum albumin

CAP- cyclase associated protein

cAPK- cAMP dependent protein kinase

cAMP- adenosine 3',5'-cyclic monophosphate

DNA- deoxyribonucleic acid

GAP- GTPase activating protein

GDP- guanosine diphosphate

GDPβS- guanosine 5'-O-(2-thiodiposphate)

GppNHp- 5'- guanylylimidodiphosphate

GTP- guanosine triphosphate

IBMX- 3- isobutyl-1-methylxanthine

kDa- kilodaltons

mRNA- messenger ribonucleic acid

OD- optical density

tS- temperature sensitive

ABSTRACT

THE CHARACTERIZATION OF THE *CYR1* AND *IRA1* POLYPEPTIDES OF THE ADENYLATE CYCLASE SYSTEM IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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The adenylate cyclase system of the yeast *Saccharomyces cerevisiae* contains many proteins, including: the *CYR1* polypeptide which is responsible for catalyzing formation of cAMP from ATP, *RAS1* and *RAS2* polypeptides, that mediate stimulation of cAMP synthesis by guanine nucleotides, and the GAP analogue *IRA1* (inhibitory regulator of the *RAS*/cAMP pathway). We have concluded that adenylate cyclase is only peripherally bound to the yeast membrane, based on the following criteria: 1. substantial activity is found in cytosolic fractions; 2. activity is released from membranes by the addition of 0.5 M NaCl; 3. antibodies to yeast adenylate cyclase identify a full length adenylate cyclase in both membrane and cytosolic fractions; 4. activity from both cytosolic fractions and NaCl extracts can be functionally reconstituted into membranes lacking adenylate cyclase activity. These results indicate the existence of a protein anchoring adenylate cyclase to the membrane.

We have also concluded that the *IRA1* polypeptide is a strong candidate for a protein involved in anchoring adenylate cyclase to the membrane, based on the following criteria: 1. A disruption of the *IRA1* gene produced a mutant with very low membrane associated levels of adenylate cyclase activity; 2. Membranes made from these mutants were incapable of binding adenylate cyclase in vitro; 3. Ira1 antibodies inhibit binding of adenylate cyclase to the membrane; 4. Ira1 and adenylate cyclase comigrate on Sepharose 4B.

Intracellular levels of cAMP play a major role in the regulation of growth in yeast, and may also be important in controlling growth arrest at the diauxic shift. The results of experiments examining the role of cAMP in growth arrest at the diauxic shift have shown: 1. yeast lower cAMP levels as they exhaust their glucose supply and shift to oxidative metabolism of ethanol; 2. a reduction in cAMP levels is essential for growth arrest at the diauxic shift; 3. this decrease in cAMP levels is accompanied by a drop in adenylate cyclase activity; and 4. the decrease in adenylate cyclase activity is associated with an increase in *IRA1* expression.

Thesis Plan

Scientists have been interested in how cells control their growth, especially as it relates to disease states such as cancer where cells can no longer control their growth. We have chosen to study the control of growth in yeast because more components involved have been identified in yeast then in mammalian cells, yet there is a great deal of conservation between the two systems. The specific regulatory system involved in controlling growth in the yeast *Saccharomyces cerevisiae*, is the *RAS*/adenylate cyclase system. This system is responsible for producing cAMP and also regulating the levels of cAMP within the yeast cell. Intracellular levels of cAMP have been shown to play a major role in the regulation of growth in yeast.

The overall goal of this project was to characterize, at the molecular level, the adenylate cyclase system in yeast. I decided to concentrate on the central component of the system, the *CYR1* polypeptide and one newly identified regulatory component, the *IRA1* polypeptide. Research in the yeast adenylate cyclase field has progressed rapidly in recent years and many new components have been identified. The genes for these components have been isolated and cloned. The problem however, is that little is known about the characteristics of the proteins these genes encode. In our lab we have tried to combine genetics, to take advantage of these cloned genes, with biochemical and molecular biology techniques to try and characterize the protein products. This approach has allowed us to learn a great deal about the *CYR1* and *IRA1* polypeptides and their roles in this system.

This thesis is divided into four parts. Part one is a general introduction comprised of two chapters. Chapter 1 explains the significance of this project and introduces the specific components of the adenylate cyclase system. Chapter 2 is devoted to describing the growth patterns in yeast and how their growth is controlled. Part two of this thesis outlines the roles of both the *CYR1* and *IRA1* polypeptides. Chapter 3 briefly introduces why we are interested in the characteristics of these two proteins. Data supporting the conclusion that adenylate cyclase in yeast is a peripheral membrane protein is presented in the fourth chapter. The fifth chapter details interactions between the *CYR1* and *IRA1* polypeptides. Part three investigates the role of the *RAS*/adenylate cyclase system in controlling yeast cell growth. Chapter 6 contains a set of experiments examining how *IRA1* expression correlates with cAMP levels and growth arrest. The final part is a summary of the overall findings of the project and the significance they might have on future studies of the yeast *RAS*/adenylate cyclase system.

PART 1

GENERAL INTRODUCTION

CHAPTER 1

The *RAS*/adenylate cyclase system of the yeast *Saccharomyces cerevisiae*

Significance

The overall goal of this project was to learn more about how cells control their growth. We hope that a better understanding of the processes involved will eventually help us to understand what happens in disease states, such as cancer, where cells can no longer control their growth. The yeast, *Saccharomyces cerevisiae*, was chosen as a model system for a number of reasons. First of all, yeast are unicellular eukaryotic organisms which have a nucleus containing chromosomes, yet they are very easy to work with and maintain in a laboratory setting. Another useful property of yeast is their ability to accept a gene that has been manipulated in vitro in place of the chromosomal copy of that gene (Rothstein 1983). Finally, regulation of growth by Ras[1] proteins in yeast is similar and relevant to the mammalian system, yet we felt that progress in the yeast system would be more rapid.

It is true that regulation of growth is a less complex process in yeast than in mammalian cells, however there definitely is a strong conservation of protein components used to regulate cellular proliferation in both systems. These include G proteins (Dietzel and Kurjan 1987; Miyajima et al. 1987), cyclic AMP dependent protein kinase (Hixson and Krebs 1980; Toda et al. 1987), Ras proteins (Papageorge et al. 1984; Powers et al. 1984), and GAP (Tanaka et al. 1989; Tanaka et al. 1990). The conservation in protein

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[1]Nomenclature: We are forced to refer to protein products that are named only by the genes that encode them. The use of the same name for gene and protein product is confusing. We will use the following conventions. When referring to genes, the letters are all the same case and italicised. *RAS*, *CYR1*, *CDC25* and *IRA1* are examples of yeast genes. The mammalian version of the ras gene is *ras*. The names of protein products of these genes will not be italicised, and the first letter will be capitalised. Thus the yeast proteins will be referred to as Ras, Cyr1, Cdc25, Ira1 etc. The product of the mammalian ras gene is called $p21^{ras}$, by convention.

components suggests that there are closely related systems controlling growth, even though the initial signals may be different.

Analysis of the Ras proteins found in yeast and mammalian cells shows the strong conservation between the two systems. Yeast have two homologous genes, *RAS1* and *RAS2*, encoding proteins which share homology with the protein products of three human ras proto-oncogenes, designated as p21^{ras} (Barbacid 1987). The Ras proteins also share sequence homology with other GTP binding proteins such as G proteins and factors involved in protein synthesis. These proteins cycle between an active GTP bound state and an inactive GDP bound state, depending on the inputs they receive (Gilman 1987). Examples of inputs that influence this cycle are G protein coupled receptors which can promote the active GTP bound state and the GTPase activating protein (GAP) which promotes the inactive GDP bound state (McCormick 1989; Trahey and McCormick 1987).

Certain mutations can block the GTPase activity of Ras proteins and lock them in an active GTP bound state. These GTPase blocking mutations, when found in p21^{ras}, are associated with many forms of cancer (Bos 1989). In mammals, oncogenic mutations, which produce activated p21^{ras} proteins appear to be an early step in malignant transformation of cells. These activated proteins may promote cellular proliferation, which then allows additional genetic defects that ultimately result in transformation of cells (Kumar et al. 1990).

Ras proteins in yeast are also important in the regulation of cellular proliferation. In *Saccharomyces cerevisiae*, Ras proteins are essential for the decision to enter mitosis. Without functional Ras proteins, yeast fail to enter mitosis and become permanently arrested in G_1 (Kataoka et al. 1984).

Normal yeast arrest in G_1 when proper nutrients are no longer available. This growth arrest state helps to protect yeast from temperature shock and starvation. An opposite effect is seen in yeast with activated Ras proteins. They fail to arrest growth in response to nutrient limitations (Kataoka et al. 1984). Conservation between the two systems is shown by the fact that mammalian $p21^{ras}$ proteins can substitute for yeast Ras proteins (DeFeo-Jones et al. 1985; Kataoka et al. 1985). Yeast which lack *RAS* genes can be rescued from G_1 arrest upon addition of a human *ras* gene and yeast containing a mutated *ras* gene (encoding an activated $p21^{ras}$ protein) are unable to arrest growth in G_1 (Kataoka et al. 1984; Toda et al. 1985).

The major problem in studying the mammalian $p21^{ras}$ system is we do not know what signals are turning on ras and we also do not know the effector for the activated ras proteins. One possible candidate for an effector is the GTPase activating protein, GAP. GAP is known to accelerate the GTPase activity of $p21^{ras}$, therefore playing a role in turning off the ras proteins. What is interesting is that the region of $p21^{ras}$ that is necessary for interaction with GAP corresponds to the "effector domain", which was previously shown to be needed for transforming activity but is not involved in GTP binding. This led to the hypothesis that GAP may be an effector for $p21^{ras}$ (McCormick 1989). In yeast, more of the components and/or signals involved have been identified. We know that nutritional signals such as the presence of glucose are at least in part responsible for turning on Ras proteins (Kappeli 1986). Also, the effector for Ras proteins in yeast is known to be adenylate cyclase (Matsumoto et al. 1985). Several other proteins have been identified which play a role in regulating the *RAS*/adenylate cyclase system in yeast; these will be discussed later.

Historical Perspective

Cellular proliferation in the yeast, *Saccharomyces cerevisiae*, is controlled at least in part, by the adenylate cyclase system. This system appears to be tightly regulated and contains numerous proteins. The main component of this system is the product of the *CYR1* gene, the adenylate cyclase enzyme, which catalyzes formation of cyclic AMP (cAMP) from ATP (Casperson et al. 1985; Kataoka et al. 1985). Acting as a second messenger, cAMP binds to the regulatory subunit of the cAMP-dependent protein kinases, encoded by the *BCY1* gene (Toda et al. 1987a), allowing the catalytic subunits, encoded by the *TPK1-3* genes (Toda et al. 1987b), to become active. The protein kinases phosphorylate proteins leading to metabolic changes within the cell and ultimately cell growth. What is the initial signal responsible for turning this system on? It is believed that nutritional signals from outside the cell, such as the presence of a fermentable carbon source, like glucose, help to turn on the system (Kappeli 1986). This initial signal then activates the Ras GTP binding proteins, encoded by *RAS1* and *RAS2* (DeFeo-Jones et al. 1983; Powers et al. 1984) which activate adenylate cyclase in the presence of GTP (Toda et al. 1985). The activation is terminated when GTP is hydrolyzed to GDP. There are additional proteins which play a role in controlling the Ras-GTP/Ras-GDP cycle. The protein product of the *CDC25* gene is thought to be a positive regulator, responsible for loading the Ras proteins with GTP and therefore activating them (Camonis et al. 1986; Robinson et al. 1987; Broek et al. 1987). In contrast, the protein products of the *IRA1* (Tanaka et al. 1989)and *IRA2* (Tanaka et al. 1990) genes (inhibitory regulators of the *RAS-*

cAMP pathway) are thought to be negative regulators, responsible for hydrolyzing active Ras-GTP to inactive Ras-GDP.

CYR1 and *CAP*

The first evidence that cAMP was essential for yeast cell growth came from the isolation of mutants that were deficient in adenylate cyclase activity and required addition of exogenous cAMP for growth (Matsumoto et al. 1982). This led to the isolation and cloning of the *CYR1* gene, which encodes adenylate cyclase, in *Saccharomyces cerevisiae* (Casperson et al. 1985; Kataoka et al. 1985) . The *CYR1* gene has an unusually long open reading frame of 2026 amino acids (Kataoka et al. 1985). Wild type yeast seem to translate the entire open reading frame to produce a 205 kDa polypeptide (Heideman et al. 1990). The *CYR1* protein can be subdivided into 3 domains; a catalytic domain comprised of the final 417 carboxyl residues (Kataoka et al. 1985), a regulatory domain at the amino terminal end of the protein (Heideman et al. 1990) and the middle region of the protein containing a set of 20 repeats of a 28 amino acid consensus sequence which may play a role in membrane binding of adenylate cyclase (Mitts et al. 1990). At low ionic strength (no NaCl) in detergent solution adenylate cyclase activity migrates on Sepharose 4B as a very large particle, approximately 2,000 kDa, while at high ionic strength (0.5 M NaCl) the activity shifts to a smaller complex of only 600 kDa (Heideman et al. 1987). This was evidence that the adenylate cyclase system has an oligomeric structure of some kind.

Another component of the system is Cap (cyclase associated protein) which was originally identified as a 70 kDa protein that copurifies with a

Cyr1 fusion protein (Field et al. 1988). Cap has also been termed Srv2 (Fedor-Chaiken et al. 1990). In membranes made from yeast lacking *CAP*, adenylate cyclase activity was not stimulated by Ras2 proteins in vitro. This would suggest that Cap may play a functional role in *RAS*/adenylate cyclase signaling pathway. Yeast with a disrupted *CAP* gene and a $RAS2^{val19}$ gene are heat shock resistant and failed to grow on rich medium (Field et al. 1990). More work needs to be done before the role of Cap is understood.

PDE1 and *PDE2*

PDE1 and *PDE2* are genes encoding low affinity and high affinity cAMP phosphodiesterases respectively (Nikawa et al. 1987; Sass et al. 1986). These genes encode proteins which are responsible for the breakdown of cAMP. Yeast lacking both *PDE* genes have intracellular cAMP levels only two to three fold higher than levels seen in wild type yeast, while yeast with an activated RAS gene ($RAS2^{val19}$) and also lacking both *PDE* genes had 1,000 fold higher cAMP levels than wild type. This shows that positive control of cAMP metabolism by Ras proteins in *Saccharomyces cerevisiae* is also influenced by negative regulatory mechanisms which activated Ras proteins are no longer responding to (Nikawa et al. 1987).

RAS1 and *RAS2*

Adenylate cyclase activity is controlled in part by *RAS1* and *RAS2* genes which encode a pair of GTP binding proteins (Toda et al. 1985). Ras1 and Ras2 proteins, which are 36 and 40 kDa respectively, share strong sequence homology with the protein products of the mammalian $p21^{ras}$ proto-oncogenes (DeFeo-Jones et al. 1983; Powers et al. 1984). The most highly

conserved residues are located in the amino terminal half of the proteins and contain important structural features. In addition to structural homology, there is also functional homology between the two systems; shown by the fact that the yeast Ras proteins can be replaced with the mammalian homologue (DeFeo-Jones et al. 1985; Kataoka et al. 1985). Disruption of the *RAS* genes produce yeast arrested in G1 and yeast producing a constitutively activated *RAS2* (a valine for glycine substitution at position 19, corresponding to a mutation at position 12 in mammalian $p21^{ras}$ which produces cellular transformation) are unable to arrest growth (Kataoka et al. 1984; Toda et al. 1985). This is evidence that the *RAS*/adenylate cyclase pathway may be important in controlling growth of yeast.

The Ras1 and Ras2 proteins are characterized by a 180 amino acid domain that is strongly conserved with the mammalian Ras and a divergent region of approximately 120 residues (DeFeo-Jones et al. 1983; Dhar et al. 1984). Either of the *RAS* genes can promote growth. A disruption of either *RAS1* or *RAS2* alone does not effect growth in rich media. However yeast with both genes deleted fail to germinate and grow (Kataoka et al. 1984). Yeast Ras proteins bind GTP and GDP with high affinity and have intrinsic GTPase activity to hydrolyze GTP to GDP (Tamanoi et al. 1985; Temeles et al. 1985). At the carboxy terminal end of the 120 amino acid divergent region are 4 conserved residues that are necessary for transport to the membrane. There is a processing step which requires the *DPR1* gene product (Tamanoi et al. 1988) followed by an acylation which appears to involve Cys-319. Deletion of the last 4 residues or substitution of Cys-319 with Ser leaves yeast Ras proteins unable to localize to the membrane and the yeast are

nonviable. However if the mutant is over expressed viability is restored even though membrane associated Ras is not found (Deschenes and Broach 1987). It appears that localization of Ras to the membrane is needed for efficient function but not a requirement.

Disruption of the *RAS2* gene leads to hyperaccumulation of glycogen and trehalose and sporulation in the absence of nutrient deprivation. Yeast with an activated *RAS2*^{val19} fail to accumulate glycogen and trehalose and don't sporulate efficiently (Kataoka et al. 1984; Fraenkel 1985; Tatchell et al. 1985). Yeast with mutated *RAS2* ^{val19} have a phenotype similar to that seen in yeast with a *bcy1* mutation. Yeast with a *bcy1* mutation bypass the need for cAMP because they express constitutively activated cAMP dependent protein kinase (Matsumoto et al. 1982). Evidence that Ras proteins are indeed regulating adenylate cyclase activity is demonstrated by the fact that adenylate cyclase activity is high in activated *RAS2*^{val19} and low in *ras2* as compared to wild type. Also cAMP levels were fourfold higher in activated *RAS2*^{val19} and fourfold lower in *ras2* cells (Toda et al. 1985). Further evidence was found in experiments where addition of purified yeast Ras to membranes lacking *RAS* genes restored adenylate cyclase activity in a GTP dependent manner (Broek et al. 1985; Field et al. 1987).

In yeast grown on glucose, *RAS1* mRNA levels and Ras1 protein synthesis decrease as cells reach the mid logarithmic phase of growth (Breviario et al. 1986) while *RAS2* mRNA levels are high during all phases of growth and synthesis of Ras2 protein is low during early logarithmic growth (Breviario et al. 1988). When cells are grown in the presence of nonfermentable carbon sources *RAS2* mRNA levels are constant and Ras2

protein synthesis is increased during early logarithmic phase while *RAS1* mRNA and protein are diminished during growth on this source (Breviario et al. 1988).

BCY1,TPK1,TPK2 and *TPK3*

Intracellular cAMP can regulate cellular functions by turning on a cAMP dependent kinase which will in turn phoshorylate proteins. In mammalian systems the cAMP dependent kinases are comprised of a catalytic subunit responsible for phosphorylating proteins and a regulatory subunit which binds cAMP (Nimmo and Cohen 1977). Yeast have a similar cAMP dependent protein kinase which is a heterotetramer made up of two identical regulatory subunits and two catalytic subunits. The identical regulatory subunits are encoded by the *BCY1* gene (bypass for cAMP requirement) (Toda et al. 1987a) which bind cAMP The catalytic subunits responsible for phosphorylating proteins can be encoded by either *TPK1*, *TPK2* or the *TPK3* gene (Toda et al. 1987b). It appears that the three *TPK* genes are functionally redundant since the deletion of any two of the three produces no significant changes to the cell. Deletion of all three of the *TPK* genes is lethal (Toda et al. 1987b). The *TPK1*, *TPK2* and *TPK3* genes can encode proteins of 397, 380 and 398 amino acids respectively. These genes have greater than 75% amino acid homology over the carboxy terminal 320 residues which shows that indeed they encode related proteins (Toda et al. 1987b). The phosphorylation of target proteins through this pathway leads to a mobilization and utilization of carbohydrate reserves and ultimately cellular proliferation (Matsumoto et al. 1985; Deschenes and Broach 1989; Tatchell 1986).

CDC25 and *SCD25*

Ras1 and Ras2 proteins are regulated in part by the protein product of the *CDC25* gene. *CDC25* has an open reading frame of 1589 amino acids and translates a 180 kDa protein which appears to be membrane associated (Camonis et al. 1986; Garreau et al. 1990) Cells lacking *CDC25* have low levels of cAMP and decreased Mg^{+2} dependent adenylate cyclase activity. It is interesting to note that the lethality of a *CDC25* disruption can be suppressed by an activated $RAS2^{val19}$ but not by high copy expression of normal *RAS1* or *RAS2* genes, suggesting that normal Ras proteins depend on functional *CDC25* (Broek et al. 1987). It appears that only the carboxy terminus (712 residues) of *CDC25* protein is essential for complementation of the *cdc25-5* allele (Camonis et al. 1986). Mutationally activated *CDC25* alleles induce a phenotype similar to that seen in yeast strains with an activated *RAS*/adenylate cyclase pathway (Broek et al. 1987).

The phenotype of *cdc25* (tS) mutants is similar to *cyr1*(Ts) mutants (Pringle and Hartwell 1981). When *cdc25* (Ts) mutants are shifted to restrictive temperature (36°), cAMP levels decrease (Camonis et al. 1986). When *cdc25*(Ts) mutants are grown at permissive temperature (24°C) adenylate cyclase is stimulated two to three fold by GppNHp but when cells are shifted to restrictive temperature the stimulation by GppNHp is almost completely lost. This change is not seen in yeast with a wild type *CDC25* gene or the active C-terminal portion of the gene (Daniel et al. 1987). This is evidence in support of the model where the C-terminal portion of the Cdc25 protein is important for guanine nucleotide sensitive adenylate cyclase activity.

A newly identified component which may play a role in the adenylate cyclase system is the *SCD25* gene (suppression of the *cdc25-5* mutation) encoding a protein of 1251 amino acids which shares homology with the *CDC25* gene product (Boy-Marcotte et al. 1989). Expression of the 3' terminal portion (584 amino acids) of the *SCD25* gene is sufficient to suppress the requirement for *CDC25*. Expression of this DNA fragment will restore a normal level of cAMP in *cdc25-2* strains. *SCD25* is not able to overcome defects of a *cyr1* mutation, or a *ras1,ras2* double mutation (Boy-Marcotte et al. 1989). It has been shown that partially purified carboxyl terminal domain of the *SCD25* gene product enhances the loading of GTP onto the Ras2 protein by stimulating the release of GDP (Cre'chet et al. 1990).

IRA1 and *IRA2*

The activity of yeast Ras proteins appears to be regulated in part by the *IRA1* polypeptide. *IRA1* mutants have elevated cAMP levels, and disruption of the gene was found to suppress the lethality of *cdc25* mutations but did not suppress the lethality of either *ras1* or *ras2* mutations (Tanaka et al. 1989). The *IRA1* gene encodes a very large protein of 2947 amino acids (Tanaka et al. 1989). One function of the *IRA1* protein is to stimulate the GTPase activity intrinsic to Ras proteins and convert active, GTP bound, Ras to inactive, GDP bound, Ras (Tanaka et al. 1990). Ira1 shares sequence homology with proteins regulating mammalian ras; GAP (GTPase activating protein) (McCormick 1989) and NF-1 protein (neurofibromatosis type 1) (Buchberg et al. 1990; Wallace et al. 1990; Xu et al. 1990). In yeast it appears that one of *IRA1*'s functions is to turn off Ras, ultimately leading to decreased cAMP levels.

Another related gene has been identified, *IRA2*, which encodes another very large protein (3079 amino acids) and is 45% identical to the Ira1 protein (Tanaka et al. 1990). This protein also contains the same region of homology conserved between Ira1 and GAP and also appears to play a role in turning off Ras proteins (Tanaka et al. 1991).

CHAPTER 2
Control of Cell Growth and Division of *Saccharomyces cerevisiae*

Historical perspective

The yeast cell cycle

Saccharomyces cerevisiae can exist as haploid or diploid organisms. We have used only haploid yeast in these studies. Haploid cells can proliferate and give rise to two identical cells by going through a mitotic cycle. With proper nutrients available, yeast cells will double in number approximately every 2 hours. During the mitotic cycle, the 17 chromosomes of the haploid cell are duplicated and distributed to each cell (Pringle and Hartwell 1981). *Saccharomyces cerevisiae* are budding yeast with the "mother" cell producing a bud which will eventually become the daughter cell (Herskowitz 1988). The daughter cell will have all new cell surface material, and since it is smaller than the mother cell it must increase in size before it can itself begin chromosome duplication. The specific stages of the *Saccharomyces cerevisiae* mitotic cycle are: G_1 phase, which precedes the beginning of DNA replication, S phase, where chromosomal DNA is replicated, G_2 phase, which precedes mitosis and an M phase, where mitosis and nuclear division takes place. There is also a position within G_1 where yeast will arrest under certain conditions. Yeast will arrest growth under environmental circumstances such as lack of nutrients or the presence of another yeast cell which it can mate with. They arrest as unbudded cells and can and arrested cells are also capable of resuming growth if supplied with the proper nutrients (Herskowitz 1988).

Along with the known stages of the mitotic cycle, numerous other stage-specific functions have been identified by studying cell-division-cycle (*cdc*) mutants. A *cdc* mutation leads to a defect in a particular stage-specific

function of the cell cycle (Hartwell 1974; Hartwell 1978; Pringle 1978). The majority of *cdc* mutations in yeast produce blockage of events that are necessary for progression through the cell cycle (Pringle and Hartwell 1981).

Characteristic growth of yeast

Metabolism of growing yeast will depend on the strain, source of nutrients used, and other environmental parameters including temperature and availability of oxygen. When yeast are categorized based on their ability to metabolize glucose under carbon and oxygen limitation *Saccharomyces cerevisiae* would be labelled glucose-sensitive yeast exhibiting aerobic ethanol production in the presence of excess glucose (Kappeli 1986; Fiechter et al. 1981). The characteristic pattern of aerobic growth of *Saccharomyces cerevisiae* which includes a diauxic shift on glucose has been studied since the 1950's (Lemoigne et al. 1954; Beck and von Meyenberg 1968). The growth pattern is characterized by two consecutive biomass increases. In the first phase yeast grow rapidly utilizing glucose as a carbon source. As the glucose is consumed, biomass and ethanol are formed. When the supply of glucose is depleted, yeast temporarily stop growing, arrest in G_1, and shift metabolism from fermentation of glucose to oxidation of ethanol. This temporary growth arrest is called the diauxic shift. During the second phase of growth, ethanol is consumed and biomass is formed (Pringle and Hartwell 1981; Kappeli 1986). The overall proliferation of cells is dependent upon the availability of essential nutrients. Once the entire nutrient supply is depleted, yeast cells arrest growth in a specific pattern and enter a stationary phase. Yeast starved for a carbon source, ammonia, sulphate, biotin, or potassium will

result in uniform arrest of cells in the unbudded phase of the cell cycle (Hartwell 1974; Johnston et al. 1977; Lillie and Pringle 1980). These cells are capable of resuming growth if they are supplied with fresh nutrients (Herskowitz 1988).

Control of cell growth

It appears that there is a specific control point in the cell cycle where mating pheremones, nutrients, and events of the preceding cell cycle are able to influence cellular proliferation. This control point has been termed Start because its completion signifies the cell's commitment to complete the phases leading to mitosis, if normal conditions are present (Hartwell et al. 1974). Start is required for DNA synthesis, bud emergence and all known phases of the cell cycle. It can also be defined as the position where yeast will arrest growth due to nutritional limitations, presence of mating pheremones, or some *cdc* mutations (Pringle and Hartwell 1981).

Role of cAMP levels

Since adenosine 3',5'-cyclic monophosphate (cAMP) has been isolated, its existence has been found to be widespread in both prokaryotic and eukaryotic organisms (Bosford 1981; Pall 1981). Studies to try and understand cAMP function in eukaryotic cells have been done with mammalian cells and also yeast cells. Powerful genetic and molecular techniques that can be used to study yeast have increased its popularity as an experimental system. If cAMP is critical for the growth of yeast cells, then there should be cAMP requiring mutants. As expected a cAMP requiring mutant, *cyr1*, was isolated. Growth of this mutant was arrested in the

absence of cAMP and the strain behaved as a *cdc* mutant (Matsumoto et al. 1982). The *cyr1* mutant produced no detectable levels of cAMP and the defect was found to be in the gene for adenylate cyclase (Matsumoto et al. 1984).

Yeast adenylate cyclase, which is responsible for producing intracellular cAMP, has been shown to be regulated in part by the two GTP binding proteins Ras1 and Ras2 (Toda et al. 1985). A mutation which constitutively activates Ras2 ($RAS2^{val19}$) has much higher cAMP levels and produces increased levels of GTP dependent adenylate cyclase activity than that seen in wild type yeast (Toda et al. 1985). When *cyr1* mutant yeast growing in the presence of cAMP are shifted to medium lacking cAMP the cells will divide for one or two generations and then arrest. Examination of these yeast has shown that they arrest in G_1 in the absence of cAMP (Matsumoto et al. 1983). This G_1 arrest seen in *cyr1* mutants appears to be similar to the point of arrest seen in yeast deprived of nutrients (Matsumoto et al. 1983). All of this information together indicated that cAMP is needed at an early point in the G_1 phase of the yeast cycle.

Intracellular cAMP can regulate cellular functions by turning on a cAMP dependent kinase which will in turn phoshorylate proteins. In mammalian systems, the cAMP dependent kinases are comprised of a catalytic subunit responsible for phosphorylating proteins and a regulatory subunit which binds cAMP (Nimmo and Cohen 1977). Yeast have a similar cAMP dependent protein kinase, (Hixson and Krebs 1980) with a regulatory subunit encoded by the *BCY1* gene (Toda et al. 1987a) which binds cAMP and three interchangable catalytic subunits encoded by the *TPK1*, *TPK2* and *TPK3* genes (Toda et al. 1987b) which can phosphorylate proteins. The

phosphorylation of target proteins through this pathway leads to a mobilization and utilization of carbohydrate reserves and ultimately cellular proliferation (Matsumoto et al. 1985; Deschenes and Broach 1989; Tatchell 1986).

Role of *IRA1*

The protein product of the *IRA1* gene (inhibitory regulators of the *RAS*-cAMP pathway) is thought to be a negative regulator of the *RAS*-cAMP pathway (Tanaka et al. 1989). One known function of the Ira1 protein is its ability to stimulate the GTPase activity intrinsic to Ras proteins, converting active, GTP bound, Ras to inactive, GDP bound, Ras (Tanaka et al. 1990). By turning off yeast Ras proteins, the Ira1 protein is indirectly decreasing adenylate cyclase activity and therefore also causing a drop in cAMP levels within the cell. We have begun to characterize the Ira1 protein in order to better understand this role. We are specifically interested in the temporary growth arrest of yeast which occurs at the diauxic shift, and how cAMP levels and Ira1 contribute to this arrest of growth.

PART 2

ADENYLATE CYCLASE IN *SACCHAROMYCES CEREVISIAE* IS A PERIPHERAL MEMBRANE PROTEIN ANCHORED BY A COMPLEX CONTAINING IRA1

CHAPTER 3
INTRODUCTION; WHY CHARACTERIZE THE *CYR1* AND *IRA1* POLYPEPTIDES?

The initial part of this project was to characterize the *CYR1* polypeptide because it is the central component of the entire system. Because of the similarities between the yeast and mammalian systems many people assumed that yeast adenylate cyclase would be an integral membrane protein since the mammalian enzyme is. Presented in Chapter 4 is evidence which has led us to conclude that the yeast enzyme is a peripheral membrane protein.

During the initial portion of this project, a new component of the *RAS*/adenylate cyclase system was identified; the *IRA1* gene. The initial report was that this gene could encode a very large protein, approximately 330 kDa, which may be a negative regulator of Ras. The Ira1 protein had homology to mammalian GAP, but the region of homology covered only approximately 10% of the Ira1 protein. Since this protein was so large, we felt it might be playing additional roles in the system; so we set out to characterize it. Experiments presented in Chapter 5 show that the Ira1 protein is an integral membrane protein, involved in anchoring adenylate cyclase to the membrane.

CHAPTER 4
Adenylate Cyclase in *Saccharomyces cerevisiae* Is a Peripheral Membrane Protein

ABSTRACT

The adenylate cyclase system of the yeast *Saccharomyces cerevisiae* contains the *CYR1* polypeptide, responsible for catalyzing formation of cAMP from ATP, and two *RAS* polypeptides, that mediate stimulation of cAMP synthesis by guanine nucleotides. By analogy to the mammalian enzyme, models of yeast adenylate cyclase have depicted the enzyme as a membrane protein. We have concluded that adenylate cyclase is only peripherally bound to the yeast membrane, based on the following criteria: 1. substantial activity is found in cytoplasmic fractions; 2. activity is released from membranes by the addition of 0.5 M NaCl; 3. in the presence of 0.5 M NaCl, activity in detergent extracts has identical hydrodynamic properties to those of cytosolic or NaCl extracted enzyme; 4. antibodies to yeast adenylate cyclase identify a full length adenylate cyclase in both membrane and cytosol fractions; 5. activity from both cytosolic fractions and NaCl extracts can be functionally reconstituted into membranes lacking adenylate cyclase activity. The binding of adenylate cyclase to the membrane may have regulatory significance; the fraction of activity associated with the membrane increases as cultures approach stationary density. In addition, binding of adenylate cyclase to membranes appears to be inhibited by cAMP. These results indicate the existence of a protein anchoring adenylate cyclase to the membrane. The identity of this protein remains unknown.

INTRODUCTION

Adenylate cyclase is a pivotal enzyme in the cells of both mammals and yeast. In mammals, the enzyme serves signalling functions that are critical in many homeostatic mechanisms (Gilman 1987). In yeast, nutritional

signals are transmitted through adenylate cyclase, which in turn regulates cellular growth by altering cAMP levels (Matsumoto et al. 1985). Activity in both systems is modulated by GTP binding proteins, G_s and G_i regulate adenylate cyclase in animals (Gilman 1987), and *RAS1* and *RAS2* regulate adenylate cyclase activity in yeast (Toda et al. 1985). In addition to sharing similar biochemical functions, the *RAS* proteins share amino acid sequence similarities with the superfamily of GTP binding proteins that includes the G proteins and $p21^{ras}$ products of the *ras* proto-oncogenes (Barbacid 1987). Models of the yeast enzyme have been based loosely on past experience with the more thoroughly studied animal adenylate cyclase. Thus, it has been suggested that the *RAS* proteins may interact with some form of receptor protein in a manner analogous to that of the G-proteins . Candidates for genes encoding such proteins have been tentatively proposed (Broek et al. 1987; Camonis et al. 1986; Robinson et al. 1987; Tanaka et al. 1989). In a similar way, it has been presumed that the yeast enzyme might be anchored directly to the phospholipid bilayer, resembling the mammalian enzyme, which is known to be an integral membrane protein. The best illustration of this assumption has been the use of detergents to solublize the yeast adenylate cyclase from the membrane (Field et al. 1988; Heideman et al. 1987).

Previous studies have indicated that adenylate cyclase in detergent extracts behaves as a large particle on gel filtration and sucrose density centrifugation. In the presence of 0.5 M NaCl, the size of this complex is reduced to approximately 600 kDa (Field et al. 1988; Heideman et al. 1987). This is considerably larger than the size of a single catalytic subunit, indicating an oligomeric structure. We have conducted experiments to

probe the structure of the yeast adenylate cyclase enzyme complex. We present evidence indicating that yeast adenylate cyclase is not an integral membrane protein, but is attached to the membrane via some unidentified macromolecule. Activity can be extracted from the membranes by addition of NaCl. These extracts were used to functionally reconstitute activity into membranes devoid of endogenous activity, prepared from *cyr1*$^-$ yeast. Membranes prepared from *cyr1*$^-$ yeast grown at high cAMP concentrations were less effective in reconstituting activity than membranes from yeast grown at lower cAMP levels. These experiments imply the presence of a component that anchors adenylate cyclase to the membrane. This component may serve a regulatory as well as a structural role.

MATERIALS AND METHODS

Abbreviations. GppNHp, 5' guanylylimidodiphosphate; GDPβS, guanosine 5'-O-(2-thiodiphosphate); IBMX, 3-isobutyl-1-methylxanthine; cAPK, cAMP dependent protein kinase; unit of adenylate cyclase activity, 1 pmol cAMP/min.

Materials. Adenylate cyclase grade [α-^{32}P]ATP (40 cpm/pmol) was from Amersham, [^{3}H]cAMP (10,000 cpm/assay) was from New England Nuclear. [^{35}S]Methionine was from Irvine Chemical and Nuclear.

Media. Yeast were grown on either rich medium (YPD) containing 1% yeast extract, 2% bacto peptone, and 2% glucose, or minimal medium (SD) containing 6.7 g/l yeast nitrogen base, 2% glucose and the appropriate factors to support the growth of auxotrophic strains.

Expression of CYR1 proteins with in-frame internal deletions. Plasmids encoding *CYR1* proteins with in-frame internal deletions were prepared from the plasmid pAC2 (Casperson et al. 1985; Heideman et al. 1987). References to residue number are based on the predicted amino acid sequence of Kataoka *et al.* (1985). pAC103 was prepared by removal of a 3.7 kb *Sac*1 fragment as previously described. This produced an in-frame deletion of amino acids 27 to 1233. pAC104 was prepared by removal of 4.06 kb between the SacI site at position 739 and the SacII site at position 4801. The ends were blunted with Mung Bean Nuclease and religated to produce an in-frame deletion from amino acids 27 to 1381. pAC202 was produced by deletion of 1.5 kb between the EcoR1 sites at positions 1066 and 2572, producing an in-frame deletion from amino acids 135 to 636. pAC 203 was produced by deletion of a 0.88 kb KpnI to HpaI fragment, blunting the ends with T4 polymerase, and religation. This produced an in-frame deletion of amino acids 42 to 333. These plasmids and the wildtype pAC2 were used to transform the *cyr1-1 cam-* yeast strain NW23-9C (Casperson et al. 1985), using the lithium acetate method (Ito et al. 1983). Transformants were grown on minimal medium lacking uracil and supplemented with 1 mM cAMP.

Preparation of yeast particulate fractions. Yeast were grown to an optical density (O.D.) of approximately 2-4, at 660 nm. The cultures were centrifuged and cells were disrupted by vortexing with glass beads in YMB buffer, containing 50 mM 2[N-morpholino] ethanesulfonic acid pH 6.0, 0.1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, and 50 mg/ml leupeptin as previously described (Casperson et al. 1983). The homogenate was centrifuged in a Beckman J6, 5 min at 1000 rpm to remove

nuclei and unbroken cells. The supernatant was then spun 30 min at 14,000 rpm to separate particulate and soluble fractions. Both particulate and soluble fractions were stored in YMB at -70° until needed.

[^{35}S]-labelling of yeast proteins. Typically, a 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° overnight to a density of approximately 1 O.D.; and the cells were disrupted as described above.

Immunoprecipitation. ^{35}S-Labelled fractions 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 mg protein and approximately 90 x 10^6 cpm were extracted in 200 ml NDET.3 for 30 min at room temperature, the mixture was centrifuged 15 min in an Eppendorf microcentrifuge at 14,000 rpm, and the supernatant was incubated overnight at 4° with 2 mg of antibody #31 (Heideman et al. 1990). Antibodies were precipitated with 15 ml 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 1 ml cushion of 30% sucrose in 0.5 x 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 resolved on 5% polyacrylamide gels, the gels were stained, treated with Enhance®, and dried for autoradiography.

Reconstitution assays. Yeast membranes from a *CYR1*$^+$ strain (381G) were extracted by incubation in YMB containing 0.5 M NaCl, 30 min on ice. The suspension was centrifuged 15 min at 14,000 rpm in an Eppendorf

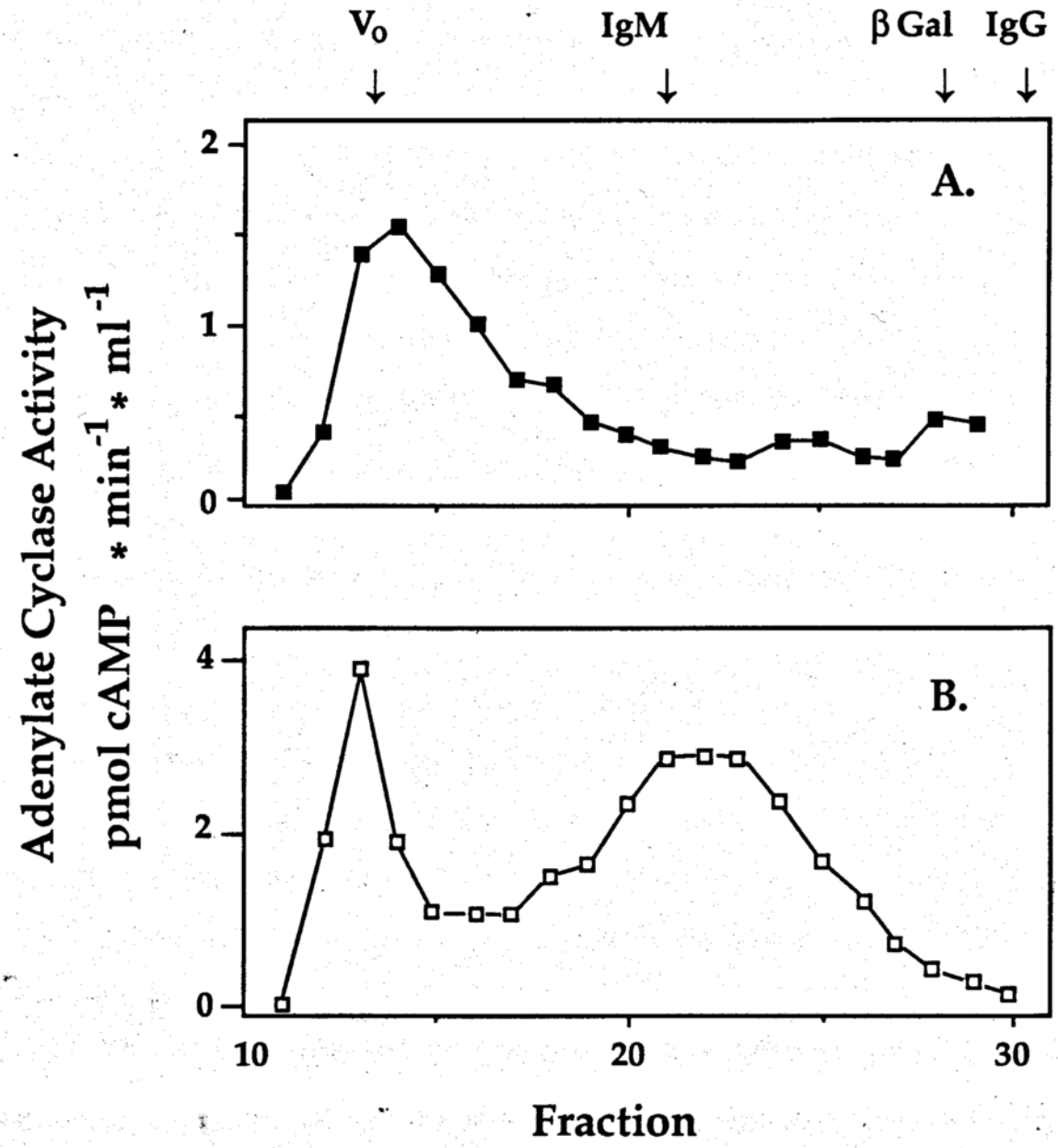
microcentrifuge (substituting 1 hr ultracentrifugation at 100,000 x g did not affect the outcome) and the supernatant was withdrawn. The extracts were then reconstituted by mixing with membranes prepared from a *cyr1*$^-$ strain (TC41-1) and reducing the NaCl concentration either by 10 fold dilution in reconstitution buffer (50 mM MES, 5 mM $MgCl_2$, pH 6.0) at 4° for 1 hr (or by dialysing against this buffer at 4° for 4 hrs where indicated). The membranes were then isolated by centrifugation for 30 min at 15,000 rpm and assayed for adenylate cyclase activity.

Adenylate cyclase assay. Adenylate cyclase was assayed by a modification of the method of Salomon et al. (Salomon et al. 1974) as described by Casperson et al. (Casperson et al. 1985). Guanine nucleotides, where present, were at 10 μM, manganese was at 10 mM. Protein was assayed by the method of Lowry et al with BSA as a standard. (Lowry et al. 1951).

RESULTS

Dissociation of adenylate cyclase activity from a large complex in detergent extracts. When we used gel filtration to measure the size of adenylate cyclase detergent solublized from the membrane with taurocholate, we found that the enzyme could exist as two different sized particles. At low ionic strength, the enzyme behaved as a very large particle of several million daltons, however, when 0.5 M NaCl was added, this size was dramatically reduced.

As shown in figure 1A, at low ionic strength, activity eluted from a Sepharose 4B column as a large particle, very near the void volume. Although beyond the range of the standards, this indicates a particle size of at least several million daltons. This does not represent incomplete

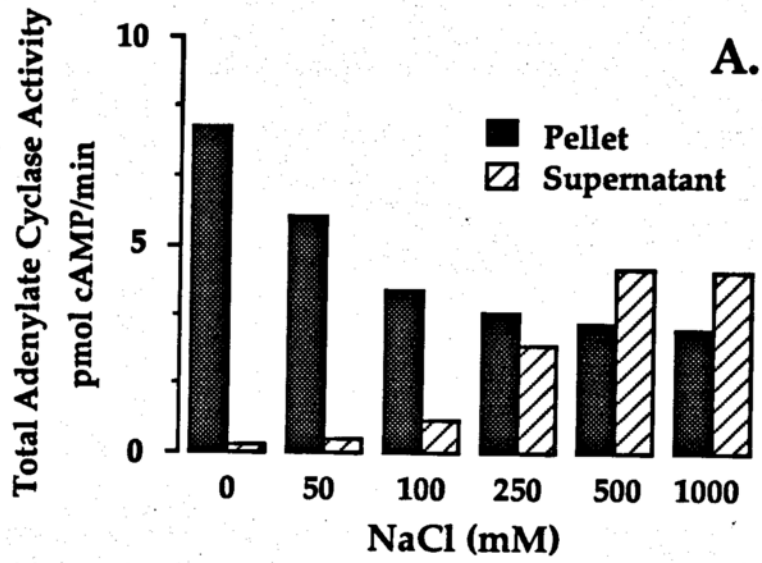

Vo
IgM
β Gal
IgG
A.
B.
Adenylate Cyclase Activity
pmol cAMP * min-1 * ml-1
Fraction
0
1
2
4
10
20
30

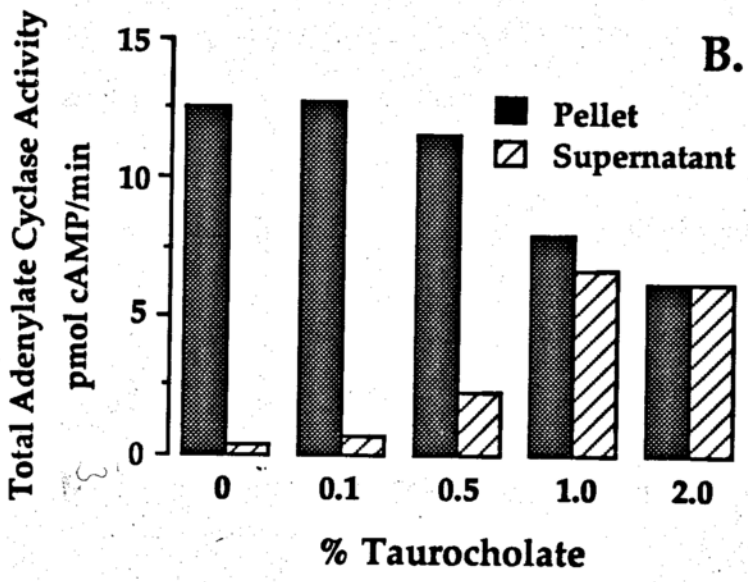
solublization; the enzyme remained soluble after centrifugation at 100,000 x g for 1 hour.

When 0.5 M NaCl was added to the detergent extracts, activity eluted as a much smaller particle, corresponding to a protein of approximately 600 kDa (fig. 1B and (Heideman et al. 1987)). A variable amount of activity generally migrated in the void volume, and apparently represents unsolublized enzyme, since it could be removed by spinning the sample at 100,000 x g prior to applying it to the column. Activity eluting in the included volume eluted at the same position when re-chromatographed (not shown). In the presence of NaCl, detergent could be omitted from the column equilibration buffer without altering the mobility of the adenylate cyclase activity.

These results indicate that in detergent extracts at low ionic strength, the catalytic *CYR1* protein is associated with a large complex, and that this association can be disrupted by addition of NaCl.

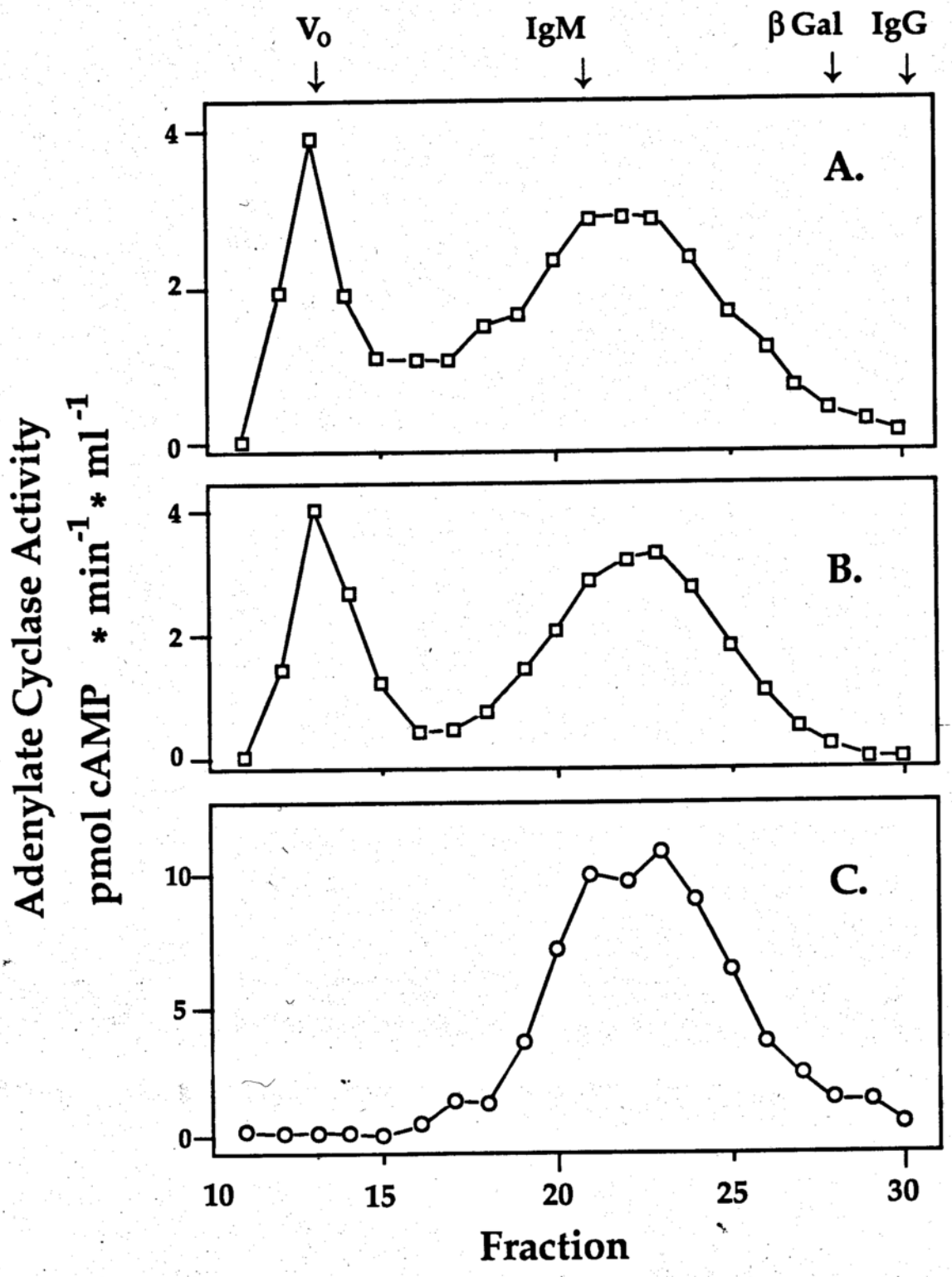
Release of soluble adenylate cyclase activity from membranes. The dissociation of adenylate cyclase activity from a larger particle induced by NaCl in detergent extracts was paralleled in membrane preparations. When crude membrane preparations were incubated with increasing concentrations of NaCl, adenylate cyclase activity was released from the membrane into the supernatant fraction (fig. 2A). Typically, we observed release of approximately 60-70% of the total activity into the supernatant. This is a rapid process, complete release took less than 5 min at 0°. Release was maximal at about 0.5 M NaCl. In most cases, a significant fraction of the total activity remained behind in the pellet fraction, and could not be released by the addition of more NaCl. As shown in figure 2B, a portion of

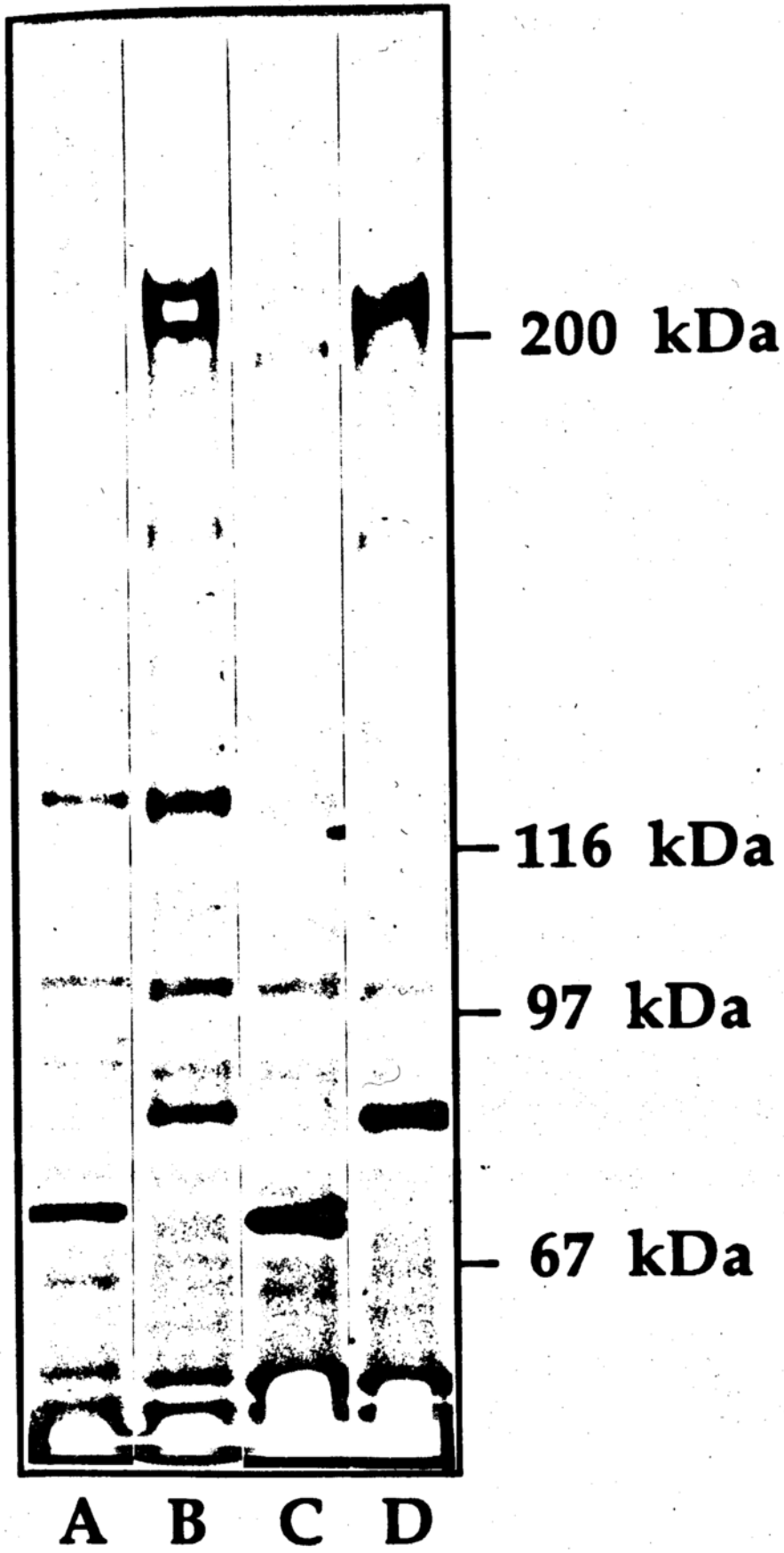

A.
Total Adenylate Cyclase Activity
pmol cAMP/min
10
5
0
Pellet
Supernatant
0
50
100
250
500
1000
NaCl (mM)


B.
Total Adenylate Cyclase Activity
pmol cAMP/min
15
10
5
0
Pellet
Supernatant
0
0.1
0.5
1.0
2.0
% Taurocholate

the particulate activity is also resistant to detergent extraction In the experiment shown, extraction with 2% taurocholate was effective in solublizing only 50% of the total activity from the pellet. We have screened several ionic and non-ionic detergents including cholate, deoxycholate, CHAPS, Lubrol PX, and Triton X-100, and have found taurocholate to be the most effective detergent for solublizing adenylate cyclase, both in terms of total amount and percent of activity released. Following extraction with NaCl, detergent extraction with 5% taurocholate was not effective in solublizing additional activity from the pellet. With either NaCl or detergent extraction, the activity remaining in the pellet must be in some way aggregated or associated with an insoluble component of the cell.

Location of adenylate cyclase activity. Because activity was so readily released from the membranes with NaCl, we examined supernatant fractions from yeast membrane preparations for adenylate cyclase activity. In all cases, we found a significant amount of activity in the soluble fraction produced during the disruption of the yeast. The percentage of soluble activity varied from 20 to 80% of the total adenylate cyclase activity, depending on the strain and conditions. Typically, about 50% of the total activity was found in the soluble fraction. We do not know whether this represents cytosolic activity, or activity released from the membrane by the cell disruption process. We will refer to this activity as cytosolic activity to distinguish it from soluble activity extracted with NaCl or detergent. Of the activity remaining in the membranes, about 60-70% (or approximately 30% of the starting total) could be released by the addition of NaCl (fig. 2A). Thus, about 80% of the total adenylate cyclase activity was either soluble upon disruption of the cell, or extractable with NaCl.


Vo
IgM
β Gal
IgG
A.
B.
C.
Adenylate Cyclase Activity
pmol cAMP * min-1 * ml-1
Fraction
0
2
4
5
10
15
20
25
30

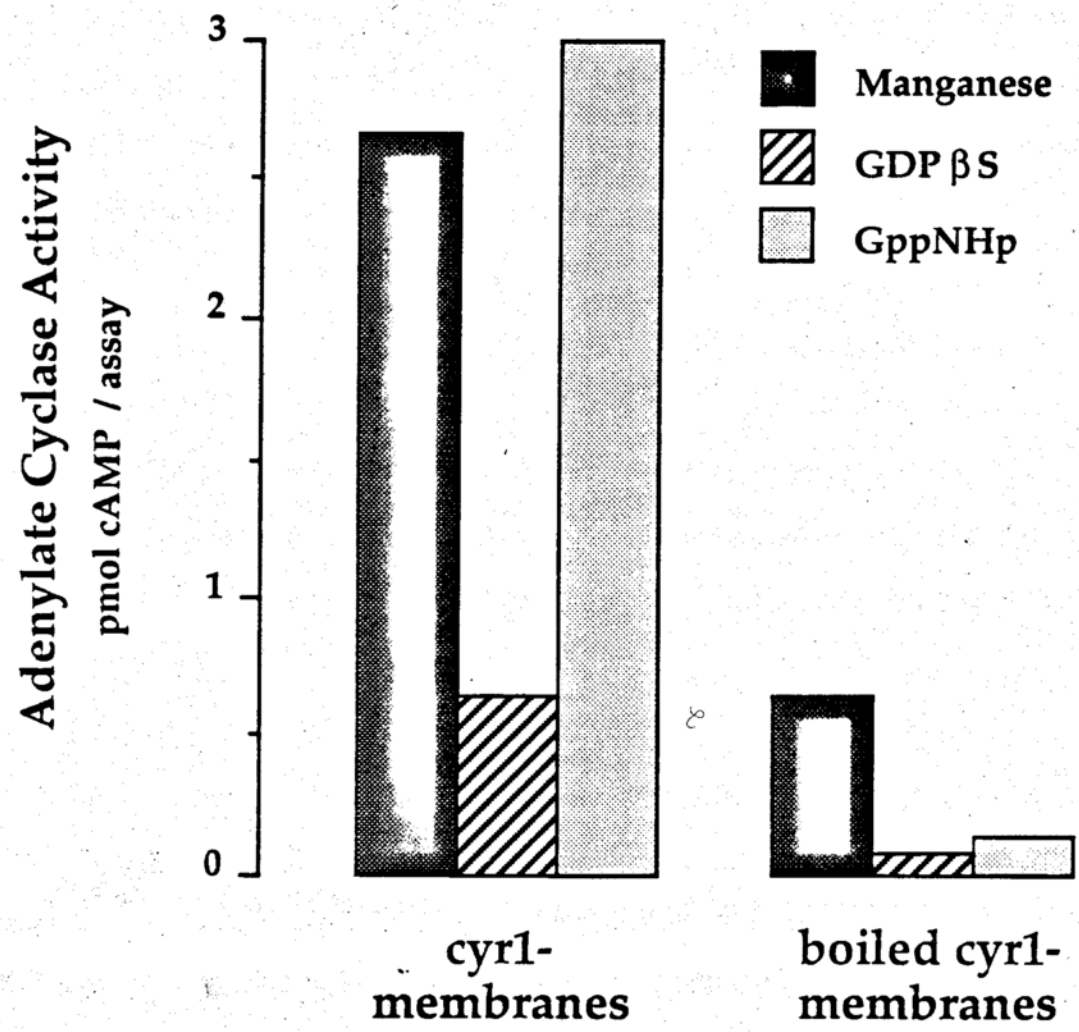

200 kDa
116 kDa
97 kDa
67 kDa
A
B
C
D

with the relative levels of adenylate cyclase activity observed in these two fractions.

Taken together, the results support a model in which adenylate cyclase is a soluble protein that can associate with a membrane bound protein to form an oligomeric complex. With detergents, adenylate cyclase can be extracted from the membrane as a part of this complex. The association of adenylate cyclase with this complex can be disrupted by NaCl, reducing the size of the enzyme in detergent extracts, or releasing activity from the membranes.

Reconstitution of soluble adenylate cyclase activity into membranes. In order to determine if dissociation of adenylate cyclase from the membrane is reversible, we reconstituted salt extracted adenylate cyclase from a wild type strain (381G) into membranes prepared from TC41-1, a *cyr1*$^-$ yeast strain (fig. 5). After mixing the wild type extract with the *cyr1*$^-$ acceptor membranes, the extraction process was reversed by diluting the NaCl concentration 10 fold, allowing adenylate cyclase to re-associate with the membranes . The addition of extract restored guanine nucleotide sensitive adenylate cyclase activity to the membranes, indicating functional coupling between the *CYR1* protein and the *RAS* proteins on the membrane. Activity in the presence of the non-hydrolysable GTP analog GppNHp was more than four fold greater than that seen with the GDP analog GDPβS.

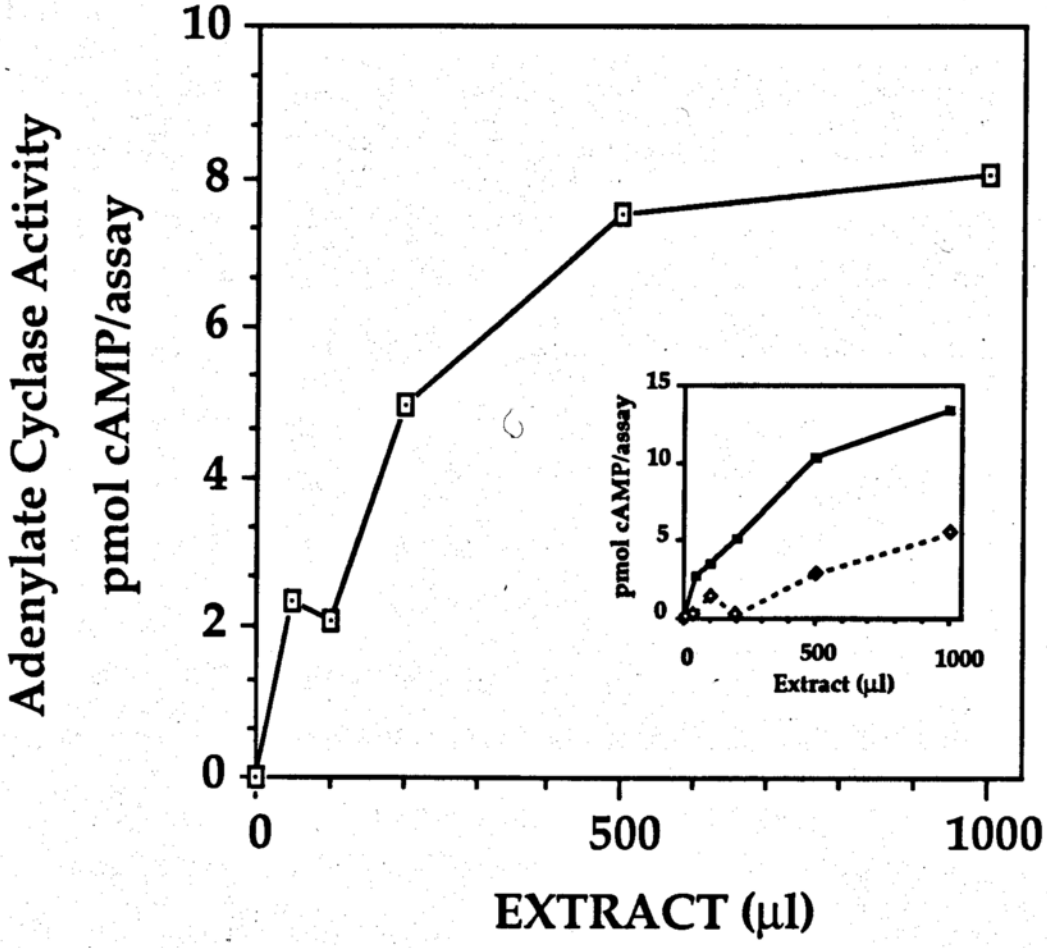
Without extract, no adenylate cyclase activity was ever observed in these membranes (not shown). The extract by itself contained Mn^{2+} dependant activity, but only very low levels of activity with Mg^{2+} ATP, and this activity was not stimulated by the addition of GppNHp. Boiling the acceptor membranes prior to reconstitution markedly diminished the amount of activity in the pellet, however, even in the complete absence of acceptor


Adenylate Cyclase Activity
pmol cAMP / assay
3
2
1
0
Manganese
GDP β S
GppNHp
cyr1-
membranes
boiled cyr1-
membranes

membranes some Mn^{2+} dependant activity always appeared in the pellet fraction. Thus, there is a component of activity in the pellet fraction that represents simple precipitation of enzyme activity, rather than functional reconstitution of adenylate cyclase into membranes.

The use of Mn^{2+} ATP as a substrate for adenylate cyclase produces high catalytic activity in the absence of any interaction with other components. However, although this activity is useful for following the catalytic subunit, it is not of physiological importance. The restoration of guanine nucleotide sensitivity to the enzyme preparation was more significant than the appearance of Mn^{2+} dependent activity in the pellet. Although some Mn^{2+} dependant activity appeared in the pellet in the absence of membranes, stimulation by guanine nucleotides was never observed without the addition of native acceptor membranes. Thus, a hallmark of reconstitution into membranes was not so much the appearance of Mn^{2+} dependant activity in the pellet fraction, but the restoration of guanine nucleotide sensitivity.

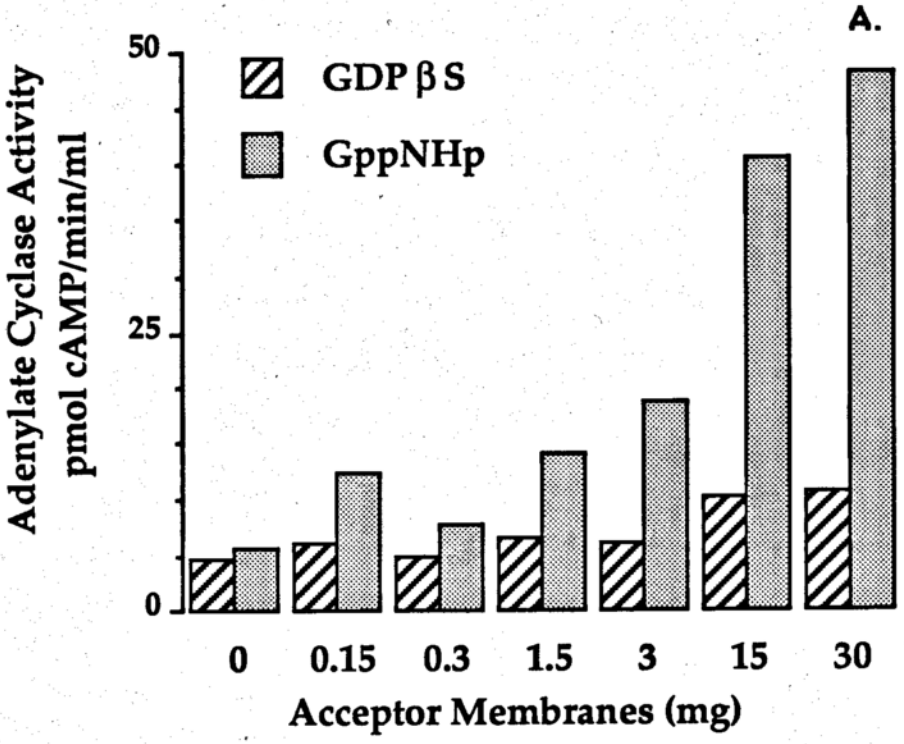
Reconstitution of soluble adenylate cyclase activity onto membranes appears to be a saturable binding process. In the reconstitution experiment shown in figure 6, the concentration of extract was varied while the quantity of acceptor membranes was held constant. Specific binding was obtained by subtracting the activity found in the pellet in the absence of acceptor membranes from the activity in the pellet with acceptor membranes present (see inset). The amount of activity associated with the membranes increased as the concentration of extract increased and reached a plateau at approximately 8 units (pmol cAMP/min) per assay (1.8 U/mg acceptor membranes).

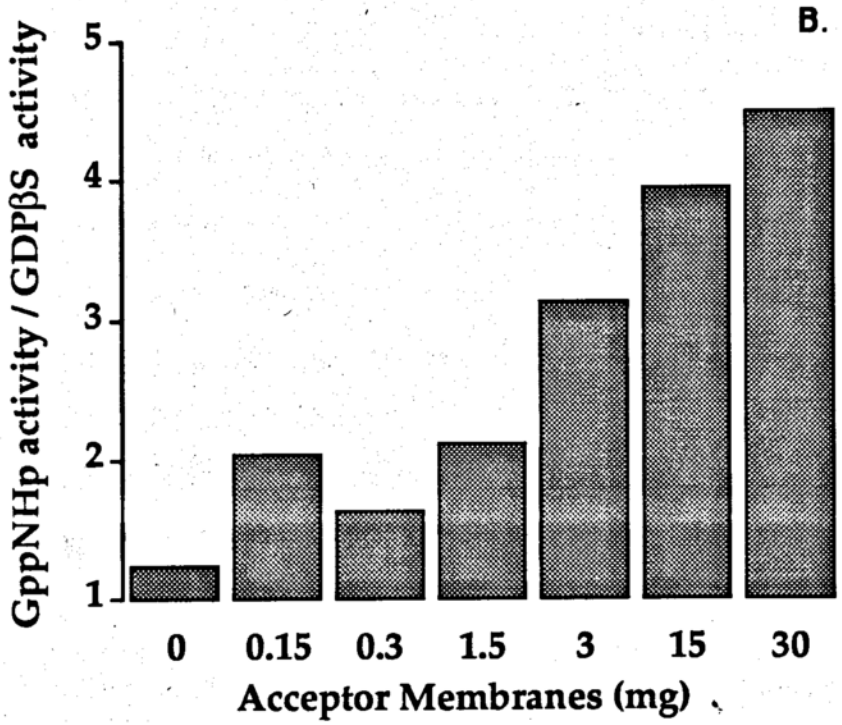

Adenylate Cyclase Activity
pmol cAMP/assay
10
8
6
4
2
0
0
500
1000
EXTRACT (µl)
15
10
5
0
pmol cAMP/assay
0
500
1000
Extract (µl)

The degree of stimulation by guanine nucleotides in reconstituted membranes was higher than that observed in the wild type membranes prior to extraction. This may be the result of a relative excess of *RAS* proteins, or some other component provided by the membranes over catalytic subunit reconstituted from the extract. When the extract was held constant while the quantity of acceptor membranes was varied, the fold stimulation by GppNHp was proportional to the amount of membranes added (fig. 7). Both the amount of activity associated with the membranes and the fold stimulation by GppNHp increased as the ratio of membranes to extract increased.

Enzyme activity appearing in the cytosol fraction after cell disruption could also be reconstituted into membranes. With these fractions, it was sufficient to simply incubate the soluble enzyme together with the membranes. After the incubation period, the membranes were collected by centrifugation, and assayed for adenylate cyclase activity. As with the NaCl extracts, the cytosolic extract was able to restore guanine nucleotide sensitive adenylate cyclase activity to the TC41-1 acceptor membranes (not shown).

Effect of cAMP on reconstitution. Our initial experiments had been based on the assumption that some intrinsic difference between the soluble and membrane bound forms of adenylate cyclase was responsible for the differences in localization. However, another possible model presumes only a single form of adenylate cyclase, but a limiting number of binding sites on the membrane. In this case, the proportion of enzyme in the cytosolic fraction would depend on the abundance of membrane binding sites. The availability of a reconstitution system allowed us to directly

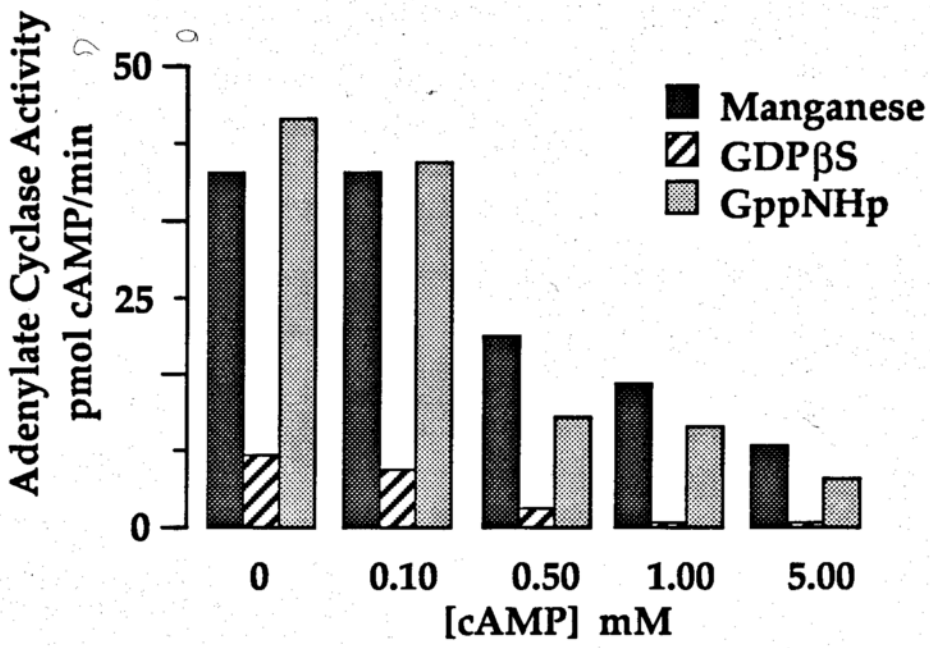

A.
Adenylate Cyclase Activity
pmol cAMP/min/ml
50
25
0
GDP β S
GppNHp
0
0.15
0.3
1.5
3
15
30
Acceptor Membranes (mg)


B.
GppNHp activity / GDPβS activity
5
4
3
2
1
0
0.15
0.3
1.5
3
15
30
Acceptor Membranes (mg)

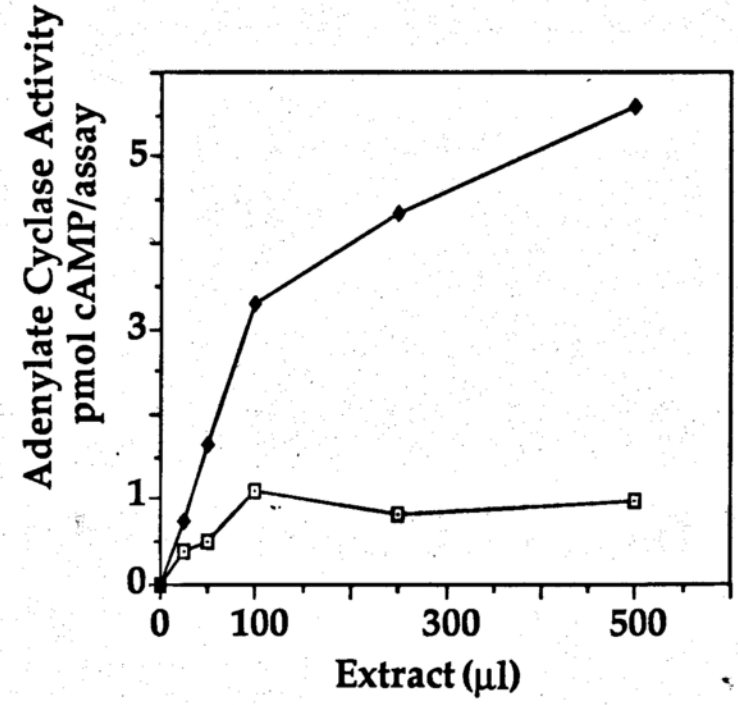
examine changes in the capacity of the membrane to accept soluble adenylate cyclase.

In order to determine if cAMP has any feedback effects on membrane association, we conducted a reconstitution experiment using acceptor membranes prepared from *cyr1*$^-$ yeast grown at several different concentrations of cAMP (fig. 8A). The yeast were first grown to late log phase in YPD medium containing 1 mM cAMP in order to obtain a sufficient quantity of cAMP dependent cells. The cells were then washed with sterile water to remove the remaining cAMP, and they were then diluted into fresh medium containing the indicated concentration of cAMP. Isobutylmethylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase activity, was also added in order to prevent intracellular degradation of the cAMP, and to allow a more effective increase in intracellular cAMP levels. Increasing concentrations of cAMP decreased the capacity of the membranes to bind adenylate cyclase in the subsequent reconstitution assay. The results in figure 8B indicate that this effect is due to a decrease in binding capacity, rather than a loss of affinity. Membranes from cells grown with low concentrations of cAMP (100μM) were able to reconstitute adenylate cyclase activity with a capacity similar to the results shown in figure 6 (approximately 2.2 U/mg acceptor membranes), while membranes grown with high cAMP (5 mM) had a greatly decreased capacity to bind adenylate cyclase (approximately 0.36 U/mg acceptor membranes). Activity that failed to bind to the membranes prepared from the 5 mM cAMP cells was fully capable of being reconstituted into membranes prepared from cells grown at lower cAMP concentrations (not shown).

A.


Adenylate Cyclase Activity
pmol cAMP/min
50
25
0
Manganese
GDPβS
GppNHp
0
0.10
0.50
1.00
5.00
[cAMP] mM

B.

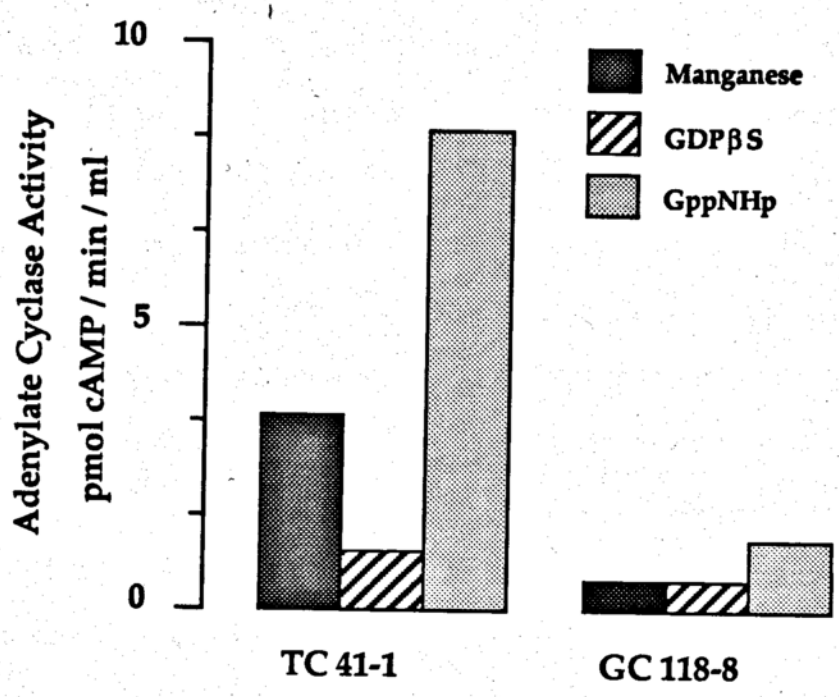

Adenylate Cyclase Activity
pmol cAMP/assay
5
3
1
0
0
100
300
500
Extract (µl)

Yeast carrying mutations in the regulatory subunit of the cAMP dependent protein kinase (cAPK), encoded by the *bcy1* gene, produce constitutively activated cAPK activity (Toda et al. 1987). Because yeast with the *bcy1* mutation resemble yeast growing at constantly elevated cAMP levels, membranes from *bcy1* strains might also be less effective in reconstituting adenylate cyclase activity. We compared the ability of membranes prepared from a *bcy1*$^-$ strain (GC118-8) to reconstitute activity with that of membranes from TC41-1 yeast (fig. 9A). The TC41-1 strain has a wild type *BCY1* gene, while the *CYR1* gene has been deleted from both strains, so that neither strain produces adenylate cyclase activity of its own. Although the strains were grown under identical conditions, the *bcy1* membranes were much less effective in reconstituting adenylate cyclase activity than the control TC41-1 membranes. In the experiment shown in figure 9B the capacity of the GC118-8 membranes to reconstitute adenylate cyclase was approximately half of that seen with the TC41-1 membranes.

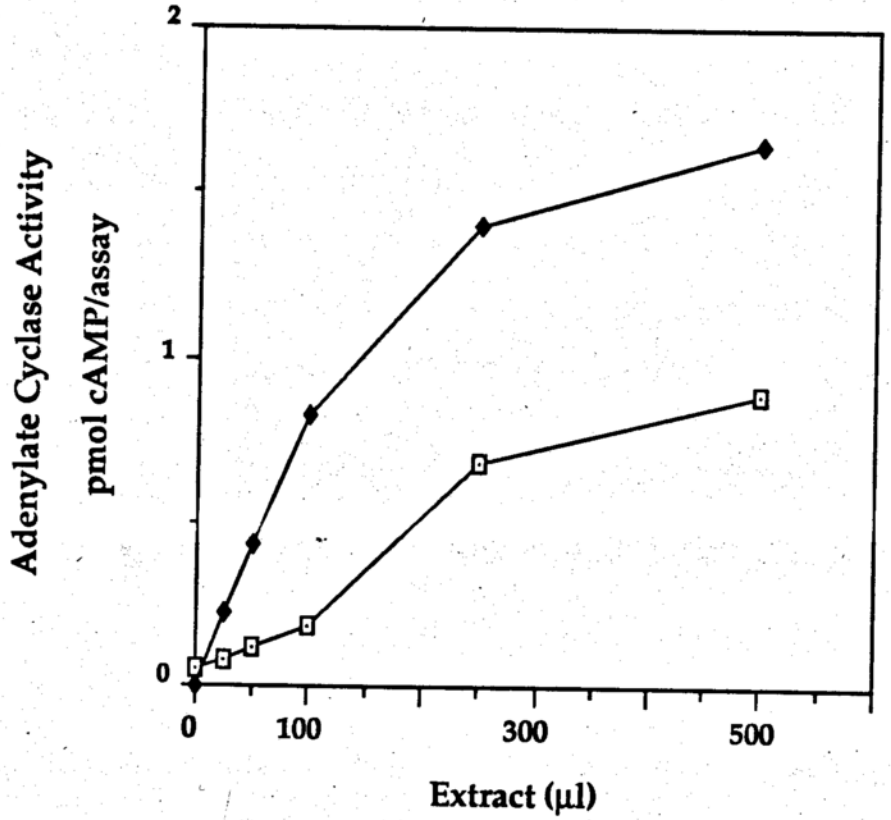
Binding of adenylate cyclase to the membrane appears to be independent of *RAS* proteins. When cells of a *ras1*$^-$, *2*$^-$ strain were disrupted, enzyme activity partitioned primarily with the membranes (not shown). Thus, *RAS* proteins are not necessary for membrane attachment of adenylate cyclase. On the other hand, it is possible that changes in membrane binding affect the amount of adenylate cyclase that can interact with *RAS* proteins; enzyme released from the membrane would be inaccessible to *RAS* proteins and thus inactive.

Localization of adenylate cyclase activity during growth. We examined the localization of yeast adenylate cyclase at various stages of growth in order to determine if culture density plays a part in the ability of the membrane to

A.


Adenylate Cyclase Activity
pmol cAMP / min / ml
10
5
0
Manganese
GDPβS
GppNHp
TC 41-1
GC 118-8

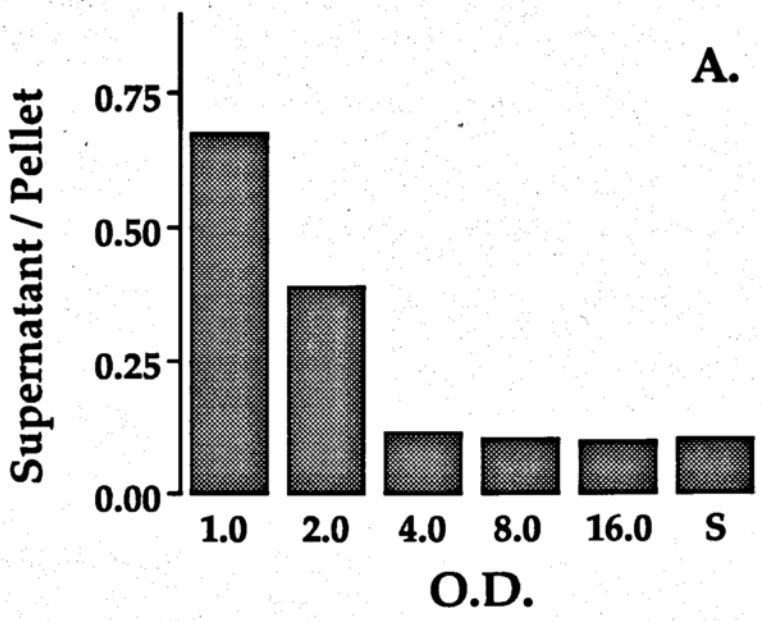
B.

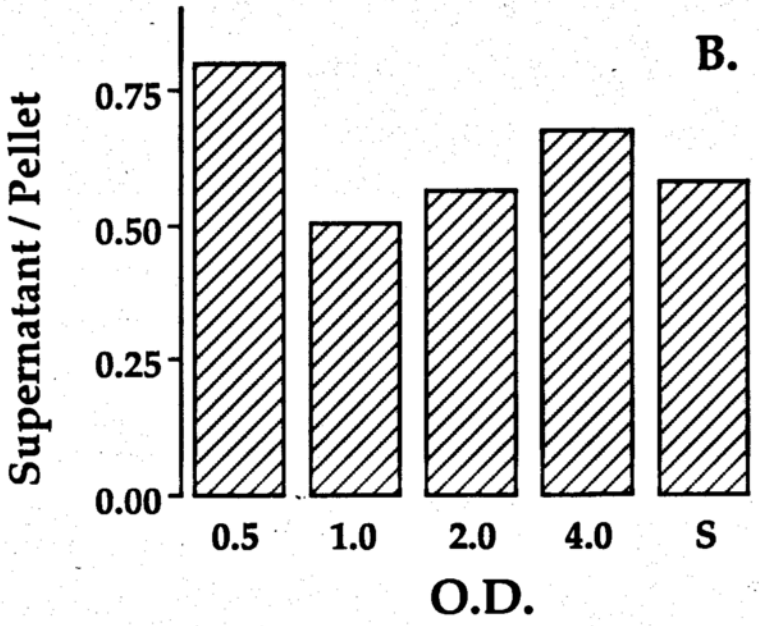

Adenylate Cyclase Activity
pmol cAMP/assay
2
1
0
0
100
300
500
Extract (µl)

bind adenylate cyclase. At low cell density, cAMP levels are presumed to be high; as cells progress through log phase growth towards stationary phase, intracellular cAMP levels fall (François et al. 1987). Thus, harvesting cells at different densities, as shown in figure 10, is similar to the *in vitro* manipulation of cAMP levels in the reconstitution experiment shown in figure 8A. Cells were harvested at varying densities, disrupted with glass beads, and separated into membrane and cytosol fractions. As wildtype cells reached increasing density, activity in the cytosol decreased, while the amount of activity associated with the membranes steadily increased. This is reflected in the decline of the ratio of supernatant activity to pellet activity (fig. 10A). Cells at lower density, where cAMP levels are higher, had a much higher proportion of activity in the soluble fraction.

The results shown in figure 10B indicate that at least part of this change is mediated by the cAMP/protein kinase system. Because yeast carrying the *bcy1* mutation have constitutively elevated cAMP dependent protein kinase activity, they are unable to sense changes in intracellular cAMP levels. Therefore *bcy1* yeast should lack the ability to regulate membrane binding of adenylate cyclase in response to cAMP. Cell fractions prepared from a *bcy1* strain (AM53-1A) should resemble those of yeast growing at constantly elevated cAMP levels. As demonstrated by figure 10B, the *bcy1* cells yielded a relatively high and constant proportion of adenylate cyclase activity in the soluble fraction, regardless of culture density.

Role of the repeated leucine rich motif. In postulating that the *CYR1* protein binds to a protein on the membrane, it is also reasonable to postulate that the *CYR1* protein has a domain that recognizes this protein. We have constructed a set of in-frame deletion mutations in the *CYR1*


A.
Supernatant / Pellet
0.75
0.50
0.25
0.00
1.0
2.0
4.0
8.0
16.0
S
O.D.


B.
Supernatant / Pellet
0.75
0.50
0.25
0.00
0.5
1.0
2.0
4.0
S
O.D.

coding region to try to produce mutant *CYR1* proteins that fail to bind to the membrane (fig. 11). These constructions are based on pAC2, a YEp24 derivative carrying the *CYR1* gene (Casperson et al. 1985). These mutations span a region of the *CYR1* gene that contains a repeating leucine rich motif (Kataoka et al. 1985) in the middle of the open reading frame. This region is indicated by the shaded area in figure 11. Deletions at the amino end of the protein (pAC202, and 203) produce a distribution of activity between pellet and supernatant that approximates the wild type enzyme. The deletions produced by pAC103 and pAC104 increase the fraction of adenylate cyclase found in the supernatant fraction. The protein encoded by pAC103 retains only 2 of the 20 leucine rich repeats, and is still partially bound to the membrane. These repeats are entirely absent in the protein encoded by pAC104, and this enzyme is found almost entirely in the supernatant. None of the altered enzymes were stimulated by GppNHp. Reconstitution experiments with the pAC103 and pAC104 encoded enzymes indicate that in the presence of saturating amounts of acceptor membranes, the pAC103 enzyme can be fully reconstituted, while the pAC104 encoded protein was not able to bind to the membranes at all (not shown). Although there are important limitations to the conclusions that can be drawn from this type of experiment (see below), the simplest interpretation is that the region containing the repeats is involved in membrane attachment.

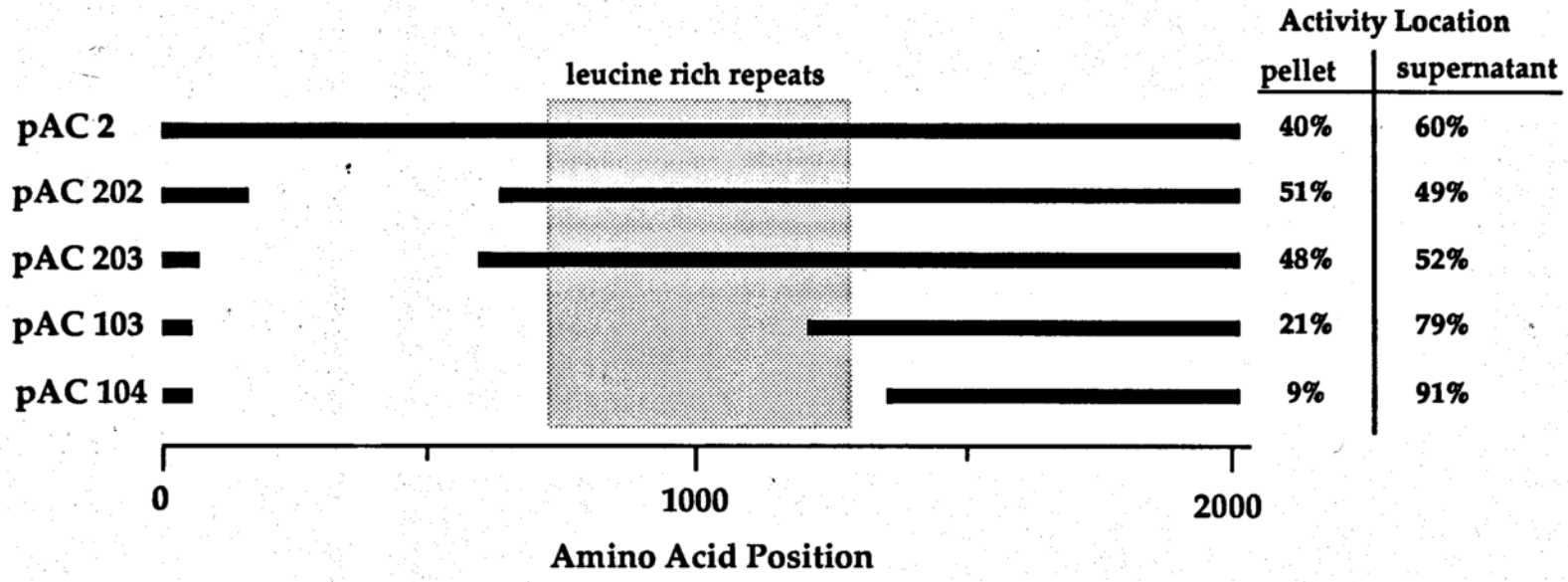

Activity Location
leucine rich repeats
pellet
supernatant
pAC 2
40%
60%
pAC 202
51%
49%
pAC 203
48%
52%
pAC 103
21%
79%
pAC 104
9%
91%
0
1000
2000
Amino Acid Position

Table 4-1. Yeast strains used in Chapter 4.

STRAIN	GENOTYPE
381 G¥ (wild type)	*MAT* **a** *ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3 cryR*
HR125* (wild type)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
NW23-9C* (*cyr1-1* in HR125)	*MAT* **a** *cyr1-1 cam leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
TC41-1§ (*cyr1* deletion in NW23-9C)	*MAT* **a** *cyr1::URA3 cam leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
GC118-8A* (*bcy1* mutant in TC41-1)	*MAT* **a** *cyr1:: URA3 bcy1 leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
AM53-1A† (*bcy1* mutant)	*MAT* **a** *bcy1 his7 gal7-2*

¥ Hartwell (1980)
* Casperson et al. (1982)
§ Heideman et al. (1990)
† K. Matsumoto

DISCUSSION

Our results support a model containing three features: First, adenylate cyclase is a soluble protein that is indirectly anchored to the membrane through an unidentified component. Second, the capacity of the membrane to bind adenylate cyclase is affected by cAMP, and may regulate interactions between the *CYR1* and *RAS* proteins. Finally, the leucine rich repeats reported by Kataoka et al (1985) may function as a domain that binds to the unidentified component on the plasma membrane. These features are described in more detail below.

Soluble Adenylate Cyclase. The following observations indicate that adenylate cyclase is not an intrinsic membrane protein:

1. *Both soluble and membrane bound activity are found when cells are disrupted.* Soluble adenylate cyclase activity made up a substantial fraction of the total in all cases examined. Although, in the experiments shown, we used a microcentrifuge to separate pellet and supernatant fractions, we have found that both the cytosolic activity and the activity in NaCl extracts remain soluble after ultracentrifugation for 1 hr at 100,000 x g. Thus, these activities are not likely to represent unsedimented particulate adenylate cyclase. Additionally, these activities migrate in the included volume on Sepharose 4B.

2. *Activity remaining bound to the membrane can be removed by the addition of NaCl.* Adenylate cyclase is anchored to the membrane through interactions that can be disrupted at high ionic strength. In addition to the experiments shown, we have successfully used other salts, including KCl, NH_4Cl, and $MgSO_4$, to remove activity from the membrane. Although most of the activity was removed by 0.5 M NaCl, a significant amount of

activity remained in the pellet. We have not been able to solublize this activity by either increasing the concentration of NaCl or disrupting the bilayer with detergents. Thus, the resistance to NaCl extraction cannot be simply explained by proposing that the enzyme is embedded in a lipid bilayer. The remaining insoluble enzyme must be associated with some insoluble element by covalent modification or by some other mechanism that is not disrupted by detergent or high ionic strength. Incubation at 30° with dithiothreitol had no effect on release.

3. *Addition of NaCl also appears to dissociate the enzyme from a large complex in detergent extracts.* Addition of NaCl to detergent extracts produced the dramatic size change demonstrated in figure 1. This appears to correspond to the dissociation from the membrane, in that a dissociation event must also be occurring in the detergent extract when NaCl is added. After NaCl was added, detergent was no longer required to maintain the solubility of the enzyme. Although we cannot positively identify the target for adenylate cyclase as a protein, these observations are consistent with dissociation of a soluble enzyme from an integral membrane protein. The limited number of binding sites on the membrane along with the decrease in reconstitution observed after heat treatment of the acceptor membranes supports the idea that adenylate cyclase associates with a protein on the membrane.

Although markedly reduced, the size of the adenylate cyclase complex at high ionic strength is still much greater than can be accounted for by a single *CYR1* polypeptide. It seems likely that even after release from the membrane this complex also includes the 70 kDa protein reported by Field et al. (1988) to be associated with a *CYR1* fusion protein. This 70 kDa protein

was immunopurified along with the *CYR1* protein from extracts containing taurocholate and 0.5 M NaCl, and is presumably also associated with adenylate cyclase in our extracts as well.

4. *A 205 kDa CYR1 protein can be immunoprecipitated from both soluble and particulate fractions with anti-CYR1 antibodies.* The most prominent *CYR1* protein identified by immunoprecipitation was a 205 kDa protein identified in both the cytosolic and membrane fractions. This indicates that the full length *CYR1* protein can be found in the soluble fraction as well as associated with the membrane. In this experiment, adenylate cyclase was overexpressed using a multicopy plasmid to improve the sensitivity of the immunoprecipitation. This produces about a five to ten fold increase in activity. The activity in these cells appears to be identical to that observed in wild type cells in terms of *RAS* coupling, solubility, and hydrodynamic properties. In cells carrying this plasmid the distribution of adenylate cyclase activity is shifted slightly towards the cytosolic fraction (see below), however the cytosolic enzyme produced by these cells remains qualitatively indistinguishable from that found in cells carrying only a single copy of the *CYR1* gene.

5. *Soluble activity can be functionally reconstituted onto membranes, regaining sensitivity to stimulation by guanine nucleotides.* Both the cytosolic activity and adenylate cyclase extracted from membranes with NaCl could be functionally reconstituted into membranes. This indicates that the soluble adenylate cyclase has not been permanently modified in some way that prevents the enzyme from re-associating with the membrane.

Regulation of Membrane Binding. The model can be further developed by suggesting that association of adenylate cyclase with the membrane is limited by the availability of sites on the membrane, and that this may be used as a mechanism for regulating the interactions between adenylate cyclase and *RAS* proteins. The evidence for this point comes from two observations:

1. *Not all of the adenylate cyclase activity in the cell appears to be associated with the membrane.* Whenever we have examined cytosolic fractions, we have found substantial levels of adenylate cyclase activity, yet the soluble enzyme appears to be capable of membrane association in reconstitution experiments. It appears that binding of soluble adenylate cyclase onto membranes lacking adenylate cyclase is a saturable process limited by the number of binding sites (fig. 6). Under normal conditions, the amount of activity that could be reconstituted into the TC41-1 membranes remained relatively constant, averaging about 2 U/mg of acceptor membranes. This is within the range of the specific activity of wild type membranes prepared from the otherwise isogenic *CYR1*$^+$ strain. Additionally, the concentration of enzyme extract needed to produce half maximal binding was approximately 1.8 U/ml. If one assumes a turnover number of 1,000/min, this corresponds to an enzyme concentration of 1.8 pM. Such an assumption is not unrealistic in light of the turnover number of 1,200/min reported for the purified mammalian adenylate cyclase, and the reported similarities between the catalytic domains of the yeast and mammalian enzymes (Krupinski et al. 1989; Smigel 1986). Regardless of the actual affinity for the membranes, complete reconstitution took place with enzyme that had been diluted well beyond the level produced during cell disruption.

Thus although it could be argued that soluble enzyme was observed as an artefact of cytosol dilution during cell disruption, reconstitution back to the membranes occurred at even greater dilutions. Together, these arguments indicate that binding to the membrane is limited by the number of sites that are available.

2. *cAMP has a negative effect on the ability of membranes to bind adenylate cyclase.* Membranes prepared from cells grown at high levels of cAMP were less effective in binding adenylate cyclase than membranes grown at lower cAMP levels. This was the result of an apparent decrease in the number of binding sites. Activity that failed to bind to membranes prepared from cells grown at high cAMP was fully capable of reconstitution when added to other TC41-1 membranes.

Additional evidence indicating that cAMP has a negative influence on membrane binding comes from strains with altered cAMP metabolism. The *bcy1* mutation results in constitutive activation of the target for cAMP, the cAMP dependent protein kinases. Thus, these mutants resemble cells growing at elevated cAMP levels. Like membranes from cells grown at high cAMP levels, *bcy1* membranes were relatively ineffective in binding adenylate cyclase activity. This reflected an apparent decrease in the number of sites available.

Over expression of adenylate cyclase tended to reduce the fraction of activity associated with the membrane. The yeast expressing *CYR1* on the multicopy plasmid pAC2 had about 50% of their total activity associated with the membrane, while the isogenic wild type strain had about 60-70% of the total activity associated with the membrane. On the other hand, strains lacking *ras* proteins produce very low levels of cAMP. These strains tended

to have a smaller proportion of adenylate cyclase activity in the soluble fraction than strains producing more activity (not shown). In strains expressing *CYR1* at high levels with a GAL10 promoter, most of the activity was in the particulate fraction, but this activity was not extractable with detergents or NaCl, nor was it responsive to guanine nucleotide stimulation.

In our experiments, the effects of cAMP were directed at the membranes, rather than at the *CYR1* protein itself. The experiments described demonstrate an effect on the capacity of the membranes to bind adenylate cyclase; we have not yet determined what effect cAMP has on the ability of the *CYR1* protein to bind to the membrane. The effects of cAMP did not seem to be directed at stimulation by *RAS* proteins. Indeed, in the reconstitution experiment shown in figure 8A, at the higher cAMP points although the total amount of enzyme reconstituted into the membranes was reduced, the fold stimulation by GppNHp was actually increased. The degree of stimulation by GppNHp observed in the reconstitution experiments was also uniformly higher than that observed in the native membranes. Stimulation by GppNHp increased as the ratio of acceptor membranes to extract increased (fig. 7). These observations may indicate that the availability of *RAS* proteins normally limits the response of adenylate cyclase to guanine nucleotides.

It is tempting to propose a model in which the *CYR1* protein is bound reversibly to the membrane in a manner that can be regulated in accordance with the needs of the cell. The response to cAMP suggests the possibility of a feedback loop. Feedback control of cAMP production has been reported previously (Nikawa et al. 1987). Inhibition of *RAS2* activity by protein

kinase A has also been reported (Resnick and Racker 1988). In addition, there are consensus sequences for cAPK phosphorylation on all of the other identified components of the system, *CYR1*, *CDC25* and *IRA1* (Broek et al. 1987; Camonis et al. 1986; Kataoka et al. 1985; Tanaka et al. 1989). Dissociation of adenylate cyclase from the plane of the membrane could be another feedback mechanism to prevent stimulation by *RAS* proteins and decrease the production of cAMP.

We also observed changes in the proportion of activity in the cytosol fraction during growth (fig. 10). As cells increased in density, the proportion of adenylate cyclase in the supernatant decreased. Although there are many possible explanations for this effect, the shift from supernatant to pellet is consistent with regulation of binding capacity by cAMP observed. Cells carrying the *bcy1* mutation failed to shift adenylate cyclase activity from the soluble fraction to the pellet fraction during growth (fig. 10). These cells are presumably insensitive to changes in cAMP, and would not be expected to regulate membrane binding in response to cAMP levels. It should be noted that the *bcy1* cells stopped growing at a much lower density than the wild type cells. The *bcy1* cells stopped growing at a density of approximately 5 o.d., while the wild type cells reached approximately 20 o.d.

The size of the complex in detergent solution at low ionic strength, where the enzyme is presumably associated with the protein(s) that anchor it to the membrane, suggests a very large complex. This is consistent with the large number of inputs that must feed into the adenylate cyclase system. The regulation of cAMP levels and cAPK activity appears to be pivotal in the decision to undergo mitosis and meiosis (Ishikawa et al. 1986; Matsumoto et al. 1983). Additionally, cAMP levels are modulated by glucose (Eraso and

Gancedo 1985) as well as during the cell cycle (Watson and Berry 1977), and proper regulation of adenylate cyclase activity is required for the appropriate response to mating factors, nitrogen starvation and heat shock (Kataoka et al. 1984; Matsumoto et al. 1985; Toda et al. 1985)]. A key to understanding the regulation of this system will be identifying proteins that make up this complex.

Binding Domain. It is reasonable to postulate that binding of adenylate cyclase to its target on the membrane occurs via a specific binding domain on the *CYR1* protein. Our experiments with in-frame deletions indicate that the loss of the leucine rich repeats roughly correlates with loss of membrane binding. This must be viewed cautiously, however. The interpretation is complicated by the effects that adenylate cyclase activity may have on its own localization; both pAC 103 and 104 express activated enzyme, and all of the mutant *CYR1* proteins are uncoupled from stimulation by *RAS* proteins. The resulting changes in intracellular cAMP concentrations might contribute to the reduction in membrane binding observed with these constructions. Reconstitution experiments with the pAC103 and 104 enzymes address this limitation. The pAC104 enzyme did not bind to the membranes at all, in agreement with the results from figure 11. On the other hand, the enzyme encoded by pAC103 which retains two of the repeats was reconstituted into the membranes. Qualitatively, the pAC103 enzyme and the wildtype enzyme bound to the membrane equally well.

Another obvious limitation to interpretation is intrinsic to deletion experiments; we do not know how the deletions alter structure. It is possible that our deletions alter protein structure at sites distant from the

leucine rich repeats. This limitation has been addressed by the recent work of Field *et al.*, (1990). In these experiments, over expression of the leucine rich repeats of the *CYR1* protein suppressed the heat shock sensitivity of the *RAS2*^{val19} mutation, suggesting that the truncated *CYR1* proteins were interfering with the coupling between *RAS2* and the wildtype adenylate cyclase also present in the cells. The authors propose that this may be due to sequestration of the mutant *RAS2* protein by the overexpressed leucine rich repeats. An alternative explanation is that the mutant *CYR1* proteins are interfering with the ability of the wild type enzyme to bind to the membrane, uncoupling adenylate cyclase from *RAS*.

The region of leucine rich repeats remains an attractive candidate for a domain that interacts with a target protein on the membrane. Positive identification of this domain, as well as identification of the protein(s) that it binds to, will be important steps to understanding the regulation of this system.

CHAPTER 5
Interactions Between Adenylate Cyclase and the Yeast GAP Protein, Ira1

ABSTRACT

The adenylate cyclase system of the yeast *Saccharomyces cerevisiae* contains many proteins, including: the *CYR1* polypeptide which is responsible for catalyzing formation of cAMP from ATP, *RAS1* and *RAS2* polypeptides, that mediate stimulation of cAMP synthesis by guanine nucleotides, and the GAP analogue *IRA1*. We have previously reported that adenylate cyclase is only peripherally bound to the yeast membrane. We have concluded that Ira1 is a strong candidate for a protein involved in anchoring adenylate cyclase to the membrane. We base this conclusion on the following criteria; 1. A disruption of the *IRA1* gene produced a mutant with very low membrane associated levels of adenylate cyclase activity; 2. Membranes made from these mutants were incapable of binding adenylate cyclase in vitro; 3. Ira1 antibodies inhibit binding of adenylate cyclase to the membrane; 4. Ira1 and adenylate cyclase comigrate on Sepharose 4B.

INTRODUCTION

Ras proteins in the yeast, *Saccharomyces cerevisiae*, share many similarities to the mammalian p21^{ras} proto-oncogene products. Both yeast and mammalian Ras proteins share structural homology with the other members of the large family of GTP binding proteins that includes the G proteins. Beyond the structural similarities, in both yeast and mammals, Ras proteins play an important functional role in regulating cell division (Barbacid 1987). The activity of Ras proteins is in turn regulated by a conserved family of proteins that inactivate Ras proteins. In mammals, this family is represented by GAP (GTPase activating protein) (McCormick 1989) and NF-1 (neurofibromatosis type 1) (Buchberg et al. 1990; Wallace et al.

1990; Xu et al. 1990), and in yeast, by Ira1 and Ira2 (inhibitory regulator of the *RAS*-cAMP pathway) (Tanaka et al. 1989; Tanaka et al. 1990). These proteins function in their respective systems by stimulating the GTPase activity intrinsic to Ras proteins, converting the active, GTP bound, Ras to the inactive, GDP bound, form (Tanaka et al. 1990; Trahey and McCormick 1987). The effector for mammalian $p21^{ras}$ has not been identified; however in yeast, the effector for Ras is adenylate cyclase. Thus in yeast, inactivation of Ras by Ira1 & 2 appears necessary for reducing cAMP levels in response to nutritional limitation. We have recently reported that adenylate cyclase is peripherally bound to the membrane through an unidentified protein anchor (Mitts et al. 1990). We now present evidence indicating that, in addition to its regulatory role, Ira1 also has a structural role; anchoring adenylate cyclase to the membrane. Our results support a model in which both the effector for Ras, and the terminator for the Ras signal are bound together.

METHODS

Abbreviations. GppNHp, 5' guanylylimidodiphosphate; GDPβS, guanosine 5'-O-(2-thiodiphosphate).

Materials. Adenylate cyclase grade [α-^{32}P]ATP (40 cpm/pmol) was from Amersham, [^{3}H]cAMP (10,000 cpm/assay) and [^{125}I] NaI were from New England Nuclear, lectin affinity resins were from Sigma.

Media. Yeast were grown on either rich medium (YPD) containing 1% yeast extract, 2% bacto peptone, and 2% glucose, or minimal medium (SD) containing 6.7 g/l yeast nitrogen base, 2% glucose and the appropriate factors to support the growth of auxotrophic strains.

Disruptions of the IRA1 gene. The 1.05 kb *Bcl*I / *Pst*I fragment of *IRA1* was inserted into the *Bam*HI / *Pst*I sites of pIC20R (Marsh et al. 1984). The resulting plasmid was then digested with *Xba*I and *Hin*DIII and the the 1.37 kb *Hin*DIII / *Xba*I fragment of *IRA1* was inserted. The resulting plasmid was then cut with *Hin*DIII and the 1.24 kb *Hin*DIII fragment of YEp24 (Botstein et al. 1979) containing the *URA3* gene was inserted. The resulting plasmid contains the *IRA1* sequence from the *Bcl*I site at position 2658 to the *Xba*I site at position 5395, with the *URA3* gene and a short sequence of synthetic polylinker inserted in place of the region between the *Pst*I site at position 3708 and the *Hin*DIII site at position 4026. This plasmid, pIKO, was used to disrupt the *IRA1* gene in the strains HR125 and NW239C, to produce strains TM101 and TM102 respectively, using one step gene disruption (Rothstein 1983). The plasmid pNR1 (Tanaka et al. 1989) provided by K. Tanaka was used to disrupt the *IRA1* gene at position 8103, in the strain HR125 resulting in the mutant TM105. Positions are indicated in reference to the sequence reported by Tanaka *et al* (1989). Disruptions were confirmed using the polymerase chain reaction and primers flanking the insertion site to amplify the region of genomic DNA disrupted by the marker.

Preparation of yeast particulate fractions. Yeast were grown to an optical density at 660 nm (OD) of approximately 2-4. The cultures were centrifuged and cells were disrupted by vortexing with glass beads in YMB buffer, containing 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 (Casperson et al. 1983). The homogenate was centrifuged in a Beckman J6, 5 min at 1,000 rpm (300 x g) to remove nuclei and unbroken cells. The supernatant was then spun 30 min at 14,000

rpm (16,000 x g) to separate particulate and soluble fractions. Both particulate and soluble fractions were stored in YMB at -70° until needed.

Reconstitution assays. Membranes from wild type yeast (381G, 17.6 mg) were extracted with 1.0 ml of YMB containing 500 mM NaCl on ice for 30 min, centrifuged 30 min at 14,000 rpm (16,000 x g) in an Eppendorf microcentrifuge, and the supernatant (extract) containing adenylate cyclase activity was collected. Samples containing varying amounts of extract (as indicated) were mixed with 5 mg of acceptor membranes prepared from *cyr1*$^-$ strains (with no adenylate cyclase activity). Reconstitution of adenylate cyclase to the acceptor membranes was initiated by diluting NaCl 10 fold with reconstitution buffer (50 mM MES, 5 mM $MgCl_2$ pH 6.0) and the mixture was incubated on ice for 30 min. The membranes were collected by centrifugation, resuspended in 250 µl of reconstitution buffer, and assayed for adenylate cyclase activity in the presence of Mn^{+2}. Values plotted represent specific binding, obtained by subtracting the activity of control samples containing no acceptor membranes (Mitts et al. 1990). Acceptor membranes used were from *cyr1*$^-$ strains with either a wild type *IRA1* (TC41-1) or a *cyr1*$^-$ strain carrying the *IRA1* disruption at codon 1058 (TM102).

Adenylate cyclase assay. Adenylate cyclase was assayed by a modification of the method of Salomon et al. (Salomon et al. 1974) as described by Casperson et al. (Casperson et al. 1985). Guanine nucleotides, where present, were at 10 µM; manganese was at 10 mM. Protein was assayed by the method of Lowry et al with BSA as a standard. (Lowry et al. 1951).

β-galactosidase/IRA1 fusion protein and preparation of antibodies. The 1.47 kb *Bam*HI/*Xba*I fragment of *IRA1* was isolated and inserted into pUR288.

This placed the *IRA1* gene fragment in frame at the carboxy-terminal end of β-galactosidase, allowing expression of amino acids 1127-1618. The resultant plasmid pIC/D was used to transform the *E. coli* strain JM105. Induction of cultures with isopropylthio-b-galactoside resulted in over expression of a 170 kDa protein in bacteria transformed with pIC/D. The protein was then partially purified by SDS polyacrylamide gel electrophoresis, dialyzed into buffer (containing; 20 mM Tris pH 7.4, 150 mM NaCl, 5mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol), and used to immunize rabbits.

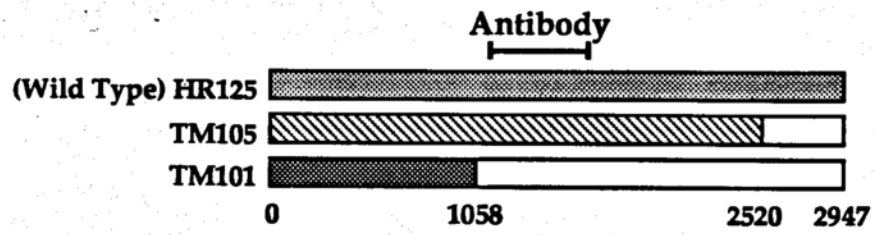
Western blots. Protein samples (yeast membranes or column fractions) were separated on 4 or 6% polyacrylamide gels, transferred to nitrocellulose and the membranes were blocked with Blotto (5% nonfat powdered milk in 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.01% sodium azide, 0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate), and probed with Ira1 antibody (1:600 dilution in Blotto). Immunoreactive proteins were visualized by the addition of [^{125}I]-labelled goat-anti-rabbit antibody (specific activity 2,000,000 cpm/μg), followed by autoradiography of the washed membrane.

Lectin-agarose. Wild type (HR125) membranes were detergent extracted (Heideman et al. 1987) with YMB containing 2% taurocholate, 1 mM Ca^{+2}, 1 mM Mn^{+2}, and samples of the extract were incubated with either Sepharose 4B, ConA-Sepharose, or lentil lectin-agarose for 4 hrs at 4°. The resin samples were then washed with either YMB or YMB with 0.5M NaCl added. Proteins were then eluted with 1% SDS, % β-mercapto-ethanol, 5 mM EDTA at 65°. Samples were run out on a 4% SDS polyacrylamide gel and prepared for Western blotting as previously described.

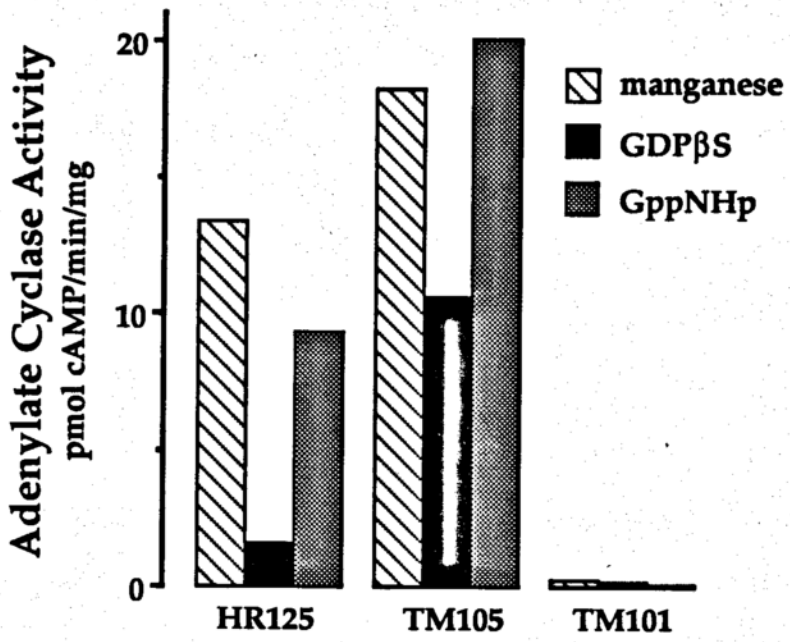
RESULTS

Effect of IRA1 disruptions on adenylate cyclase activity and subcellular localization. We created two disruptions of the *IRA1* coding region by inserting auxotrophic markers at positions 3708 and 8103, (codons 1058 and 2520, fig. 1A). We then measured adenylate cyclase activity in membrane and cytosolic fractions from cells carrying either the wild type, or the disrupted *IRA1* genes (Fig. 1B). The downstream disruption at codon 2520 (strain TM105) produced an increase in adenylate cyclase activity consistent with the other phenotypes reported for this disruption. Adenylate cyclase activity in the presence of Mn^{+2}.ATP (a measure of catalytic activity of the enzyme independent of Ras stimulation) was slightly elevated, compared with values from wild type yeast (HR125). However, a more striking feature of this mutation was the substantial elevation of basal activity, as measured with GDPβS. This is consistent with reports that this disruption raises cAMP levels and blocks the ability of Ira1 to accelerate the GTPase activity of Ras proteins (Tanaka et al. 1990). This was in contrast to the results obtained with the second *IRA1* disruption, farther upstream at codon 1058. This disruption (TM101) produced membranes with strikingly low levels of adenylate cyclase activity compared with wild type, or TM105 membranes.

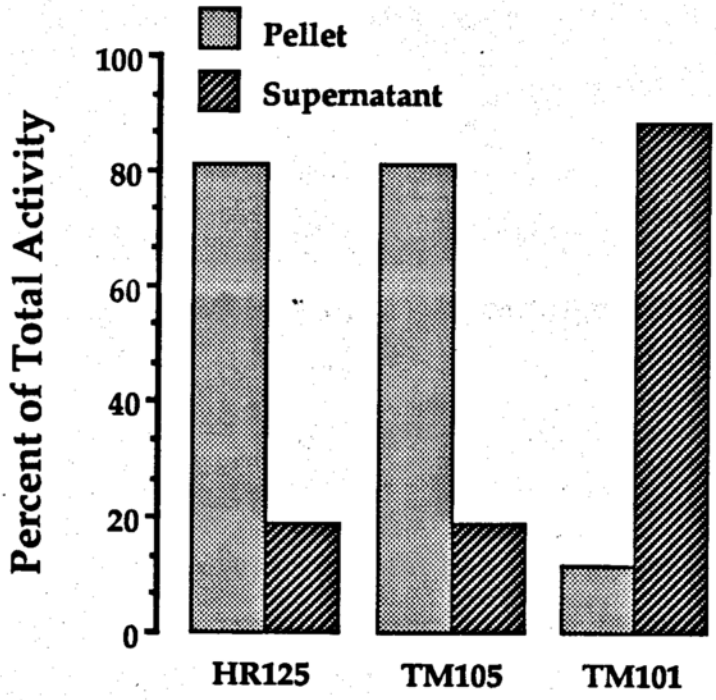
When membrane and cytosol fractions were compared, the majority of adenylate cyclase activity in wild type yeast (HR125) was associated with the membrane fraction (80%) and only a small portion (20%) was located in the soluble fraction (Fig. 1C). This distribution remained unchanged in TM105, carrying the *IRA1* disruption at codon 2520. However, this pattern changed dramatically in cells carrying the disruption at codon 1058 (TM101). In these


Antibody
(Wild Type) HR125
TM105
TM101
0
1058
2520
2947

A.


Adenylate Cyclase Activity
pmol cAMP/min/mg
20
10
0
manganese
GDPβS
GppNHp
HR125
TM105
TM101

B.


Pellet
Supernatant
Percent of Total Activity
100
80
60
40
20
0
HR125
TM105
TM101

C.

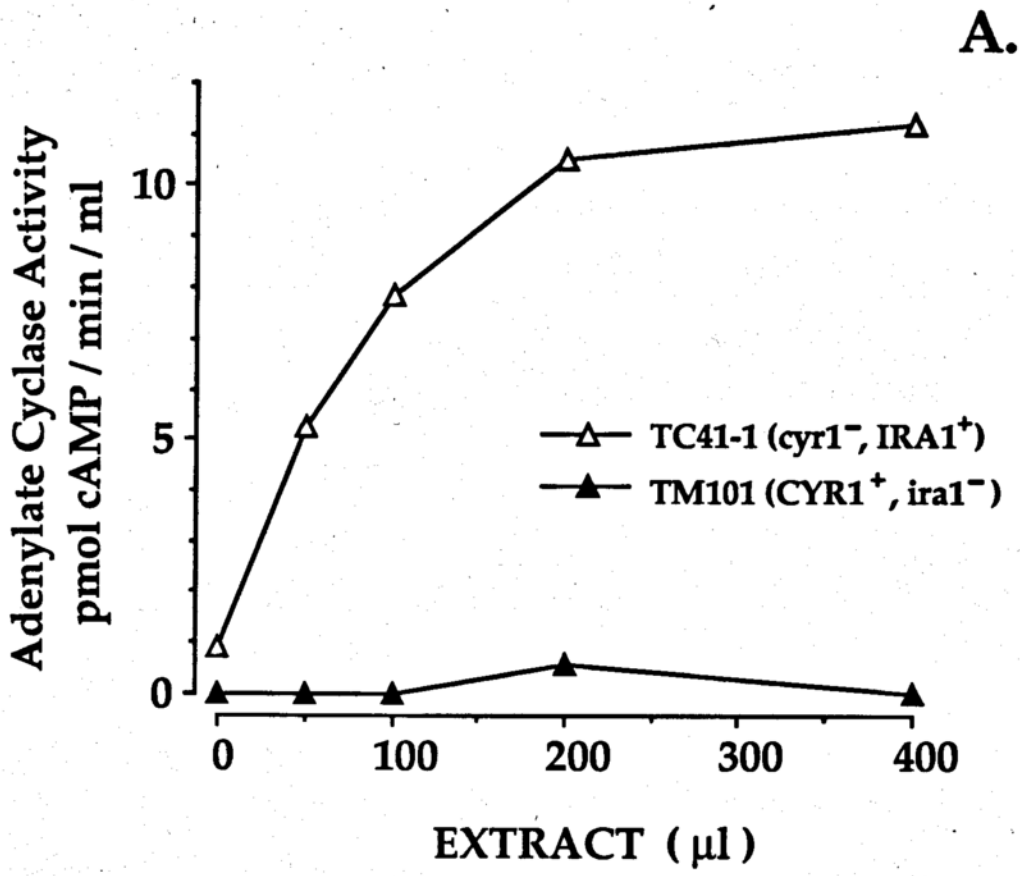
cells, adenylate cyclase activity was found almost entirely in the soluble fraction (90%) and only a small portion (10%) was associated with the membrane fraction. The total activity in the TM101 fractions was always lower than that found in wild type fractions; membrane specific activity was typically reduced by approximately 200 fold, to barely measurable levels. The specific activity of the cytosolic fraction was also reduced, but by only a factor of 2; from an average of 0.70 Units (pmol cAMP/ min^{-1}) per mg to an average of 0.34 U/mg. This indicates two changes in adenylate cyclase activity in the TM101 mutants; a marked reduction in total activity, and a dramatic shift of the remaining activity from the membrane to the cytosol. The change in adenylate cyclase levels could result from a number of causes, including a decrease in message level, or reduced stability of the enzyme. The latter possibility could be secondary to the change in location, adenylate cyclase may be more stable when associated with the membrane. We do not know what causes the decrease in adenylate cyclase expression, but we have examined the shift from the membrane to the cytosol in more detail.

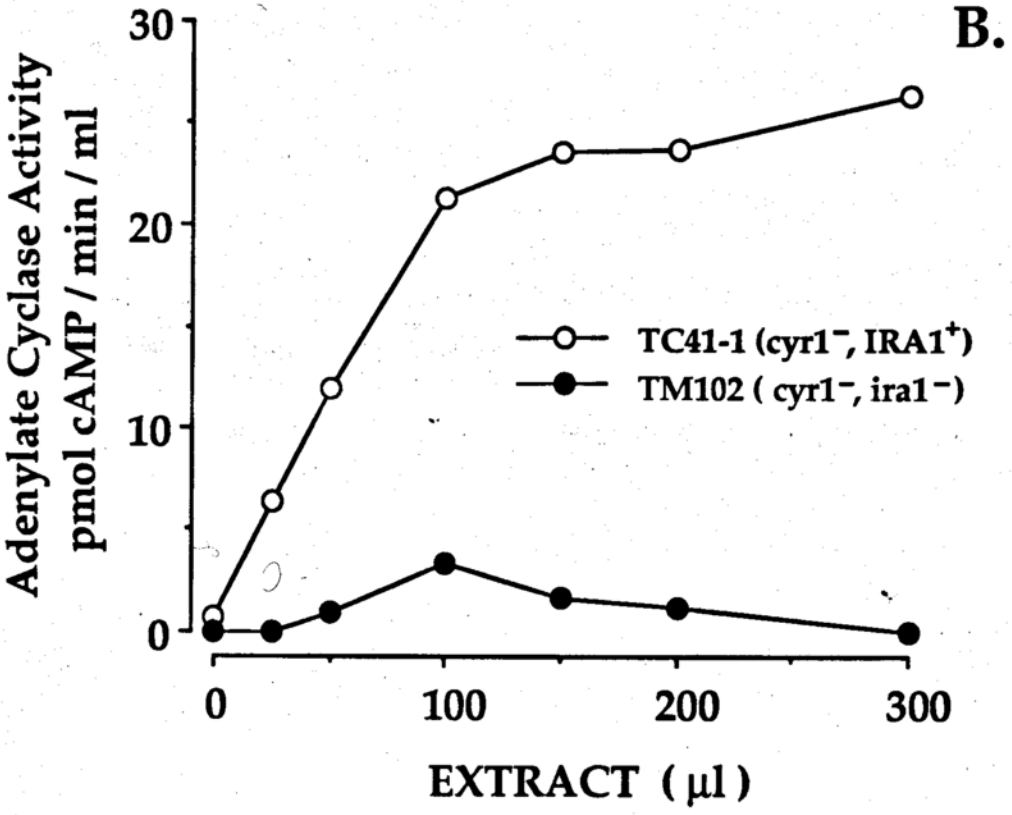
IRA1 disruption prevents membrane binding of adenylate cyclase. A reconstitution assay revealed that the *IRA1* disruption at codon 1058 produced membranes that had lost the ability to bind adenylate cyclase. Adenylate cyclase activity can be extracted from wild type membranes with 0.5M NaCl, and then reconstituted onto membranes prepared from *cyr1*$^-$ yeast (TC41-1), lacking adenylate cyclase activity. We have previously used this type of reconstitution assay to measure the capacity of membranes to bind adenylate cyclase (Mitts et al. 1990). Such a reconstitution experiment, comparing membranes from *IRA1* disrupted cells with membranes from

cells carrying a normal *IRA1* gene, is shown in Figure 2. In this experiment, acceptor membranes were incubated with varying concentrations of salt extracted adenylate cyclase from wild type cells, and the NaCl concentration was then lowered by dilution, to allow the enzyme to bind to the membranes. Membranes were then collected by centrifugation, and assayed for reconstituted adenylate cyclase activity. Values plotted represent specific binding, obtained by subtracting the activity of control samples containing no acceptor membranes. The activity in these control samples averaged approximately 20% of that obtained with the TC41-1 samples (Mitts et al. 1990).

Membranes prepared from *cyr1*$^-$ yeast with a wild type *IRA1* gene (TC41-1) bound increasing amounts of adenylate cyclase activity as the concentration of extract increased. In contrast, membranes prepared from *cyr1*$^-$ yeast carrying the *IRA1* disruption at codon 1058 (TM102) failed to bind adenylate cyclase activity. This experiment indicated that the disruption of *IRA1* produced membranes lacking binding sites for adenylate cyclase activity. An alternate possibility was that the mutant membranes bound adenylate cyclase, but inhibited the activity of the enzyme. However, the total activity observed in these experiments was the same with *IRA1* disrupted membranes and wild type membranes, indicating that the TM102 membranes did not inhibit adenylate cyclase activity.

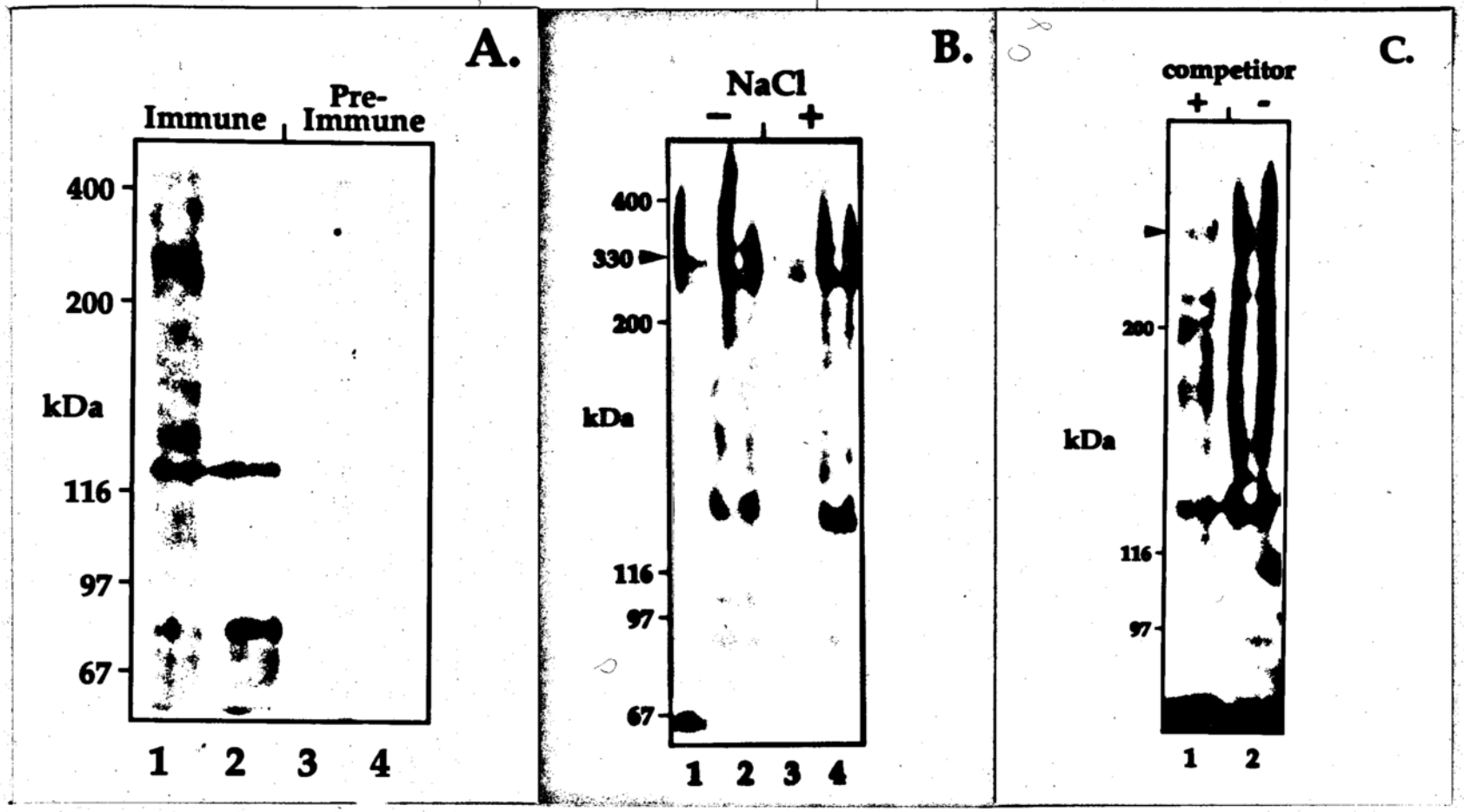
Antibodies against IRA1 recognize a 330 kDa membrane protein. We have developed polyclonal antibodies against an *IRA1*/β-galactosidase fusion protein (described in Methods). Western blots show that these antibodies recognize a 320-350 kDa protein matching the size predicted by the Ira1


A.
Adenylate Cyclase Activity
pmol cAMP / min / ml
10
5
0
TC41-1 (cyr1⁻, IRA1⁺)
TM101 (CYR1⁺, ira1⁻)
0
100
200
300
400
EXTRACT (µl)


B.
Adenylate Cyclase Activity
pmol cAMP / min / ml
30
20
10
0
TC41-1 (cyr1⁻, IRA1⁺)
TM102 (cyr1⁻, ira1⁻)
0
100
200
300
EXTRACT (µl)

amino acid sequence. This band was observed in samples from wild type yeast membranes (fig 3A. lane 1), however no immunoreactive bands were observed with *IRA1* disrupted membranes (TM101, lane 2). This result was expected, since the *IRA1* disruption in TM101 is upstream of the portion of the Ira1 protein that the antibodies were raised against. No immunoreactive bands were seen with preimmune serum (lanes 3 and 4). Immunoreactive proteins were found only associated with membrane fractions and were not observed in cytosolic fractions. We were unable to extract the Ira1 protein from membranes with NaCl, however some of the 330 kDa protein was solubilzed with 5% taurocholate.

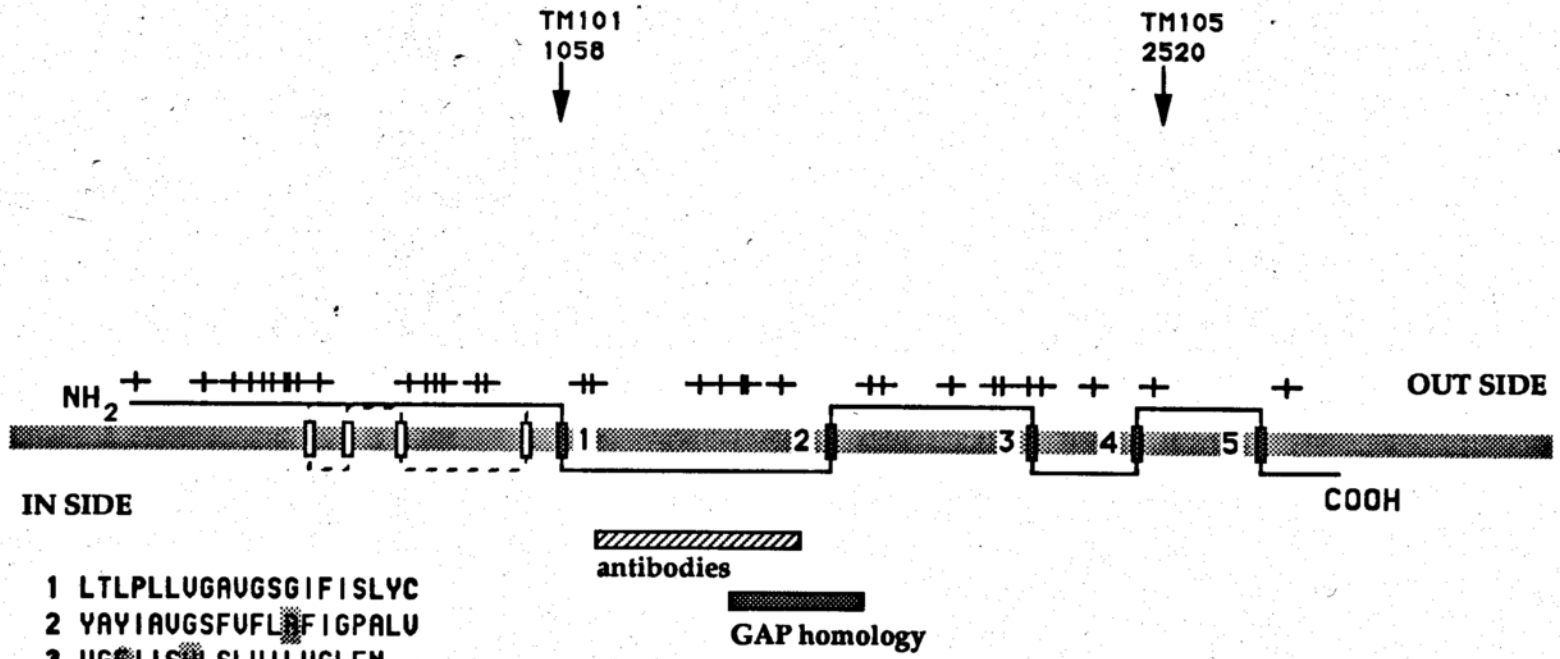
Ira1 in detergent extracts bound to Concanavalin A (Figure 3B) indicating that Ira1 is glycosylated. Detergent extracts of wild type membranes were incubated with either Concanavalin A linked to Sepharose 4B (ConA-Sepharose), or underivatized Sepharose 4B as a control. The resin was washed with buffer, and bound proteins were removed by treatment with reducing SDS gel sample buffer at 65°. The proteins were then separated with SDS PAGE for Western blotting with Ira1 antibodies. As shown in lane 1, a small amount of Ira1 was associated with the control Sepharose 4B, but this decreased when 0.5 M NaCl was added to the buffer (lane 3). In contrast, a much more intense Ira1 signal was associated with the ConA-Sepharose (lanes 2 and 4). Ira1 also bound to lentil lectin-agarose, as shown in figure 3C. Lentil lectin has similar sugar specificity to Con-A, and the binding to the lectin resin was inhibited by the addition of methyl-α-D mannopyranoside, and methyl-α-D glucopyranoside as competetive ligands

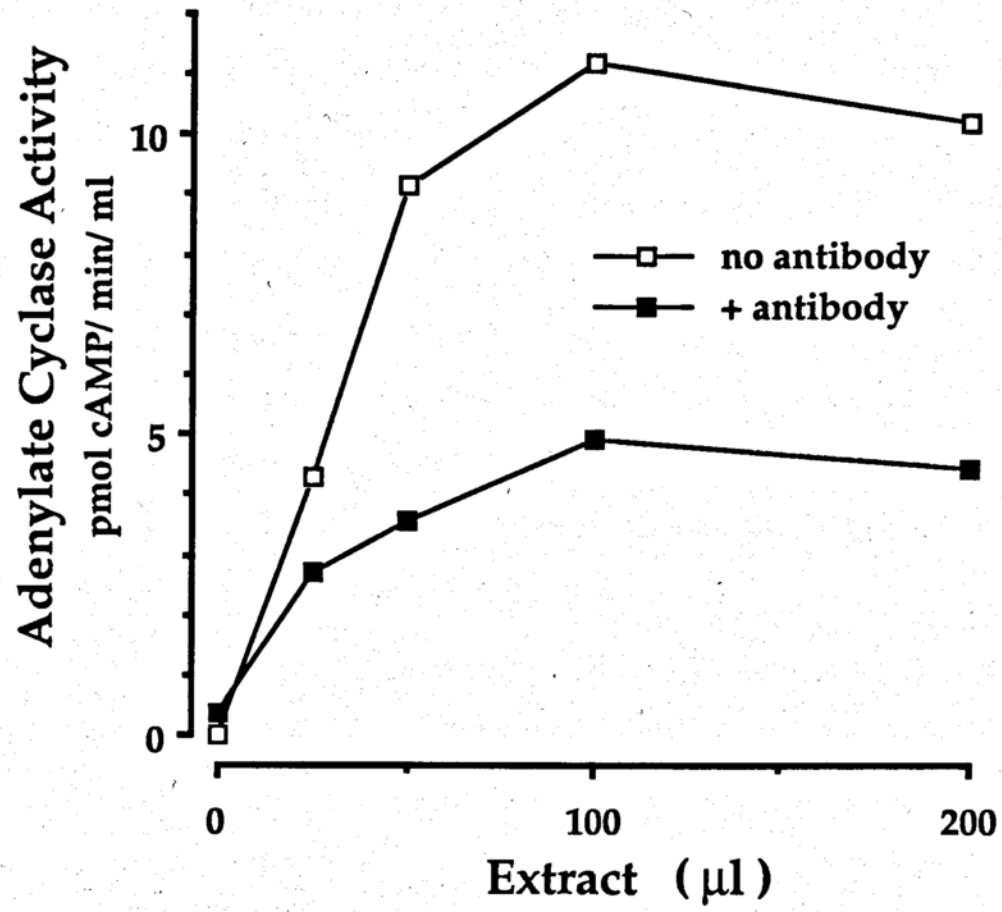

A.
Immune
Pre-Immune
400
200
kDa
116
97
67
1 2 3 4
B.
NaCl
– +
400
330
200
kDa
116
97
67
1 2 3 4
C.
competitor
+ -
200
kDa
116
97
1 2

(lane 1). Thus, on the basis of its diffuse appearance on gels, and its interactions with two different lectins, Ira1 appears to be glycosylated.

Glycosylation of Ira1 indicates at least one extracellular domain. The Ira1 protein sequence contains numerous sites for N-linked glycosylation, as well as potential transmembrane regions (Fig 4). As indicated by the + symbols, Ira1 contains 34 potential sites for N-linked glycosylation. Half of these sites are found in two clusters in the first 900 residues. A Kyte-Doolittle hydrophobicity plot using a window of 19 amino acids identified five potential transmembrane domains in which the hydrophobic index exceeded 1.6 (Fig 4, shaded bars). The locations and sequences of these segments is indicated in figure 4. In addition to these regions, several other stretches of hydrophobic sequence came close to this value, and may also be considered as potential transmembrane sequences (Fig 4, open bars).

IRA1 antibodies inhibit membrane binding of adenylate cyclase. In a reconstitution experiment similar to that described for figure 2, antibodies to *IRA1* inhibited the binding of adenylate cyclase activity to acceptor membranes (Fig. 5). When acceptor membranes from a strain lacking adenylate cyclase activity were incubated with antibodies against Ira1 prior to use in a reconstitution assay, adenylate cyclase binding was inhibited by approximately 50%. We have been unable to completely inhibit reconstitution with our antibodies. The fact that these *IRA1* antibodies did not entirely inhibit reconstitution suggests the possibility of a second class of Ira1 binding sites, not recognized by the antibodies. The antiserum had no direct effect on adenylate cyclase activity, and pre-immune serum did not inhibit reconstitution of adenylate cyclase activity (not shown).


TM101
1058
TM105
2520
NH2
OUT SIDE
1
2
3
4
5
IN SIDE
COOH
antibodies
GAP homology
1 LTLPLLVGAVGSGIFISLYC
2 YAYIAVGSFVFLAFIGPALV
3 VGEIISHLSLVILVGLFN
4 SPLLSQMYLPEILFAVSLLI
5 IILGFIANGLSIPVVKGAAL


Adenylate Cyclase Activity
pmol cAMP/ min/ ml
10
5
0
no antibody
+ antibody
0
100
200
Extract (µl)

Comigration of IRA1 and adenylate cyclase. In detergent solution, adenylate cyclase activity and the Ira1 protein comigrated on a Sepharose 4B gel filtration column. Wild type (381G) membranes were extracted with 5% taurocholate and the extract was applied to a Sepharose 4B column. Fractions were collected and assayed for adenylate cyclase activity. Samples from these fractions were also run on a 4% SDS gel for Western blotting with Ira1 antibodies. Figure 6 allows comparison of the elution position of adenylate cyclase activity (lower box) and the 330 kDa Ira1 protein (upper boxes). At low ionic strength (open triangles) both adenylate cyclase levels and Ira1 protein peaked in the fraction immediately following the void volume, indicating a large complex. When 0.5M NaCl was added to the membrane extract and the column buffer (closed triangles), both adenylate cyclase and Ira1 migrated as smaller particles, and they no longer migrated with the same elution profile.

We interpret these results to indicate that, at low ionic strength, Ira1 and adenylate cyclase are physically associated. However, an alternate interpretation cannot be discounted; the proteins might co-migrate by chance. Ira1 and adenylate cyclase might be independently associated with separate large complexes that are dissociated by high ionic strength. As a more specific test of association between Ira1 and adenylate cyclase, we measured the size of the Ira1 complex in both the presence and absence of adenylate cyclase. If Ira1 is associated with adenylate cyclase, then the size of the complex containing Ira1 should be reduced in the absence of adenylate cyclase. As shown in figure 7, we have found this to be the case. Detergent extracts were prepared from three different strains, a wild type strain

(HR125), a strain carrying a deleted *CYR1* gene (TC41-1), and a strain carrying *CYR1* on YEp24, a multi copy plasmid (HR125::pAC2). TC41-1 has no adenylate cyclase activity or protein, and HR125::pAC2 expresses approximately five fold higher levels of adenylate cyclase than the isogenic wild type strain. The extracts were fractionated on the Sepharose 4B column, and the elution position of Ira1 was measured by Western blotting samples from each fraction. The top portion of Figure 7 shows the 330 kDa regions of the Western blots, and the lower portion shows scanning densitometer measurements of each band. In the absence of adenylate cyclase (TC41-1, open squares) practically all of the Ira1 protein migrated well within the included volume of the column, while in the wild type extracts a significant fraction of the Ira1 protein eluted near the void volume, indicating the formation of a larger complex. The proportion of Ira1 travelling as the large complex was increased even further in the extract from HR125::pAC2 cells that produce elevated levels of adenylate cyclase. The elution position of Ira1 in the absence of adenylate cyclase matched that observed in wild type cell extracts run at high ionic strength.

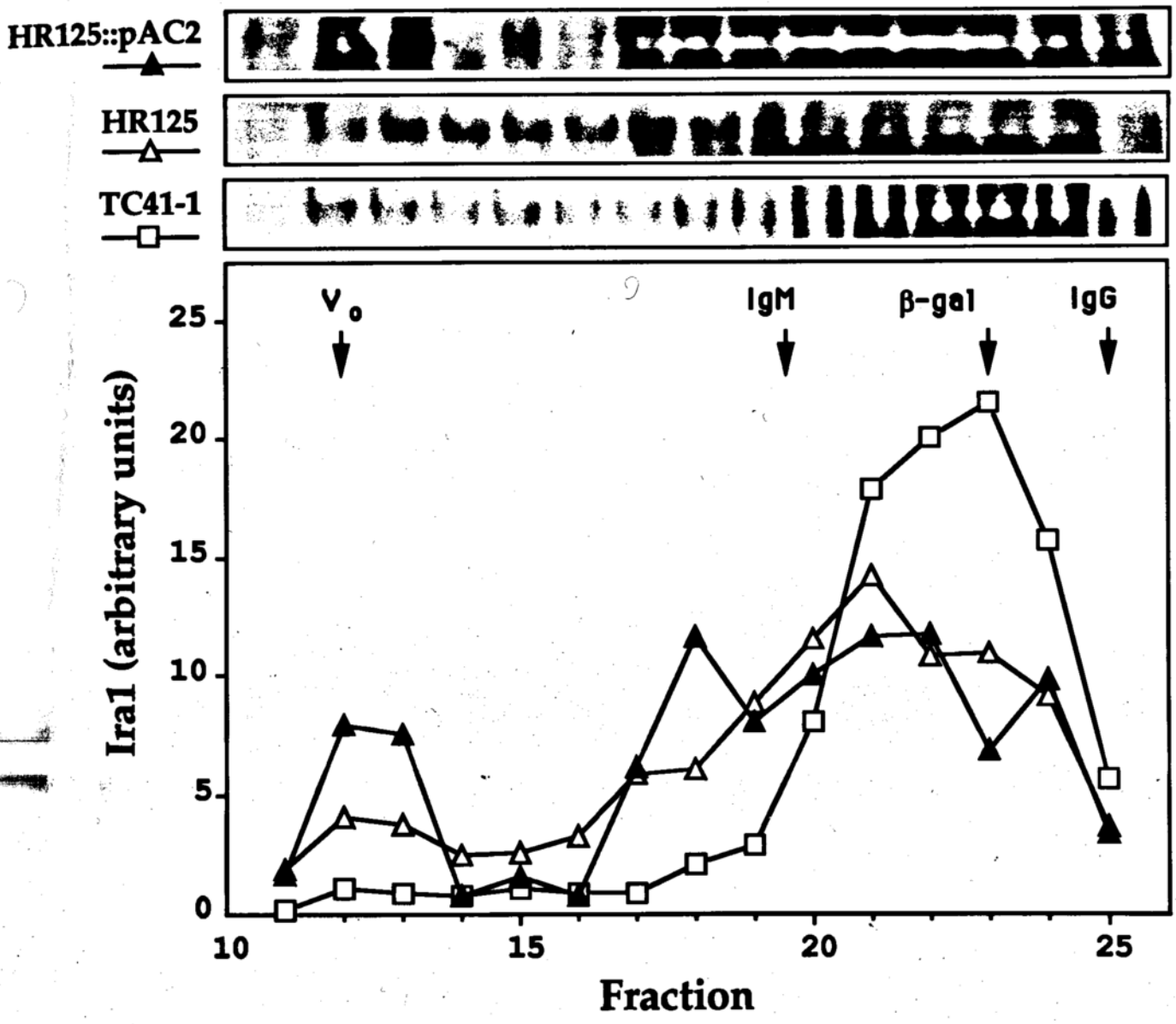

HR125::pAC2
HR125
TC41-1
V0
IgM
β-gal
IgG
Ira1 (arbitrary units)
25
20
15
10
5
0
10
15
20
25
Fraction

Table 5-1. Yeast strains used in Chapter 5.

STRAIN	GENOTYPE
381 G¥ (wild type)	*MAT* **a** *ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3 cryR*
HR125*(wild type)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
NW23-9C*(*cyr1-1*)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1-1 cam*
HR125::pAC2*(multicopy *CYR1*)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ::YEp24:CYR1:URA3*
TC41-1§ (*cyr1* deletion)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1::URA3 cam*
TM101 (*IRA1* disruption at codon 1058)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira1::URA3*
TM102 (*IRA1* disruption at codon 1058, *cyr1*$^-$)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1-1 cam ira1::URA3*
TM105 (*IRA1* disruption at codon 2520)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira1::LEU2*

¥ Hartwell (1980)
* Casperson et al. (1982)
§ Heideman et al. (1990)
† K. Matsumoto

DISCUSSION

Our results support a model in which adenylate cyclase and the *IRA1*polypeptide are associated with each other. This conclusion is based on multiple lines of biochemical and genetic evidence. We propose that Ira1 plays a membrane anchoring role for adenylate cyclase in addition to its previously known regulatory role (Tanaka et al. 1989; Tanaka et al. 1990).

Effect of IRA1 disruptions on adenylate cyclase activity. Previous disruptions of the *IRA1* gene, including the disruption at codon 2520 in TM105, have produced a phenotype characterized by heat shock sensitivity and elevated cAMP levels (Tanaka et al. 1989). This phenotype is characteristic of mutations that activate the Ras/adenylate cyclase pathway. In our hands, the codon 2520 mutation produced substantial elevation of basal adenylate cyclase activity. These observations are consistent with the reports that this disruption blocks the ability of Ira1 to accelerate the GTPase activity of Ras proteins (Tanaka, 1989). The disruption at codon 1058 (strains TM101 and TM102) produced an almost opposite phenotype. Rather than resembling cells with an activated Ras/adenylate cyclase system, these mutants appear deficient in cAMP production. These contrasting phenotypes can be reconciled by taking into account the change in subcellular location of adenylate cyclase in these mutants. Presumably, *all* of the *IRA1* disruptions produce activated Ras proteins, but the disruption at codon 1058 also releases adenylate cyclase from the membranes into the cytosol, where it can no longer be efficiently coupled to Ras. Thus in our model, two functions can be assigned to Ira1; regulation of Ras GTPase activity, and complex formation with adenylate cyclase. This interpretation

is supported by the finding that membranes from the TM101 and TM102 mutants have a greatly reduced capacity to bind adenylate cyclase *in vitro*. Some low level of binding must remain in the TM101 disruptants, since the cells grow, albeit slowly. They must therefore retain some limited ability to couple Ras to adenylate cyclase, in that cells lacking Ras function will not grow at all.

Characterization of the Ira1 protein. Previous experiments using gel filtration to measure the size of taurocholate solublized adenylate cyclase have shown that the enzyme can exist as two different sized complexes. At low ionic strength, adenylate cyclase behaves as a very large particle of several million daltons. However, at higher ionic strength, (0.5 M NaCl) this complex dissociates, and the enzyme migrates as a smaller particle of approximately 600,000 daltons. Adenylate cyclase can also be dissociated from this complex, without prior detergent solublization, by extracting membranes with NaCl. The salt extracted enzyme is indistinguishable from the 600,000 dalton complex seen in salt treated detergent extracts. Thus, NaCl appears to dissociate adenylate cyclase from a complex that anchors the enzyme to the membrane.

If Ira1 is the membrane anchor for adenylate cyclase, then we should observe the two proteins co-migrating at low ionic strength, where adenylate cyclase is associated with the complex, but not at high ionic strength. This is what we have observed; at low ionic strength, the peak of both Ira1 and adenylate cyclase activity eluted in the fraction immediately following the void volume. Although these proteins eluted in the included volume, the resolution of the column in this region is very limited. The

proteins could be fortuitously travelling together. Therefore, although this result is consistent with our model, it falls short of demonstrating association between adenylate cyclase and Ira1.

The experiments shown in figure 7, demonstrating that adenylate cyclase contributes to the size of the Ira1 complex provide more compelling evidence indicating association between the two proteins. In wild type extracts, or in extracts prepared from cells over-expressing adenylate cyclase, the elution position of some of the Ira1 is shifted to that of the larger particle. In extracts prepared from cells lacking adenylate cyclase, Ira1 elutes well in the included volume as a particle similar in size to β-galactosidase (520 kDa). The elution pattern of Ira1 in the absence of adenylate cyclase is similar to the elution position of Ira1 in wild type extracts at high ionic strength. Though dissociated from the larger complex, even the smaller form of Ira1 migrates as a particle larger than expected for monomeric Ira1. This could represent a very elongated Ira1 monomer, or Ira1 involved in some sort of oligomeric structure.

The proportion of Ira1 eluting as the large complex in the wild type (381G) Ira1 elution profiles shown in figure 6 (low ionic strength, open symbols) is greater than that shown in figure 7 (HR125). The profile from figure 6 shows almost all of the Ira1 protein migrating as the large form, indicating that in this extract all of the Ira1 is involved in some large oligomeric complex. While in figure 7, it appears that a smaller proportion of the Ira1 protein migrates as the larger complex. Some of this may be due to strain-to-strain variation in adenylate cyclase activity. We have used the wild type strain 381G shown in figure 6 because it has high levels of adenylate cyclase activity, and because this activity is easily extracted from the membrane.

Thus 381G is more suitable for experiments following adenylate cyclase activity through column chromatography. We have used the strain shown in figure 7 (HR125) for genetic work. HR125 has lower adenylate cyclase activity than 381G, even in the isogenic strain over-expressing *CYR1* (HR125::pAC2). Beyond this difference between strains, we have observed some variation in Ira1 levels between membrane preparations. The variations in the levels of these two proteins may account for some of the changes in the amount of Ira1 that migrates as the larger complex.

Ira1 structure. The lectin binding experiments indicate that Ira1 is glycosylated, and is therefore likely to traverse the plasma membrane at least once. As indicated by the Kyte-Doolittle hydropathy plot in figure 4, there are several candidates for transmembrane sequences in the Ira1 predicted amino acid sequence (Kyte et al. 1982; Jähnig 1990). Five regions meet the criteria of reaching a peak value of at least 1.6 when averaged over a window of 19 amino acids. Additional transmembrane regions that do not quite reach an index of 1.6 can be postulated, four of these are indicated in figure 4. Ira2 also has peaks of hydrophobicity in these regions. Clearly the actual number of trans-membrane segments will only be determined by much more thorough experimentation; however with the evidence at hand, we can begin to make a preliminary model of Ira1 membrane topology. Such a model will be of value in developing tests to further refine our picture of Ira1 structure. As indicated by the + symbols in figure 4, N-linked glycosylation sites are clustered in several regions spread across the sequence; however there is an unusually high concentration of potential glycosylation sites at the amino end of the protein, with 11 sites located

within the first 500 amino acids. We therefore propose that the amino terminus is likely to be on the outside of the membrane.

Tanaka *et al* (1989) have reported a disruption of *IRA1* at codon 1430 that produces the activated Ras phenotype. It is therefore likely that adenylate cyclase in these mutants remains coupled to Ras, and is associated with the membrane. This indicates that some structure between position 1430 and our disruption at codon 1058 is important for proper localization of adenylate cyclase. It is interesting to note that our antibodies that block adenylate cyclase binding also recognize this region. These antibodies were raised against a fusion protein expressing amino acids 1127 to 1618 of Ira1. Since the interaction between Ira1 and adenylate cyclase must take place within the cell, this region of Ira1 is likely to occupy the cytoplasmic face of the membrane. Additionally, the nearby region of GAP homology, occupying positions 1471 to 1780, must interact with the cytoplasmic Ras proteins, is also likely to be located in the interior of the cell. A model of Ira1 membrane topology based on the above constraints is shown in figure 4. It should be emphasized that there are alternative interpretations of the data that lead to different models of Ira1 structure. In particular, the actual number of membrane spanning domains will have a large impact on the model.

Ira1/adenylate cyclase complex. Our results indicate that Ira1 and adenylate cyclase are associated in a large oligomeric complex. This is based on the results obtained with mutations, antibodies and gel filtration chromatography. If such a complex exists, what proteins are included? Presumably the complex includes at least the Cyr1 catalytic subunit, Ira1, and CAP, (cyclase associated protein, also identified as the product of the SRV2

gene). Ras proteins must also be associated with the complex, at least on a transient basis. Are there other proteins involved? By virtue of physical and functional similarities to Ira1, the *IRA2* gene product is a good candidate to serve a similar function to that of Ira1. Inclusion of Ira2 in the complex might explain several observations. Antibodies against Ira1 were effective in blocking only half of the membrane capacity to bind adenylate cyclase, while disruption of *IRA1* almost entirely abolished binding of adenylate cyclase to the membrane. This might be explained by a second class of binding sites (such as Ira2) that is not blocked by the antibodies, but is dependent on a functional Ira1 protein for stability. The disruption of *IRA1* at codon 1058 may have decreased the stability of all of the members of the complex. In the absence of adenylate cyclase, Ira1 migrates as a particle of approximately twice the size expected for monomer Ira1. This is approximately the size that would be expected of an Ira1/Ira2 dimer.

Role of IRA1 in regulating cAMP production. Association between Ira1 and adenylate cyclase implies that Ras proteins interact with both adenylate cyclase and Ira1 either simultaneously, or in quick succession. Since Ira1 turns off Ras activity, this indicates that termination of Ras activation does not begin until the Ras protein encounters the enzyme complex (unless there is a substantial excess of Ira1). This would prevent a futile cycle of Ras proteins being activated and inactivated without finding their target.

To date, Ira1 is the only protein identified in the Ras/adenylate cyclase pathway that appears to span the membrane. Although a transmembrane protein is not absolutely necessary, such a protein would be well suited for receiving signals originating outside of the cell. Perhaps related to this model is the finding that the smaller GAP, which is cytoplasmic, interacts

with a transmembrane mitogen receptor, the platelet derived growth factor (PDGF) receptor (Kazlauskas et al. 1990). The model we have described allows more regulatory input into the Ras system. Multiple inputs are appropriate to a system that must regulate growth in response to many different environmental conditions, a need that is held in common between yeast and animal cells.

PART 3

THE ROLE OF THE *RAS*/ADENYLATE CYCLASE SYSTEM IN THE CONTROL OF CELL GROWTH IN YEAST

CHAPTER 6

IRA1 gene expression; correlation with cAMP levels and growth arrest in the yeast *Saccharomyces cerevisiae*.

ABSTRACT

Levels of cAMP play an important role in the decision to enter the mitotic cycle in the yeast, *Saccharomyces cerevisiae*. In addition to growth arrest at stationary phase, *S. cerevisiae* transiently arrest growth as they shift from fermentative to oxidative metabolism (the diauxic shift). Experiments examining the role of cAMP in growth arrest at the diauxic shift show: 1. yeast lower cAMP levels as they exhaust their glucose supply and shift to oxidative metabolism of ethanol; 2. a reduction in cAMP is essential for traversing the diauxic shift; 3. this decrease in cAMP levels is accompanied by a drop in adenylate cyclase activity; 4. the decrease in adenylate cyclase activity is associated with an increase in *IRA1* expression.

INTRODUCTION

As yeast grow and divide in glucose medium, the culture shows a characteristic growth pattern. Initially, the yeast grow rapidly, utilizing glucose as a carbon source, until the supply of glucose becomes depleted. The cells then temporarily arrest growth in the G_1 phase of the mitotic cycle, while shifting metabolism from fermentation of glucose to oxidation of ethanol (the diauxic shift). They then resume proliferative growth. This pause in growth produces a plateau in the growth curve, separating fermentative from oxidative growth (Kappeli 1986). The diauxic shift therefore normally involves two processes, a mitotic cycle exit and re-entry, and derepression of the synthesis of enzymes needed for oxidative growth. Intracellular levels of cAMP have been implicated in controlling the decision to enter mitosis, and mutations in genes regulating the cAMP pathway have been shown to block either entry or exit from the mitotic cycle (Matsumoto et al. 1983). Mutations that elevate cAMP produce cells

that have difficulty arresting proliferation, while mutations that block cAMP production produce cells that cannot enter the mitotic cycle (Matsumoto et al. 1985). As an example, *bcy1* mutants, producing a constitutively activated cAMP dependent protein kinase (cAPK), do not properly arrest growth (Matsumoto et al. 1983). Changes in cAMP levels might then be expected to play a role in the transient growth arrest at the diauxic shift. However, very little is known about how cAMP levels are regulated, or how this regulation affects growth at the diauxic shift.

In *S. cerevisiae*, cAMP is produced by adenylate cyclase, encoded by the *CYR1* gene, (Matsumoto et al. 1982). Adenylate cyclase is in turn stimulated by the GTP binding products of the *RAS1* and *RAS2* genes (DeFeo-Jones et al. 1983; Powers et al. 1984; Toda et al. 1985). Downstream in the pathway, cAMP binds to the regulatory subunit of the cAMP dependent protein kinase (encoded by *BCY1*) and initiates a phosphorylation cascade. Upstream in the pathway are positive and negative regulators of Ras, *CDC25* and *IRA1&2* respectively. The *CDC25* gene product has Ras GDP/GTP exchange activity which serves to activate Ras proteins. Ira1 and Ira2 are homologs of the mammalian GAP (GTPase activating protein) these proteins stimulate the GTPase activity intrinsic to Ras proteins, thereby converting active Ras -GTP to inactive Ras -GDP. In yeast this decreases adenylate cyclase activity, and ultimately reduces intracellular levels of cAMP (Tanaka et al. 1989; Tanaka et al. 1990).

We present experiments demonstrating that cAMP levels drop sharply in *S. cerevisiae* as they arrest growth at the diauxic shift. This drop in cAMP is associated with induction of Ira1 and a decrease in GTP stimulated adenylate cyclase activity. This is consistent with a model in which growth is actively

arrested at the diauxic shift by changes in the expression levels of components of the Ras/adenylate cyclase system.

METHODS

Abbreviations. GppNHp, 5' guanylylimidodiphosphate.

Materials. Adenylate cyclase grade [α-^{32}P]ATP (40 cpm/pmol) was from Amersham, [^{3}H]cAMP (10,000 cpm/assay) and [^{125}I] NaI were from New England Nuclear.

Media. Yeast were grown on either rich medium containing 1% yeast extract, 2% bacto peptone, and either 2% glucose (YPD) or 2% ethanol (YPE), or on minimal medium (SD) containing 6.7 g/l yeast nitrogen base, 2% glucose and the appropriate factors to support the growth of auxotrophic strains.

Disruptions of the IRA1 gene. The plasmid pNR1 (Tanaka et al. 1989) provided by K. Tanaka was used to disrupt the *IRA1* gene at position 8103, in the strain HR125 resulting in the mutant TM105. Disruption was confirmed using the polymerase chain reaction and primers flanking the insertion site to amplify the region of genomic DNA disrupted by the marker.

Preparation of yeast particulate fractions. Yeast were grown to an optical density (O.D.) of approximately 2-4, at 660 nm. The cultures were centrifuged and cells were disrupted by vortexing with glass beads in YMB buffer, containing 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 (Casperson et al. 1983). The homogenate was centrifuged in a Beckman J6, 5 min at 1,000 rpm to remove

nuclei and unbroken cells. The supernatant was then spun 30 min at 14,000 rpm to separate particulate and soluble fractions. Both particulate and soluble fractions were stored in YMB at -70° until needed.

Adenylate cyclase assay. Adenylate cyclase was assayed by a modification of the method of Salomon et al. (Salomon et al. 1974) as described by Casperson et al. (Casperson et al. 1985). Guanine nucleotides, where present, were at 10 μM. Protein was assayed by the method of Lowry et al with BSA as a standard. (Lowry et al. 1951).

Western blots. Protein samples (yeast membranes) were separated on 5% polyacrylamide gels, transferred to nitrocellulose and the membranes were blocked with Blotto (5% nonfat powdered milk in 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.01% sodium azide, 0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate), and probed with *IRA1* antibody (1: 600 dilution in Blotto). Immunoreactive proteins were visualized by the addition of [^{125}I]-labelled goat-anti-rabbit antibody (specific activity 2,000,000 cpm/μg), followed by autoradiography of the washed membrane.

Preparation of RNA and Northern blots. Yeast cells (approximately 20 OD units) were resuspended with 300 μl lysis buffer (0.1 M Tris, pH 7.5; 0.1 M LiCl; 0.01 M dithiothreitol) and added to tubes containing glass beads (500μl), 1% SDS (60 μl), and phenol/chloroform (600 μl). The cells were disrupted by six cycles of 30 second bursts of vortexing and 30 seconds on ice. Lysis was followed by multiple phenol chloroform extractions and ethanol precipitations as described (Ellwood and Craig 1984). Fifteen microgram samples were electrophoresed and transfered onto a Gene Screen Plus membrane according to the suppliers directions (New England Nuclear). Blots were probed with two EcoR1 fragments (1255 and 1048 bp) from IRA1

radiolabelled with ^{32}P by the random primer method (Promega Prime-A-Gene system). To confirm uniform loading and transfer, ribosomal RNA was also probed.

cAMP Measurements. Cells (approximately 4-10 OD units /sample) were harvested, and extracts were prepared by resuspending pellets in 1 ml of 10% trichloroacetic acid (TCA). This extract was freeze thaw cycled 3 times and centrifuged in an eppendorf microcentrifuge (14,000 RPM) for 2 min. The supernatant was extacted with 5 washes of ether (2 ml each). The aqueous sample was then dried in a Speed Vac and resuspended in assay buffer. The samples were assayed for cAMP content with a Rianen cAMP [^{125}I] radioimmunoassay kit (New England Nuclear) according to the manufacturers instructions.

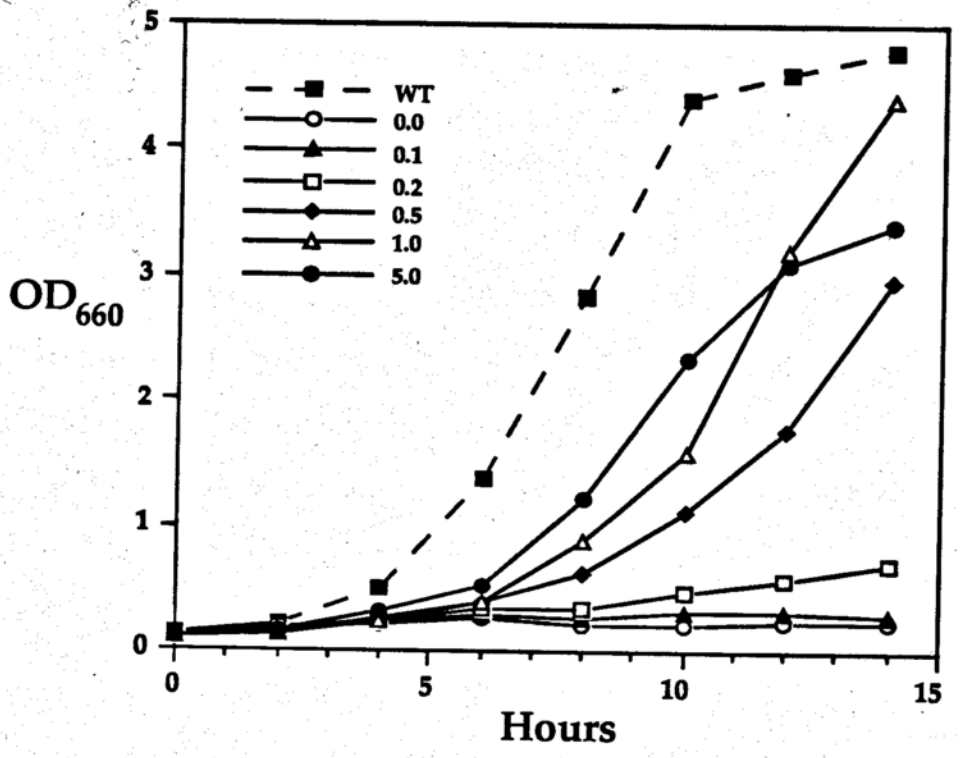
RESULTS

Role of cAMP in the diauxic shift. In order to examine the effects of cAMP at the diauxic shift, we used a strain in which we could manipulate cAMP levels. This strain (TC41-1) cannot make its own cAMP because it carries a deletion in *CYR1*, the structural gene for adenylate cyclase. These cells are able to take up cAMP from the growth medium by virtue of *cam* mutations, and can be propagated by adding exogenous cAMP. Thus, cAMP levels in these cells can be manipulated by changing the cAMP concentration of the growth medium. When TC41-1 cells were grown in rich (YPD) medium with varying amounts of cAMP, two effects became apparent. The first effect was observed in the lag phase of the growth curve. Increased cAMP concentrations shortened the lag between dilution into fresh medium and

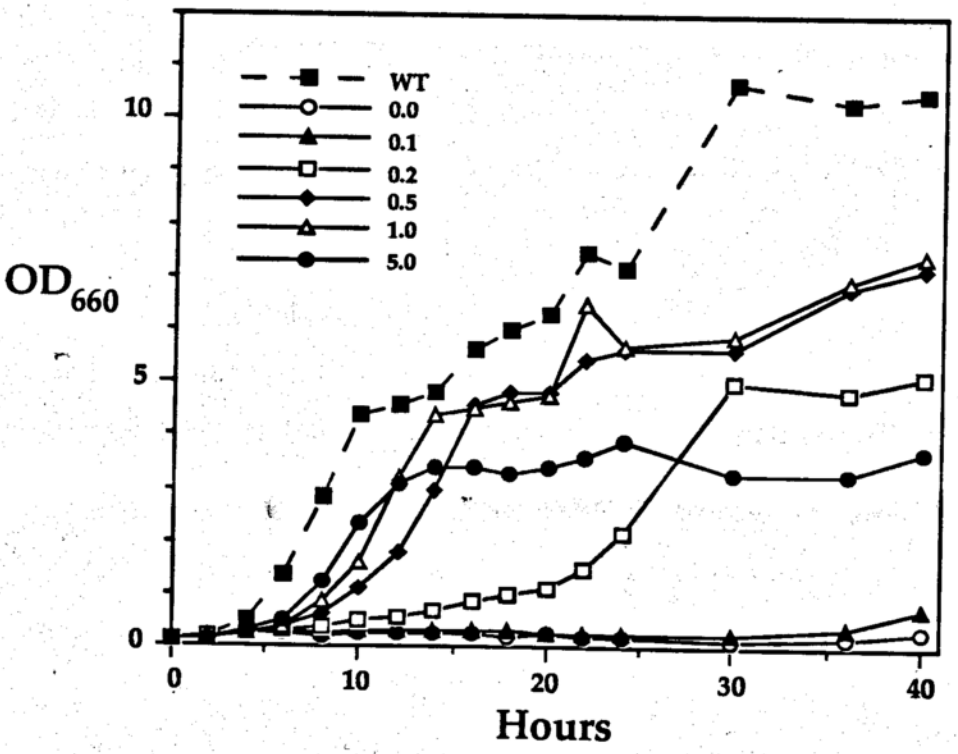
growth (Fig 1A). The TC41-1 cells grown at the highest cAMP concentrations had the shortest lag phase, while cells grown at lower cAMP concentrations took longer to begin proliferating. The isogenic wild type cells (HR125) invariably had an even shorter lag phase, indicating that we may not be able to duplicate normal peak cAMP levels in the *cyr1*$^-$ cells. Although cAMP had a pronounced effect on the lag phase, the rate of growth that the cultures ultimately achieved was approximately the same, regardless of cAMP concentration. Cultures at low cAMP, showing an obviously greater lag, eventually reached the same growth rate as wild type cells. Thus, cAMP levels appeared to have a more pronounced effect on the decision to leave stationary phase and enter the mitotic cycle than on the actual rate of cell division.

The second effect was manifested as cells reached the diauxic shift. Invariably, cells cultured at higher cAMP concentrations stopped growing at lower densities (Fig 1B). Additionally, although cells grown at 5 mM cAMP most closely resembled the wild type strain in the fermentative phase of growth, these cells failed to grow at all in the oxidative phase of the growth curve. When examined, these cells had stopped growth at all stages of the cell cycle. Cells grown at lower cAMP concentrations were more able to traverse the diauxic shift and grow in the second phase of the growth curve. However, even the cultures at lower cAMP concentrations failed to achieve the rate of growth seen in the wild type culture after the diauxic shift. None of the cultures grown at constant cAMP displayed a real growth plateau. Rather, cells grown at lower cAMP appeared able to grow slowly

A.


WT
0.0
0.1
0.2
0.5
1.0
5.0
OD660
Hours
0
1
2
3
4
5
0
5
10
15

B.

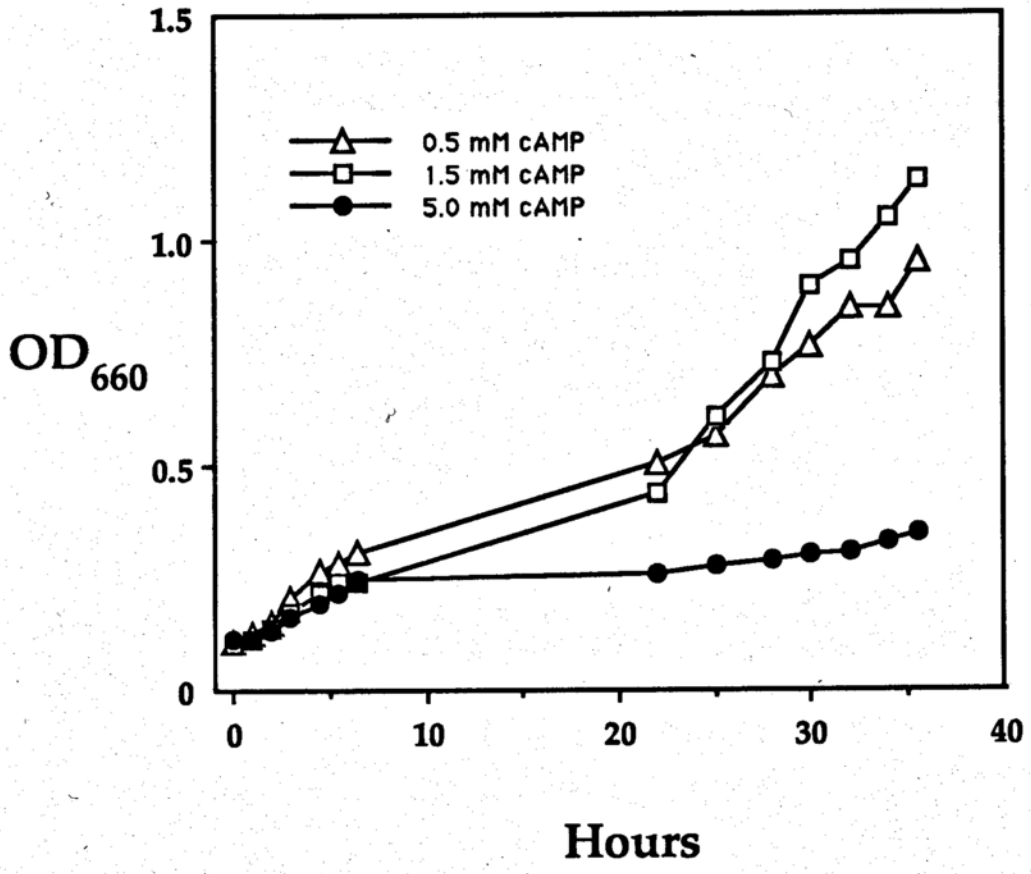

WT
0.0
0.1
0.2
0.5
1.0
5.0
OD660
Hours
0
5
10
0
10
20
30
40

but steadily on ethanol immediately upon reaching the second phase of growth.

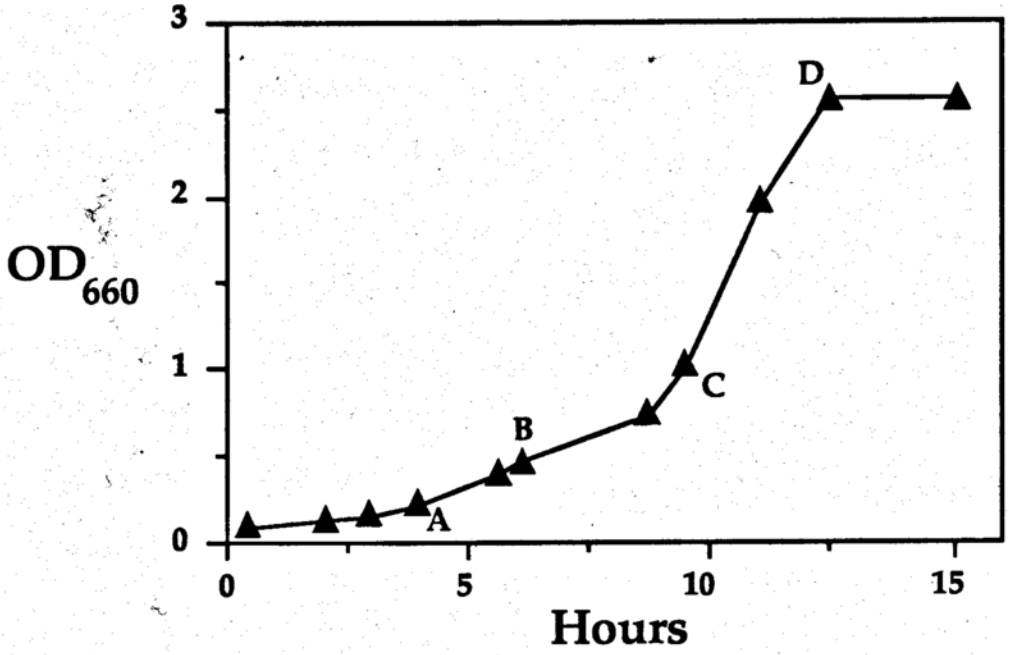
Our data indicates that no fixed concentration of cAMP allows normal growth rates, and that the ability to control cAMP production is needed for optimal growth. In all cases, cells that could control cAMP production (wild type cells) grew more rapidly than TC41-1 cells provided with exogenous cAMP. None of the cultures grown at fixed concentrations of cAMP displayed a normal diauxic shift. Cells at high cAMP showed no second growth phase, while the cultures at lower cAMP showed no apparent pause, but appeared already able to utilize ethanol.

These results are consistent with the idea that although yeast grow most vigorously at high cAMP levels in the early stages of culture, they require cAMP to drop below a certain concentration in order to utilize a non-fermentable carbon source. We tested this by varying the cAMP concentration as cells growing in glucose were shifted into ethanol medium. Yeast were grown in glucose medium at 5 mM cAMP, and then transferred to ethanol medium at varying cAMP concentrations (Fig 2). Cells maintained at high cAMP in the ethanol medium failed to grow, but if the cAMP level was reduced to at least 1.5 mM in the ethanol medium, the yeast were able to proliferate. This demonstrates that a drop in cAMP is necessary for successful progression to oxidative growth.

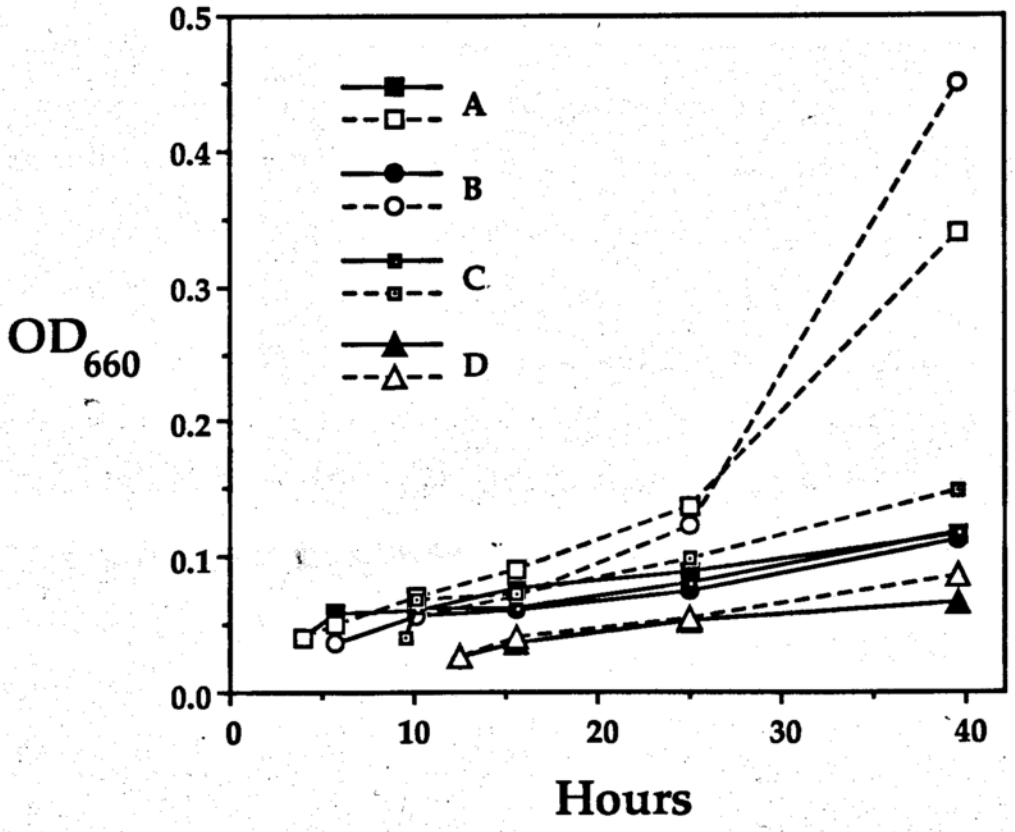
Further experiments showed that this drop in cAMP level must occur at the right time in order to allow growth on ethanol medium (Fig 3). Cells were grown in YP-glucose (panel A) at high cAMP (5 mM), and at various points in the growth curve, as indicated, the samples were transferred to YP-


1.5
1.0
0.5
0
0.5 mM cAMP
1.5 mM cAMP
5.0 mM cAMP
OD660
0
10
20
30
40
Hours

A.


3
2
1
0
OD660
A
B
C
D
0
5
10
15
Hours

B.

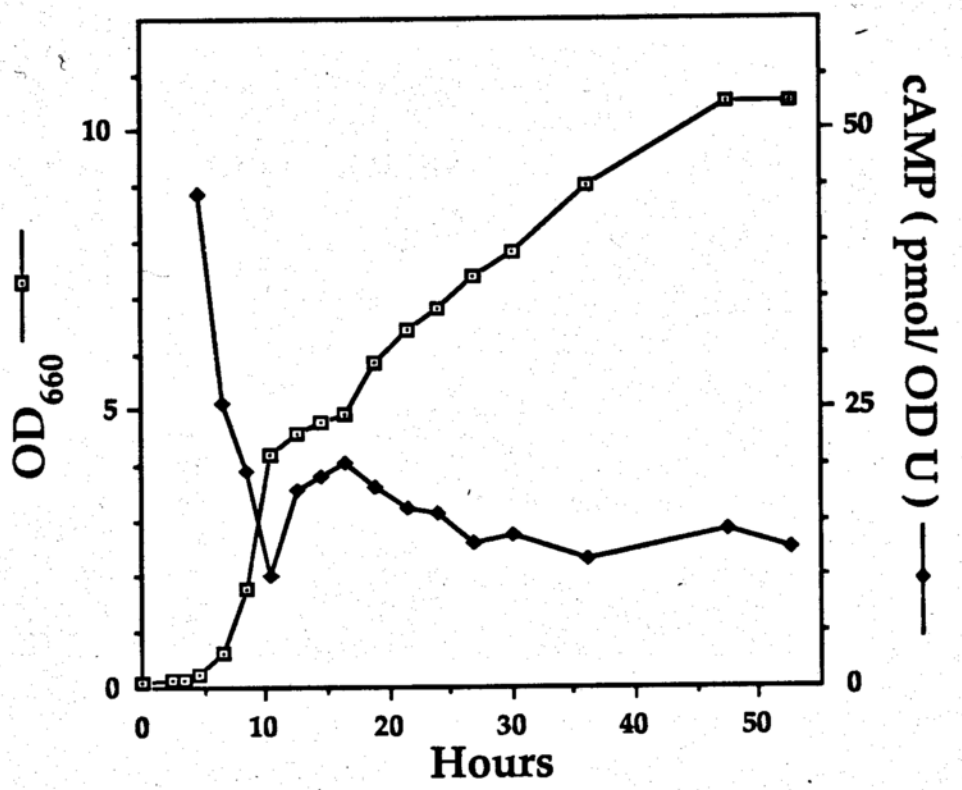

0.5
0.4
0.3
0.2
0.1
0.0
OD660
A
B
C
D
0
10
20
30
40
Hours

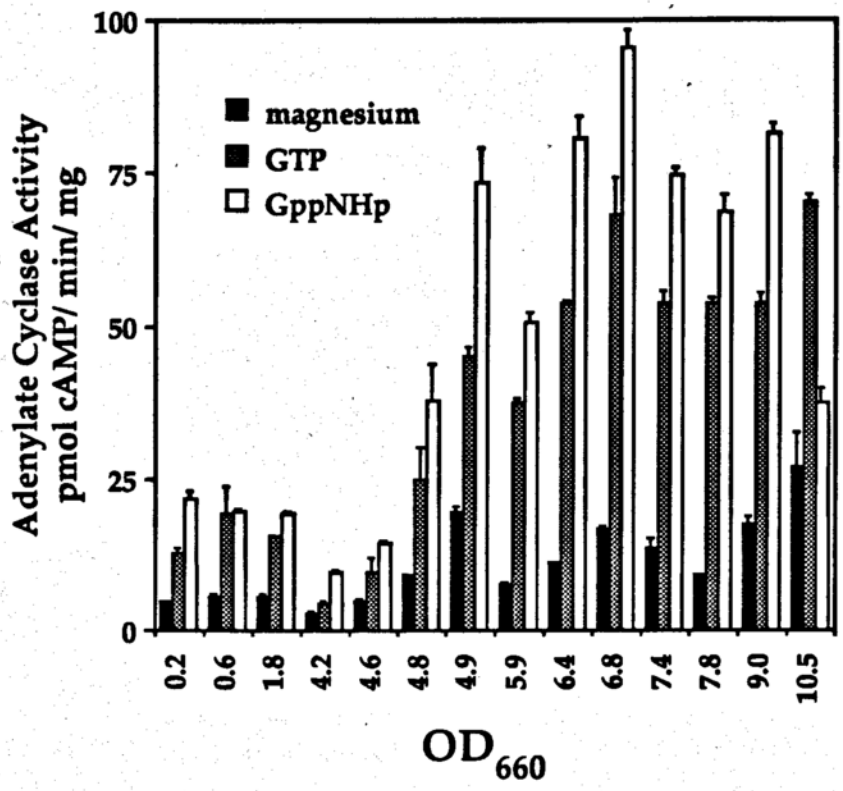
ethanol (panel B) at either the same high cAMP concentration, or at reduced cAMP (1 mM). As was the case in the experiment shown in figure 2, if cells were shifted into ethanol early in the growth curve (below 0.5 OD), they were able to grow if cAMP was reduced (1 mM, dashed lines) but not at high cAMP (5 mM, solid lines). However, if the cells were allowed to proceed farther in the glucose growth curve (1-3 OD) before the shift to ethanol, dropping the cAMP no longer allowed growth on ethanol medium. The critical density appeared to be during the last generation before the glucose was exhausted; between 1 and 3 OD.

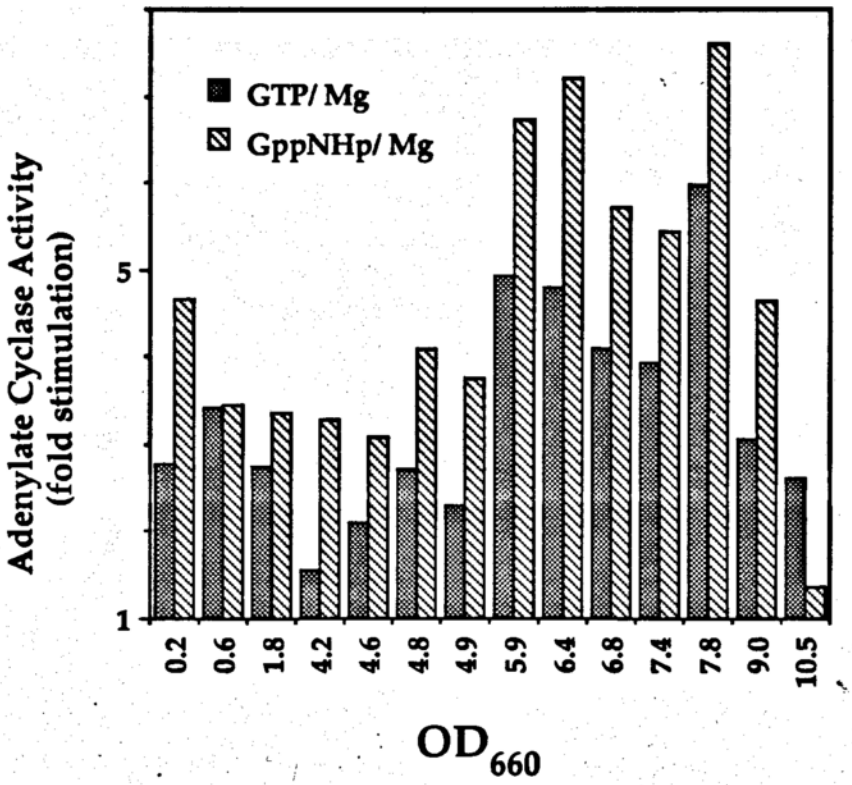
It is reasonable to suppose that the drop in cAMP allows the cells to halt cell division long enough to shift metabolic machinery. Cells that do not halt division for this process grow to a point where they no longer have sufficient resources to effect the shift; they are then forced to halt growth for lack of nutrient supply. Left in this condition, these cells will lose viability much faster than cells arrested in G_1. Although unable at this point to be converted to growth on ethanol, these cells could be rescued with fresh medium containing glucose.

Changes in cAMP production during growth. Measurement of cAMP levels in cells harvested at various points during the culture of a wild type (HR125) strain confirmed that yeast do indeed drop their cAMP levels as they approach the diauxic shift (Fig 4). cAMP levels showed a sharp minimum at about 4 OD, as cells stopped dividing. As the cells resumed growth, cAMP levels rose again; however, neither cAMP, nor growth rate returned to the levels observed during the fermentative phase.

The capacity to produce cAMP also fell as the yeast reached the diauxic shift (Fig 5). Membranes were prepared from cells grown in the experiment


OD660
cAMP (pmol/ OD U)
Hours
0
5
10
0
25
50
0
10
20
30
40
50


A.
Adenylate Cyclase Activity
pmol cAMP/ min/ mg
100
75
50
25
0
magnesium
GTP
GppNHp
0.2
0.6
1.8
4.2
4.6
4.8
4.9
5.9
6.4
6.8
7.4
7.8
9.0
10.5
OD660


B.
Adenylate Cyclase Activity
(fold stimulation)
5
1
GTP/ Mg
GppNHp/ Mg
0.2
0.6
1.8
4.2
4.6
4.8
4.9
5.9
6.4
6.8
7.4
7.8
9.0
10.5
OD660

shown in figure 4, and assayed for adenylate cyclase activity with either Mg^{2+} alone, to measure basal activity; with the non-hydrolysable GTP analog, GppNHp, to measure adenylate cyclase maximally stimulated by Ras proteins; or with GTP to measure the activity that would be produced at normal GTP levels within the cell. Adenylate cyclase was lowest in membranes prepared from the cells at the beginning of the plateau in the growth curve (4.2 OD). These were also the cells with the lowest cAMP levels.

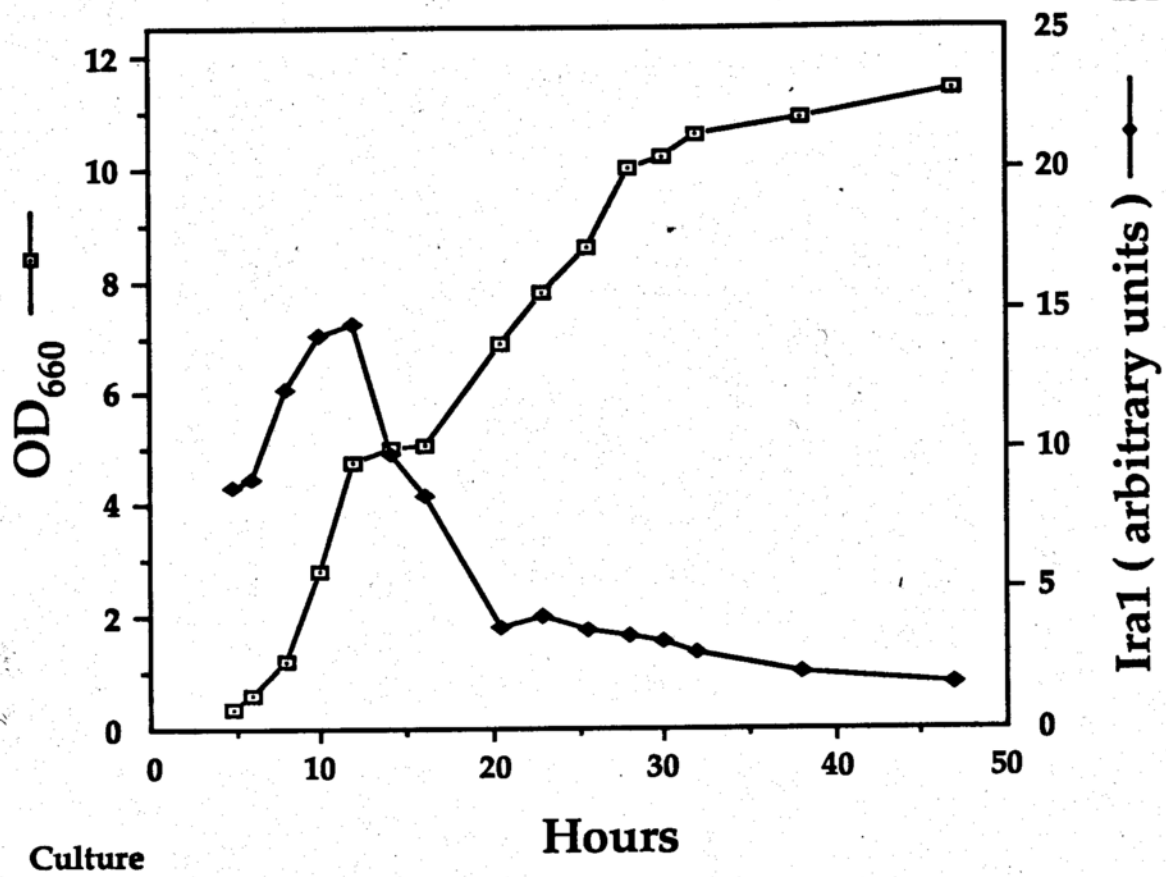
Each density point in Figure 5A involved a separate membrane preparation. Because there is some variability in the specific activity of adenylate cyclase between membrane preparations (even when prepared from the same culture of cells) it is difficult to determine exactly how the membrane specific activity varies with growth. However, when activities are expressed as fold stimulation by either GTP or GppNHp over basal activity, the variability due to changes in specific activity is eliminated (Fig 5B). Fold stimulation by guanine nucleotides decreased as the culture approached the diauxic shift, and increased as the cells resumed growth. This effect was very reproducible; in three separate experiments, stimulation by GppNHp, and GTP, was consistently lowest in cells taken from the generation just before the diauxic shift. GTP stimulated activity was especially reduced as cells reached the diauxic shift.

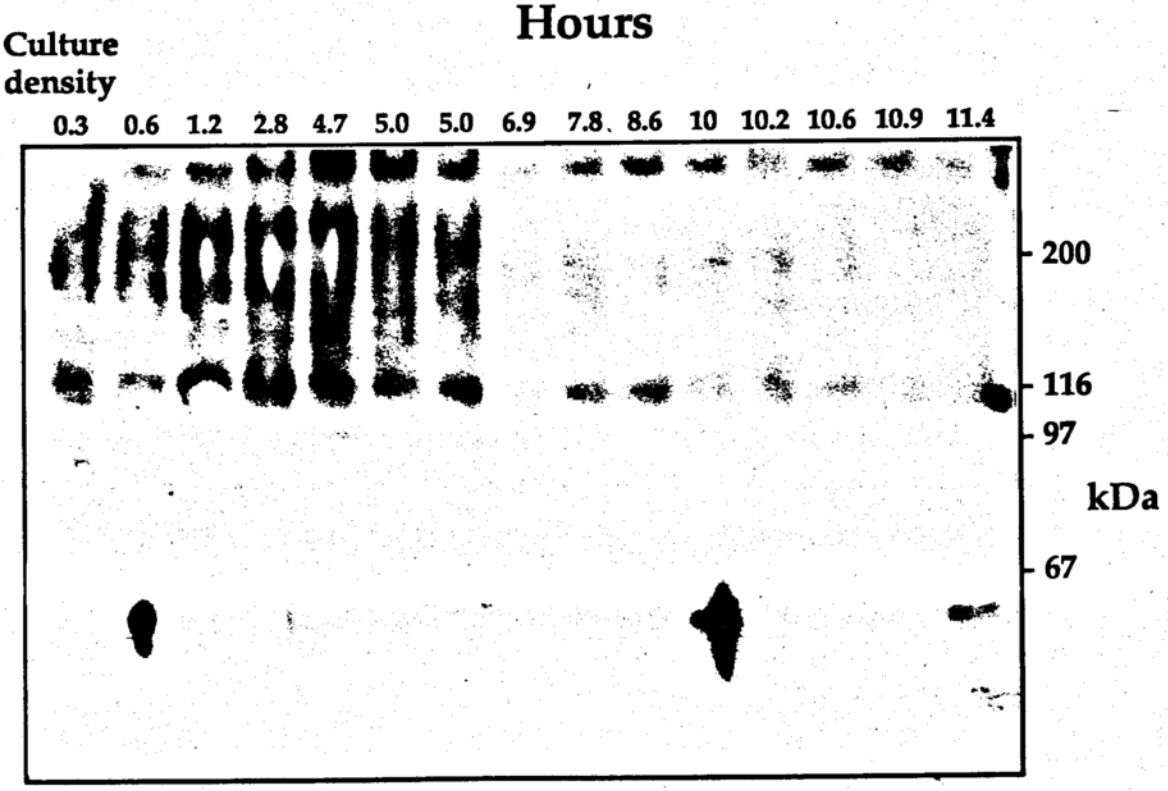
Expression of IRA1. A number of factors could contribute to the decrease in guanine nucleotide sensitivity observed at the diauxic shift. These include changes in guanine nucleotide exchange activity initiating Ras activation, changes in the coupling between Ras and adenylate cyclase, or changes in the rate of termination of the Ras signal. We have examined one possible

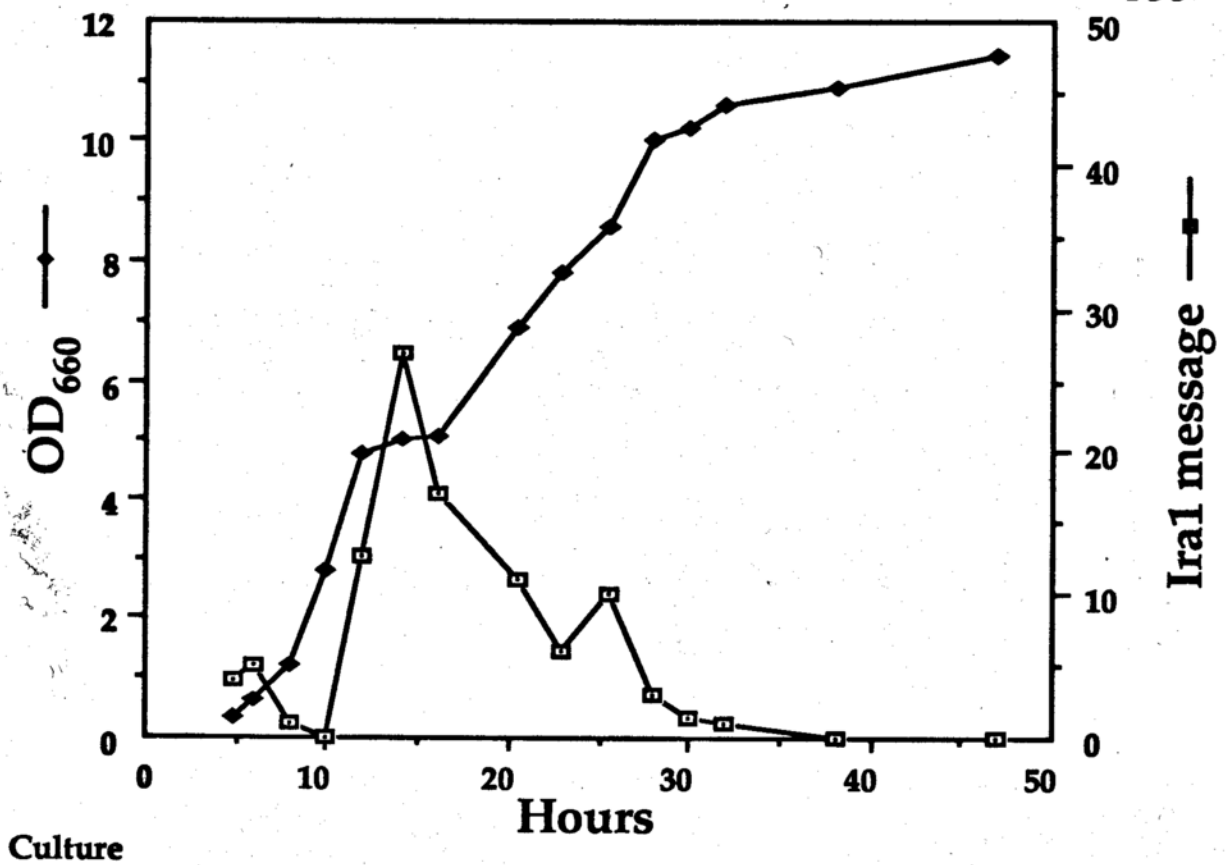
factor in the regulation of guanine nucleotide sensitivity, expression of the product of the *IRA1* gene. Membranes prepared from cells harvested at varying densities were prepared for Western blotting with antibodies against Ira1 (Mitts et al. 1991). Levels of Ira1 protein varied at least 15 fold over the course of culture growth (Fig 6). The top panel shows a plot of culture density as well as Ira1 levels as measured by densitometer scanning of the Western blot. Ira1 levels peaked in the generation leading up to the diauxic shift, and diminished markedly as the culture resumed growth in the oxidative phase. Thus, Ira1 levels peaked as cAMP, and Ras stimulated adenylate cyclase activity reached their lowest levels.

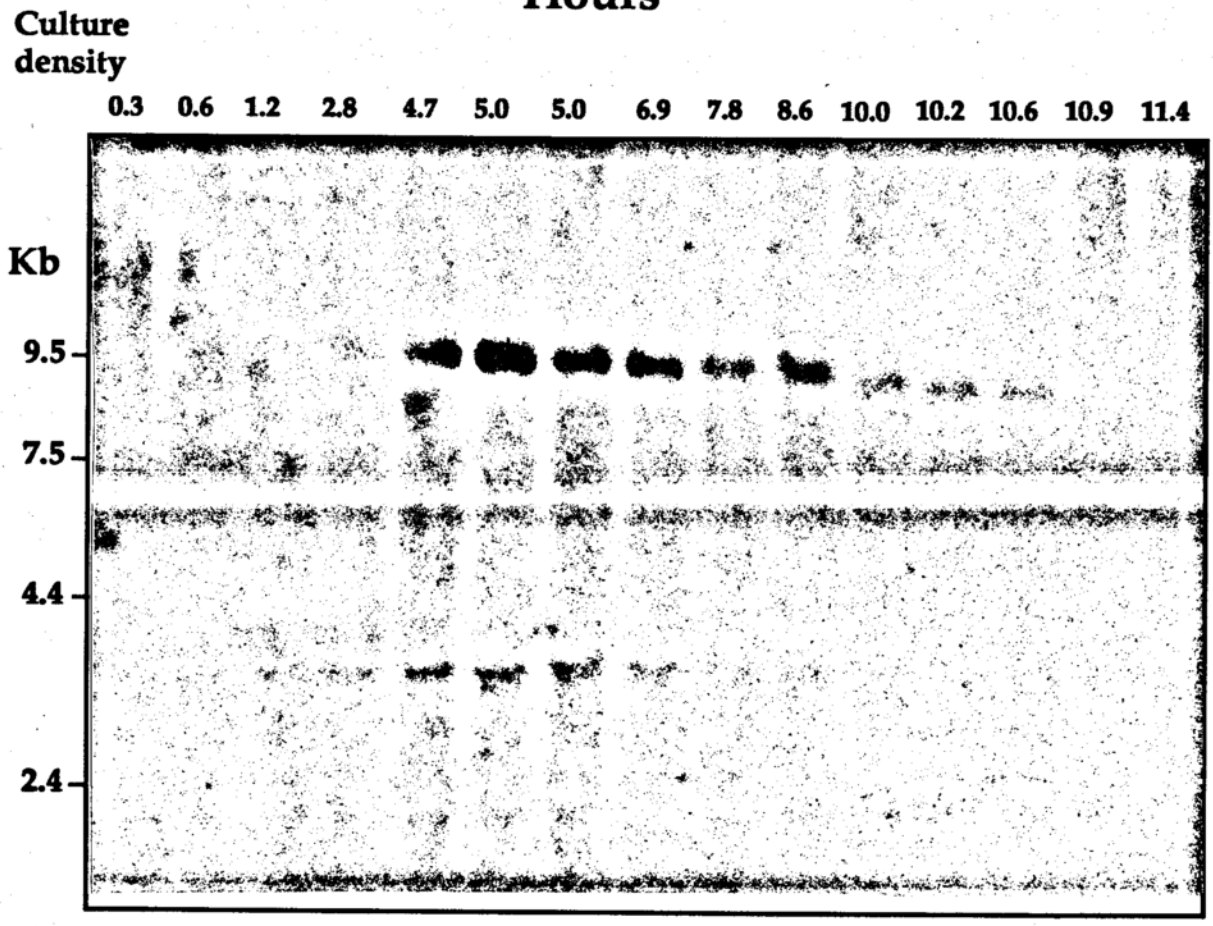
IRA1 message levels also varied with culture density (Fig 7.) Northern blots of total RNA prepared from samples harvested during the growth of wild type yeast (HR125) showed that the *IRA1* message level was highest at about the midpoint of the diauxic shift. The *IRA1* message gradually disapeared as cells resumed growth, in parallel with changes in Ira1 protein levels. Although both Ira1 message and protein levels were increased at the diauxic shift these did not exactly coincide; the *IRA1* message level reached a peak after the peak of Ira1 protein. This indicates that although message levels may play a large role in *IRA1* expression, Ira1 protein levels are not regulated by the levels of *IRA1* message alone. The smaller band observed at about 3 kb was not specific to the *IRA1* probe.

Effect of IRA1 mutation on diauxic shift. Our results are consistent with a model in which induction of Ira1 at the diauxic shift lowers cAMP production and halts proliferation. In order to test this, we used a disruption of *IRA1* at codon 2025 that has been shown to abolish the ability of Ira1 to activate the Ras GTPase (Tanaka et al. 1990). We grew cells


OD660
Ira1 (arbitrary units)
Hours
0
2
4
6
8
10
12
0
5
10
15
20
25
0
10
20
30
40
50


Culture density
0.3
0.6
1.2
2.8
4.7
5.0
5.0
6.9
7.8
8.6
10
10.2
10.6
10.9
11.4
200
116
97
67
kDa


12
10
8
6
4
2
0
OD660
50
40
30
20
10
0
Ira1 message
0
10
20
30
40
50
Hours


Culture density
0.3
0.6
1.2
2.8
4.7
5.0
5.0
6.9
7.8
8.6
10.0
10.2
10.6
10.9
11.4
Kb
9.5
7.5
4.4
2.4

carrying this disruption (TM105) on rich medium in order to see what effect the loss of the GAP function of Ira1 would have as the cells approached the diauxic shift. As illustrated in Figure 8, the mutants were able to traverse the diauxic shift; however the growth profile of these mutants was altered. The mutants started growth with a shorter lag than the wild type cells, and were reproducibly less successful in utilizing ethanol as a carbon source. The TM105 mutants stayed longer in the growth plateau and grew more slowly in the second phase than the wild type cells. The Tm105 mutants also never reached the same density at stationary phase as wild type cells. This is more evident when the growth curves are aligned to compensate for the shorter lag phase in the TM105 culture (dashed line). Overall, the growth curve of the TM105 cells resembled that of cells growing at elevated cAMP, and is consistent with the increased cAMP levels reported for this disruption. The loss of Ira1 GTPase did alter the diauxic shift, indicating a role in the normal regulation of this process. However, the Ira1 mutation did not entirely block the transition to growth on ethanol. This indicates that either the disruption did not entirely block Ira1 function (the altered protein is produced at normal levels), or that other pathways are also important in the process.

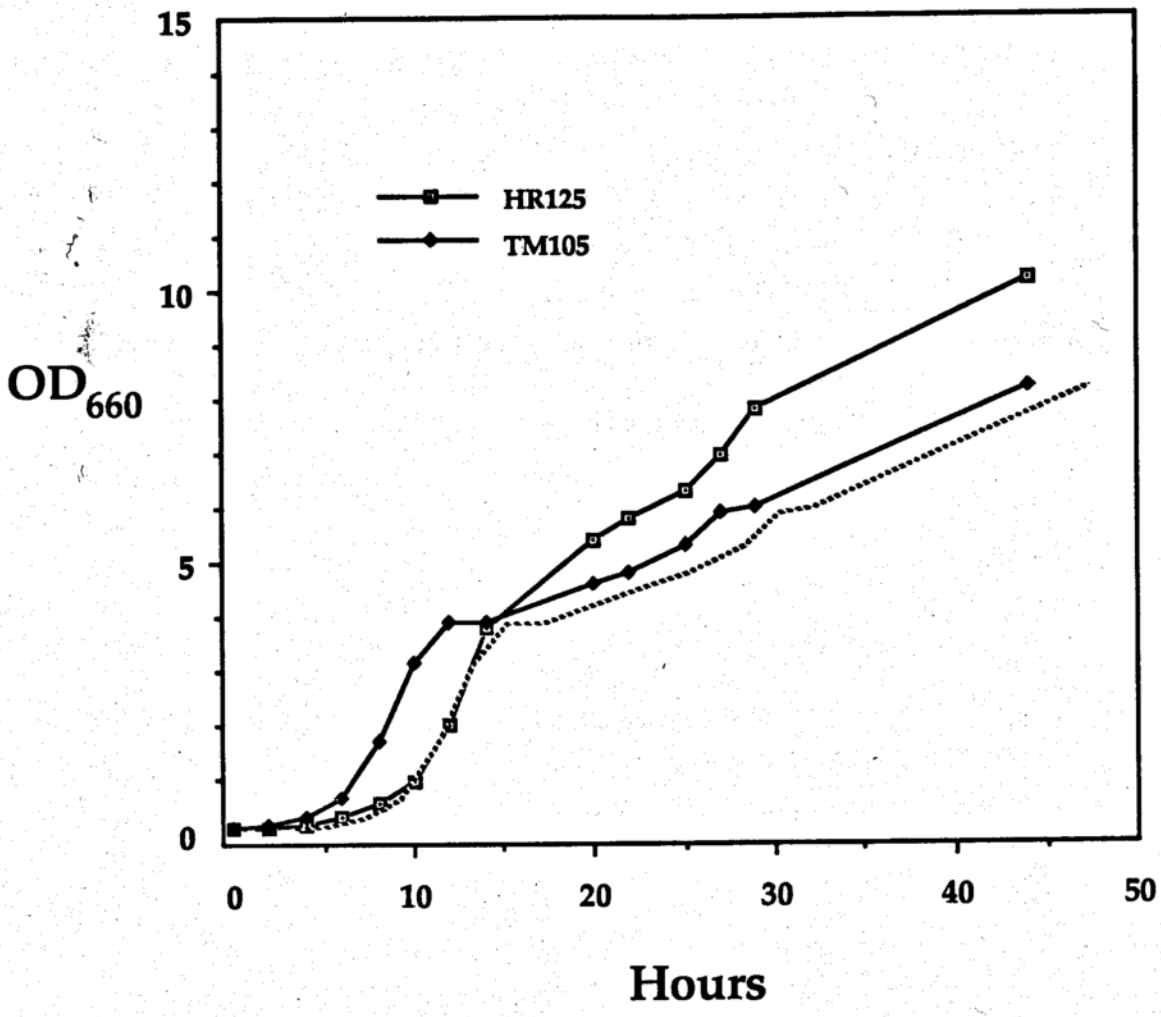

15
10
5
0
HR125
TM105
OD660
0
10
20
30
40
50
Hours

Table 6-1. Yeast strains used in Chapter 6.

STRAIN	GENOTYPE
HR125*(wild type)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*
TC41-1§ (*cyr1* deletion)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1::URA3 cam*
TM105 (*IRA1* disruption at codon 2520)	*MAT* **a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ira1::LEU2*

* Casperson et al. (1982)
§ Heideman et al. (1990)

DISCUSSION

Our results illustrate the following points: 1) Yeast lower cAMP levels as they exhaust their glucose supply and shift to oxidative metabolism of ethanol. This reduction in cAMP is associated with transient arrest of proliferation as unbudded cells. 2) The reduction of cAMP levels is essential for traversing the diauxic shift. Without this drop in cAMP, cells halt growth at all stages of the cell cycle, and lose viability rapidly. 3) The decrease in cAMP levels is accompanied by a drop in Ras stimulated adenylate cyclase activity. 4) The decrease in adenylate cyclase activity is associated with an increase in *IRA1* expression. 5) Mutants carrying an *IRA1* mutation that blocks GAP activity also have difficulty traversing the diauxic shift.

These points can be assembled into a model in which yeast lower cAMP in order to halt cell division while changing metabolic machinery. Cells that have either elevated cAMP levels, or mutations such as *BCY1*, do not arrest growth in G_1, and do not become able to grow on ethanol. These cells do stop growing, but the fact that they do so at all stages of the cell division cycle indicates that this halt is forced by lack of metabolic resources. Thus, it is important that yeast cease the process of cell division while there is sufficient glucose to allow production of the enzymes needed to oxidize non-fermentable carbon sources.

In our model, the fall in cAMP is produced, at least in part, by induction of Ira1. The increased Ira1 levels could have this effect by two different mechanisms. First, Ira1 accelerates the GTPase step that inactivates Ras, thus an increase in Ira1 would inactivate Ras proteins, and lower cAMP production. Additionally, an excess of Ira1 might sequester Ras proteins,

preventing them from stimulating adenylate cyclase. These two mechanisms would both decrease cAMP production *in vivo*, but they can be distinguished *in vitro* by using the non-hydrolysable GTP analog, GppNHp. The GTPase activating function of Ira1 would be blocked by GppNHp, thus the GAP activity of Ira1 would decrease GTP stimulation of adenylate cyclase, while stimulation by GppNHp would be unaffected. On the other hand, sequestration of Ras proteins would decrease activity regardless of whether Ras was bound to GTP or GppNHp. Our results indicate that both mechanisms may play a role in lowering cAMP levels. At the diauxic shift, as Ira1 levels peaked, GTP stimulation of adenylate cyclase was almost completely eliminated, while stimulation by GppNHp, although affected to a lesser degree, was also reduced. The greater loss of GTP stimulated activity indicates that the first mechanism is at play, while the reduction in GppNHp stimulation that was observed is consistent with the second mechanism.

Disruption of the Ira1 GAP activity retarded the shift to ethanol metabolism indicating that Ras GTPase activation plays some role in the process. However, this disruption only delayed the shift to ethanol metabolism, therefore the GAP activity of Ira1 is not absolutely necessary in traversing the shift. Other mechanisms must also be important. Although the *IRA1* disruption in the TM105 mutants blocks GTPase activation, it still allows production of a truncated Ira1 protein that may still be capable of binding Ras. If so, the disruption would not block the second mechanism, sequestration of Ras by an excess of Ira1. We do not know if Ira1 is indeed in excess at the diauxic shift. However, the intensity of the Cyr1 signal on Western blots is very low; roughly equivalent to the lowest levels of Ira1

intensity observed in figure 6. This is hardly a quantitative argument, and the actual ratios of Ira1 to Cyr1 proteins remain to be measured. Previous work has indicated that Ira1 is necessary for membrane binding of adenylate cyclase (Mitts et al. 1991), and that at low culture density the number of binding sites for adenylate cyclase on the membrane is limiting, allowing only about half of the adenylate cyclase in the cell to associate with the membrane (Mitts et al. 1990). This limitation in binding sites disappears rapidly as cells approach the diauxic shift, perhaps due to the increased abundance of Ira1.

It is difficult to explain why yeast might need to produce a 330 kDa protein simply to sequester Ras. Ira1 may be interacting with other targets besides adenylate cyclase. Halting cell division is just one step in the diauxic shift. In addition to lowering cAMP, there may be other signals that originate from Ira1. We will need to identify the proteins that interact with Ira1 in order to address this question.

The model presented above fits our results well, but it must be emphasized that other factors affect cAMP levels, and these factors may very well play important roles as yeast halt growth at the diauxic shift. For example, the break-down of cAMP, catalysed by phosphodiesterases (PDE), could also regulate cAMP levels at the diauxic shift. Our results using exogenous cAMP indicate that although PDE activity may play a role in lowering cAMP, regulation of PDE activity alone does not suffice to allow cells to traverse the diauxic shift. In the experiments shown in figure 1, the phosphodiesterases were intact, yet cells grown at constant high cAMP were unable to grow on ethanol. Additionally, the decrease in adenylate cyclase activity measured in figure 5 could not be caused by changes in PDE activity.

It is interesting to note that PDE deficient mutants were not found to have greatly elevated cAMP levels unless they also contained a *RAS2^{val19}* mutation (Nikawa et al. 1987). The second mutation greatly elevated cAMP in a PDE deficient background, indicating the loss of a mechanism that could compensate for loss of PDE activity. Ira1 is able to inactivate wild type Ras2, but not Ras2^{val19} (Tanaka et al. 1990).

The observed changes in adenylate cyclase activity *could*, however be explained by changes in Ras GDP/GTP exchange activity mediated by Cdc25. We cannot rule this out, and indeed both GDP/GTP exchange and GTPase activity may be regulated in concert to halt proliferation as glucose is depleted. However, we do not see comparable changes in *CDC25* message levels at the diauxic shift (not shown). Therefore, if Cdc25 is playing a role in this process, it is regulated in a different manner than Ira1.

Neither can we rule out changes in Ira2. By virtue of its similarities to Ira1 in structure and GAP activity, Ira2 would be expected to produce similar effects on Ras activity, and cAMP production. We have not measured *IRA2* expression.

Regardless of whether Ira1 is the sole factor regulating cAMP at the diauxic shift, the experiments presented here provide evidence for an active process for exiting the mitotic cycle as glucose is depleted. Models for Ras function have centered around regulation of the GTPase cycle that is central to both Ras and G proteins. The finding that Cdc25 functions by promoting guanine nucleotide exchange has strengthened the idea that Ras proteins might be regulated along the lines of the G proteins. In this sort of model, nutrient levels would be coupled to cAMP levels. Although this produces a useful working model, such a system is too simple to meet the needs of growing

yeast. Yeast are under strong selective pressure to proliferate rapidly. A slight increase in doubling rate will provide a large selective advantage. On the other hand it is perilous for a yeast cell to traverse the mitotic cycle with inadequate nutrient supplies. Thus for yeast, growth on glucose is like a race to the edge of a cliff; the cells must grow and divide at the fastest possible pace and stop at the last moment. A system that directly couples growth rate to nutrient levels would have growth gradually slow as the nutrient supply diminshed. Such a system is much better suited to maintaining a homeostatic balance than it is to controlling the decision to halt growth. The evidence presented above indicates that the Ras/adenylate cyclase system is not static, but rather changes with growth conditions. Induction of Ira1 demonstrates an active process for halting growth. It also demonstrates yet another signal affecting growth — that which induces Ira1 expression. We will need to identify this signal before we understand growth regulation in *S. cerevisiae*,

The regulation of cell division is a complex process, even for a unicellular eukaryote. Our finding that Ira1 levels vary during culture growth indicates that in addition to allosteric mechanisms for regulating Ras and adenylate cyclase, the regulation of *IRA1* gene expression is an important mechanism for regulating the Ras pathway.

PART 4

SUMMARY

CHAPTER 7
The Overall Findings of This Project and the Significance Those Findings May Have on Future Studies of the Yeast *RAS*/Adenylate Cyclase System

The overall goal of this project was to characterize the adenylate cyclase system in yeast, at the molecular level. This general goal was then focused and I decided to concentrate on two components of the system, the *CYR1* and *IRA1* polypeptides. The studies we have conducted have given us a great deal of information about these proteins, and the roles they play in this vital system, which acts to regulate yeast cell growth. The major findings are: 1. yeast adenylate cyclase is a peripheral membrane protein; 2. the *IRA1* polypeptide is involved in anchoring adenylate cyclase to the membrane; 3. *IRA1* plays a role in reducing cAMP levels at the diauxic shift, where yeast temporarily arrest growth.

The finding that yeast adenylate cyclase is a peripheral membrane protein is very significant since this protein is the central component of the entire system. Since this enzyme is responsible for producing cAMP, which is nessecary for yeast cell growth, characterization of the protein will certainly help us learn more about growth control in yeast. Information about the *IRA1* polypeptide has been limited since it is a recent addition to the system, so the finding that this protein is involved in anchoring adenylate cyclase to the membrane is also important. This shows that Ira1 has a structural role, in addition to its previously known regulatory role. Information about Ira1 has relavance to the mammalian system, since it is possible that the target for p21^{ras} might be associated with GAP or NF-1.

The conclusions from the final portion of the project have given us insight into the role of cAMP levels in growth arrest at the diauxic shift and also how *IRA1* expression is related to those cAMP levels. We have shown that yeast need to decrease cAMP levels in order to temporarily arrest

growth at the diauxic shift. This decrease in cAMP is paralled by a decrease in adenylate cyclase activity and an increase in *IRA1* expression. This adds information about the regulatory role of the *IRA1* polypeptide in this system. The concept of Ras regulation by changes in gene expression may also be applicable to the mammalian system.

The information that this project has provided builds on previous knowledge of the system and allows us to strengthen previously described models. There are definitely important additional components involved which we have not had the time to characterize, but we feel that the characterization of the *CYR1* and *IRA1* polypeptides will aid in future studies involving these other components. Information about the *CYR1* polypeptide is crucial, since it is the central component of the entire system, so models must be built around it. Characterization of the *IRA1* polypeptide is important because it is a negative regulator of adenylate cyclase activity and information learned about it will hopefully help to characterize another new component, a second negative regulator, *IRA2*. Understanding how these negative regulators work may also give insight into the roles of the positive regulators, *CDC25* and *SCD25*.

The significance of this work goes beyond learning about the yeast *RAS*/adenylate cyclase system and how it regulates growth. Because of the strong conservation between the yeast and mammalian systems controlling growth, we hope that information learned about the yeast system will also help unravel the puzzle of growth control in mammalian cells.

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