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Cloning and Functional Analysis of MCH1 - a Mammalian Homolog of the Yeast Adenylyl Cyclase-Associated Protein, CAP.

by

Audrey Zelicof

A dissertation submitted to the Graduate Faculty in Biomedical Sciences, in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York.

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ABSTRACT

The Cloning and Functional Analysis of MCH1 - a Mammalian Homolog of the Yeast Adenylyl Cyclase-Associated Protein, CAP.

by Audrey Zelicof

Adviser: Dr. Jeffrey E. Gerst

The RAS pathway of the budding yeast *S.cerevisiae* has been well characterized using genetic and biochemical means. This signaling cascade is distinguished by the RAS-dependent activation of adenylyl cyclase which ultimately results in enhanced cell growth and proliferation.

One of the requirements for RAS-mediated stimulation of adenylyl cyclase activity is the presence of the CAP protein. CAP consists of three domains; an amino terminus, a carboxyl terminus and a proline-rich stretch that connects the two regions. The amino terminus mediates RAS signaling through adenylyl cyclase while the carboxyl terminus regulates cellular morphology in response to environmental extremes. In contrast to the amino-terminus, the function of the carboxyl-terminal domain is not well understood. Thus, the objectives of this work were to clarify the role of the carboxyl terminus of CAP and, furthermore, to examine if CAP and its functions have been conserved in mammals.

In order to identify a mammalian homolog of *CAP*, or genes that encode functionally similar proteins, we transformed a rat embryo cDNA library into a strain deficient for CAP. We then looked for proteins which could suppress phenotypes incurred due to the loss of the carboxyl terminus. This strategy yielded a mammalian homolog of *CAP*, which we named *MCH1* (Mammalian Cap Homolog 1). Structurally, MCH1 is similar to the yeast CAP proteins with the greatest similarity in the proline-rich and carboxyl-domains.

Functional characterization of MCH1 reveals that it is capable of both actin binding and dimerization. Furthermore, these properties are conserved between CAP and MCH1, and are mediated by separate regions on the carboxyl terminus. The actin binding properties of MCH1 and CAP, described here, define a role for these proteins in actin-cytoskeleton regulation, although, the functions mediated through protein dimerization have not yet been determined.

The bifunctional nature of CAP suggests that it couples proliferative signals with changes in cell growth and proliferation. Many of these effects are mediated through the carboxyl-terminal domain. Here, we have demonstrated two novel properties of the carboxyl terminus of CAP and, furthermore, have shown that these functions remain conserved between yeast and mammals.

Dedication.

To my family whose love, teaching and encouragement have made all this possible.

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CHAPTER I

Introduction

Ras proteins have been both functionally and structurally conserved in eukaryotic cells. Generally, Ras serves to translate signals from the external milieu into two sets of signaling pathways -- one pathway enhances cellular proliferation while the other results in changes in the actin cytoskeleton (Prendergast *et al.*, 1993). In the budding yeast *S.cerevisiae*, RAS transmits these signals by activating adenylyl cyclase through the protein, CAP (Toda *et al.*, 1985, Field *et al.*, 1990). CAP is a bifunctional protein that consists of three domains, an amino terminus that mediates RAS signaling, a carboxyl terminus that controls cellular morphology and maintains homeostatic growth, and a proline-rich domain that connects the two regions (Gerst *et al.*, 1991). The manner by which the carboxyl-domain of CAP affects the cytoskeleton and nutrient response is unknown. Therefore, the goals of this study were to resolve the functions of the carboxyl-terminal domain of CAP and to determine if CAP proteins or their functions have been conserved in mammals. An introduction to the *ras* signaling pathways in yeast and mammals, and a discussion of the known aspects of CAP-function are given below.

1.1 Mammalian ras proteins.

Ras proteins are members of the family of small GTP-binding proteins. These proteins both bind GTP and have slow intrinsic GTPase activity (reviewed by Barbacid, 1987). Thus far, over 50 small, Ras-like, GTP-binding proteins have been identified. Overall these proteins modulate processes such as cellular proliferation, vesicular transport and the cytoskeleton (Boguski and McCormick, 1993).

In mammals, Ras is encoded by the genes *H-ras, N-ras and K-ras*. All three genes encode 21 kilodalton (kDa) proteins that are highly homologous except in the

hypervariable carboxyl-terminal domain (Barbacid, 1987). Structural and mutational analysis divides Ras into four regions; the effector domain, the GTP-GDP binding domain, the hypervariable region, and the carboxyl-terminal site for membrane attachment. Post-translational modification of the latter region serves to direct Ras to the plasma membrane where it is situated to participate in the process of signal transduction (Hancock *et al.*, 1991).

1.2 Regulation of ras function.

Like other GTP-binding proteins, Ras exists in two conformational states -- an active GTP-bound state and an inactive GDP-bound state (reviewed by Bollag and McCormick, 1991). Conversion between the GTP and GDP-bound forms of Ras is accomplished in the presence of regulatory proteins that either enhance GTPase activity or accelerate the rate of GTP-GDP exchange.

1.2a. Ras GTPase Activating Proteins.

Proteins that stimulate intrinsic GTPase activity are known as GAPs or GTPase Activating Proteins (Bollag and McCormick, 1991). At least two specific GAPs have been identified which regulate mammalian Ras. These include p120 GAP and NF1 (Ballester *et al.*, 1990; Trahey and McCormick *al.*, 1987).

p120 GAP was the first GAP protein to be identified. It was originally isolated as a factor in cytosolic extracts that dramatically enhances the intrinsic GTPase activity of Ras (Trahey *et al.*, 1987). NF1-GAP is similar to p120 GAP in its ability to down regulate Ras activity; and is homologous to p120 GAP in the GTPase catalytic domain. NF1 is encoded by the *NF1* gene that, when mutated, results in type 1 neurofibromatosis (Viskochil *et al.*, 1990). This is a fairly common genetic disease that is associated with benign tumors of the peripheral nervous system, as well as, an increased predisposition towards systemic malignancies (McCormick, 1995). Sequence analysis of the *NF1* gene reveals extensive homology to the *S.cerevisiae* yeast genes, *IRA1* and *IRA2* (Ballester *et al.*, 1990). *IRA1* and *IRA2* encode yeast

equivalents of the mammalian *GAP* genes and accelerate the rate of yeast RAS GTPase activity (Tanaka *et al.*, 1990). Both p120 GAP and NF1 can functionally complement the loss of IRA function in *S.cerevisiae* thus demonstrating that GAP proteins have remained conserved throughout evolution (Ballester *et al.*, 1990).

1.2b. Ras guanine nucleotide exchange factors.

Since the intracellular concentration of GTP exceeds that of GDP, GDP-bound Ras should rapidly convert to the active GTP-bound state. However, Ras, and other small-GTP binding proteins, have a high affinity for GDP, and GTP-binding is limited by the slow rate of GDP-dissociation. In order for Ras to rapidly respond to external mitogenic signals, additional regulatory factors are necessary to stimulate GDP-GTP exchange on Ras. Such proteins have been characterized and are described as GEF's (GDP-GTP Exchange Factors). The first GDP-GTP exchange factor to be identified was the *CDC25* gene in *S.cerevisiae* (Camonis *et al.*, 1986). *CDC25* was isolated genetically as a factor upstream of *RAS* that was necessary for its activation (Broek *et al.*, 1987). Since the discovery of *CDC25*, additional Ras-GEF proteins have been identified including the Drosophila *sos* gene and its mammalian homologs, *m-sos* and *h-sos* (Bowtell *et al.*, 1992; Chardin *et al.*, 1993). Structurally, the *sos* genes are similar to *CDC25* within the catalytic GEF domain (Quilliam *et al.*, 1995). However, Sos proteins possess an SH3 binding domain downstream of the catalytic site. SH3 domains are non-catalytic regions that interact with specific proline-rich polypeptide sequences. These domains are found on proteins involved in either signal transduction or the cytoskeleton and are believed to play a role in protein localization (Schlessinger *et al.*, 1994). The SH3-binding region of Sos has been shown to complex with the SH2-SH3 adapter protein, GRB2, in the cytosol . (Aronheim *et al.*, 1994). Upon growth factor stimulation, GRB2 is believed to localize Sos to the membrane where Sos activates Ras by stimulating guanine nucleotide exchange (Aronheim *et al.*, 1994).

This results in the conversion of Ras to its GTP-bound state where it can now activate a number of downstream signaling pathways.

1.3 Ras signaling pathways in mammals

Recent work has shown that Ras regulates at least two signal transduction cascades -- one involves cellular proliferation and differentiation, while the other modulates cytoskeletal organization. As shown in Figure 1, Ras effects changes in cell growth and division through the mitogen activated protein kinase (MAPK) cascade, and cytoskeletal remodeling through the Rho/Rac pathway (Prendergast *et al.*, 1993a).

1.3a. The Ras MAP Kinase Cascade.

The well-characterized MAP kinase pathway appears to be the primary or best-established mode of signal transmission through receptor-tyrosine kinases. Components of this signaling cascade include Raf kinase, MAP kinase kinase (MEK), and MAP kinase (MAPK) (Marshall, 1994). Based on recent work, a simplified model has been proposed for the MAP kinase signaling pathway (Figure1). In growth-factor stimulated cells, Ras recruits Raf kinase to the membrane by physically interacting with the Raf amino terminus (Leevers *et al.*, 1994; Vojtek *et al.*, 1993). Raf is then activated by an unknown protein, possibly Src, which allows Raf to stimulate MEK activity by serine/threonine phosphorylation. MEK, in turn, activates MAPK through threonine/tyrosine phosphorylation (Marshall, 1994).

RAS SIGNALING IN MAMMALS

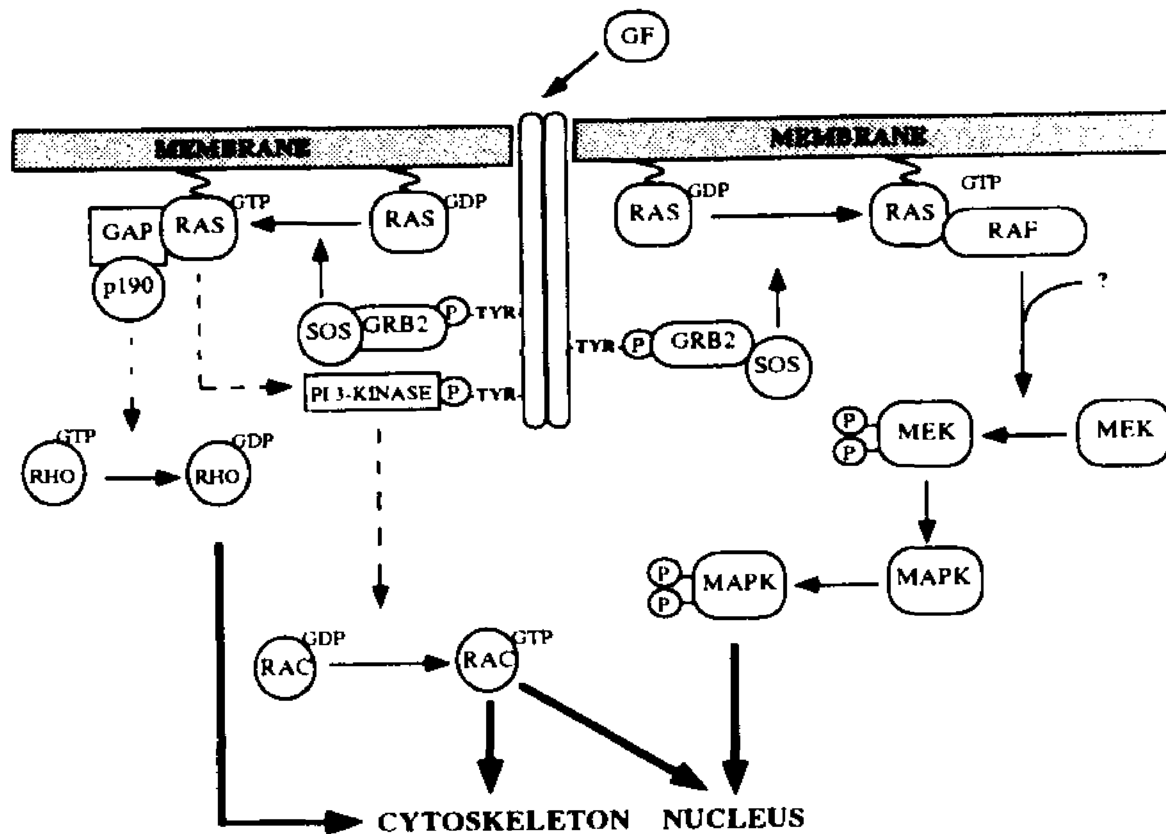


Figure 1. Ras proteins affect both proliferative and cytoskeletal signaling pathways in mammals. As shown here, Ras activates cell proliferation through the MAP kinase cascade that ultimately stimulates the transcription of growth-specific genes. In addition, Ras may effect cytoskeletal changes through the down regulation of Rho and the activation of Rac activity (Parker *et al.*, 1995; Settleman *et al.*, 1992a). Ras dependent-inactivation of Rho occurs through the p190 GAP protein that binds to p120 GAP. Inactivation of Rho results in the loss of actin stress fibers and focal adhesions (Ridley and Hall, 1992). Stimulation of Rac is mediated by PI-3 Kinase which is thought to be a target for Ras activation (Parker *et al.*, 1995). Thus, Ras may activate PI-3 kinase which then activates Rac. Rac, in turn, stimulates cell ruffling and lamellipodium formation as well as activating a number of nuclear factors. ABBREVIATIONS; **GF**= Growth Factor; **MAPK**= Map Kinase; **MEK**= MAP kinase kinase; **GAP**= GTPase Activating Protein.

Once activated, MAPK phosphorylates cytoskeletal and cytosolic proteins, and also migrates into the nucleus where it serine/threonine-phosphorylates a number of transcriptional regulatory components (Seger and Krebs, 1995). These include c-jun, c-myc and p62TCF (Treismann, 1994). Phosphorylated p62 is part of the ternary complex that binds to the c-fos serum response element in conjunction with SRF (serum response factor) and activates *c-fos* transcription (Gille *et al.*, 1992). Thus phosphorylation by MAPK results in the activation of immediate early gene transcription and ultimately cell growth and division. While many regulatory aspects of this pathway are still unresolved, the partial characterization of the Ras-activated MAP kinase cascade provides an explanation of the mechanism by which Ras affects cellular proliferation.

1.3b. Ras and the cytoskeleton.

The role of Ras in cytoskeletal regulation is less well-defined. Evidence that Ras modulates cellular morphology is seen in *ras*-transformed cells which display a disorganized actin cytoskeleton, loss of focal contacts, an increase in cell volume and enhanced cellular motility (Dartsch *et al.*, 1994). Furthermore, micro-injection of either c-Ras or v-Ras proteins into fibroblasts leads to high levels of membrane ruffling and pinocytotic activity (Bar-Sagi *et al.*, 1986). Until recently, it was not known how Ras effected these morphological changes. However, with the discovery of the Rho family of small GTP-binding proteins, many models are being developed that link Ras and Rho proteins to the regulation of the actin cytoskeleton.

As mentioned previously, over 50 small GTP binding proteins have been identified. These proteins have been classified into 5 groups based on functional and structural similarities (see Figure 2A). Rab and Arf proteins regulate vesicular transport; Ran proteins mediate nuclear transport; and Rho proteins effect changes in the actin cytoskeleton (Hall, 1994). The family of Rho proteins can be further

subdivided into the Rac proteins, Rho proteins and the CDC42-related proteins (Hall, 1994). Each of these groups mediates distinct morphological changes that can either function coordinately to regulate cell movement or separately in response to independent mitogenic factors. For example, in response to lypophosphatidic acid (LPA), Rho stimulates actin stress fiber and focal adhesion formation (Ridley and Hall, 1992). Rac proteins cause membrane ruffling and lamellipodium formation downstream of PDGF/ insulin, and CDC42Hs has recently been shown to stimulate filopodium formation (Ridley and Hall, 1992; Nobes and Hall, 1995). In addition, CDC42Hs activates Rac which, in turn, stimulates Rho (Nobes and Hall, 1995 and Figure 2B). Thus CDC42Hs may function to initiate a series of events necessary for cellular movement.

As described, *ras*-transformed cells exhibit the loss of actin stress fibers, increased cell membrane ruffling and enhanced motility. It is possible that these effects are mediated through the Ras-dependent down-regulation of Rho activity, as well as, through the activation of Rac and/or CDC42Hs.

It has been suggested that Ras mediates inhibition of Rho activity in the following manner (Takai *et al.*, 1995). Rho, like other small GTP binding proteins, possesses GAPs which convert Rho to the inactive, GDP-bound state. One of these proteins has been identified as p190 GAP (Settleman *et al.*, 1992b). p190 GAP has been shown to directly complex with the amino terminus of p120 GAP in growth-factor stimulated cells (Settleman *et al.*, 1992a; Moran *et al.*, 1991). Thus, in the presence of GTP-bound Ras, p190 GAP activity may be stimulated and, in turn, down-regulate Rho (McGlade *et al.*, 1993). This would then result in the loss of actin stress fibers and focal adhesions that is characteristic of cells possessing constitutively activated *ras*.

A. **SMALL GTP-BINDING PROTEINS**

Subfamily	biological functions
ras	growth and differentiation
rab	vesicular transport
arf	vesicular transport PLD activation
ran	nuclear protein import
rho	actin cytoskeleton NADPH oxidase

B.

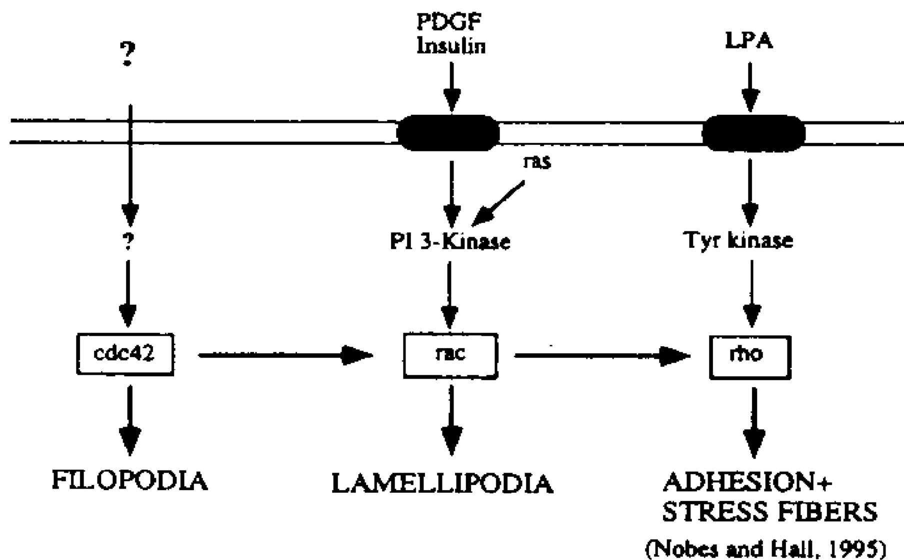


Figure 2. The Rho family of small GTP-binding proteins. (**A**) The family of Ras-like small GTP binding proteins include over 50 members and can be divided into 5 classes based on structural or functional homologies (Boguski and McCormick, 1993). (**B**) The Rho proteins, Rac, Rho and CDC42Hs can either act independently along separate signaling pathways or coordinately to affect cell movement. In the latter case, CDC42Hs has been shown to activate Rac activity which then stimulates Rho (Nobes and Hall, 1995).

At the same time, GTP-bound Ras can also activate Rac activity through a different pathway thought to involve PI3-kinase. PI3-kinase is a heterodimer consisting of two subunits, p85 and p100 (Downes *et al.*, 1991). p85 is the adapter subunit that binds to the activated PDGF receptor through its SH2 domain; p100 is the catalytic subunit which phosphorylates and converts PIP_2 to PIP_3 (Downes *et al.*, 1991). Recently, Ras has been shown to directly bind to PI3-kinase and stimulate its activity in an unknown manner (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994). It was also demonstrated that PI3- kinase increases the intracellular levels of GTP-bound Rac (Hawkins *et al.*, 1995; Kotani *et al.*, 1995). Thus it is possible that in the presence of activated Ras, PI3-kinase is activated which, in turn, results in the formation of GTP-bound Rac (Parker, 1995; Figure 1). Activated Rac can then stimulate the formation of membrane ruffles and lamellipodia that are prominently seen upon exposure to PDGF or activated *ras*. Additional evidence supports the role of Rac in Ras-mediated cell ruffling. Microinjection of the dominant negative form of Rac along with Ras prevents the formation of lamellipodia and cell ruffles, and Rac has been shown to potentiate cellular transformation by Ras (Ridley and Hall, 1992; Qui *et al.*, 1995). Thus it appears that Ras participates in multiple signaling pathways. This is not surprising given the diverse phenotypes displayed by *ras*-transformed fibroblasts.

1.4 Ras signaling pathways in *S.cerevisiae*.

Ras proteins not only exist in mammals but are also conserved in a number of other organisms including Aplysia, Drosophila and yeast (Barbacid, 1987). The budding yeast *S.cerevisiae* contains two ras genes, *RAS1* and *RAS2* , which encode proteins of 36 and 40 kd respectively (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984). *RAS1* and *RAS2* display striking homology to the mammalian genes, particularly within the amino terminus and the conserved CAAX box (Powers *et al.*, 1984). Furthermore, these genes appear to be functionally conserved. For example, NIH3T3 cells transfected with activated yeast *RAS1* become morphologically transformed, and

mammalian *H-ras* can partly complement the loss of RAS function in yeast (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985).

Genetic and biochemical characterization of the Ras signal transduction pathway in *S.cerevisiae* has revealed many similarities to the mammalian Ras signaling pathway.

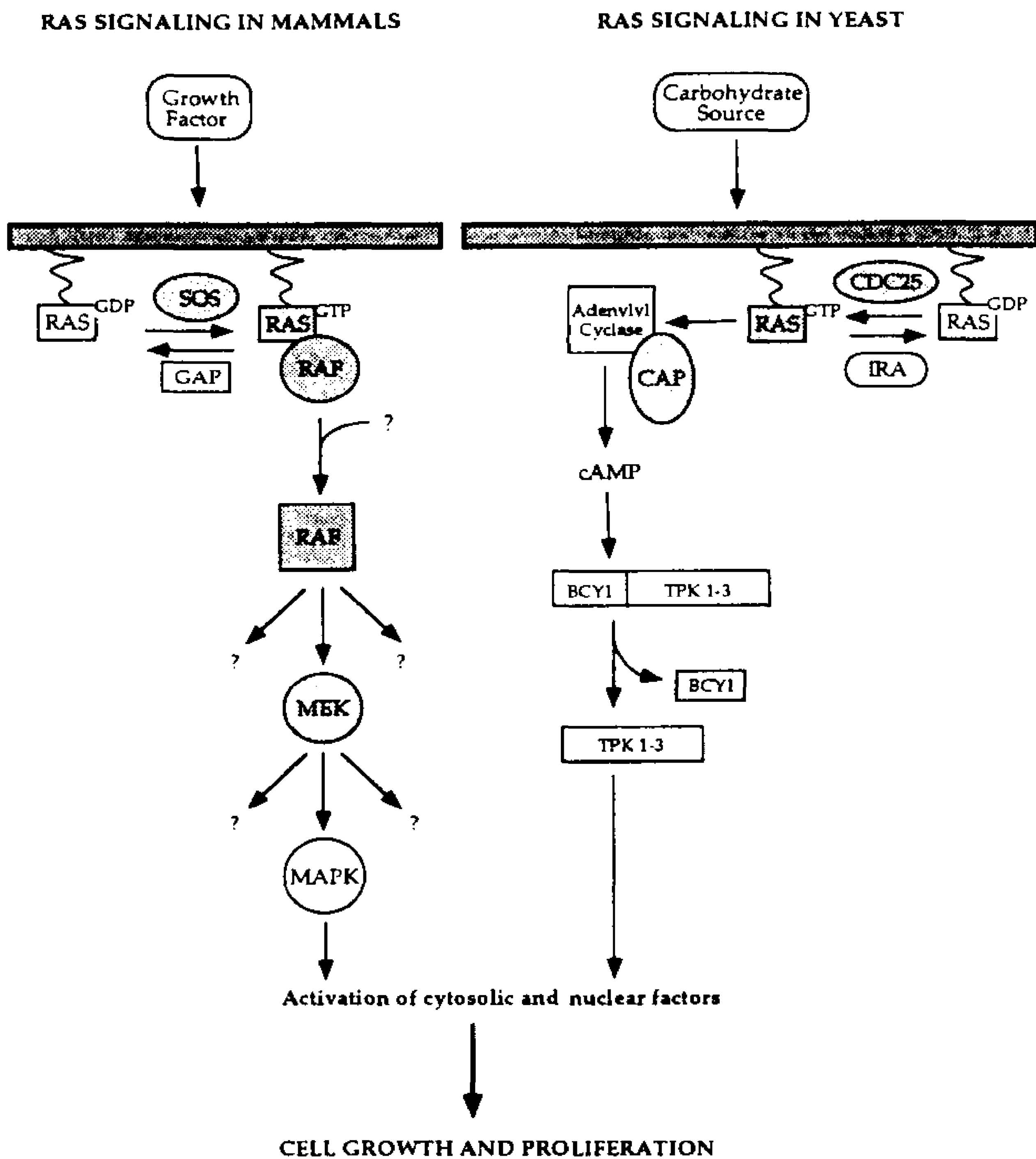


Figure 3. Similarities between mammalian and yeast Ras signaling pathways

Figure 3. Similarities between mammalian and yeast Ras signaling pathways. The mammalian and yeast Ras proteins are both structurally and functionally conserved. First, both Ras proteins associate with the plasma membrane. Second, yeast and mammalian Ras proteins respond to an external mitogenic signal to induce cell growth and proliferation. Third, both Ras proteins are activated by homologous nucleotide exchange factors; Sos activates mammalian Ras and CDC25 activates yeast RAS. In addition, both RAS proteins are negatively regulated by GAPs. Yeast RAS is down regulated by the IRA proteins, while mammalian Ras is inactivated by GAP. Yeast RAS differs from mammalian Ras in its effector protein which is adenylyl cyclase. Activation of adenylyl cyclase results in the production of cAMP. cAMP then binds to the negative regulatory subunit, BCY1, and stimulates its dissociation from the protein kinase A complex, TPK 1-3. The protein kinase complex then phosphorylates a number of factors involved in cell growth and proliferation. Mammalian Ras proteins activate a kinase cascade through Raf and have not been shown to stimulate adenylyl cyclase activity directly.

This is particularly true at the level of Ras regulation (see Figure 3). As described, Ras is a small GTP-binding protein with a slow intrinsic GTPase activity. This activity is stimulated in the presence of GAP, while GDP-GTP exchange is enhanced in the presence of Sos. Yeast RAS proteins are regulated in an identical manner (Broach, 1991). The GAP homolog in yeast is encoded by the genes *IRA1* and *IRA2*, and the corresponding Sos homolog in yeast is the cell cycle protein, CDC25 (Tanaka *et al.*, 1990; Camonis *et al.*, 1986). Furthermore, as in mammalian cells, yeast Ras proteins must be localized to the membrane to function properly. Localization is accomplished by a series of biochemical modifications at the highly conserved CAAX site (Broach, 1991). These modifications include farnesylation and palmitoylation and are performed by the products of the *RAM1* and *RAM2* genes (reviewed by Broach, 1991).

Because yeast and mammalian Ras proteins are well-preserved structurally and biochemically, it was thought that *S.cerevisiae* would provide a strong model for deciphering the Ras signaling pathway in *vivo*. Like mammalian Ras, *S.cerevisiae* RAS proteins play an important role in the regulation of cell growth response to extracellular signals. Yeast lacking both *RAS* alleles are incapable of vegetative growth, while strains transformed with the constitutively activated *RAS* allele, $RAS2^{val19}$, exist in a hyperproliferative state (Kataoka *et al.*, 1984). Phenotypically, the presence of activated *RAS* results in the activation of glycolysis, the inability to store glycogen, inhibition of sporulation under conditions of nutrient deprivation and the inability to arrest in G1 (Kataoka *et al.*, 1984). Moreover, these strains are sensitive to heat shock and nitrogen starvation (Kataoka *et al.*, 1984). These characteristics are indicative of an inherent loss of growth control, and are reminiscent of mammalian cells that have been transformed by oncogenic *ras*.

In contrast to the mammalian Ras proteins, which act through a kinase cascade initiated by Raf, *S.cerevisiae* RAS has been shown to directly activate adenylyl cyclase (Toda *et al.*, 1984; see Figure 4). Yeast adenylyl cyclase differs from the mammalian

A MODEL FOR CAP FUNCTIONS IN *S.CEREVISIAE*

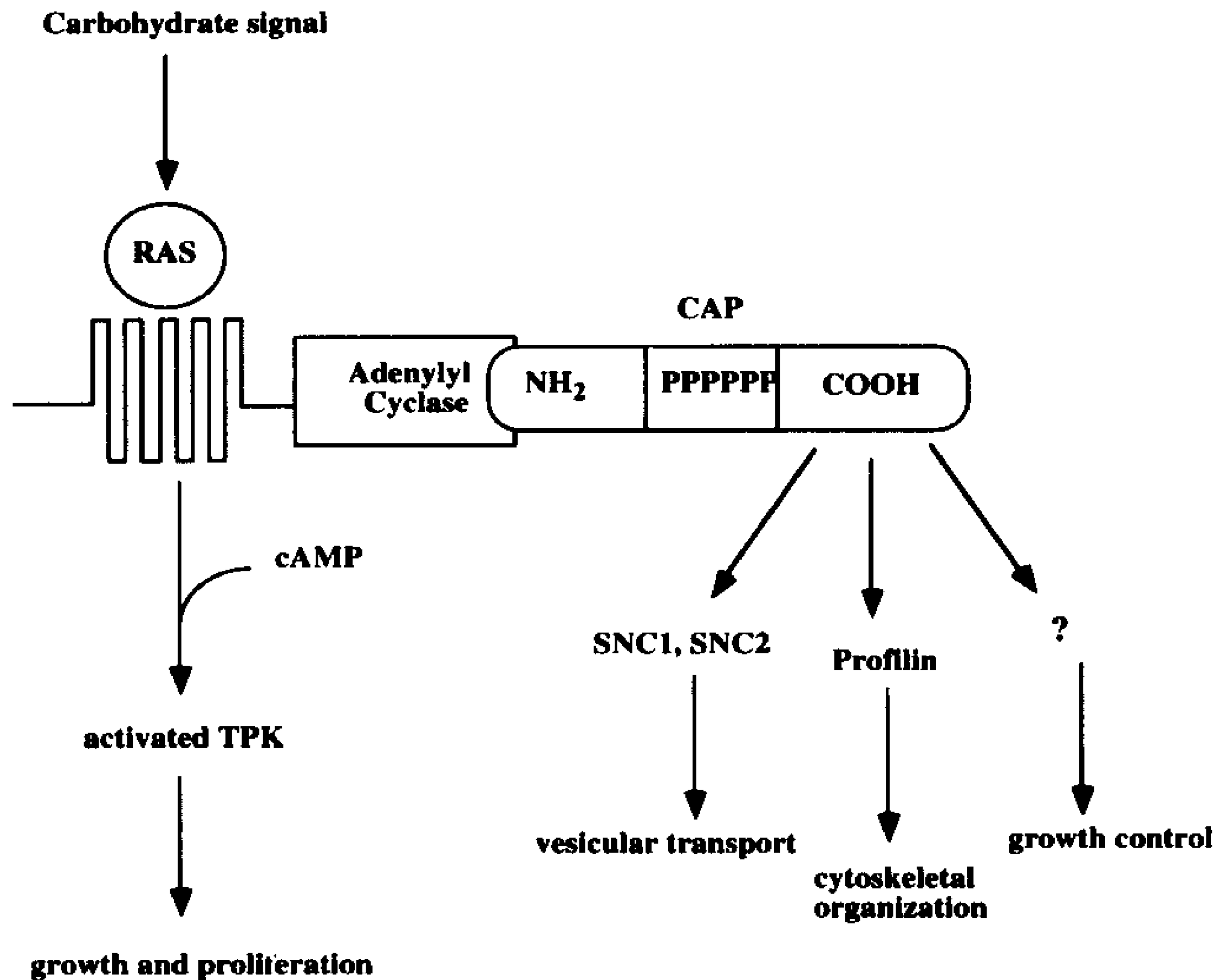


Figure 4. A schematic representation of yeast CAP protein function. CAP proteins coordinate proliferative signals with changes in cell growth and morphology. The amino terminus interacts with, and mediates RAS signal transduction through adenylyl cyclase. In contrast, the carboxyl terminus of CAP activates an unknown signal which affects cell growth and morphology. Cloned genes which suppress the loss of the carboxyl-domain include *SNC1* and *SNC2*, as well as, profilin (*PFY*). Snc proteins participate in vesicle trafficking while profilin binds actin and phosphoinositide bis-phosphate (PIP_2). These proteins may relate signal transduction through CAP to processes controlling vesicular transport and the integrity of the cytoskeleton.

adenylyl cyclase protein in that both proteins are structurally unrelated and that yeast adenylyl cyclase is a peripheral membrane protein while mammalian adenylyl cyclase consists of two domains each containing six membrane spanning segments (Mitts *et al.*, 1990). Furthermore, mammalian adenylyl cyclase is regulated by heterotrimeric G proteins and not small ras-like GTP binding proteins (Boguski and McCormick, 1993). Nonetheless, both cyclase proteins catalyze the formation of cAMP. In *S.cerevisiae*, cAMP activates protein kinase A by binding to the negative regulatory subunit, encoded by *BCY1*, and stimulating its dissociation from the protein kinase A catalytic subunits (TPK 1-3) (Toda *et al.*, 1987). The activated catalytic subunits of protein kinase A in turn phosphorylate numerous cytoplasmic and nuclear targets involved in the metabolism of storage carbohydrates, the activation of glycolysis and the transcriptional activation of certain growth specific genes (Broach, 1991). Interestingly, protein kinase A may serve to down-regulate the RAS signaling pathway possibly through phosphorylation of the RAS and CDC25 proteins (Resnick and Racker, 1988; Sreenath *et al.*, 1988; Gross *et al.*, 1992).

Both genetic and biochemical evidence support the role of RAS in adenylyl cyclase activation. The biochemical evidence that RAS stimulates adenylyl cyclase activity comes from three observations: (1) cAMP levels are much lower in *bcy1 ras1 ras2* strains relative to wild-type; (2) yeast transformed with an activated *RAS* allele have much higher levels of cAMP relative to wild-type strains, and (3) yeast fed with glucose show a biphasic accumulation of cAMP that only occurs when at least one *RAS* allele is functional (Wigler *et al.*, 1988). Furthermore, evidence that RAS indirectly activates the cAMP-dependent kinase activity consists of the following: wild-type yeast strains transformed with any of the protein kinase A subunits, or strains lacking the *BCY1* gene, display the same set of phenotypes as seen in an activated RAS setting, and second, yeast lacking both *RAS* alleles can be rescued by the disruption of

BCY1 or by the overexpression of *PKA* catalytic subunits (TPK 1-3) (Toda *et al.*, 1987).

1.5 The CAP protein in yeast.

While both genetic and biochemical studies demonstrate that RAS stimulates adenylyl cyclase activity, it was not known whether RAS directly activates adenylyl cyclase activity or if another component is involved. In order to address this question, the RAS-responsive adenylyl cyclase complex was purified and found to consist of at least two subunits; one is a 200kDa protein that corresponds to adenylyl cyclase (Field *et al.*, 1988). The other is a 70kDa protein called CAP for adenylyl cyclase-associated protein (Field *et al.*, 1988; Field *et al*, 1990). CAP's role as an effector for activated *RAS* was confirmed independently in a genetic screen. This screen relied upon the identification of mutants that suppressed phenotypes associated with activated *RAS* (Field *et al.*, 1990; Fedor-Chaiken *et al.*, 1990). One of the suppressors isolated was the gene termed *supC*, which is an allele of *CAP* (Field *et al.*, 1990)

Disruptions of the *CAP* gene reveal that CAP is a bifunctional protein, since CAP-depleted cells exhibit two sets of apparently unrelated phenotypes (Field *et al.*, 1990, and Figures 4 and 5). One set relates to the loss of cellular responsiveness to RAS and the second set pertains directly to cellular growth and the integrity of the cytoskeleton. Each set of phenotypes can be ascribed to separate domains of the CAP protein (Gerst *et al.*, 1991). Structurally, CAP is a 526 amino acid protein that can be divided into three domains: an amino-terminal domain, a carboxy-terminal domain, and a proline-rich domain that separates the two regions (Field *et al.*, 1990, and Figure 5).The polyproline stretch is highly reminiscent of the stretch of residues known to interact with SH3 domains (Ren *et al.*, 1993). However, this region is not essential for either the stimulation of adenylyl cyclase in cells expressing activated RAS or for signals

mediated through the carboxyl terminus of CAP. Therefore, the function of this domain of CAP remains unresolved.

Deletion of the amino terminus is associated with the loss of cellular responsiveness to activated RAS (Gerst *et al.*, 1991). Cells lacking the amino terminus are insensitive to heat shock and nitrogen starvation in the presence of an activated *RAS* allele (Gerst *et al.*, 1991). This domain has also been shown to interact with the carboxyl terminus of adenylyl cyclase and CAP has been shown, by gel filtration, to associate with the RAS-dependent cyclase fraction (Wang *et al.*, 1992). Interestingly, however, RAS has been shown to activate adenylyl cyclase *in vitro* in a CAP-independent fashion. Therefore, the amino terminus of CAP is not absolutely required for RAS-mediated cyclase activation *in vitro*, although this domain is necessary for the penetrance of the activated RAS allele *in vivo*. Thus it is thought that the amino terminus of CAP may serve to stabilize the interaction between RAS and adenylyl cyclase.

Loss of the carboxyl terminus of CAP in wild-type cells results in an array of phenotypes that are not directly linked to ras/cAMP signaling. Cells lacking this domain are temperature-sensitive, unable to grow in nutrient-rich medium, sensitive to nitrogen starvation, and display a severely disrupted actin cytoskeleton (Gerst *et al.*, 1991 and Figure 5). Moreover, these phenotypes are not complemented by overexpression of the catalytic subunit of PKA, or other members of the cAMP-effector pathway; however, overexpression of protein kinase A has been shown to restore phenotypes associated with loss of the amino-terminal domain (Field *et al.*, 1990). Thus it is thought that the two regions of CAP may act upon separate signaling pathways. The amino terminus acts upon the pathway related to RAS-mediated cell proliferation, while the carboxyl terminus acts upon a cAMP-independent pathway that appears to affect cellular growth control and the cytoskeleton. Thus, as seen in Figure 4, CAP may act as a molecular bridge to connect processes that are involved with coordinating cytoskeletal changes in response to a proliferative signal.

Proteins that suppress the loss of the carboxyl-terminal domain of CAP, when overexpressed, include the actin/phosphoinositide binding protein, profilin, and the yeast homologs of the synaptobrevin family of synaptic vesicle proteins, named Snc1 and Snc2 (Vojtek *et al.*, 1991; Gerst *et al.*, 1992; Protopopov *et al.*, 1993). Snc proteins are essential for normal cellular secretion and are likely to be involved in vesicle fusion with the plasma membrane (Protopopov *et al.*, 1993). Profilin is though to directly influence changes in the actin cytoskeleton by regulating the formation of actin filaments (Sohn and Goldschmidt-Clermont, 1994). While initially characterized as an actin-sequestering protein which affects actin filament depolymerization, profilin is now believed to stimulate actin polymerization by enhancing the rate of nucleotide exchange on G-actin molecules (Sohn and Goldschmidt-Clermont, 1994). Because of the strong connection between CAP function and processes relating to cytoskeletal regulation, CAP may be seen as a protein that can coordinate proliferative signals mediated by RAS with changes in cell growth and morphology.

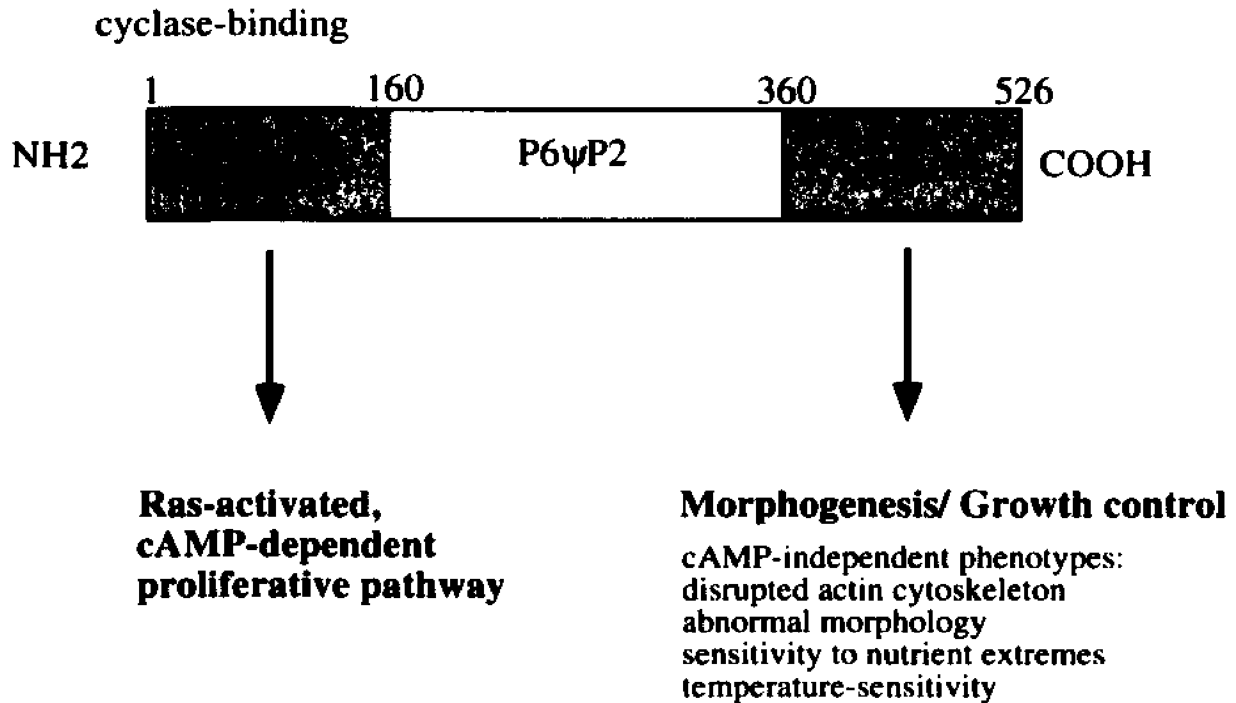


Figure 5. CAP is a bifunctional protein. CAP proteins can be divided into three domains--an amino terminus, a carboxyl domain and a proline-rich stretch. The amino terminus mediates RAS signaling through adenylyl cyclase, while the carboxyl terminus regulates a number of cAMP-independent processes. Deletion of the amino terminus is associated with the loss of RAS-mediated heat shock sensitivity, and loss of the carboxyl-domain results in sensitivity to environmental extremes, as well as, gross alterations of the cytoskeleton.

1.6 Homologs of *CAP*

Homologs of CAP have been found in a multitude of organisms, including *S.pombe* and mammals (Kawamuki *et al.*, 1992; Gieselmann and Mann, 1992). Comparative analysis reveals that these proteins are structurally similar and bear the highest degree of

homology in regions comprising the carboxyl terminus and the proline-rich stretch located between the amino and carboxyl domains. In the fission yeast *S.pombe*, many functional aspects of Cap have been preserved which reflect the structural similarities between the two proteins. Thus, while the amino terminus of *S.pombe* cap does not mediate ras signaling in either *S.cerevisiae* or *S.pombe*, functions associated with the carboxyl domain of Cap have been completely conserved (Kawamuki *et al.*, 1992). Deletion of the carboxyl terminus of *S.pombe* cap is associated with an inability to grow in nutrient-rich medium, temperature sensitivity, and a disrupted actin cytoskeleton (Kawamuki *et al.*, 1992). These are essentially the same phenotypes seen in *cap S.cerevisiae*. Furthermore, heterologous expression of *S.pombe* cap in *S.cerevisiae cap* strains successfully complements phenotypes associated with the loss of the carboxyl terminus of *CAP* (Kawamuki *et al.*, 1992). Thus CAP proteins in *S.cerevisiae* and *S.pombe* both link signaling pathways to changes in cell growth and morphology, and these changes are mediated by the well-conserved carboxyl-terminal domain.

Preliminary evidence to suggest that this function has been conserved in mammals came from the discovery of the porcine actin-binding protein, ASP-56 (Gieselmann and Mann, 1992). Partial peptide sequencing of this protein revealed that it is highly homologous to yeast CAP with the greatest similarity in the carboxyl-terminal region (Gieselmann and Mann, 1992). Although the complete sequence of this protein is lacking, it seems likely that CAP proteins and their functions have been maintained through evolution. Furthermore, it appears that the best-preserved functions are those

associated with the carboxyl terminus as demonstrated in *S.pombe* cap and ASP-56. Nonetheless, little is known about the signaling pathway downstream of the well-conserved carboxyl terminus or the mechanism by which this domain coordinates growth response and cellular morphology. Thus the goals of this work are to clarify the role of the carboxyl domain of CAP, and to examine if CAP and its functions have been preserved in mammals.

CHAPTER 2
Materials and Methods

2.1 DNA Manipulations.

DNA restriction endonucleases, *Taq* polymerase, and T4 DNA ligase were used as recommended by the suppliers (New England Biolabs, Inc, Promega and Perkin Elmer Cetus Instruments). Molecular cloning, Southern blotting, and hybridization techniques were performed as described by Sambrook *et al.* (1989). DNA sequencing was performed using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Standard methods were used for the introduction of DNA into the various bacterial strains (Sambrook *et al.*, 1989). *Escherichia coli* strains XL1 and DH5α were used for the plasmid transformations and plasmid DNA preparations.

2.2 Media and Genetic Manipulations.

Yeast were grown in medium containing 2% glucose as a carbon source. Standard rich medium (YPD: yeast extract/Bactopeptone/dextrose), synthetic minimal medium (SC), and SC drop-out minimal medium, lacking an essential amino acid or nucleotide base, were prepared essentially as described by Rose *et al.* (1990). DNA was introduced into yeast by LiOAc transformation, and yeast spheroplasts were prepared using standard procedures (Rose *et al.*, 1990). Rat-6 fibroblasts were cultured in DMEM (low glucose) medium (GIBCO) containing 10% bovine calf serum (Hyclone).

2.3 Yeast strains.

For the two-hybrid selection assay, yeast strain Y153 (*Mata gal4 gal80 his3 trp-902 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-lacZ LYS2::Gal-HIS3*) was used (Durfee

et al., 1993). *cap* strains SKN50 (*Mat1 leu2 trp1 ade8 can1 ira1::HIS3 cap::URA3*), and SKN37 (*Mata leu2 ura3 trp1 ade8 can1 cap::HIS3 RAS2^{val19}*) were used for testing functional expression of both the myc- and HA-tagged forms of MCH1 and CAP (Gerst *et al.*, 1992). SKN50 was also used for library screening. A wild type strain, SP1 (*Mat1 leu2 ura3 trp1 ade8 can1 his3*), has been described and was used in the immunoprecipitation experiments (Toda *et al.*, 1985).

2.4 Phenotypic Assays and Selections.

Screening of a rat embryo cDNA library in yeast was accomplished by transforming the *cap* strain SKN50 with a plasmid-based cDNA library constructed by Drs. R. Ballester and J. Camonis. This library was created from poly $(A)^+$ RNA derived from whole rat embryos and consisted of $>10^6$ independent clones. These cDNA clones were subcloned into the *NotI* site of a YEp-based expression plasmid, pLFADA13, which bears the *LEU2* selectable marker and the *ADH1* promoter. Constitutive cDNA gene expression from this plasmid is under the control of the *ADH1* promoter. This library was the generous gift of Drs. R. Ballester, J. Camonis, and M. Wigler. SKN50 cells were transformed with the library and were plated on YPD plates to select for growth on rich medium. The transformants were grown for 72 hrs before replica plating onto selective medium. In an initial screen of 25,000 Leu+ transformants, 4 were YPD+, only one of which was found to grow on rich medium in a plasmid-dependent manner. Plasmids were isolated from yeast according to standard methods (Rose *et al.*, 1990). Candidate plasmids were re-transformed back into SKN50 cells and were examined for their ability to confer growth on rich medium. Assays for cell growth on rich medium, temperature-sensitive growth at 37° C, and cellular sensitivity to nitrogen starvation, or heat shock at 55° C, were performed as described in Gerst *et al.* (1991).

2.5 RNA Preparation and Northern Analysis.

Two Sprague Dawley rats were killed by CO_2 asphyxiation, and tissue samples were removed by dissection. RNA extraction from homogenized tissue samples was performed using the guanidinium thiocyanate procedure followed by cesium chloride gradient centrifugation (Sambrook *et al.*, 1989). Aliquots of 30ug of total RNA were electrophoresed on formaldehyde gels, transferred to nylon membranes and probed with the ^{32}P-labeled 2.1-kb *NotI* fragment from pADH-MCH1. A 1-kb fragment of glyceraldehyde-phosphate dehydrogenase was used as a control probe for loaded RNA samples.

2.6 Plasmids

2.6a. Yeast vectors:

Yeast plasmids used in this study include: YCp50, a yeast centromeric plasmid bearing the *URA3* selectable marker (Rose *et al.*, 1987); pLFADA13, a YEp-based plasmid bearing the *LEU2* selectable marker and the yeast *ADH1* promoter (Colicelli et al., 1991); pAD4Δ, a YEp-based plasmid bearing the *LEU2* selectable marker and the yeast *ADH1* promoter (Ballester *et al.*, 1989); pAD54, a plasmid derived from pAD4Δ, containing an oligonucleotide encoding 22 amino acids of the influenza hemagglutinin antigen (HA) 5' to a polycloning site; and pAD6, another plasmid derived from pAD4Δ, that contains an oligonucleotide encoding 10 amino acids of the myc epitope 5' to the polycloning site.

Previously described plasmids include: pADH-CAP, which expresses *CAP* under the control of the *ADH1* promoter; pADH-CAPΔ4 and pADH-CAPΔ15, which express the carboxyl- and amino-terminal domains of CAP, respectively; pADH-CAPΔ7, which expresses a mutant CAP protein containing the amino- and carboxyl-terminal domains, but lacking the polyproline stretch ; pADH-CAPΔ11 which lacks the

last 27 amino acids of CAP; and pADH-MCH1 which expresses *MCH1* under the control of the *ADH1* promoter (Gerst *et al.*, 1991; Zelicof *et al.*, 1993).

Vectors used in the two-hybrid selection assay included: pPC86, which bears the sequence encoding the transactivating domain of *GAL4* cloned upstream to a polycloning site; and pPC97, which encodes the DNA binding domain of *GAL4* cloned upstream to the polycloning site. These centromeric plasmids contain the *TRP1* and *LEU2* selectable markers respectively, and were created by Pierre Chevray.

2.6b. Mammalian expression vectors:

Mammalian expression vectors used included pMV12 which contains a hygromycin-selectable marker with the polycloning site downstream of the HSV-thymidine kinase promoter.

2.6c. Bacterial plasmids:

Cloning vectors and bacterial expression plasmids used included pUC118, pBluescript SKII, pT7-Blue (Novagen), and pMAL-c2. pMAL-c2 (New England Biolabs) is a bacterial expression vector used for the IPTG-induction of MalE fusion proteins.

2.7 Plasmid Construction.

pUC118-MCH1, pMV12-MCH1 and Bluescript-MCH1 were created by subcloning a gel-purified *NotI*-digested MCH1-fragment isolated from the plasmid pLFADA13, into the alkaline-phosphatase treated *NotI* site of pUC118, pMV12 and Bluescript, respectively. Constructs were verified by restriction endonuclease analysis. All remaining constructs were created using gene fragments synthesized in the polymerase chain reaction (PCR). Standard conditions for PCR were employed and included 25 cycles of denaturation (94°C-1.5 minutes), annealing (45°C-2.5 minutes), and extension (72 ^{o}C-3.5 minutes). Unless otherwise specified, the resulting PCR products were gel purified and subcloned into the pT7-Blue cloning vector (Novagen).

Following subcloning into pT7-Blue, the inserts were subcloned into the appropriate yeast expression vectors using standard cloning techniques. All constructs were verified using restriction endonuclease analysis and are listed in Table II.

2.7a. Bacterial expression constructs:

In order to create an in-frame MalE-MCH1 fusion protein, an *MCH1 EcoR*I-*Sal*I fragment was cloned into the *EcoR*I-*Sal*I sites of the pMalc2 vector. The *MCH1 EcoR*I-*Sal*I in-frame fragment was created through PCR amplification from the pADH-MCH1 template. pADH-MCH1 is the pLFADA13 plasmid containing *MCH1* cloned into the *Not*I site. Forward oligonucleotide AZ1, which contains an *EcoR*I site 5' to the initiation site, and reverse oligonucleotide AZ2 , which contains a *Sal*I site 3' to the termination site, were used to amplify *MCH1* from the pADH-MCH1 template. Exact oligonucleotide sequences are given in Table I. After PCR, the amplified fragment was gel purified using electroelution, and the isolated DNA was then digested with *EcoR*I and *Sal*I The digested fragment was then ligated to the *EcoR*I-*Sal*I digested pMalc2 vector.

2.7b. Constructs created for the two-hybrid assay:

Appropriate oligonucleotides were used to create in-frame gene fusions between the sequences encoding either the DNA binding domain or transactivating domain of *GAL4* and genes of interest, (e.g. *CAP*, *CAP* deletion mutants and *MCH1*). The oligonucleotides used for PCR amplification are listed in Table I. All *GAL4-CAP* fusions were created by subcloning the appropriate *CAP* fragment into the *Sal*I and *Sac*I sites of either pPC86 or pPC97. To create gene fusions between *GAL4* and full-length *CAP*, a forward oligonucleotide, JG260, which encodes a *Sal*I restriction endonuclease site 5' to the initiation codon of *CAP*, and a reverse oligonucleotide, JG151, which encodes a *Sac*I restriction endonuclease site 3' to the termination codon were used in the PCR reaction. Plasmid pUCAP was used as a template and contains full-length *CAP* cloned into pUC118 (Gerst *et al.*, 1991). Plasmids pPC86-CAP and

pPC97-CAP were created by subcloning the subsequent PCR product into pPC86 and pPC97, respectively. Plasmids pPC86-CAP$^{2-269/369-526}$ and pPC97-CAP$^{2-269/369-526}$, which encode the amino- and carboxyl- domains of CAP in-frame to the domains of Gal4, were created by subcloning the PCR product obtained when pADH-CAPΔ7 was used as template, into plasmids pPC86 and pPC97, respectively. Plasmids pPC86-CAP$^{186-384}$ and pPC97-CAP$^{186-384}$, which encode the middle polyproline-rich domain of CAP in-frame to the Gal4 domains, were created by subcloning the PCR product obtained from oligonucleotides XY1 and XY2, when used with pUCAP as template in the PCR reaction, into pPC86 and pPC97, respectively. Oligonucleotide XY1 encodes an initiation codon at amino acid residue 185, 3' to the *Sal*I restriction endonuclease site, while oligonucleotide XY2 encodes a termination codon at amino acid position 385.

To create gene fusions between *GAL4* and *MCH1*, a forward oligonucleotide, AZ3, that encodes a *Sal*I restriction endonuclease site 5' to the *MCH1* initiation codon, and a reverse oligonucleotide, AZ2, which encodes a *Sal*I restriction endonuclease 3' to the termination codon, were used in the PCR reaction with plasmid pADH-MCH1 as template. The *GAL4-MCH1* gene fusions were created by subcloning the resulting *Sal*I fragment of *MCH1* into the *Sal*I site of pPC86 and pPC97, to yield pPC86-MCH1 and pPC97-MCH1, respectively.

2.7c. Constructs created for immunoprecipitation experiments:

To create epitope-tagged forms of CAP, the *CAP* gene was cloned downstream of, and in-frame to, the sequence encoding either the HA or myc epitopes in plasmids pAD54 and pAD6, respectively. Oligonucleotides JG260 and JG151 were used to amplify *CAP* for the creation of both constructs. PCR fragments were amplified, gel purified, and subcloned in the manner described above. The *Sac*I-*Sal*I fragments were subcloned into pAD54, to yield pADH-HACAP, or pAD6, to yield pADH-mycCAP. A single copy plasmid bearing the *ADH1-HA-CAP* fragment was created by subloning

a *Bam* HI fragment from pADH-HACAP into YCp50 to yield YCp-HACAP. To design epitope-tagged forms of CAP lacking the proline-rich region, oligonucleotides JG260 and JG151 were used with pADH-CAPΔ7 as template. Constructs pADH-HACAPΔ7, pADH-mycCAPΔ7 and YCp-HACAPΔ7, were generated by subcloning the resulting PCR fragment into pAD54 and pAD6, respectively. A single copy plasmid expressing HACAPΔ7 (YCp-HACAPΔ7) was constructed as described for YCp-HACAP. To create epitope-tagged forms of the carboxyl-terminal domain of CAP, the carboxyl-terminal region of *CAP* was amplified with oligonucleotides C-CAPF, which contains a *Sal*I site 5' to an initiation codon at position 184, and C-CAPR which encodes a *Sac*I site 3' to the termination codon of *CAP*. The resulting PCR fragments were subcloned into pAD54, pAD6, and YCp50, as described above, to yield plasmids pADH-HACAPΔ4, pADH-mycCAPΔ4, and YCp-HACAPΔ4. To create epitope-tagged forms of the amino-terminal domain of CAP, the amino-terminal region of *CAP* was amplified with oligonucleotides JG260 and NtermR, which encodes a *Sac*I site 3' to the termination codon at position 193. The resulting PCR fragments were subcloned into pAD54, pAD6, and YCp50, as described above, to yield plasmids pADH-HACAPΔ15, pADH-mycCAPΔ15, and YCp-HACAPΔ15. To create epitope-tagged forms of CAPΔ11 which lacks the last 27 amino acids, CAPΔ11 was amplified with oligonucleotides JG260 and CAPΔ11R using pADH-CAPΔ11 as a template. Oligonucleotide CAPΔ11R encodes a *Sac*I site 3' to the termination codon at position 1495. The resulting PCR fragment was subcloned into pAD54, pAD6 and YcP50, as discussed.

To create epitope-tagged forms of MCH1, the *MCH1* gene was cloned downstream of, and in-frame to, the sequence encoding either the HA or myc epitopes in plasmids pAD54 and pAD6, respectively. Oligonucleotides AZ3 and AZ2 were used in this reaction to amplify *MCH1* for subcloning into pAD54, to yield pADH-HAMCH1, or into pAD6 to yield pADH-mycMCH1. To create the epitope-tagged forms of the

carboxy-terminal of MCH1, we used oligonucleotides CtermF, which encodes a *Sal*I site 5' to an initiation codon at amino acid residue 277, and CtermR, which encodes a *Sal*I site 3' to termination codon of *MCH1*, in the PCR reaction with pADH-MCH1 as a template. Subcloning of the resulting PCR product into pAD54 and pAD6 yielded plasmids pADH-HAMCH1$^{277-474}$ and pADH-myc-MCH1$^{277-474}$, respectively. YCp-HAMCH1 and YCp-HAMCH1$^{277-474}$ were generated as described previously.

To create epitope tagged forms of the profilin gene, *PFY*, we used the forward oligonucleotide HAPFY, which encodes a *Sma*I site 5' to the translational start site and the reverse oligonucleotide PFYR which encodes a *Bgl* II site 3' to the termination site. This fragment was amplified from a plasmid containing the profilin gene, *PFY1* and was then subcloned into the *Sma*I-*Bgl*II sites of pAD54 to create pADH-HAPFY.

TABLE I.

Oligonucleotides used in this study

For CAP constructs		
JG260	forward	5'-CCGTGTGAAAGTCGACCATGCCTGACT-3'
JG151	reverse	5'-ACAATTAGAGCTCCTCGC-3'
C-CAPF	forward	5'-CCCACCAGCGTCGACCATGGAAATCTCT-3'
C-CAPR	reverse	5'-CAATTAGAGCTCGCAATA-3'
JG35	reverse	5'-TTTCAAAGACGGGAGCTCATGGGGCTGCTG-3'
XY1	forward	5'-GTGGACACTCCGTCGACTATGGTCACA-3'
XY2	reverse	5'-ATTCAGTTTCGAGCTCTTAATTCTCAAT-3'
CAPD11	reverse	5'-CAGGGATTGGGAGCTCTACTTAATCATCGT-3'
For MCH1 constructs		
AZ-3	forward	5'-GAGCAGGTGGTCGACTATGGCTGACA-3'
AZ-2	reverse	5'-CCCATGCAGTCGACTTATCCAGC-3'
AZ-1	forward	5'-AGCAGGTGGGAATTCATGGCTGAC-3'
C-termF	forward	5'-GAAACATGTGTCGACTGACATGAAG-3'
C-termR	reverse	5'-CCATGCAATGAGCTCATCCAGCGA-3'
N-termR	reverse	5'-GTTCTGTCCGGATCCTCTAGTAAGCTC-3'
For Profilin constructs		
HAPFY	forward	5'-AACATAAACCCGGGTACGATCGCA-3'
PFYR	reverse	5'-AAGAAAACTAGATCTGCAAATTA-3'

TABLE II.

Plasmid	Vector	Promoter	Gene expressed
Yeast plasmids			
YcP-HACAP	YcP50		*HACAP*
YcP-HACAPΔ7			*HACAPΔ7*
YcP-HACAPΔ15			*HACAPΔ15*
YcP-HACAPΔ4			*HACAPΔ4*
YcP-HACAPΔ11			*HACAPΔ11*
YcP-HAMCH1			*HAMCH1*
pADH-HACAP	pAD54	*ADH1*	*HACAP*
pADH-HACAPΔ7			*HACAPΔ7*
pADH-HACAPΔ15			*HACAPΔ15*
pADH-HACAPΔ4			*HACAPΔ4*
pADH-HACAPΔ11			*HACAPΔ11*
pADH-HAMCH1			*HAMCH1*
pADH-HAMCH1$^{277-474}$			*HAMCH1*$^{277-474}$
pADH-HAPFY			*HAPFY*
pADH-mycCAP	pAD6	*ADH1*	*mycCAP*
pADH-mycCAPΔ7			*mycCAPΔ7*
pADH-mycCAPΔ15			*mycCAPΔ15*
pADH-mycCAPΔ4			*mycCAPΔ4*
pADH-mycCAPΔ11			*mycCAPΔ11*
pADH-mycMCH1			*mycMCH1*
pADH-mycMCH1$^{277-474}$			*mycMCH1*$^{277-474}$
pADH-CAP	pAD4Δ	*ADH1*	*CAP*
pADH-CAPΔ7			*CAPΔ7*
pPC86-CAP$^{186-384}$	pPC86	*ADC1*	*CAP*$^{186-384}$
pPC86-CAP$^{2-269/369-526}$			*CAP*$^{2-269/369-526}$
pPC86-MCH1			*MCH1*
pPC97-CAP	pPC97	*ADC1*	*CAP*
pPC97-CAP$^{186-384}$			*CAP*$^{186-384}$
pPC97-CAP$^{2-269/369-526}$			*CAP*$^{2-269/369-526}$
pPC97-MCH1			*MCH1*
pADH-MCH1	pLFADA13	*ADH1*	*MCH1*

Plasmid	Vector	Promoter	gene expressed
Bacterial plasmids			
pUC-CAP	pUC118	T7	*CAP*
pUC118-MCH1	pUC118	T7	*MCH1*
pMAL-c2-MCH1	pMAL-c2	pMal	*MCH1*
pBluescript MCH1	pBluescript	T7	*MCH1*
Mammalian expression plasmids			
pMV12MCH1	pMV12	HSVthymidine kinase	*MCH1*

2.8 Antibodies.

A polyclonal antiserum against MCH1 was raised in rabbits using a MalE-MCH1 fusion protein as the antigen. The gene fusion encoding the MalE-MCH1 protein was created by subcloning an *Eco*RI-*Sal*I fragment of *MCH1* into pMalc2 (New England Biolabs). This construct, pMalE-MCH1, was expressed in bacteria and MalE-MCH1 fusion protein was isolated by affinity chromatography, using the procedure recommended by the supplier. We did notice, however, that the fusion protein appeared to be truncated upon bacterial expression and had an apparent mobility of ~70kDa in acrylamide gels. We calculate that the truncated MCH1 protein was approximately 35kDa in size. After injection of the fusion protein into rabbits and successive boosting using standard procedures, a polyclonal anti-MCH1 antiserum (#30358) was obtained. This antiserum detects a single protein band of approximately 60kDa in lysates prepared from rat fibroblasts (see Figure 6). Similarly, this antiserum could recognize a protein of equal molecular weight in yeast expressing *MCH1* from the plasmid pADH-MCH1 (see Figure 6). In contrast, the antiserum did not cross react with any protein in wild-type yeast cells. Thus this antiserum is MCH1-specific.

Figure 6. MCH1 antibody specifically recognizes a protein of ~60 kDa. Total cell lysates (30μg) from SKN50 cells transformed with pLFMCH1, untransformed SKN50 cells, and Rat-6 fibroblasts were resolved on a 10% SDS-acrylamide gel. The gel was then transferred to a nylon membrane that was probed with anti-MCH1 antibody (1:2500 dilution). As seen here, anti-MCH1 antibody specifically recognizes a protein of ~60 kDa in mass which is absent in SKN50 cells that were not transformed with pLFMCH1. As shown, MCH1 is also recognized in Rat-6 fibroblasts.

Other antibodies used in this study included an affinity-purified monoclonal antibody against the influenza virus HA epitope (12CA5) and a monoclonal antibody against the myc epitope (9E10) in ascites fluid. An anti-actin monoclonal antibody was purchased from Boerhinger Mannheim.

2.9 Synthetic peptides

Peptides corresponding to the Hemagglutinin Antigen (HA) and myc epitopes (NH_2-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser-Leu-COOH and NH_2-Met-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-COOH) respectively, were synthesized commercially (Biosynthesis Inc.).

2.10 Two-hybrid Selection Assays:

The two-hybrid selection assay was performed as described (Durfee *et al.*, 1993). Briefly, Y153 cells were grown to log phase prior to transformation with plasmids bearing genes encoding either full length yeast CAP or rat MCH1, or deletion mutants thereof. Plasmids containing no insert were used as negative controls for interaction and were transformed into Y153 cells in conjunction with the aforementioned plasmids. Individual transformants were patched onto double selective medium, lacking leucine and tryptophan, and were grown for 2 days prior to replica plating onto fresh double selective plates or triple selective medium, which contain 25mM 3AT and lack histidine. Replicas were allowed to grow for 2 days prior to examination for growth on triple selective medium.

Quantitative assays for β-galactosidase activity were performed on cell lysates prepared from growing cultures of yeast. Briefly, between 25-50 O.D.600 units of cells in log phase growth were harvested and lysed in an ice-cold extraction buffer containing 0.1M Tris HCl pH8.0, 20% glycerol (vol/vol), 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, and 10μg per ml of aprotinin, soybean trypsin

inhibitor, and leupeptin. Cell lysates were prepared by first adding an equal volume (e.g., 250μl) of 0.5mm washed glass beads, followed by 10-15min of constant vortexing at 1800 rpm in a IKA-Vibrax shaker at 4°C. Cell extracts were clarified by centrifugation at 10,000xg for 3 min and the protein concentration of the resulting supernatants was determined. Protein concentration was assayed using the MicroBCA method (Pierce). β-galactosidase activity was determined using the hydrolysis of *o*-nitrophenolgalactoside (ONPG) as an indicator, as according to Miller (Miller, 1972). β-galactosidase activity in Y153 cell lysates was found to be linear with time (0-24hrs) and protein (50-1000ug protein) in this assay system. Samples used to measure β-galactosidase activity were normalized for protein and contained between 250-500ug protein per experiment. Units of β-galactosidase activity are expressed in nmol ONPG cleaved/mg protein/hour.

2.11 Immunoprecipitation and Immunoblot Analysis.

Yeast cells were grown to log phase, and 7.5 O.D. 600 units of cells were harvested and resuspended in ice-cold lysis buffer. Lysis buffer is composed of a phosphate buffered saline (PBS) solution with 1% Triton X-100, leupeptin (10μg/ml), soybean trypsin inhibitor (10μg/ml), aprotinin (10μg/ml), and 100μM phenylmethylsulfonyl fluoride (PMSF). Cell extracts were prepared by first adding an equal volume of 0.5mm washed glass beads to the cells, followed by 20 minutes of constant shaking at 1800 rpm in a IKA-Vibrax shaker at 4°C. Cell lysates were then clarified by centrifugation at 10,000xg for 10 minutes and the protein concentration of the supernatants was determined using the Micro-BCA method (Pierce). Between .35 and .5 mg of total protein were incubated with 10μg of affinity-purified anti-HA antibody (12CA5), 1.5 μl anti-myc (9E10) ascites fluid, or 3 μl of anti-MCH1 polyclonal antiserum in the absence and presence of 75μg of HA or myc peptide, respectively. Immunoprecipitations were performed at 4°C with constant rotation for 1.5 to 2 hours.

The antibody complexes were then precipitated by adding a 35μl bed volume of protein A-agarose (Boehringer Mannheim), pre-washed in lysis buffer, to the IP reaction. Following a 1.5 hour incubation at 4°C with constant rotation, the complexes were washed three times with ice-cold lysis buffer. Precipitated complexes were resuspended in SDS-sample buffer and boiled 5 minutes prior to electrophoresis on 10% acrylamide gels (Sambrook *et al.*, 1989).

For immunoblotting, gels were transferred onto GeneScreen Plus nylon membranes (DuPont NEN). Membranes were then blocked in a 5% non-fat dry milk-phosphate buffered saline solution (PBS) for 1 hour to overnight. After blocking, the blots were washed repeatedly in a 1.5% bovine serum albumin, 0.1% Tween-20 solution in PBS (TBS). Blots were then incubated with primary antibody diluted in TBS for 1.5 hours. After incubation with primary antibody, the blots were washed and incubated with a 1:5000 dilution of either peroxidase labeled-anti-rabbit or anti-mouse antibody for 1 hour. The blots were washed again in TBS, and the proteins detected using the ECL chemiluminescent assay (Amersham Corp).

2.12 Immunofluorescence Methods.

In experiments designed to aid in the localization of MCH1 in mammalian cells, Rat-6 fibroblasts were seeded at a density of 5×10^4 on pre-sterilized cover slips. After 24 hours in medium containing 10% bovine calf serum, the coverslips were gently washed (x1) with PBS (phosphate-buffered saline) and fixed in a solution of formaldehyde (2%) for 15 minutes. Coverslips were then rinsed (x3) with PBS and the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes on ice. After incubation with permeabilization buffer, the coverslips were washed (x3) in PBS and were treated for 5 minutes with 50mM NH_4Cl in PBS to quench the aldehyde fluorescence. Coverslips were washed (x2) with PBS and then incubated with primary antibody. Primary and secondary antibody dilutions were prepared in PBS. Anti-

MCH1 polyclonal antiserum was diluted 1:1000 in PBS; anti-vinculin antibody was diluted 1:200 as recommended by the supplier (Sigma). After dilution of the antibodies, they were aliquoted onto coverslips containing cells that had been permeabilized and washed as described above. After a 60 minute incubation with primary antibody at room temperature, the cover slips were washed (x3) in PBS for 10 minutes. The cover slips were then incubated in the dark for 45 minutes with FITC-conjugated goat anti-rabbit antibody (1:500 dilution in PBS) or Texas Red-conjugated goat anti-mouse antibody (1:50 dilution in PBS) (Molecular Probes). To determine if MCH1 co-localizes with actin stress fibers, FITC-conjugated goat anti-rabbit antibody was added together with rhodamine-conjugated phalloidin (0.8 mg/ml) (Sigma). After secondary antibody incubation, the cells were washed (x3) with PBS for 10 minutes. The cover slips were then mounted with anti-fade medium, and viewed using a Zeiss fluorescence microscope.

In order to visualize the distribution of MCH1 shortly after cellular attatchment to a fibronectin-coated surface, we seeded freshly-trypsinzed rat-6 fibroblasts onto fibronectin-cover slips in 10%BCS-containing medium. The cover-slips were then incubated at 37°C for 45 minutes. Following incubation, the coverslips were washed in PBS, and the localization of MCH1 or vinculin was assessed by immunofluorescence using the methods described above.

2.13 Electron microscopy and immunogold labeling.

Yeast were grown to log phase in the appropriate liquid synthetic medium. Cells were harvested by centrifugation, washed, and fixed in a phosphate-buffered saline (PBS) solution containing 3% paraformaldehyde and 0.75% glutaraldehyde. Cells were washed, resuspended in a solution of 1% sodium metaperiodate, and incubated for 45 min at 25° C, to improve free diffusion through the yeast cell wall. The fixed cells were treated with 50mM NH_4Cl, dehydrated sequentially in ethanol, and

embedded in Lowicryl K4M resin. Fixed cells were sectioned and mounted on 200-mesh grids for morphology and immunogold labeling procedures. Grids were stained with 5% uranyl acetate in 25% ethanol for 40 min and quickly washed with a solution of 5mM lead citrate in 0.01N NaOH. Electron microscopy was performed on a Hitachi TEM 7000.

Immunogold labeling of thin-sectioned yeast was performed using 20nm Protein A-gold (E-Y Laboratories). Prior to uranyl acetate/lead citrate staining, grids containing thin sections were incubated with PBST (phosphate-buffered saline containing 0.05% Tween) for 15min before incubation in PBST and 1% BSA (PBST-BSA). The grids were then incubated for 2hr at room temperature with anti-CAP antiserum (1:500 dilution) (Field *et al.*, 1990) in PBST-BSA. Following incubation, the grids were washed 5 times with PBST and further incubated with Protein A-gold diluted 1:50 in PBST-BSA for 1hr at room temperature. The grids were then washed, as described above, and fixed with 0.25% glutaraldehyde in PBS. Staining of the sections with uranyl acetate and lead citrate, and electron microscopy was performed as described above. (All electron microscopy procedures were performed by V. Protopopov. Dept. of Cell Biology and Anatomy, Mount Sinai Medical Center.)

2.14 Mammalian Cell Transfection Procedures.

Rat-6 fibroblasts were seeded at a density of 5×10^4 24 hours prior to transfection. Transfections were performed using standard calcium phosphate methods as originally described by Wigler *et al.*, (1978). Cells were transfected with 30µg of either pMV12MCH1 or pMV12 plasmid DNA plus 10µg of Rat-6 carrier DNA. DNA was prepared by cesium-gradient ultracentrifugation. After transfection, fibroblasts were rinsed with phosphate buffered saline solution and refed overnight with 10%BCS in DMEM. Confluent cell-cultures were then trypsinized and seeded at a 1:10 dilution in 10% BCS medium containing 350µg/ml hygromycin. Cells were fed with

hygromycin-containing medium every three days over a period of 12 days. At this point, colonies were visualized with Giemsa stain and counted.

2.15 Protein phosphorylation in fibroblasts.

In order to determine whether MCH1 exists as a phosphoprotein in Rat-6 cells, cells were seeded at a density of $5x10^4$ in 10%BCS/DMEM 24 hours prior to phosphate-labeling. The next day, the cells were rinsed twice in a Tris-Glycine buffer (TGB) and then prestarved in labeling medium lacking phosphate for 2 hours at 37°C. Labeling media contains 5% dialyzed calf serum and 10% phosphate-free DMEM. Afterwards, the cells were treated with 1mCi of [^{32}P]-orthophosphate in 5 mls of labeling media for 4 hours. The cells were then rinsed twice with TGB and lysed in a lysis buffer contained 150mM NaCl, 50mM Tris pH 8.0, 1% Triton X-100, protease inhibitors, as well as, 1mM Na_3VO_4 and 50mM sodium pyrophosphate. After solubilization, the cells were centrifuged at 12000xg for 10 minutes to remove protein aggregates and nuclei. Proteins were then quantitated using the Micro BCA assay (Pierce). 250μg of total cell extract was used for immunoprecipitation with anti-MCH1 antibody. Immunoprecipitations were performed in the presence and absence of 15μg of exogenous unlabeled MCH1 peptide using conditions already described. IPs were run on a 10% SDS acrylamide gel and were transferred to a nylon membrane. The membrane was probed with anti-MCH1 antibody, as described, and detection was done by ECL chemiluminescence. Afterwards, the membrane was exposed to film for 24 hours to visualize phospholabeling.

CHAPTER 3

Results

3.1 Isolation and Cloning of MCH1.

As described, *cap* yeast strains display two sets of phenotypes--one set of phenotypes is attributed to the loss of the amino terminus of *CAP*; the second set of phenotypes is associated with the loss of the carboxyl-terminal domain (Field *et al.*, 1990). Deletion of the amino terminus results in heat shock resistance in cells possessing an activated allele of *RAS*, while removal of the carboxyl terminus results in the inability to grow on (YPD) nutrient-rich medium, temperature sensitivity at 37°C, and other phenotypes (Gerst *et al.*, 1991). The sensitivity of *cap* cells to extremes in temperature or amino-acid concentrated media may reflect an overall inadequacy in protein synthesis or protein assembly. Such a defect would result in the cell's inability to tolerate conditions that would stress the metabolic machinery such as growth in the presence of excess amino acids or in the presence of elevated temperatures.

We transformed a *cap* null yeast strain (SKN50) with a mammalian cDNA library in an effort to isolate genes that would suppress phenotypes associated with the deletion of the carboxyl terminus of CAP (Figure 7). In this manner, we might identify either mammalian homologs of yeast *CAP* or possible downstream effectors of the carboxyl-terminal pathway.

Use of a Genetic Screen to Isolate effectors of the Carboxyl-terminal Domain of CAP (Adenylyl Cyclase-Associated Protein).

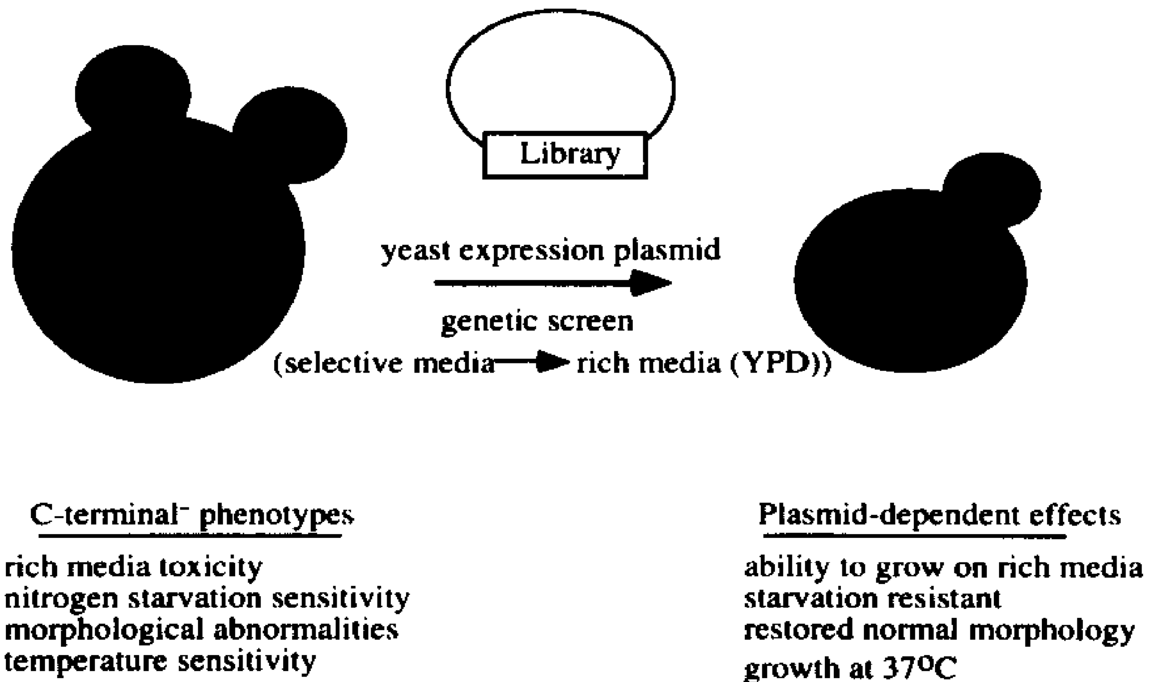


Figure 7. A genetic screen was used to identify mammalian homologs of yeast CAP or proteins downstream of the carboxyl-terminal signaling pathway. The *cap* SKN50 yeast strain was transformed with a mammalian cDNA library, and the transformants were plated on nutrient rich media (YPD), in order isolate clones that can suppress phenotypes associated with the loss of the carboxyl-domain of CAP. Suppressors were then grown on synthetic minimal medium in order to lose the plasmid and were reassayed for growth on YPD to determine whether the resulting suppression was plasmid-dependent.

The library used for transformation was a rat embryonic cDNA library cloned into a multicopy expression vector. After transformation of the library into the *cap* SKN50 yeast strain, cells were plated on amino-acid rich medium (YPD). Of 25,000 transformants, one was found to confer growth on YPD in a plasmid-dependent manner. This was confirmed by attempting to lose the plasmid by growth on synthetic medium and then re-testing for growth on YPD and on selective medium. Following confirmation of the plasmid-dependent effect, the plasmid was recovered from yeast and retransformed into SKN50 cells. Transformed strains were assayed once again for phenotypes which result from the loss of the carboxyl terminus of CAP. These included temperature sensitivity, growth on YPD, cellular morphology and sensitivity to nitrogen starvation. Results of this transformation can be seen in figures 8 and 9. The plasmid, as shown, confers suppression of all phenotypes associated with loss of the carboxyl terminus, while not affecting characteristics associated with loss of the amino terminus (Figures 8, and 9).

Following isolation of the plasmid, we subcloned the 2.1kb *Not* I insert into the pUC derivative plasmid, pUC118, for sequencing purposes. Sequencing revealed an open reading frame of 474 amino acids with a predicted molecular weight of approximately 55 kDa (Figure 10). Analysis of the protein sequence revealed that this gene encodes a protein highly homologous to other CAP proteins (Figure 11). Thus, we named this gene *MCH1* (Mammalian CAP Homolog 1). MCH1 is organized similarly to other CAP proteins with an amino terminus and a carboxyl terminus connected by a proline-rich region. Comparison of the amino acid sequence of MCH1 to other sequences reveals 36% overall homology to *S.cerevisiae* CAP and 35% homology to *S.pombe* cap. The regions of greatest conservation are the proline-rich region, as well as, the carboxyl-terminal domain. In addition to isolation of the yeast CAP protein, a human homolog was identified and is shown in comparison to the rat

and porcine homologs (Matviw *et al.*, 1992; see Figure 11). MCH1 is 93% homologous to the porcine homolog and 96% homologous to human CAP (Figure 11).

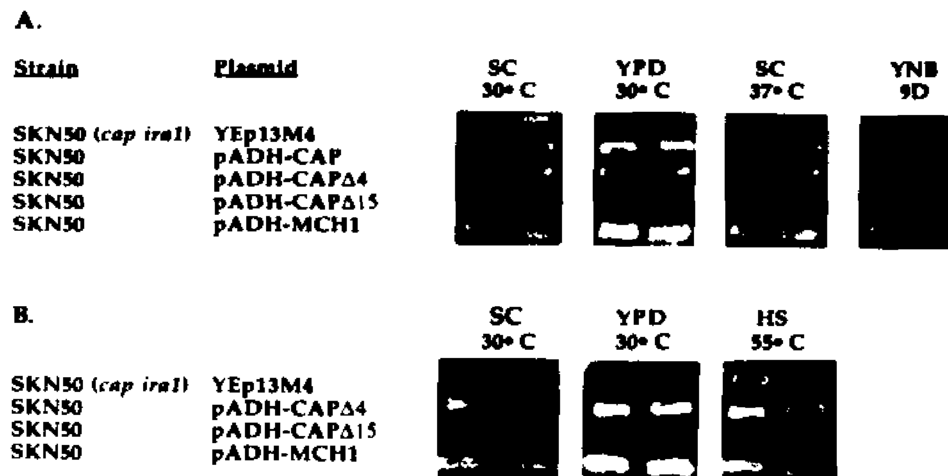


Figure 8. Effect of MCH1 expression on *cap* null phenotypes in *S. cerevisiae*. The *cap* strain SKN50 was transformed with plasmids expressing full length CAP (pADH-CAP), the carboxyl terminus of CAP (pADH-CAPΔ4), the amino terminus of CAP (pADH-CAPΔ15), MCH1 (pADH-MCH1), or a control plasmid (YEp13M4). Experiment A: Transformed yeast strains were grown on synthetic medium prior to replica plating onto synthetic medium at 30° C (SC 30° C) as a control, synthetic medium at 37° C (SC 37° C) to test for temperature-sensitive growth, or YPD (YPD 30° C) to test for growth on rich medium. Other transformants were plated onto YNB plates for 9 days (YNB 9D) to test for sensitivity to nitrogen starvation, followed by replica plating onto SC plates for recovery. Strains were allowed to recover for 72hrs. Experiment B: Transformed yeast strains were grown as in Experiment A, but some transformants were replica plated onto pre-heated (55° C) SC plates for 10 min (HS 55° C) to test for heat shock sensitivity.

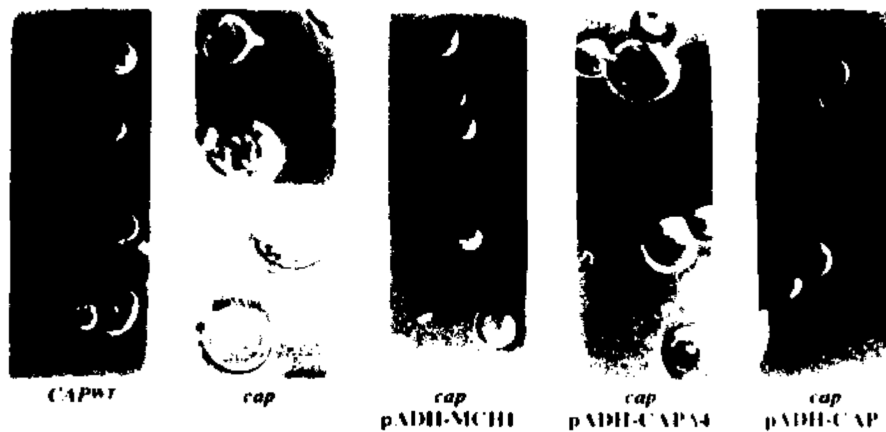


Figure 9. Effect of MCH1 on the cellular morphology of *cap* yeast. The*cap* strain SKN50 transformed with plasmids expressing full length CAP (pADH-CAP), the carboxyl terminus of CAP (pADH-CAPΔ4), or MCH1 (pADH-MCH1) was examined for cellular morphology. A *CAP^{wt}* strain, SP1, and the *cap* strain SKN50 were used as controls for wildtype yeast and *cap* yeast, respectively. Yeast strains were grown in liquid synthetic medium for two days prior to visual examination.

```
 001   ATGGCTGACATGCAAAATCTTGTAGAAAGATTGGAGAGGGCAGTGGGCCGCCTGGAGGCG
 001    M  A  D  M  Q  N  L  V  E  R  L  E  R  A  V  G  R  L  E  A
 061   GTGTCACATACTTCTGACATGCACTGTGGATATGGAGACAGTCCTTCAAAAGGAGCAGTT
 021    V  S  H  T  S  D  M  H  C  G  Y  G  D  S  P  S  K  G  A  V
 121   CCATATGTGCAAGCATTTGACTCGCTGCTTGCCAATCCCGTGGCGGAGTACTTGAAGATG
 041    P  Y  V  Q  A  F  D  S  L  L  A  N  P  V  A  E  Y  L  K  M
 181   AGTAAGGAGATTGGGGGAGATGTGCAGAAACACGCGGAGATGGTCCACACAGGCCTGAAG
 061    S  K  E  I  G  G  D  V  Q  K  H  A  E  M  V  H  T  G  L  K
 241   TTGGAGCGAGCTCTCCTGGTTACAGCTTCTCAGTGCCAGCAGCCAGCCGGTAATAAACTC
 081    L  E  R  A  L  L  V  T  A  S  Q  C  Q  Q  P  A  G  N  K  L
 301   TCGGATTTGTTGGCCCCTATCTCGGAGCAGATCCAGGAAGTTATAACCTTCCGGGAGAAG
 101    S  D  L  L  A  P  I  S  E  Q  I  Q  E  V  I  T  F  R  E  K
 361   AACCGAGGCAGCAAGTTTTTCAATCATCTATCAGCTGTCAGTGAAAGCATCCAGGCCCTG
 121    N  R  G  S  K  F  F  N  H  L  S  A  V  S  E  S  I  Q  A  L
 421   GGCTGGGTGGCTCTGGCTGCGAAACCCGGCCCCTTTGTGAAAGAGATGAATGATGCCGCC
 141    G  W  V  A  L  A  A  K  P  G  P  F  V  K  E  M  N  D  A  A
 481   ATGTTTTACACAAACCGAGTCCTCAAGGAGTACAGAGATGTGGATAAAAAGCATGTGGAC
 161    M  F  Y  T  N  R  V  L  K  E  Y  R  D  V  D  K  K  H  V  D
 541   TGGGTTAGAGCTTACTTGAGTATATGGACAGAACTGCAGGCTTACATCAAGGAGTTCCAT
 181    W  V  R  A  Y  L  S  I  W  T  E  L  Q  A  Y  I  K  E  F  H
 601   ACCACTGGCCTGGCCTGGAGCAAGACGGGGCCTGTGGCAAAAGAACTGAGTGGATTGCCA
 201    T  T  G  L  A  W  S  K  T  G  P  V  A  K  E  L  S  G  L  P
 661   TCTGGACCCTCTGTGGGATCAGGCCCACCTCCTCCCCCACCAGGCCCTCCTCCTCCCCCA
 221    S  G  P  S  V  G  S  G  P  P  P  P  P  P  G  P  P  P  P  P
 721   GTTCCTACCAGTTCTGGCTCTGACGACTCTGCTTCACGCTCAGCACTGTTTGCACAGATT
 241    V  P  T  S  S  G  S  D  D  S  A  S  R  S  A  L  F  A  Q  I
 781   AATCAGGGGGAAAGCATCACACATGCCCTGAAACATGTATCTGATGACATGAAGACTCAC
 261    N  Q  G  E  S  I  T  H  A  L  K  H  V  S  D  D  M  K  T  H
 841   AAGAACCCTGCCCTGAAAGCTCAGAGTGGTCCAGTTCGGAGTGGCCCCAAACCATTCTCT
 281    K  N  P  A  L  K  A  Q  S  G  P  V  R  S  G  P  K  P  F  S
 901   GCACCTAAACCCCAAACTAGCCCCTCCCCCAAACCAGCCACAAAGAAGGAGCCAGCTCTG
 301    A  P  K  P  Q  T  S  P  S  P  K  P  A  T  K  K  E  P  A  L
 961   CTGGAACTGGAAGGCAAGAAATGGAGAGTGGAAAACCAGGAGAATGTTTCCAACCTGGTG
 321    L  E  L  E  G  K  K  W  R  V  E  N  Q  E  N  V  S  N  L  V
1021   ATCGATGACACTGAGCTGAAGCAGGTGGCTTACATCTACAAGTGTGTCAACACGACATTG
 341    I  D  D  T  E  L  K  Q  V  A  Y  I  Y  K  C  V  N  T  T  L
1081   CAAATCAAGGGCAAAATCAATTCCATTACAGTAGATAACTGTAAGAAGCTTGGCCTGGTG
 361    Q  I  K  G  K  I  N  S  I  T  V  D  N  C  K  K  L  G  L  V
1141   TTTGATGACGTGGTGGGCATTGTGGAGATAATCAATAGTAGGGATGTCAAAGTTCAGGTG
 381    F  D  D  V  V  G  I  V  E  I  I  N  S  R  D  V  K  V  Q  V
1201   ATGGGAAAAGTGCCAACCATTTCCATTAACAAAACAGATGGCTGCCACGCTTACCTGAGT
 401    M  G  K  V  P  T  I  S  I  N  K  T  D  G  C  H  A  Y  L  S
1261   AAGAACTCCCTGGACTGCGAGATAGTCAGTGCCAAATCTTCTGAGATGAACGTCCTCATT
 421    K  N  S  L  D  C  E  I  V  S  A  K  S  S  E  M  N  V  L  I
1321   CCTACAGAAGGCGGTGATTTTAACGAGTTCCCAGTCCCTGAGCAGTTCAAGACCTTGTGG
 441    P  T  E  G  G  D  F  N  E  F  P  V  P  E  Q  F  K  T  L  W
1381   AACGGACAGAAGTTGGTCACCACAGTGACAGAAATCGCTGGATAA
 461    N  G  Q  K  L  V  T  T  V  T  E  I  A  G  *
```

Figure 10. Sequence of rat *MCH1* and its encoded product. The DNA sequence of *MCH1* cDNA and its corresponding amino acid sequence is given. The coordinates for nucleotides and codons are indicated on the left. The single-letter amino acid code for MCH1 is given below each 3-bp codon.

```
                         **                  *     *   *         *
SC     001 MPDSKYTMQ-GYNLVKLLKRLEEATARLEDVTIYQEGYIQNKLEASKNNKPS-------------------
SP     001 MSDMINIRETGYNFTTILKRLEAATSRLEDLVESGHYPLPNMHRPSRDSNSQTHNISFNIGTPTAPTVSTGS
ASP-56                                 LEAVSHASDTHYGYGDSAAK
HU     001 MADMQN----------LVERLERAVGRLEAVSHTSDMHRGYADSPSKAGAAP-------------------
RA     001 MADMQN----------LVERLERAVGRLEAVSHTSDMHCGYGDSPSK-GAVP-------------------

                        *                     *  *  **   *   * *      ** *    *     *
SC     052 -------DSGADANTTNEPSAENAPEVEQDPKCITAFQSYIGENIDPLVELSGKIDTVVLDALQLLKGGFQS
SP     073 PAVASLHDQVAAAISPRNRSLTSTSAVEAVPASISAYDEFCSKYLSKYMELSKKIGGLIAEQSEHVEKAFNL
ASP-56                                                        EIGGDV----LKHAEMVET
HU     043 ------------------------------YVQAFDSLLAGPVAEYLKISKEIGGDV----QKHAEMVET
RA     042 ------------------------------YVQAFDSLLANPVAEYLKMSKEIGGDV----QKHAEMVET

              *    ***          *    *    ** *  ***       *       *      *      *
SC     117 QLTFLRAAVRSRKPDYS-SQTFADS-LRPINENIIKLGQLKESNRQSKYFAYLSALSEGAPLFSWVAVD-TP
SP     145 LRQVLSVALKAQKPDMD-SPELLEF-LKPIQSELLTITNIRDEHRTAPEFNQLSTVMSGISILGWVTVEPTP
ASP-56     GLK
HU     079 GLKLERALLVTASQCQQPAENKLSDLLAPISEQIKEVITFREKNRGSKLFNHLSAVSESIQALGWVAMAPKP
RA     078 GLKLERALLVTASQCQQPAGNKLSDLLAPISEQIQEVITFREKNRGSKFFNHLSAVSESIQALGWVALAAKP

             *  *  **           *    ***      **  *  * **        *        **        *  *    *
SC     186 VSMVTDFKDAAQFWTNRILKEYRESDPNAVEWVKKFLASFDNLKAYIKEYHTTGVSWKKDGMDFADAMAQST
SP     215 LSFMSEMKDSSQFYANRVMKEFKGKDDLQIEWVRSYLTLLTELITYVKTHFKTGLTWSTKQDAVPLKTALAN
ASP-56                                EYKDVDK                                   XGPVAKELSGLPS
HU     151 GPYVKEMNDAANFYTNRVLKEYKDVDKKHVDNVKAYLSINTELQAYIKEFHTTGLANSKTGPVAKELSGLPS
RA     150 GPFVKEMNDAANFYTNRVLKEYKDVDKKHVDNVRAYLSINTELQAYIKEFHTTGLANSKTGPVAKELSGLPS

              *                                            *      *      *   *         *
SC     259 KNTGATSSPSPASATAAPAPPPPPPAPPASVFEISNDTPATSSDA----NKGGIGAVFAELNQGENITKGLK
SP     287 LGASKTQAPSSGDSANGGLPPPPPPPPP-----SNDFWKDSNEPAPADNKGDHGAVFAEINKGEGITSGLR
ASP-56     GPSAGSG------------PPPP                              SALFAQINQGESITHALK
HU     223 GPSAGSG------------PPPPPPGPPPPP-------VSTSSGS---DESASRSALFAQINQGESITHALK
RA     222 GPSVGSG------------PPPPPPGPPPPP-------VPTSSGS---DDSASRSALFAQINQGESITHALK

                              *  *  *                                *          *       *  ***
SC     326 KVDKSQQTHKNPELRQSSTVSSTGSKSG--PPPRPKKPSTLKTKRPPRK-ELVGNKWFIENYENETESLVID
SP     353 KVDKSEMTHKNPNLRK-TGPTP-GPKPKIKSSA-PSKPAETAPVKPPRI-ELENTKWFVENQVDNH-SIVLD
ASP-56     HVADDEKTHKNPADKAQSGPVRXGPKPFSASKPGISPSPKPVTK            KWRXENXENVSNLVIDD
HU     273 HVSDDMKTHKNPALKAQSGPVRSGPKPFSAPKPQTSPSPKRATKKEPAVLELEGKKWRVENQENVSNLVIED
RA     272 HVSDDMKTHKNPALKAQSGPVRSGPKPFSAPKPQTSPSPKPATKKEPALLELEGKKWRVENQENVSNLVIDD

                     ** *       *      * ****         * * ***    *    *    *      *        *
SC     395 ANKDE-SIFIGKCSQVLVQIKGKVNAISLSETESCSVVLDSSISGMDVIKSNKFGIQVNHSLPQISIDKSDG
SP     420 SVELNHSVQIFGCSNCTIIIKGKLNTVSMSNCKRTSVVVDTLVAAFDIAKCSNFGCQVMNHVPMIVIDQCDG
ASP-56     TELK        SVNSTLQIK   INSITVDNYK                              VPXISINKXDG
HU     345 TELKQV-AYIYKCVNTTLQIKGKINSITVDNCKKLGLVFDDVVGIVEIINSKDVKVQVMGKVPTISINKTDG
RA     344 TELKQV-AYIYKCVNTTLQIKGKINSITVDNCKKLGLVFDDVVGIVEIINSRDVKVQVMGKVPTISINKTDG

                       *  *  *   *  *  *  *       *      *          *      * *
SC     466 GNIYLSKESLNTEIYTSCSTAINVNLPIGEDDDYVEFPIPEQMKHSFAD-G-KFKSAVFEHAG.
SP     492 GSIYLSKSSLSSEVVTSKSTSLNINVPNEEG-DYAERAVPEQIKHKVNEKGE-LVSEIVRHE.
ASP-56     RHIYLSKNSLDCEIVSAKSSEMNVLIPTEGG-DFNEFPVPEQXKXIWN--GQK
HU     416 CHAYLSKNSLDCEIVSAKSSEMNVLIPTEGG-DFNEFPVPEQFKTLWN--GQKLVTTVTEIAG.
RA     415 CHAYLSKNSLDCEIVSAKSSEMNVLIPTEGG-DFNEFPVPEQFKTLWN--GQKLVTTVTEIAG.
```

Figure 11. Comparison of MCH1 to yeast and mammalian Cap proteins. The amino acid sequence of MCH1 (RA) was aligned with the sequences of the two yeast Cap proteins (*S. cerevisiae* (SC) and *S. pombe* (SP)) and those of the other known mammalian Cap homologs: ASP-56 and human CAP (HU). Amino acid coordinates are given on the left. Identities between amino acid residues are given in bold-face print, while conserved substitutions are denoted by an asterisk above the one letter code. The X marks in the ASP-56 sequence denote unspecified amino acid residues.

3.2 Northern analysis of MCH1 expression.

We examined the expression of *MCH1* mRNA in a variety of rat tissues to determine whether *MCH1* expression is unique to a particular tissue or is expressed ubiquitously (Figure 12). Briefly, two Sprague Dawley rats were sacrificed by CO_2 asphyxiation and tissue samples were removed by dissection. Total RNA extraction was performed using guanidinium thiocyanate followed by cesium chloride centrifugation. Aliquots of 30µg of total RNA were then electrophoresed on formaldehyde gels, transferred and probed with a P^{32}-labelled gene fragment from pADH-MCH1. To control for RNA-loading, glyeraldehyde phosphate dehydrogenase (*GAPDH*) was also used as a probe. Results from the Northern blot are seen in Figure 12. A single transcript was detected in all major tissue types tested. Furthermore, the levels of expression paralleled *GAPDH* expression (not shown). Thus *MCH1* appears to be ubiquitously expressed.

In order to determine if *MCH1* expression was altered in *ras*-transformed cells, we examined *MCH1* expression in a series of wild type and transformed rat fibroblast cell lines. These include Rat-6, a rat embryo fibroblast cell line, PKC-3-A5, a transformed cell line that overexpresses the PKC-β1 isoform and the T-24 *H-ras* oncogene, and R1-a, a spontaneous revertant of the PKC-3-A5 cell line. While PKC-3-A5 cells exhibit all the phenotypes associated with a transformed cell line such as large colony formation on soft agar and tumor formation in nude mice, R1-a cells are normal in phenotype and are resistant to transformation by a host of oncogenes including *v-src*, *v-fos* and *v-raf* (Krauss *et al.*, 1992). MCH1 appears to be expressed at similar levels in the various cell lines examined (Figure12).

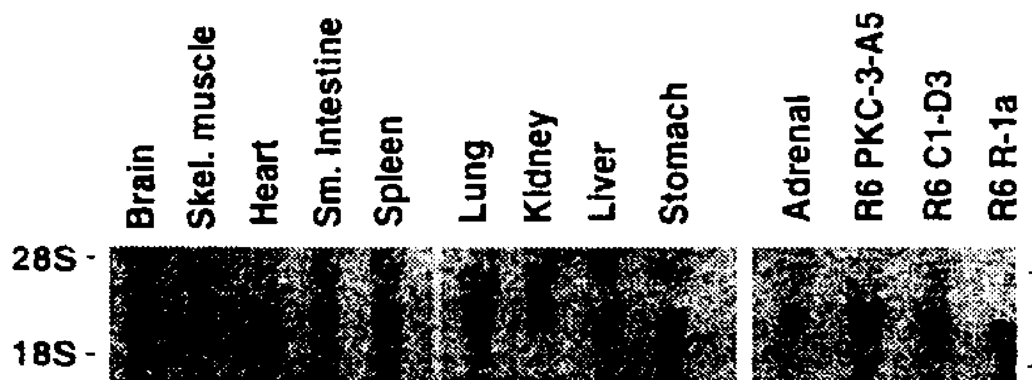


Figure 12. Expression of MCH1 in rats . The 2.1kb *NotI* fragment of *MCH1* was radiolabeled and used as a probe for the expression of *MCH1*. Total RNA isolated from a variety of rat tissues and cell types was electrophoresed, blotted onto nylon membranes, and probed. The tissue and cell types tested included: brain, skeletal muscle, heart, small intestine, spleen, lung, kidney, liver, stomach, adrenal and three rat 6 fibroblast cell lines, C1-D3, PKC-3-A5, and R-1a. Blots were imaged using a Molecular Dynamics Phosphorimager.

In order to better understand the cellular functions of MCH1, we used three different approaches. First, we wanted to determine which proteins interact with MCH1. Second, we wanted to assess the cellular localization of MCH1, and third, we were interested in examining the effect of MCH1 overexpression on cellular proliferation.

3.3. MCH1 Antibody

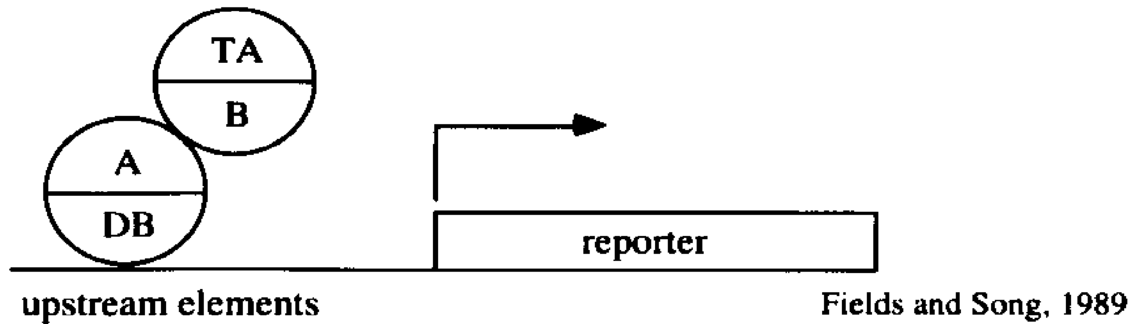
To help address these questions, we first raised rabbit polyclonal antisera against a MalE-MCH1 fusion protein. This fusion protein couples the bacterial maltose binding protein, malE to MCH1. Protein expression was induced from the pMalc2-MCH1 plasmid (New England Biolabs) in bacteria by the addition of IPTG, and the protein was subsequently purified on an amylose resin column. The fusion protein has an electrophoretic mobility of approximately 70 kDa in mass with the estimated size of MCH1 being ~35 kDa. Since this is smaller than the expected size of MCH1, it would appear that the protein was either translated incompletely or is partially degraded in bacteria. After protein purification, the fusion protein was injected into rabbits and successive bleeds were collected. The antisera specifically recognizes a protein of approximately ~60 kDa that corresponds to MCH1 (Figure 6). As seen from this Western blot, no band is detected at 60 kDa in SKN50 cells which lack MCH1. Thus the MCH1 antibody is specific to the MCH1 protein.

3.4 Protein interactions of CAP and MCH1 proteins.

Protein-protein interactions are a necessary component of almost all biological processes. Therefore, the elucidation of a protein's function can be clarified by determining which proteins interact with it. Experimental procedures designed to

investigate protein-protein interactions include, among others, the recently-established two-hybrid assay, as well as co-immunoprecipitation.

The principle behind the two-hybrid system is as follows (see review Luban and Goff, 1995). Transcriptional activators can be separated into two functional domains - the DNA binding domain and the trans-activating domain. When these two regions are brought in contact with one another, either directly or through other proteins, transcriptional activation will occur. A model for this is shown in Figure 13. Briefly, one protein (protein A) is fused to the amino terminus of the DNA binding domain, while the second protein (protein B) is fused to the carboxyl terminus of the trans-activating domain. If proteins A and B physically interact, transcriptional activation will occur, and this is evidenced by the activation of a reporter gene (Fields and Song, 1989). The two-hybrid assay has been used reliably to demonstrate the interaction of other proteins that form tight complexes with one another, such as the retinoblastoma susceptibility gene product (Rb) and protein phosphatase 1α (PP1α) (Durfee *et al.*, 1993). We utilized both the two-hybrid assay, as well as, coimmunoprecipitation experiments to identify proteins that bind to MCH1. In addition, we examined whether these interactions are conserved between MCH1 and CAP proteins.



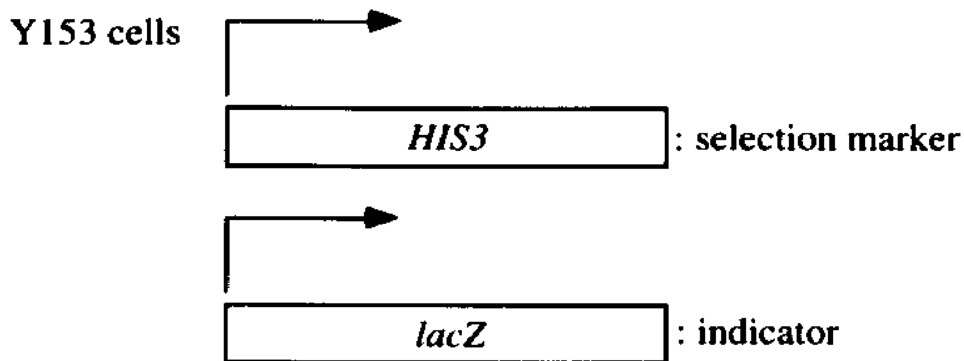


Figure 13. The Two-Hybrid System for detecting protein-protein interactions. Shown here are two fusion proteins, TA-B, and DB-A. TA=transactivating domain and DB=DNA-binding domain. If proteins A and B interact, this results in the transcriptional activation of a reporter element such as *lac Z* or *HIS* 3. The *Lac Z* reporter gene results in the production of B-galactosidase, and the *HIS 3* reporter stimulates the production of histidine thus bypassing the need for the histidine biosynthetic pathway that is inhibited in the presence of 3-aminotriazole.

3.4a. MCH1 and CAP proteins undergo dimerization.

Partial purification of the RAS-dependent adenylyl cyclase complex by gel exclusion chromatography shows that it is a protein complex of ~280 kDa (Wang *et al.*, 1992). Yet, fractionation of the RAS-dependent adenylyl cyclase complex from a *cap* strain reveals a decrease in size of approximately 280 kDa relative to the same complex wild type strains (Wang *et al.*, 1992). Since the predicted molecular mass of the CAP protein is 70 kDa, it appears that *in vivo* CAP may exist in a complex with other proteins. An additional possibility is that CAP, itself, may form homomeric complexes. In order to investigate these possibilities, we first began to use the two-hybrid assay to determine whether CAP proteins are capable of dimerization and if this property is conserved in MCH1 proteins.

The two-hybrid system.

As detailed in the Materials and Methods, we created gene fusions between *CAP* and either the DNA binding domain (DB) or the transactivating domain (TA) of the yeast transcription factor, *GAL4*. Similar gene fusions were made with *MCH1*. These constructs were transformed into yeast, and the expressed fusion proteins were assayed for protein-protein interactions in a yeast strain bearing the *GAL4*-inducible reporter elements *lacZ* and *HIS3*. Productive protein interactions in this strain result in the expression of lacZ reporter activity as well as the ability to survive growth in the presence of 3-aminotriazole (3AT), a metabolic inhibitor of histidine biosynthesis. Survival of this metabolic block correlates with *HIS3* reporter activity, which is required to overcome the toxicity of 3AT (Kishore et al., 1988).

Co-expression of the *GAL4-MCH1* gene fusions resulted in *lacZ* and *HIS3* reporter activities that were comparable to those seen between DB-Rb and TA-PP1α which served as a positive control (Figure 14A and B). We could also demonstrate significant *lacZ* reporter activity, as well as, robust growth in the presence of 3AT, in cells co-expressing TA-MCH1 and DB-CAP (Figure 14A and B). In contrast, co-expression

of the Gal4-CAP fusion proteins gave little to no ß-galactosidase activity with minimal growth in the presence of 3AT (Figure 14A and B). None of the gene fusions yielded ß-galactosidase activity or growth on 3-AT containing medium, when expressed individually in cells (Figure 14A and B). These results imply that MCH1 can exist in complexes either with itself or with CAP. This is supported by the subsequent screening of a Hela cell cDNA library in the two-hybrid system. Proteins identified in this screen that were capable of interacting with MCH1 included the human homolog of CAP (D.David and J.Gerst, unpublished results).

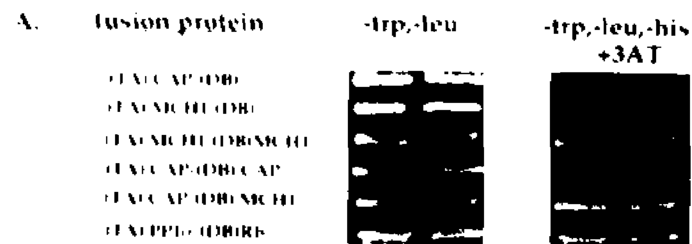
CAP has previously been described as a bifunctional protein (Gerst *et al.*, 1991). The amino terminus of CAP interacts with adenylyl cyclase and effects RAS signaling, while the carboxyl domain is involved in growth control and the maintenance of normal cellular morphology (Gerst *et al.*, 1991). A third, proline-rich region, connects the two domains, and bears significant homology to known SH3 binding sites (Ren *et al.*, 1993 and Figure 15).

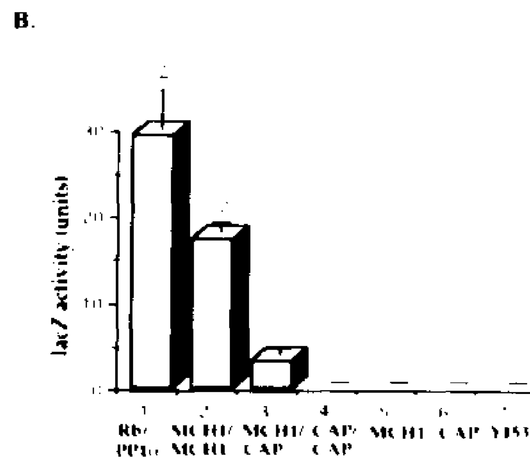
In order to determine which domain of CAP mediates dimerization, we tested the ability of various deletion mutants of CAP to form dimers with MCH1 and with full length CAP. Thus, gene fusions were created between the transactivating or DNA binding domains of *GAL4* and different *CAP* deletion mutants. These included *DB* and *TA* $CAP^{1-169/369-526}$,as well as, *DB*-and *TA*-$CAP^{186-384}$. The first set of gene fusions expresses a deletion mutant of CAP that lacks the polyproline stretch and is equivalent to the previously described CAPΔ7 mutant (Gerst *et al.*, 1991). The second set of gene fusions express the middle, proline-rich domain of CAP (Gerst *et al.*, 1991).

Co-expression of DB-$CAP^{1-169/369-526}$ with TA-MCH1, or with TA-$CAP^{1-169/369-526}$, resulted in histidine auxotrophy and survival in the presence of 3AT. Furthermore, we reproducibly detected *lacZ* reporter activities in cells co-expressing these gene fusions (Figure 14C). In contrast, we were unable to detect growth in the

absence of histidine or lacZ reporter activity in cells co-expressing fusions between Gal4 and the proline-rich domain of CAP. Similarly, we were unable to detect an interaction between this domain and either full length CAP or MCH1. None of these fusion proteins was able to confer histidine auxotrophy or enzymatic activity when expressed individually.

These results imply that MCH1 is able to form a tight complex with itself and can form heteromeric complexes with yeast CAP. Furthermore, both the amino- and carboxyl-terminal domains of CAP appear to mediate this interaction, while the proline-rich region does not appear necessary for dimerization.





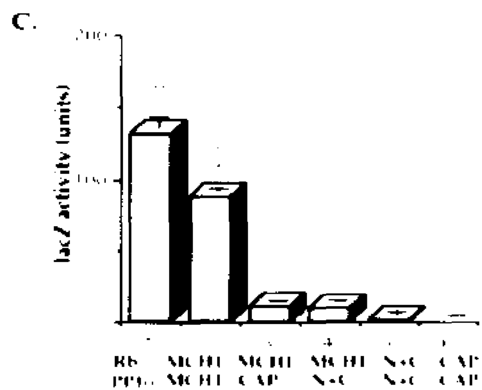


Figure 14. MCH1 and CAP form homodimers and heterodimers with each other as shown by two-hybrid analysis.

Figure 14. MCH1 and CAP form homodimers and heterodimers with each other as shown by two-hybrid analysis. (A) Two-hybrid selection for auxotrophic growth on medium lacking histidine. Plasmids expressing full length *MCH1* and *CAP* fused with either the transactivating domain (TA) or the DNA binding domain (DB) of Gal4 were transformed into Y153 cells. Expressed plasmids included: (TA)CAP, (TA)MCH1, (DB)CAP, (DB)MCH1. A plasmid expressing the Gal4 transactivating domain alone is represented as "TA". Similarly, a plasmid expressing the Gal4 DNA binding domain alone is defined as " DB ". Plasmids were maintained by growth on synthetic double selective medium (-trp,-leu). To select for protein-protein interactions that lead to auxotrophic growth in the absence of histidine, and in the presence of a metabolic block for histidine synthesis, patches were replica plated onto triple selective medium containing 25mM 3AT (-leu,-trp,-his +3AT), and allowed to grow for three days. Yeast bearing plasmids which express Gal4-Rb and Gal4-PP1α ((DB)Rb and (TA)PP1α, respectively) were used as a positive control in this experiment. (**B**) Two-hybrid selection for lacZ reporter activity. Cell extracts were made from strains expressing Gal4(TA) and Gal4(DB) fusion proteins described in section A and were assayed for β-galactosidase activity (see Materials and Methods). Units of β-galactosidase activity are expressed in nmol ONPG cleaved/mg protein/hour. The average of two separate experiments are given. Error bars indicate the standard error of the mean. (**C**) Two-hybrid selection analysis: Regions of cyclase-associated proteins required for interaction. Yeast cell extracts were made from strains expressing different CAP and MCH1 fusions with the TA or DB domains of Gal4, and included the CAP deletion mutant CAPΔ7 ($CAP^{2-269/369-526}$) (N+C) fusion protein. Cell lysates were assayed for enzymatic activity as detailed in Materials and Methods. Units are expressed as described above. The average of two separate experiments performed in duplicate are given. Error bars indicate the standard error of the mean.

A Comparison of SH3 Binding Domains

αrENaC	P	P	L	A	L	T	A	P	P	P
3BP1-10	A	P	T	M	P	P	P	L	P	P
Sos			V	P	P	P	V	P	P	R
m4 mAChR	P	P	A	L	P	P	P	P	R	P
CAP	P	P	P	P	P	A	P	P	A	S
MCH1	P	P	P	P	P	G	P	P	P	P
Estimated Consensus Sequence	P	P	Ψ	Ψ	P	X	Ψ/P	Ψ/P	P	Ψ/P

Figure 15. A comparison of the proline-rich domains of CAP and MCH1 to known SH3 binding domains of Sos, 3BP-10, m4 mAChR, and arENaC (the epithelial sodium channel) (Cohen *et al.*, 1995; Rotin *et al*, 1994).

Immunoprecipitation experiments.

The two-hybrid system has been instrumental in defining many novel protein-protein interactions; however, this system cannot detect all protein interactions in the cell. Some proteins fold improperly or cannot be imported into the nucleus. Certain fusion proteins are even toxic to the cell. Thus, this assay can result in false negatives. In addition, other proteins will interact as fusion proteins in the nucleus even though they would never bind in their native state. Therefore, results obtained through the two-hybrid system must be validated through other means. For this reason, we also employed standard immunoprecipitation (IP) procedures to demonstrate physical interactions of CAP and MCH1 proteins.

First, to confirm that MCH1 forms homologous interactions, we co-expressed HA-tagged and myc-tagged forms of MCH1 in yeast, and performed IP's on cell lysates using the anti-hemagglutinin antigen (HA) antibody. Detection with anti-myc antibody reveals that myc-MCH1 is present in these immunoprecipitates (Figure 16A). Thus as seen with the two-hybrid system, MCH1 forms homologous associations in yeast. We next examined whether MCH1 and CAP can form heterologous associations as shown in the two-hybrid system. As shown in Figure 16B, native CAP can also be detected along with HA-MCH1 in immune complexes precipitated by the anti-HA antibody. Furthermore, the detection of native CAP is eliminated when IPs are performed in the presence of excess HA peptide. Thus we could confirm that MCH1 associates with CAP.

Although we originally determined that CAP interacts poorly with itself in the two hybrid system, we tested whether we could detect a physical association between the proteins for two reasons. Because MCH1 can form a tight complex with itself, as well as with CAP, it seems likely that the property of dimerization is well-preserved. Second, as discussed, false negatives have been known to occur in the two-hybrid assay for a number of reasons including improper protein folding or degradation.

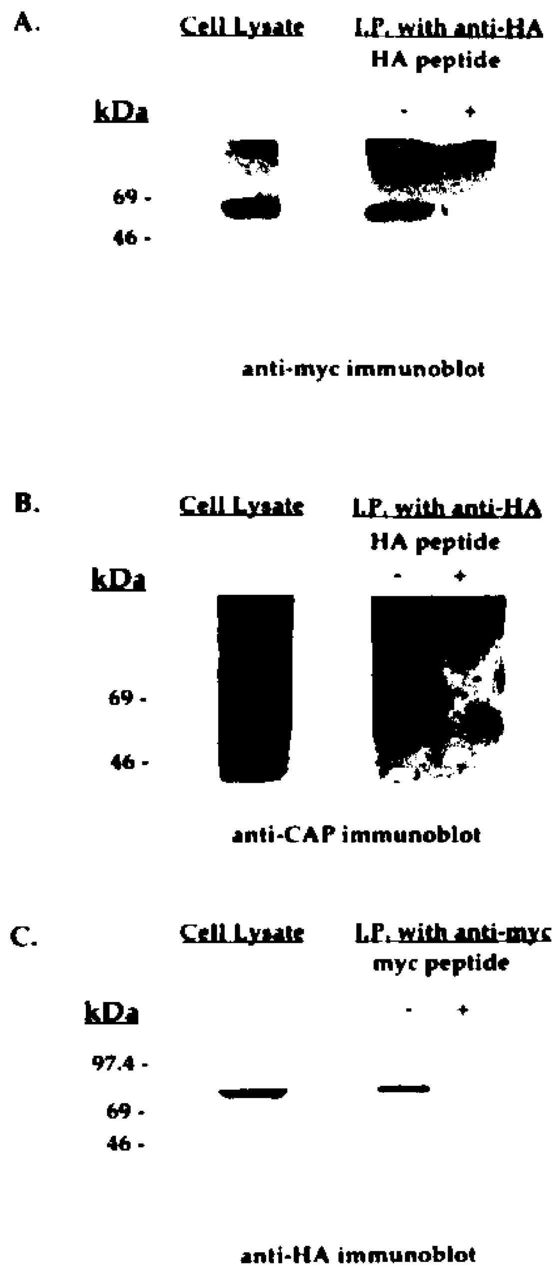


Figure 16. MCH1 and CAP form homodimers and heterodimers with each other, as shown by co-immunoprecipitation.

Figure 16. MCH1 and CAP form homodimers and heterodimers with each other, as shown by co-immunoprecipitation. (**A**) MCH1 forms homodimers. Total cell lysates from wild type yeast co-expressing HA-tagged and myc-tagged MCH1 were subject to immunoprecipitation with anti-HA antibody. Immune complex formation was blocked by the addition excess HA peptide (75μg) . The resulting immune complexes were resolved on SDS-acrylamide gels, transferred to nylon membranes, and were probed with anti-myc antibody (1:2500). (**B**) MCH1 and CAP form heterodimers. Cell extracts from wild type yeast expressing HA-tagged MCH1 were subjected to immunoprecipitation with anti-HA antibody. Immune complex formation was blocked by the addition of excess HA peptide (75μg). Immune complexes were resolved on SDS-acrylamide gels and transferred to nylon membranes. The immunoblot was probed with anti-CAP antibody at a dilution of 1:1000. (**C**) CAP forms homodimers. Total cell lysates from strains co-expressing HA-tagged CAP and myc-tagged CAP were subjected to immunoprecipitation with anti-myc antibody. Immune complex formation was blocked by the addition of excess myc peptide (50μg). Immunoblots were incubated with anti-HA antibody at a 1:5000 dilution. In all cases, detection of antigen was performed using the ECL chemiluminescent assay.

Therefore, we expressed both HA-tagged and myc-tagged CAP in wild-type yeast and performed IP's on cell lysates using the anti-HA antibody. In contrast to the results from the two-hybrid system, we could detect the presence of myc-CAP in these immune complexes and could block its detection by the addition of excess HA peptide to the IP reaction (Figure 16C). Therefore, CAP, like MCH1 forms homologous associations.

In order to determine which domains of CAP mediate protein dimerization, we used cells that co-express HA-CAP and various myc-tagged domains of CAP. Thus we examined the interaction of full length CAP with the amino terminus of CAP, the carboxyl-terminal region, the proline-rich domain, and a deletion mutant that lacks this domain. These deletion mutants include CAPΔ15 (CAP^{1-192}); CAPΔ4 ($CAP^{291-526}$); $CAP^{186-384}$; and CAPΔ7 ($CAP^{1-169/369-526}$). Both protein expression and function of the individual domains were verified using *cap* cells. We found that HA-tagged CAP co-precipitates with myc-tagged CAPΔ7, as well as, with myc-tagged CAPΔ4 (figure 17A). In contrast, HA-CAP does not co-precipitate with the amino terminus of CAP or the middle proline-rich domain (Figure 17A). Thus it appears that dimerization is mediated primarily through the carboxyl terminus.

We also examined the interaction of HA-tagged $CAP^{1-169/369-526}$ with myc-tagged $CAP^{1-169/369-526}$, and other myc-tagged CAP domains. We observed that deletion mutants lacking the proline-rich domain interact tightly with each other and with the carboxyl terminus of CAP (CAPΔ4) (Figure 17B). Moreover, an interaction was also noted between the $CAP^{1-169/369-526}$ mutant and the amino terminus of CAP. Thus, the $CAP^{1-169/369-526}$ mutant interacts more tightly with the two functional domains of CAP than does native CAP. This suggests that the middle proline-rich domain could act to inhibit the ability of CAP to form multimeric complexes.

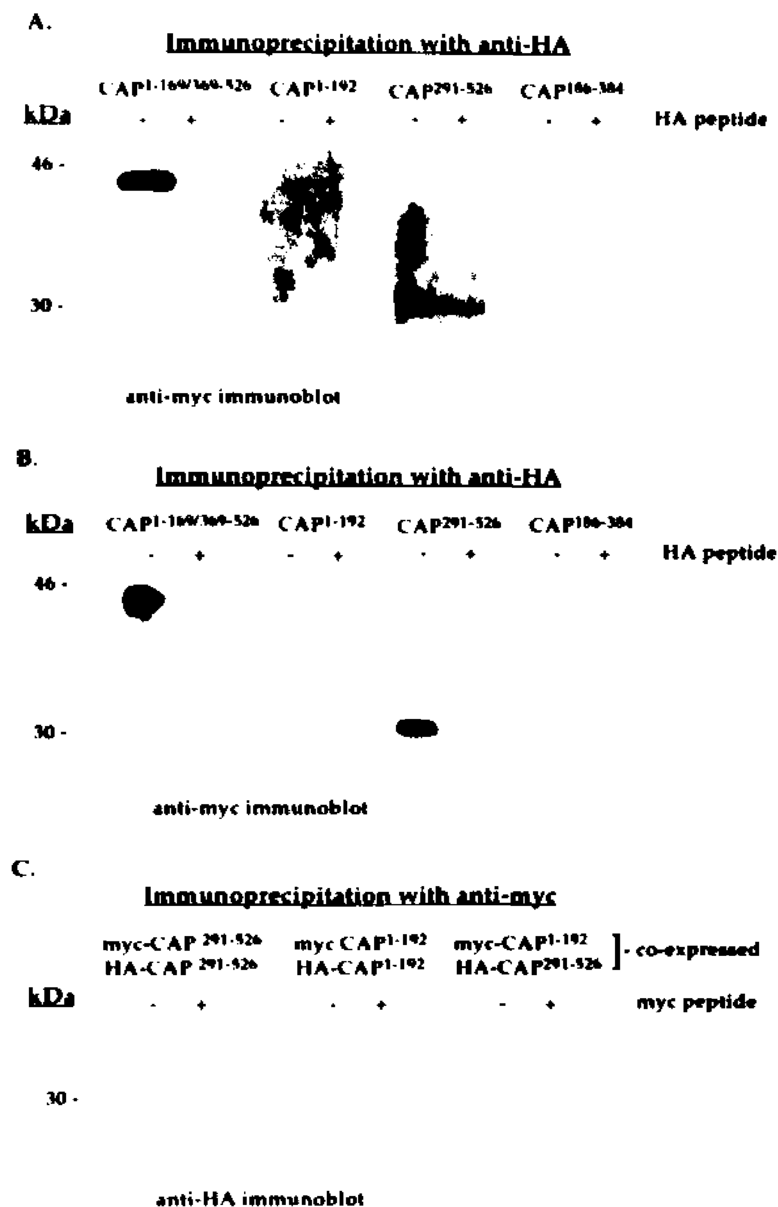


Figure 17. Domains of CAP involved in dimer formation, as shown by co-immunoprecipitation.

Figure 17. Domains of CAP involved in dimer formation, as shown by co-immunoprecipitation. (**A**) Full length CAP interacts with the carboxy-terminal domain of CAP. Whole cell lysates from wild type yeast co-expressing HA-tagged CAP and different myc-tagged domains of CAP (i.e. CAP^{1-192}, $CAP^{291-526}$, $CAP^{1-169/369-526}$ and $CAP^{186-384}$) were subjected to immunoprecipitation with anti-HA antibody. Immune complex formation was blocked by the addition of excess HA peptide (50µg). Following immunoprecipitation, the immune complexes were resolved on SDS-acrylamide gels and were transferred to nylon membranes. The resulting immunoblot was probed with anti-myc antibody (1:2500) . (**B**) $CAP^{1-169/369-526}$ interacts with itself as well as the amino-terminal and carboxy-terminal domains of CAP. Total cell lysates from yeast co-expressing HA-tagged $CAP^{1-169/369-526}$ and various myc-tagged domains of CAP were subjected to immunoprecipitation with anti-HA antibody. Immune complex formation was inhibited by the addition of excess HA peptide (75µg). Immunoblots were incubated with anti-myc antibody (1:2500). (**C**) The carboxy-terminal domain of CAP alone interacts with itself and the amino-terminus. Total cell extracts from yeast co-expressing either the HA-tagged carboxy-terminal domain of CAP or the HA-tagged amino-terminal domain of CAP, and different myc-tagged domains of CAP, were subjected to immunoprecipitation with anti-myc antibody. Specific immune complex formation was blocked by the addition of excess myc peptide (50µg). Immunoblots were probed with anti-HA antibody (1:5000). In all cases, antigen detection was perfomed using ECL chemiluminescent assay.

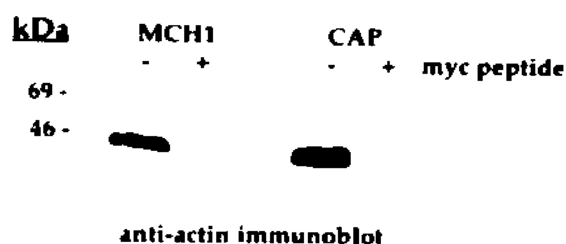
Because we observed a direct interaction between the carboxyl terminus of CAP and either full length CAP or $CAP^{1-169/369-526}$, we examined whether this domain alone could mediate dimerization. As shown in Figure 17C, the carboxyl domain could precipitate with itself, or with the amino terminus. Together these results imply that dimerization is mediated principally by the carboxyl terminus. Similar results were obtained with the carboxyl domain of MCH1, and thus, this property appears to have been conserved through evolution.

3.4b. MCH1 and yeast CAP are actin-binding proteins.

Three lines of evidence suggested that CAP may be involved in cytoskeletal organization. First, profilin, an actin-binding protein, was isolated as a suppressor of the deletion of the carboxyl terminus of CAP (Vojtek *et al.*, 1991). Removal of the carboxyl terminus results in the mislocalization of actin and leads to enlarged cells with gross cytoskeletal abnormalities (Gerst *et al.*, 1991). Overexpression of profilin in *cap* cells corrects the cytoskeletal abnormalities and leads to near-normal cellular morphology (Vojtek *et al.*, 1991). Second, a porcine CAP homolog, ASP-56, was isolated directly as an actin-sequestering protein (Gieselmann and Mann, 1992). Finally, immunofluorescent localization of MCH1 in Rat-6 cells revealed that MCH1 is concentrated at the lamellipodium, where extensive cytoskeletal remodeling is known to occur. Therefore, we directly tested whether CAP or MCH1 are actin-binding proteins.

We performed IP experiments to assay for the presence of actin in precipitated complexes formed with either epitope-tagged MCH1 or CAP. As shown in Figure 18, a protein of ~42 kDa can be detected in protein complexes formed in the presence of HA-tagged CAP or HA-tagged MCH1, using an anti-actin antibody. Furthermore, this interaction occurs with the CAP deletion mutant, $CAP^{1-169/369-526}$ (CAPΔ7), as well as, with the carboxyl terminus of CAP (CAPΔ4) (Figure 18). In contrast, neither the amino terminus nor the middle proline-rich domain of CAP, could display this property, although expression of these tagged domains was verified by immunoblot

A. Immunoprecipitation with anti-myc



B. Immunoprecipitation with anti-myc

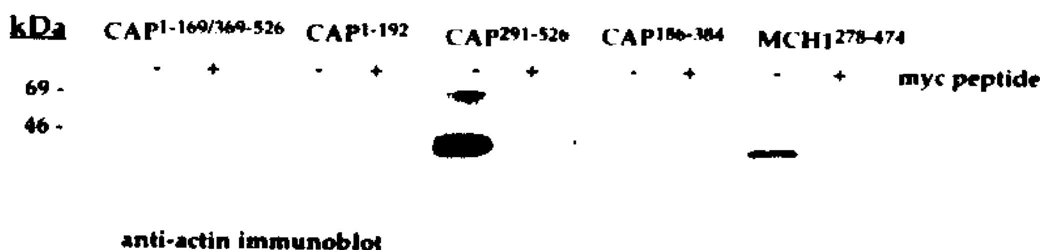


Figure 18. MCH1 and CAP are actin-binding proteins. (**A**) Full length MCH1 and CAP bind actin. Yeast cell lysates from cells expressing either myc-tagged CAP or myc-tagged MCH1 were subjected to immunoprecipitation with anti-myc antibody. Specific protein interactions were blocked by the addition of excess myc peptide (50mg). Immune complexes were resolved on SDS-acylamide gels and transferred to nylon membranes. Immunoblots were probed with anti-actin antibody (1:500) (**B**) The carboxy-terminal domain of CAP, and MCH1, is involved in actin binding. Yeast cell lysates from cells expressing various myc-tagged domains of CAP, or the carboxy-terminal region of MCH1 were subjected to immunoprecipitation as described above. Immunoblots were incubated with anti-actin antibody (1:500). Antigen detection was perfomed by ECL chemiluminescent assay.

analysis (Figure 18). Thus we conclude that both CAP and MCH1 are actin-binding proteins and the domain required for this interaction localizes to the carboxyl-terminal regions of both CAP and MCH1. Recently, another group has independently shown that the carboxyl terminus of CAP binds actin monomers, and that CAP acts as an actin-monomer sequestration protein with the stoichiometry of actin-binding roughly 1:1 (Freeman *et al.*, 1995).

3.4c Protein dimerization and actin-binding can be separated.

Thus far we have defined two separate functions mediated by the carboxyl terminus of both CAP and MCH1. These include actin-binding and dimerization. In order to determine whether these functions localize to the same or different regions of the carboxyl terminus, we examined whether a specific CAP deletion mutant, CAPΔ11, could bind to actin or dimerize. This mutant lacks the last 27 amino acids of the CAP protein and is unable to confer phenotypic suppression of the loss of the carboxyl terminus of CAP (Gerst *et al.*, 1991). Thus CAPΔ11 cells are sensitive to nutrient extremes and display an abnormal cytoskeleton. As one might expect, co-precipitation experiments with myc-tagged CAPΔ11 revealed that this mutant is unable to bind actin (Figure 19A).

In order to determine if this mutant is capable of forming homologous protein interactions, myc-tagged CAPΔ11 and HA-tagged CAP were co-expressed in the *cap* yeast strain, SKN34. Immunoprecipitations were performed on total cell lysates using the anti-myc antibody. As seen in Figure 19B, the HA-tagged CAP protein is present in these immunoprecipitates. Therefore, CAP does not need to bind actin in order to form dimers. Furthermore, these functions appear to be mediated by distinct regions on the carboxyl domain of CAP.

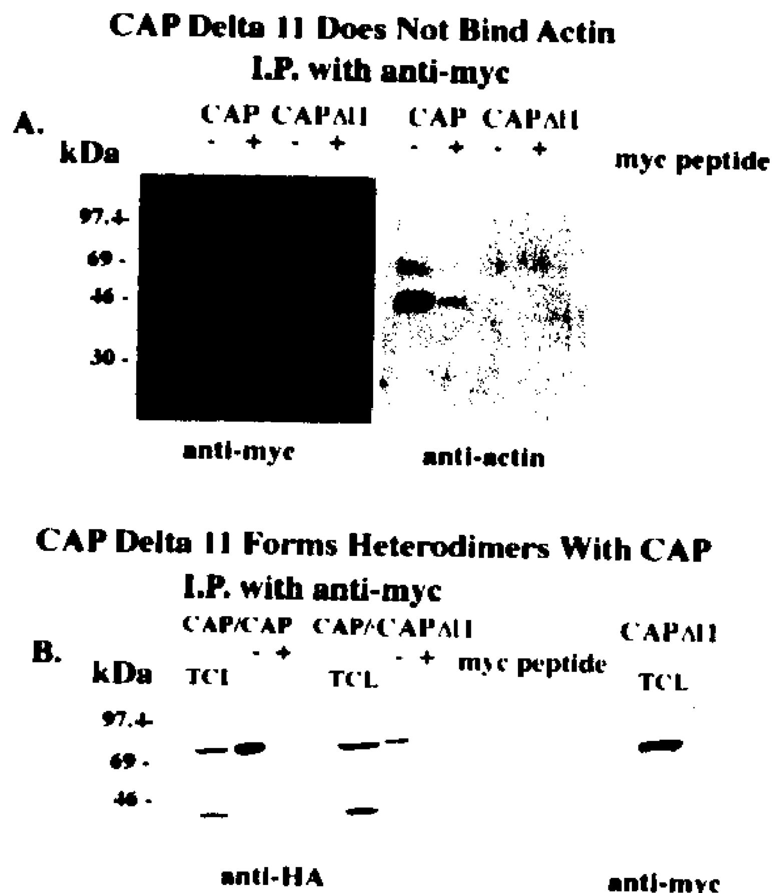


Figure 19. Actin binding and dimerization are mediated by separate regions on the carboxyl-domain of CAP. (A) CAPΔ11 cannot bind actin. Total cell lysates from SKN34 yeast expressing either myc-tagged CAP or myc-tagged CAPΔ11 were subject to immunoprecipitation with anti-myc antibody. Immune complex formation was blocked by the addition of excess myc peptide (75ug). The resulting immune complexes were resolved on SDS-acrylamide gels, transferred to nylon membranes, and were probed with anti-actin antibody (1: 500) or anti-myc antibody (1:5000) (B). CAPΔ11 can form heterodimers with HA-tagged CAP. Total cell lysates from SKN34 yeast expressing either myc-tagged CAP and HA-tagged CAP or myc-tagged CAPΔ11 and HA-tagged CAP were subject to immunoprecipitation with the anti-myc antibody. Immune complex formation was blocked with the addition of excess myc peptide (75ug). Immune complexes were resolved on SDS acrylamide gels and transferred to nylon membranes. The immunoblot was probed with anti-HA antibody at a dilution of 1:5000. In all cases, detection of antigen was performed using the ECL chemiluminescent assay.

3.4 d. CAP does not physically interact with profilin.

Based on previous findings which demonstrated that profilin suppresses the loss of the carboxyl terminus of *CAP* (Vojtek *et al.*, 1991) and the knowledge that profilin can be purified on a poly-proline affinity column (Sohn and Goldschmidt-Clermont, 1994), we were interested in determining whether profilin interacts physically with CAP. In order to address this question, we first used the two-hybrid system to determine if CAP and profilin interact. However, we could detect neither *lacZ* reporter activity nor cell growth in the presence of 3AT. We then co-expressed HA-tagged profilin and myc-tagged CAP in yeast and attempted to detect CAP in profilin immunoprecipitates that were precipitated using anti-HA antisera; expression of HA-tagged profilin was verified by Western blotting (data not shown). We were unable to visualize myc-CAP in these samples. Therefore, we conclude from these experiments that CAP and profilin do not physically interact.

3.5 Cellular localization of CAP proteins.

In order to further characterize MCH1 and CAP proteins, we examined the localization of the CAP in yeast and MCH1 in Rat-6 fibroblasts.

3.5a Localization of CAP proteins and deletion mutants in yeast.

As described, CAP proteins can be divided into three domains - an amino terminal-region, a carboxyl terminus, and a proline-rich stretch located between the two domains. This proline-rich region bears significant homology to known SH3 binding domains. Because SH3 domains appear to play a role in protein localization, we wanted to determine if the polyproline region of CAP was required for proper cellular localization.

We examined the requirements for the cellular localization of CAP in yeast using thin-section microscopy and immunogold labeling to detect the presence of CAP deletion mutants. Native CAP protein was found to localize primarily to the cytosol in wild-type cells (data not shown). Similarly *cap* cells expressing the carboxyl terminus of CAP (CAPΔ4; $CAP^{291-526}$) are also labeled in the cytosol (Figure 20, panel 2). In contrast, the $CAP^{1-169/369-526}$ mutant was not distributed throughout the cytosol, but localized to non-nuclear electron-dense aggregates (Figure 20, panel 3) that were often surrounded by small (~50nm) vesicle-like structures (Figure 20, panel 4). These structures are unique to cells expressing the $CAP^{1-169/369-526}$ deletion mutant and were not seen with any of the other CAP mutants. Thus it appears that removal of the proline-rich domain of CAP results in the mislocalization of the protein and, perhaps, aggregation.

3.5b. Localization of MCH1 in Rat-6 fibroblasts.

In both *S.pombe* and *S.cerevisiae*, disruption of the carboxyl terminus of CAP is associated with dramatic changes in the actin cytoskeleton. Cells are rounder and greatly enlarged with random budding patterns and the loss of actin cables. Thus, it seems that the carboxyl-terminal domain of CAP plays a significant role in remodeling

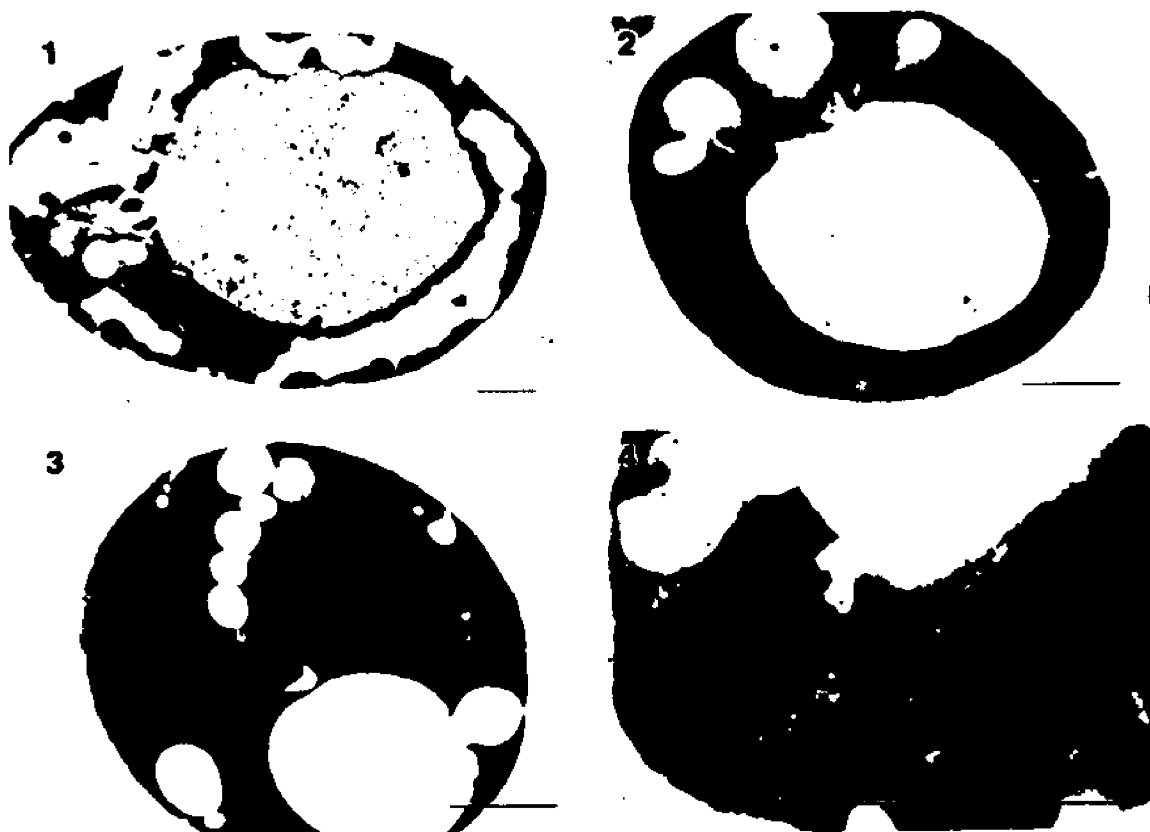


Figure 20. Cellular localization of CAP in yeast by immunogold labeling. *cap* yeast strains expressing the carboxy-terminal domain of CAP ($CAP^{291-526}$), CAP lacking the proline-rich region ($CAP^{1-169/369-526}$), or cells bearing a control plasmid were processed for immunogold labelling and electron microscopy (see Materials and Methods). Panel (1) *cap* cells; panel (2) *cap* cells expressing the carboxy-terminal domain of CAP; panels (3) and (4) *cap* cells expressing the $CAP^{1-169/369-526}$ deletion mutant. Thin-sectioned yeast were treated with an anti-CAP antiserum (1:500 dilution) and immunogold labeled, as described in Materials and Methods. The size bar in all panels corresponds to 1.0mm.

of the cytoskeleton. As discussed, MCH1 and CAP are homologous proteins with the greatest similarity in the middle proline-rich stretch and carboxyl domains. Furthermore, we have demonstrated that the carboxyl domains of CAP and MCH1 are functionally conserved.

In accordance with these observations, we were interested in examining the cellular localization of MCH1 and its association with aspects of the actin cytoskeleton through indirect immunofluorescence. Rat-6 cells were seeded onto sterile coverslips for 12 hours. The following day, the cells were rinsed with PBS (phosphate-buffered saline) and fixed with formaldehyde. The cells were then incubated with anti-MCH1 antiserum followed by incubation with FITC-conjugated goat anti-rabbit antibody in the presence or absence of rhodamine-conjugated phalloidin. Through the use fluorescence microscopy, we determined that MCH1 localizes primarily to the cytosol, with particularly intense staining in the cellular region that corresponds to the lamellipodium and membrane ruffles (see arrows in Figure 21, panels a and c).

Both lamellipodia and membrane ruffles are dynamic, transitory structures which contain a high percentage of actin-binding proteins as well as actin (Bray, 1992). Many of the actin-binding proteins identified in this region appear to enhance actin polymerization and often co-localize to actin stress fibers. Thus, we were interested in determining whether MCH1 co-localized with actin stress fibers. As seen in Figure 21, panels a,b and c, d, MCH1 does not appear at the same site as actin stress fibers in the cell, but does appear to localize with actin at the cell edges. Therefore, MCH1 does not associate with pre-formed actin filaments, but does co-localize where filament formation and restructuring of the cytoskeleton is thought to occur.

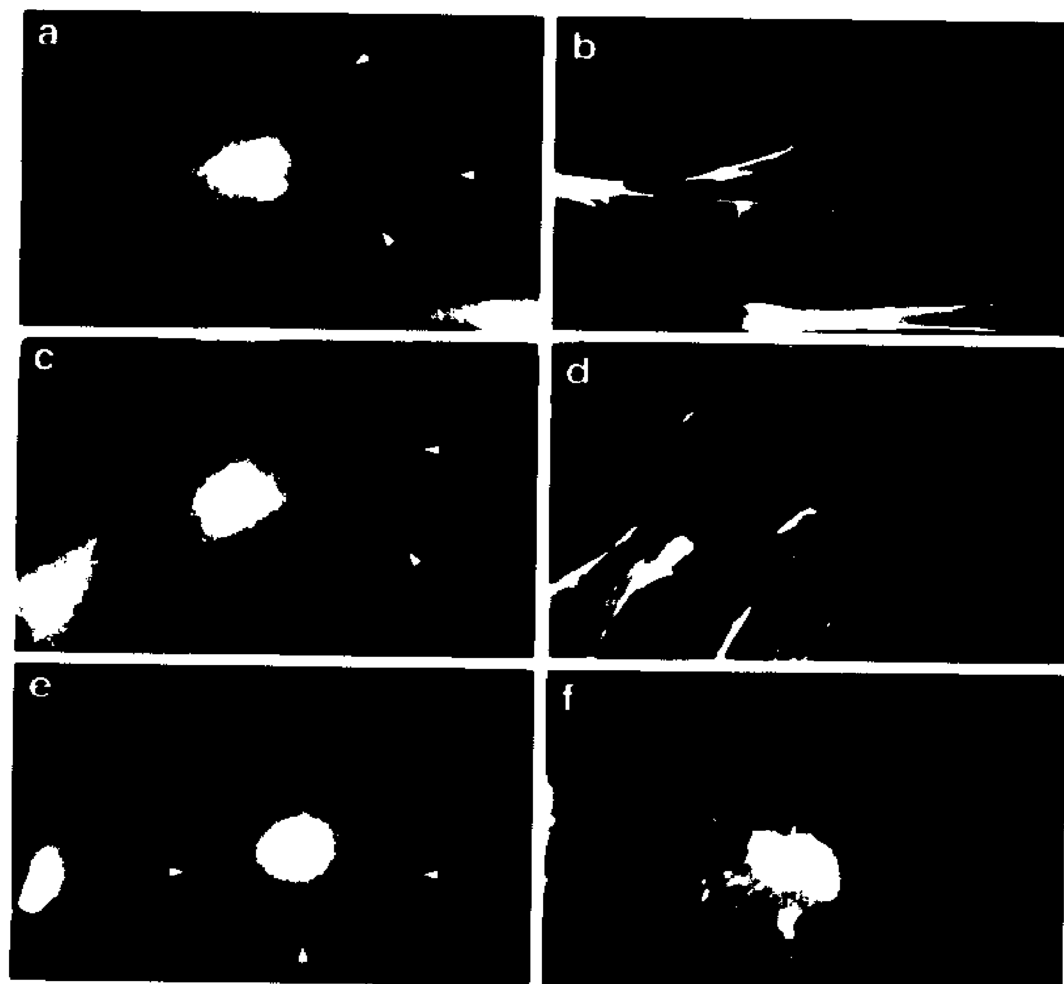


Figure 21. Immunolocalization of MCH1 in Rat-6 fibroblasts.

Figure 21. Immunolocalization MCH1 in Rat-6 fibroblasts. (A and C) MCH1 co-localizes to the cytosol and leading edge of fibroblasts. Rat-6 fibroblasts were first incubated with anti-MCH1 antibody (1:1000) and, after washing, with FITC-conjugated goat anti-rabbit antibody (1:500). See Materials and Methods for details. (B and D) MCH1 does not localize to actin stress fibers. Rat-6 cells were first incubated with anti-MCH1 antibody (1:1000), washed, and then incubated with FITC-conjugated goat anti-rabbit (1:500) antibody and rhodamine-phalloidin. FITC fluorescence is shown in panels A and C, while rhodamine fluorescence is shown in panels B and D. (E) MCH1 localizes to membrane ruffles in fibroblasts that have been recently seeded onto fibronectin-coated cover slips. Rat-6 cells were trypsinized and then seeded onto fibronectin-coated cover slips for 45 minutes. The cells were then incubated with anti-MCH1 antibody (1:1000) and, after washing, with FITC-conjugated goat anti-rabbit antibody (1:500). (F) MCH1 does not co-localize with vinculin nor does MCH1 appear at focal adhesions. Rat-6 cells seeded onto fibronectin-coated cover slips for 45 minutes, were incubated with anti-vinculin antibody (1:200). The cells were then incubated with Texas red-conjugated goat anti-mouse antibody (1:50). After immunolabeling, cells were viewed using a Zeiss fluorescence microscope.

In order to determine if MCH1 localizes to focal adhesions, Rat-6 fibroblasts were seeded onto fibronectin-coated cover slips for 45 minutes. We then examined whether MCH1 co-localized with vinculin - a prominent focal adhesion protein. Under these conditions, MCH1 labeling clearly stains the entire rim of the fibroblast plasma membrane (Figure 21, panel e). In contrast, vinculin staining is restricted to well-defined contact points throughout the cell. Thus, MCH1 does not appear to exist as part of the focal adhesion complex and does not co-localize with vinculin.

3.6 MCH1 function in mammalian cells.

While the role of CAP in the RAS signaling pathway has been extensively studied in *S.cerevisiae*, virtually nothing is known about the function of MCH1 in mammalian cells or its role in Ras signal transduction.

Therefore, we sought to overexpress MCH1 in Rat-6 fibroblasts in order to assay its biological activity. *MCH1* was cloned into the retroviral expression vector, pMV12. This vector contains the hygromycin-resistance gene, *hph*^{r}, as well as, the *HSV thymidine kinase* promoter. The resulting plasmid, pMV12-MCH1 was stably transfected into Rat-6 fibroblasts by using standard calcium phosphate techniques. At the same, separate Rat-6 cultures were transfected with pMV12 alone as a negative control. After 12 days of growth in selective, hygromycin-containing medium, the resulting colonies were stained with Giemsa and then counted. The results of this can be seen in Figure 22. In three separate experiments, we can see that MCH1 inhibits the formation of colonies by an average of 74%. The striking effect of MCH1 on colony formation is also demonstrated in Figure 22 B. Thus, overexpression of MCH1 clearly has either a growth inhibitory role or cytotoxic effect in Rat-6 cells.

3.6 b. MCH1 is not phosphorylated in Rat-6 fibroblasts.

Many signaling proteins are regulated by phosphorylation. Thus, we wanted to determine whether MCH1 exists as a phosphoprotein in Rat-6 cells. In order examine

the phosphorylation state of MCH1 in Rat-6 fibroblasts, we incubated semi-confluent Rat-6 cell cultures with [P^{32}]-orthophosphate for 4 hours. We then rinsed the cells and performed IP's using anti-MCH1 antibody in the presence or absence of unlabeled MCH1-peptide. As seen in Figure 23, MCH1 is not phosphorylated under the cellular conditions we examined. This does not exclude the possibility that MCH1 is phosphorylated under other growth conditions; however, in growing Rat-6 fibroblasts, MCH1 does not appear to exist as a phosphoprotein.

A. TABLE III. Inhibition of Colony Formation by MCH1

Transfected Plasmid	Number of colonies/dish for Experiment number		
	1	2	3
pMV12	99±14	143±7.5	145±17
pMV12MCH1	20±2.5	60±14.5	21±3.5

B. Giemsa-stained, transfected Rat-6 fibroblast colonies.

pMV12

pMV12 MCH1

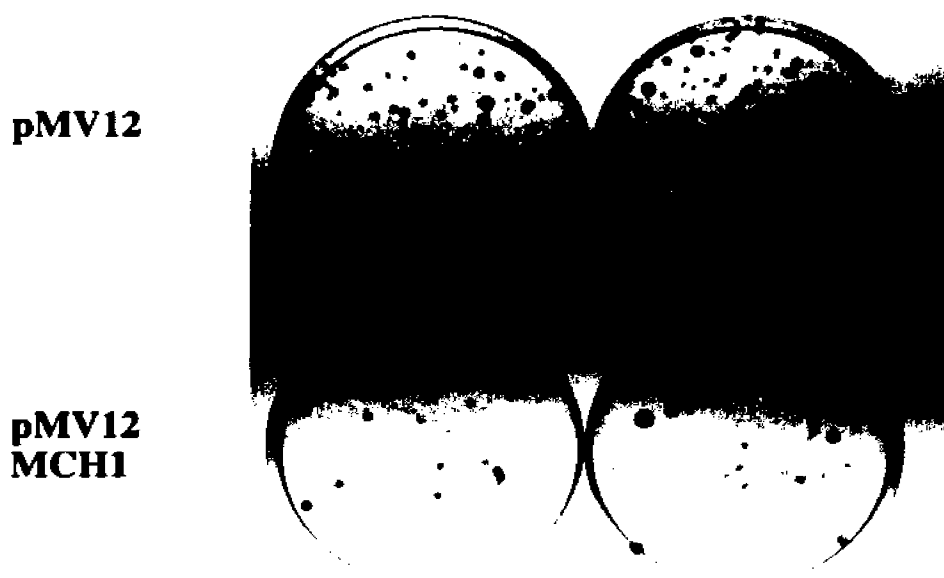


Figure 22. MCH1 overexpression in Rat-6 fibroblasts inhibits colony formation.

Figure 22. MCH1 overexpression in Rat-6 fibroblasts inhibits colony formation. (A) Rat-6 cells were transfected with 30μg of either pMV12 plasmid DNA or pMV12MCH1 plasmid DNA. pMV12 is a retroviral expression vector which expresses the hygromycin resistance gene under the control of the HSV thymidine kinase promoter. Colonies were selected for growth in hygromycin-containing media (Materials and Methods), and were then stained with Giemsa. After Giemsa staining, the visualized colonies were counted. Data are presented as the average number of colonies per dish ± standard error of the mean from 4 recipient culture dishes. (B) Rat-6 transfected colonies were grown for 12 days in hygromycin-containing media and the surviving colonies were stained with Giemsa. Shown here are the Giemsa-stained colonies from cells transfected with either pMV12 (control) or pMV12MCH1 plasmid.

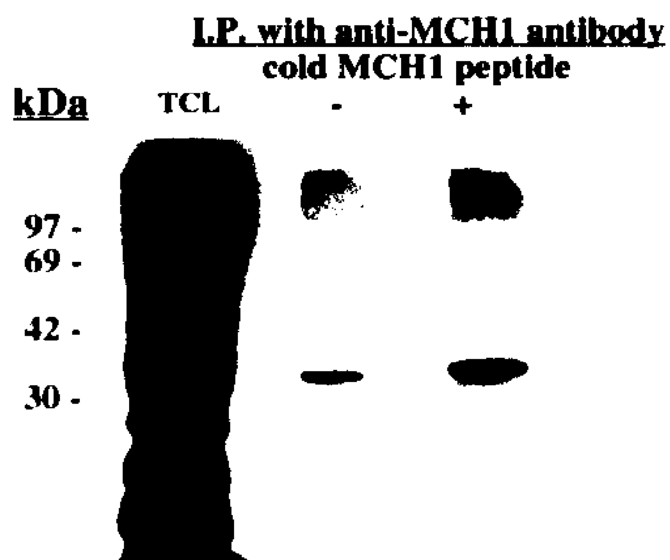


Figure 23. MCH1 is not phosphorylated in growing Rat-6 fibroblasts.

Figure 23. MCH1 is not phosphorylated in growing Rat-6 fibroblasts. Rat-6 cells were grown in phosphate-depleted medium for 2 hours. Afterwards, the cells were washed and radiolabelled with 1mCi of [^{32}P]-orthophosphate in medium lacking cold phosphate for 4 hours. The cells were then rinsed, and cell extracts were prepared as described in Materials and Methods. 250μg of total cell lysate was inubated with anti-MCH1 antibody in the presence or absence of 15μg of excess unlabelled MalE-MCH1 fusion protein. Following immunoprecipitation, 30μg of total cell lysate, as well as, the immunoprecipitants were run on a 10% SDS-acrylamide gel. The proteins were then transferred to a nylon membrane which was immunoblotted with anti-MCH1 antiserum (1:2500). Following ECL-detection to identify the presence of the MCH1 protein in the lysates, the membrane was exposed to film for 24 hours and then developed. As shown above, MCH1 does not appear to be phosphorylated in Rat-6 cells under the growth conditions examined.

CHAPTER 4

Discussion

The yeast cyclase-associated protein, CAP, is thought to affect separate and distinct functions for cell proliferation, cell growth, and morphology. CAP has been shown to interact with adenylyl cyclase and is involved in its regulation (Field *et al.*, 1990; Wang *et al.*, 1993). In addition, CAP mediates cellular responses to environmental extremes and may coordinate cellular morphology with cell growth (Field *et al.*, 1990). This second function, which localizes to the carboxyl terminus is less well understood; however, this domain appears to be the best conserved among *CAP* homologs.

4.1 Identification of the mammalian *CAP* homolog, *MCH1*.

We sought to determine whether Cap proteins and their carboxyl-terminal functions are conserved in mammals. Through screening a rat cDNA library in yeast cells lacking *CAP*, we isolated a clone that fully suppresses the loss of the carboxyl terminus of CAP. DNA sequencing indicated that this clone encodes a rat homolog of yeast Cap proteins which we named *MCH1* (Mammalian Cap Homolog 1). The rat *MCH1* gene encodes a 474 amino-acid protein that is expressed in all tissue types examined as seen by Northern hybridization. The protein sequence is 36% identical to *S.cerevisiae* CAP, 35% identical to *S.pombe* cap and 93% identical to the partial peptide sequence of the porcine actin-sequestering protein over shared residues.

Since the identification of *MCH1*, additional mammalian homologs have been cloned including human *CAP1* (Matviw *et al.*, 1993) and *CAP2* (Yu *et al.*, 1994), rat *CAP2* (Swiston *et al.*, submitted), and mouse *CAP* (Vojtek *et al.*, 1993). It is not known yet whether the two rat *CAP* alleles serve different functions; however, *MCH1* and rat *CAP2* do appear to be differentially expressed in adult tissues (D. Young, unpublished results). While *MCH1* is expressed ubiquitously, the rat *CAP2* mRNA

transcript appears to be present at high levels in the testes and low to undetectable levels in the liver and spleen (D. Young, unpublished observations).

The two human *CAP* genes have already been shown to function somewhat differently in *S.pombe* (Yu *et al.*, 1994). Disruption of *cap* in *S.pombe* results in two sets of phenotypes. One set, mediated by the loss of the amino terminus, results in hypersporulation due to decreased adenylyl cyclase activity. The second set of phenotypes includes altered cell growth and morphology and is associated with the loss of the carboxyl-domain (Kawamukai *et al.*, 1992). Transformation of a *cap* null *S.pombe* strain with human *CAP1* suppresses the phenotypes associated with the loss of both the amino- and carboxyl-terminal domains of *cap* (Yu *et al.*, 1994). Human *CAP2* suppresses only the loss of the carboxyl-domain (Yu *et al.*, 1994). Thus, CAP proteins may have evolved to serve separate functions, perhaps where one gene interacts with a mammalian protein structurally similar to the yeast adenylyl cyclase.

4.2 Functional characterization of the carboxyl terminus of CAP and MCH1.

CAP homologs appear to be most similar to one another within the proline-rich segment and the carboxyl-terminal domain. Furthermore, the carboxyl terminus is also functionally conserved. In order to resolve the function of the carboxyl-domain of CAP and MCH1, we examined possible protein-protein interactions between CAP, MCH1 and other cellular factors. Using the two-hybrid system and co-immunoprecipitation procedures, we have defined two separate and specific molecular interactions conferred by CAP proteins. First, both CAP and MCH1 interact to form homodimers and also heterologously interact with each other, when co-expressed in yeast. Second, both CAP and MCH1 are actin-binding proteins. Both these functions are mediated by the well-conserved carboxyl domain. According to data from gel exclusion chromatography, the predicted size of the CAP protein complex is estimated

to be ~280kDa (Wang *et al.*, 1993). Interestingly, the sum of two CAP molecules and two actin monomers yields a predicted mass of ~230kDa. Therefore, CAP may exist *in vivo* in a dimeric complex with actin monomers.

Because dimerization is conserved in both yeast and mammalian CAP proteins, we had predicted that this interaction would be mediated solely by the carboxyl terminus. However, we cannot rule out participation of the amino terminus in this process. Co-immunoprecipitation studies using CAP deletion mutants reveal that the carboxyl-terminal domain can interact either with itself or with the amino terminus of CAP. The same set of experiments demonstrates that the amino terminus does not possess the same properties. Thus, the amino-terminal domain cannot interact with itself.

Computer analysis of the primary sequence of CAP proteins leads us to predict that the carboxyl terminus contains several potential beta-strands (Rost *et al.*, 1993). Therefore the carboxyl-terminal regions of CAP proteins could potentially interact to form a beta sheet. Examination of the amino terminus reveals the existence of several potential amphipathic helices. In the case of yeast CAP, these structures may allow for the interaction of CAP with adenylyl cyclase. Furthermore, without the polyproline stretch, the alpha-helical regions of the amino terminus may mediate protein-protein interactions with the carboxyl terminus as demonstrated in Figure 17. Alternatively, if one regards CAP as a flexible molecule with the proline-rich stretch as a hinge, such flexibility might allow for interactions of the carboxyl-terminal region with another carboxyl-domain, as well as, with the amino terminus. The ability of the carboxyl terminus of CAP to interact with both the amino- and carboxyl-terminal sites could allow for the formation of a large protein complex where CAP acts as a molecular scaffold for other protein-protein interactions.

4.3 The role of the polyproline domain in CAP dimerization and localization.

In both the two-hybrid assay and co-immunoprecipitation experiments, we have shown that the middle proline-rich domain is not required for dimerization (Figure 14). Furthermore, data from the two-hybrid assay indicates that this domain may, in fact, inhibit dimerization. This idea is supported by protein localization studies of the deletion mutant *CAPΔ7* that lacks the proline-rich domain. In *cap* cells expressing this mutant allele, we observed the formation of mislocalized protein aggregates (Figure 21, panel C). Thus the polyproline domain may somehow restrict the formation of CAP multimers and prevent the formation of these electron-dense regions. Moreover, this domain may also serve to target CAP protein complexes to their proper cellular location. The accumulation of small vesicle-like structures in these cells is particularly intriguing, considering the indirect link between CAP and the secretory pathway in yeast. As mentioned earlier, the *SNC* genes are conditional suppressors of phenotypes associated with the loss of the CAP carboxyl terminus (Protopopov *et al.*, 1993). Thus mislocalization of the CAP protein might result in a parallel mislocalization of secretory vesicles.

The role of the polyproline stretch in protein localization may reflect its function as a potential SH3 binding site since such proline-rich regions are often recognized by SH3-domain containing proteins (Ren *et al.*, 1993). SH3 domains are non-catalytic, conserved segments found in many signaling molecules (Schlessinger *et al.*, 1994). While the function of these domains is not as clearly defined as that of SH2 domains, SH3 domains are believed to play an important role in cytoskeletal regulation and protein localization. For example, micro-injection of the SH3 domains of PLC-γ or GRB2 into fibroblasts localizes these proteins to the actin cytoskeleton, while mutation of these domains results in mislocalization of these proteins as revealed by diffuse cytoskeletal staining (Bar-Sagi *et al.*, 1993). MCH1 and CAP are likely to interact with SH3 containing proteins as they not only contain a putative SH3 binding site,as

predicted by sequence analysis (Figure 15), but also bind to actin and regulate the actin cytoskeleton.

4.4 MCH1 and CAP are actin-binding proteins.

The finding that MCH1 and CAP associate with actin was predicted by deletional analysis of the CAP protein, and in an earlier study by Gieselmann and Mann who partially sequenced a porcine actin-binding protein, ASP-56, and noted its homology to CAP (Gieselmann and Mann, 1992). Through co-immunoprecipitation, we have ascribed the property of actin-binding solely to the carboxyl-domain of both CAP and MCH1 (Figure 18). Two recent reports have confirmed this interaction with the two-hybrid assay and co-immunoprecipitation experiments; however, both studies only examine yeast CAP (Amberg *et al.*, 1994; Freeman *et al.*, 1995). We have demonstrated that this interaction is conserved in mammalian CAP proteins as well. Hence, CAP proteins appear to constitute novel members of the growing family of actin-binding proteins.

In order to deterimine if actin binding and dimerization are mediated by the same site on the carboxyl terminus, we utilized the CAP deletion mutant, CAPΔ11. This mutant lacks the last 27 amino acids of the carboxyl-domain and displays all the phenotypes associated with a disrupted carboxyl terminus (Gerst *et al.*, 1991). Thus, it seemed likely that this mutant would be unable to bind actin. As shown in Figure 19, this mutant is incapable of actin-binding; however, CAPΔ11 is still capable of dimerization. Therefore, we have shown that actin-binding and dimerization are separate processes, and that actin-binding is mediated by the last, well-conserved, 27 amino acids of CAP. We compared this region to the actin-binding domains of other known actin-binding proteins by using the MACAW protein sequence homology program. However, neither MCH1 nor CAP displayed any homology to other actin-binding proteins within the carboxyl terminus.

Thus far, actin-binding proteins have been classified into at least 10 groups based on how they affect actin filament polymerization or depolymerization within the cell (Kreis and Vale, 1993; see Table IV). Regulation of the state of actin polymerization in the cell is mediated by a diverse array of proteins. This is understandable, given that the actin cytoskeleton is implicated in a wide variety of functions ranging from cellular locomotion and cell division to secretory processes and cell signaling. The effect that actin-binding proteins have on actin-filament formation can be determined *in vitro* through assays that measure the viscosity of an actin-filament solution in the presence or absence of an actin-binding protein or through pyrene fluorescence assays (Bray,

Table IV. Actin-Binding Proteins

Monomer Binding Proteins

Thymosin-beta-4
Actobindin
Profilin
DNaseI
ABP-50
Vitamin D-Binding Protein

Small severing proteins

Depactin

Cofilin

Cofilin

Barbed End Capping Proteins

Capping Protein
Radixin
Insertin

Barbed End Capping/Severing Proteins

gCAP39/MCP
Fragmin
Fragmin 60
Scinderin
Gelsolin
Villin

Membrane Associated Actin-Binding Protein

Ponticulin
Synapsins
Actolinkin

Miscellaneous

Yeast ABP-1
Annexins
Coronin
Ezrin
Band 4.1

Microtubule Binding Proteins

Tau
MAP-2

Myosins

Myosin-I
Myosin-II

Lateral Binding Proteins

Hisactophilin
Calponin
Troponin
Tropomyosin
Caldesmon
Adducin

Crosslinking Proteins

ABP-30
Dematin
Fascin
Fimbrin
MARCKS
ABP-120

(Kreis and Vale, 1993)

1992). In pyrene fluorescence assays, actin molecules are covalently linked to a pyrene hapten. This hapten will show a pronounced shift in fluorescence during actin polymerization. Two very recent reports have shown, through the use of these assays, that *in vitro*, CAP is an actin monomer-sequestering protein which binds actin in roughly a 1:1 stoichiometric ratio (Freeman *et al.*, 1995; Gottwald, unpublished results). Other proteins included in this category include profilin and thymosin-ß4.

Thymosin-ß4 has been identified in vertebrates as the predominant actin-monomer sequestering protein. Intracellular concentrations of thymosin-ß4 range from 200uM in leukocytes to 600uM in stimulated-platelets -- concentrations comparable to that of intracellular G-actin. Thus the primary function of this protein is to maintain a reserve of G-actin in the cell. In contrast, the intracellular concentration of CAP is estimated to be ~ 5uM which is ~20 fold less than the G-actin concentation in the cell (Gottwald, unpublished results). Therefore, CAP proteins do not appear to be the primary actin-sequestering proteins *in vivo*. It is more likely that CAP proteins and their homologs regulate the actin cytoskeleton under specific signaling conditions and in localized regions of the cell. For example, MCH1 may regulate the polymerization state of actin in the lamellipodium during cell movement. Likewise, CAP may stimulate reorganization of the actin cytoskeleton in the presence of an activated RAS protein or other growth signals.

4.5 CAP function and profilin.

It has been shown previously that profilin functionally complements loss of the carboxyl-terminal domain of CAP. Profilin has been classically described as a polyphosphoinositide binding protein, and an actin-sequestering protein that prevents the nucleation of actin filaments. The role of profilin in actin monomer-sequestration was supported by results from the *in vitro* assays, described above, which showed the stoichiometric binding of profilin to actin at a 1:1 ratio (Carlsson *et al.*, 1977).

Recently, however, the role of profilin in actin polymerization has become more complex. Profilin has been demonstrated to act as an adenine nucleotide exchange factor for actin (Goldschmidt-Clermont *et al.*, 1992). Thus actin, like the small GTP binding proteins, is "active" when bound to ATP and "inactive" when bound to ADP. ATP-bound actin polymerizes much more readily than ADP-actin; therefore, profilin acts to increase the amount of "activated" actin in the cell (Goldschmidt-Clermont *et al.*, 1992).

Experimental evidence has accumulated to support profilin's role in stimulating actin polymerization, as well as, depolymerization. First, CHO cells that overexpress profilin show a higher level of F-actin (Finkel *et al.*, 1994), and second, the bacteria *Listeria monocytogenes* requires profilin to promote an actin-based motility system that involves actin polymerization in infected cells (Theriot *et al.*, 1994). Third, like *cap* cells, yeast deficient in profilin lack actin cables-possibly indicative of faulty actin polymerization (Haarer *et al.*, 1990). Profilin has also been shown to stimulate actin depolymerization *in vivo*. Micro-injection of rat kidney cells with profilin results in a decrease in F-actin (Cao *et al.*, 1992). Thus, it seems that profilin can either enhance or decrease actin polymerization depending on the cell context. As profilin suppresses phenotypes associated with the loss of the carboxyl terminus of CAP, consideration should be given to the possibility that CAP, like profilin, may serve to modulate actin polymerization or depolymerization depending on cellular localization. Indirect immunofluorescence in yeast shows that CAP and profilin both localize to cortical actin patches in buds. Immunofluorescence in fibroblasts, reveals that MCH1, like profilin, localizes to the lamellipodia and indicates that MCH1 may play a role in cellular locomotion (Figure 22; Buß *et al.*, 1992). Such a function requires constant cytoskeletal remodeling, consistent with the hypothesis that MCH1, and CAP, may serve to modulate actin polymerization through both inhibitory and stimulatory means.

Because profilin seems to functionally interact with CAP, we chose to investigate whether profilin physically interacts with CAP as well. Evidence to support such an interaction came from data showing that profilin binds to polyproline stretches *in vitro*. Thus, we considered the possibility that profilin binds to the proline-rich stretch of CAP. We examined the potential interaction between CAP and profilin using both two-hybrid and co-immunoprecipitation methodologies; however, we were unable to demonstrate any physical interactions between these proteins. While the lack of a physical interaction between CAP and profilin does not rule out the possibility that these proteins act along a comon pathway, it may be that CAP and profilin only share overlapping functions and act independently of one another.

Alternatively, a third possibility is that these proteins interact in a highly transitory fashion. Recently, the protein, VASP has been shown to bind profilin through a proline-rich domain that is almost identical to the polyproline stretch in CAP and MCH1 (Reinhard *et al.*, 1995). Unlike the CAP proteins which possess a single proline-rich domain, the VASP protein possesses multiple proline-rich repeats. Thus an interaction between VASP and profilin might be stronger and thus easier to detect. Interestingly, immunostaining of the VASP protein in human fibroblasts appears almost identical to that of MCH1 in Rat-6 cells (Reinhard *et al.*, 1995). Therefore, it is possible that VASP and MCH1 proteins serve similar functions in mammalian cells.

4.6 Models of CAP and MCH1 function in yeast and mammalian cells.

Based on the data discussed here, we have presented a model for CAP function in yeast and MCH1 function in mammals. CAP mediates two separate sets of functions. One set relates to the activation of the RAS signaling pathway, while the other set is involved in the regulation of growth and cell morphology. The amino terminus of CAP acts along the RAS signaling cascade at the level of adenylyl cyclase. As shown in Figure 24 and 25, the amino-terminal domain physically associates with adenylyl

cyclase and may stabilize its interaction with RAS (Wang *et al.*, 1992). In addition, CAP may bind to the CDC25 protein through its SH3 domain (M.Wigler, unpublished results). Thus CAP could allow for the functional association of a number of RAS signaling proteins by acting as a molecular scaffold. This idea is further supported by our data showing that CAP proteins form dimers in yeast, since the formation of dimers or multimers in the cell would allow for numerous protein interactions. For example, the formation of CAP complexes results in the presence of multiple SH3 binding domains (Figure 25). Therefore, while CDC25 may interact with one SH3 domain, another unidentified protein may interact with the remaining available SH3 binding sites. Furthermore, this protein (X) may serve to localize CAP dimers to the appropriate location for signal transduction. This prediction is supported by the observation that CAP mutants lacking the proline-rich region form mis-localized protein aggregates in the cell.

The carboxyl domain mediates functions associated with cell growth and cytoskeletal regulation. Here, we show that CAP regulates the cytoskeleton by binding to actin through the last 27 amino acids of the carboxyl domain. In addition, the carboxyl terminus mediates dimerization. We have shown that actin-binding is separate from the site of dimerization, and dimerization is not dependent on CAP's association with the cytoskeleton.

MCH1 was isolated as a suppressor of phenotypes associated with the loss of the carboxyl-domain of CAP. Therefore, it is not surprising to note that many of the properties associated with the carboxyl terminus of CAP have been conserved in MCH1. As shown in Figure 26, the carboxyl terminus of MCH1 mediates both actin binding and dimerization in the same manner as CAP. The role for MCH1 in cytoskeletal regulation is further supported by immunofluoresence studies that localize MCH1 to the membrane lamellipodia. Thus the carboxyl-terminal functions of CAP appear to have been conserved through evolution. Functions associated with the amino

terminus and polyproline stretch of MCH1 have not yet been defined. However, given the role of the amino-terminal domain of CAP in the regulation of cell proliferation, it is possible that this function extends to mammalian CAP as well. As discussed the proline-rich stretches of both CAP and MCH1 are highly reminiscent of SH3 binding domains. Therefore, this region may bind cellular signaling molecules that contain SH3 domains. In addition, the proline-rich stretch is almost identical to the profilin-binding site of the mammalian protein, VASP. Therefore, it is possible that MCH1 associates with profilin, although this interaction was not detectable with CAP.

A comparison of the structural and functional organization of CAP and MCH1 leads us to predict that these proteins act as molecular scaffolds for protein complexes that are involved in cell proliferation and growth. Furthermore, the role of the carboxyl terminus in actin-binding suggests that CAP proteins may serve to translate proliferative signals into morphological changes in the cell.

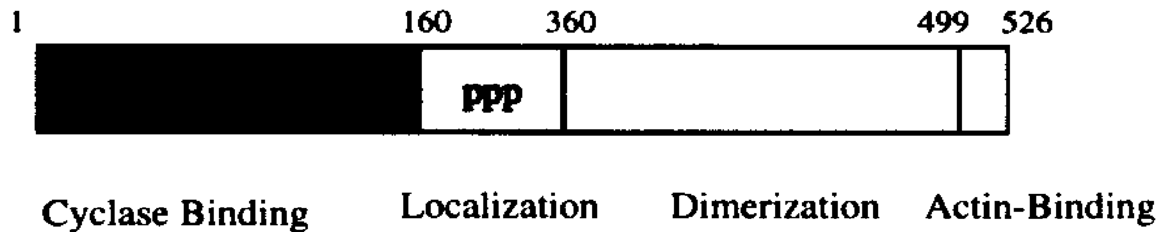


Figure 24. Functional domains of the adenylyl cyclase-associated protein. Shown here are the three domains of CAP. The amino terminus binds to adenylyl cyclase. The proline-rich region plays a role in protein localization and the carboxyl-domain mediates both dimerization and actin binding. Actin binding has been localized to the last 27 amino acids of CAP.

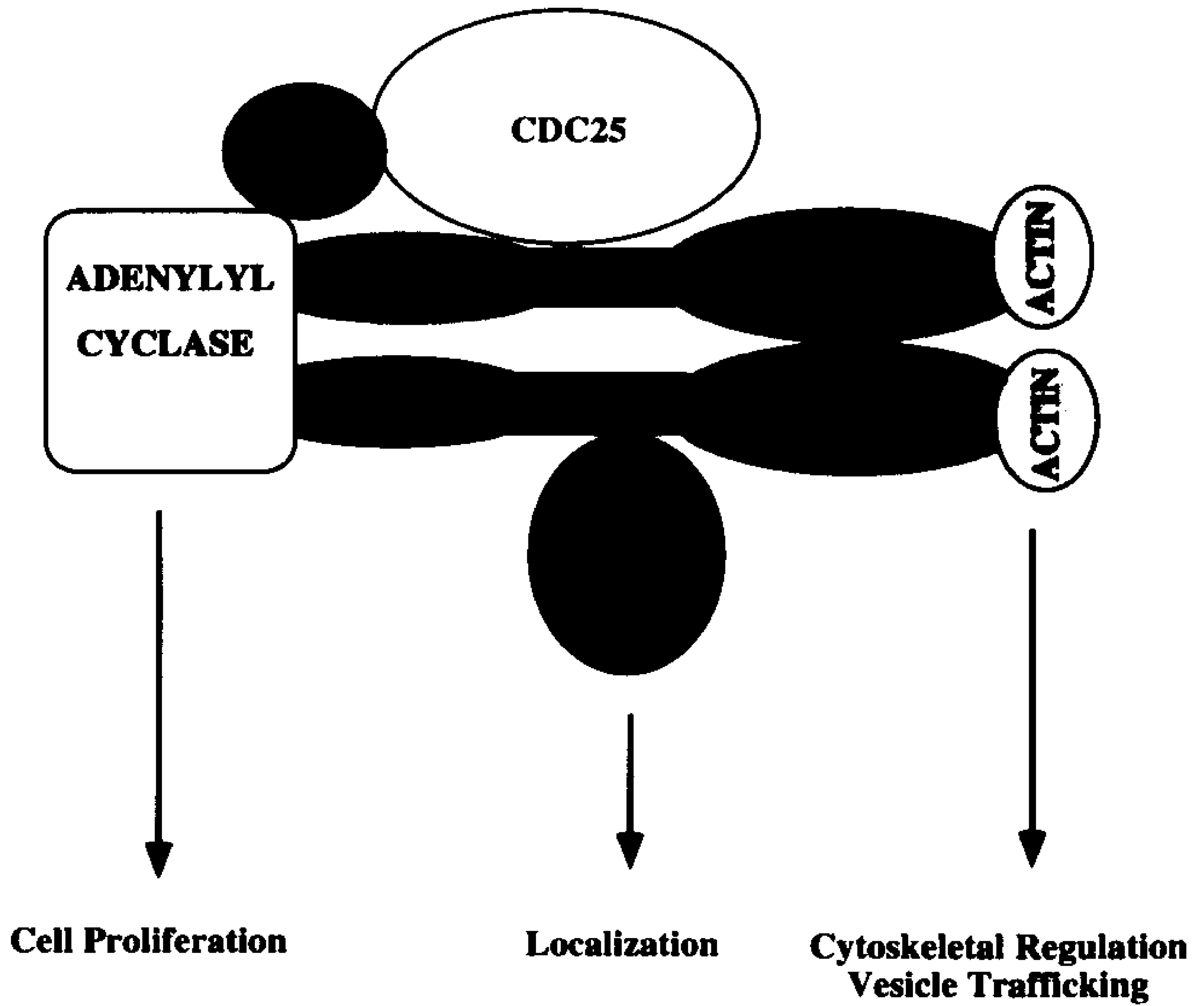


Figure 25. A model for CAP function in yeast.

Figure 25. A model for CAP function in yeast. CAP proteins can be divided into three domains -- an amino terminus. a carboxyl terminus and a proline-rich region. The amino terminus participates in signaling pathways affecting cell proliferation through RAS and physically associates with adenylyl cyclase. The proline-rich domain is similar to SH3 binding domains and may interact with adenylyl cyclase as well as other SH3 domain-containing proteins. In addition, removal of this domain appears to affect localization of the CAP protein. Proper localization of CAP may be mediated by the interaction of an unknown protein, X, with the CAP SH3 binding site. The carboxyl terminus binds actin monomers and mediates dimerization. As shown, actin binding and dimerization occur through different regions on the carboxyl-domain. Furthermore, dimerization allows CAP to mediate multiple protein-protein interactions whereby CAP can act as a molecular scaffold for a variety of signaling proteins.

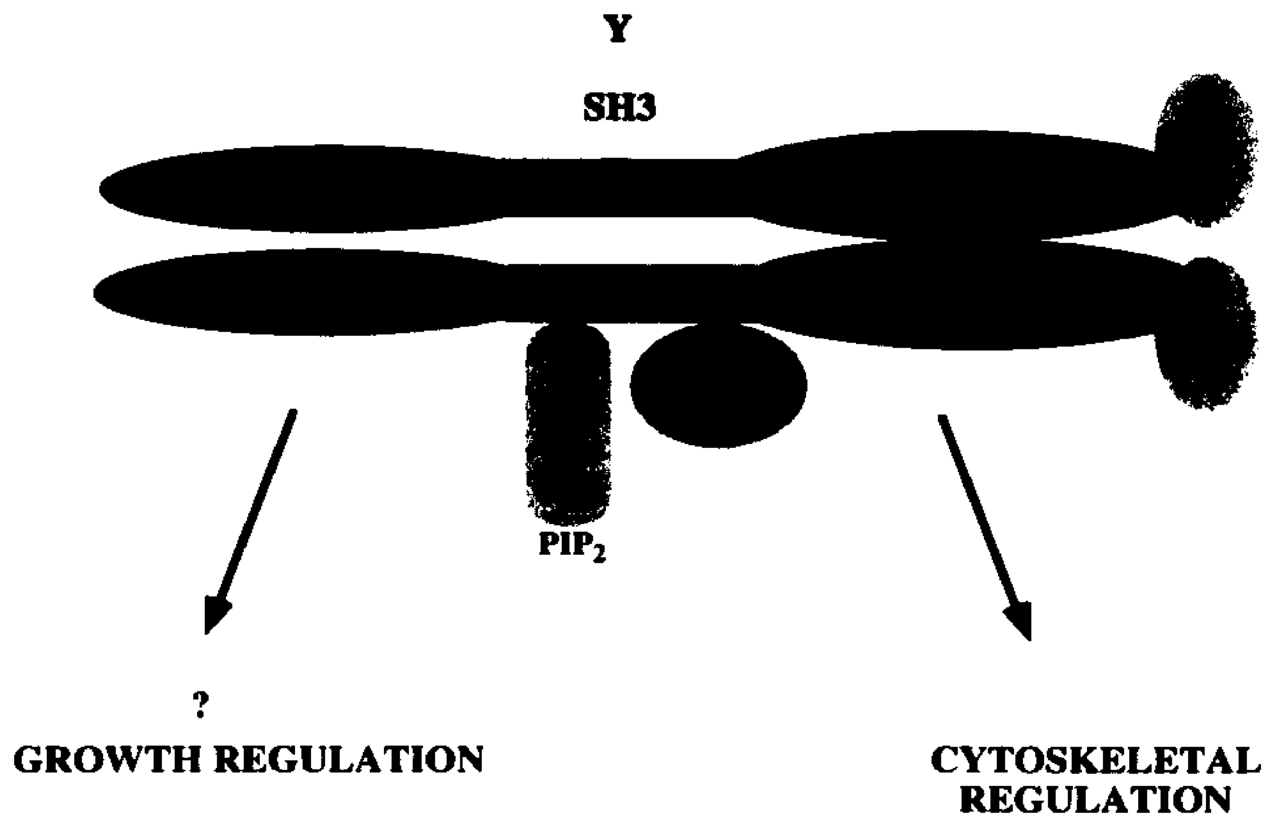


Figure 26. A model for MCH1 function in mammals

Figure 26. A model for MCH1 function in mammals. In this model, MCH1 functions as a site for multiple protein interactions. These proteins are involved in cytoskeletal regulation, and unknown factors may be involved in the regulation of signal transduction. Similar to yeast CAP, MCH1 proteins form dimers and also bind to actin monomers through the carboxyl terminal-domain. The actin-binding site is separate from the region responsible for dimerization. This prediction is based upon data obtained from CAP protein interactions since the carboxyl-domains of CAP and MCH1 are extremely well-conserved. Unlike the amino terminus of CAP, the amino-terminal domain of MCH1 has not been functionally characterized. However, based upon the CAP model, it is possible that this region mediates the regulation of cellular proliferation. Finally, the proline-rich region of MCH1 is similar to known SH3 binding domains. Therefore, it is possible that this site binds to SH3 domain-containing proteins. This region is also highly similar to the profilin-binding site of the protein VASP. Therefore, we have included profilin as a potential binding ligand for MCH1.

Ultimately, we predict that MCH1, like CAP, serves as a molecular scaffold that may allow for the functional interaction of protein complexes. Furthermore, MCH1 may serve to coordinate regulation of the actin cytoskeleton with changes in cell growth and proliferation.

4.7 Future Directions

We have demonstrated that CAP proteins are conserved in mammals, and, moreover, that these proteins are most conserved in the carboxyl-terminal regions. The carboxyl terminus of both CAP and MCH1 binds actin and mediates dimerization. Thus, we can say that CAP proteins constitute a novel class of actin binding proteins that may serve to connect cell signaling to cytoskeletal regulation. Nonetheless, many aspects of MCH1 and CAP function remain to be examined.

First, while CAP proteins have been well-defined as actin-monomer sequestering proteins, the role of dimerization in cell function has not yet been determined. We predict that CAP protein dimerization may facilitate numerous protein interactions. However, we have not determined which aspects of cell signaling are affected by protein dimerization. Mutational analysis of the carboxyl terminus to define the region necessary for dimerization may provide an answer to the role of CAP complex formation in the cell.

Second, in yeast, loss of the carboxyl domain of CAP correlates with gross morphological changes including disruption of the actin cytoskeleton as well as random budding. It is possible that CAP proteins help to establish cell polarity and indirectly play a role in bud site selection through the rearrangement of the actin cytoskeleton. In order to investigate if there is a role for CAP in bud site selection, genetic interactions between CAP and genes responsible for bud-site selection and formation could be examined. Genes responsible for bud selection include *BUD1-4*. *BUD1* encodes a small ras-like protein which is epistatic to *BUD 3* and *BUD4* (Drubin, 1991). A connection between CAP and the BUD proteins might then be extended to mammalian cells possibly through the rac/rho pathway since the *BUD* genes share some homology to Rho family members. In addition, genetic interactions between CAP and other actin binding proteins could be examined to determine whether CAP acts along a common or parallel pathway with other actin-binding proteins in the regulation of the cytoskeleton.

Third, through genetic and biochemical studies, the role of CAP in yeast has been well-characterized. In contrast, the function of MCH1 in mammalian cells is largely unknown. Preliminary studies we have done reveal that MCH1 overexpression dramatically inhibits colony formation in Rat-6 cells. However, the mechanism by which this occurs is unknown. One possibility is that MCH1 overexpression is cytotoxic because MCH1 interferes with the cytoskeleton. Two alternative possibilities are that MCH1 is a growth inhibitor or that MCH1 activates apoptosis. The idea that MCH1 is involved in the regulation of cell proliferation is intriguing since CAP proteins have already been shown to mediate RAS signaling through the amino-terminal domain. While we have focused on characterizing the carboxyl-domain functions of MCH1, the functional role of the amino terminus has not yet been investigated. An examination of protein-protein interactions of the amino-terminal domain, as well as, overexpression in fibroblasts may serve to clarify the role of this region in cellular function.

Both MCH1 and CAP are actin binding proteins, and from genetic analysis CAP appears to play a critical role in the regulation of the cytoskeleton. Furthermore, CAP mediates cell signaling. Recently, a number of mammalian proteins have been identified that affect both signal transduction cascades as well as changes in cell morphology. Among these proteins are the small GTP-binding proteins Rac and Rho, as well as, the focal adhesion protein, Focal Adhesion Kinase (FAK). A more detailed characterization of the effect of MCH1 overexpression, perhaps using microinjection studies, may determine the role of MCH1 in mammalian cytoskeletal regulation. Results from these studies may help to place MCH1 along a known cytoskeletal signaling pathway and serve to elucidate its role in mammalian cell signal transduction.

APPENDIX I

FREQUENTLY USED ABBREVIATIONS

ATP	Adenosin 5'- triphosphate
BCS	Bovine Calf Serum
BSA	Bovine Serum Albumin
cAMP	cyclic 3'5'-Adenosine monophosphate
CAP	Adenylyl Cyclase-Associated Protein
DB	DNA binding
DMEM	Dulbecco's Modified Eagles Medium
DTT	Dithiothreitol
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
HA	Influenza Hemagglutinin Antigen
kDa	Kilodalton
MCH1	Mammalian CAP Homolog 1
PBS	Phosphate-Buffered Saline
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phospharidylinositol tri-phosphate
PMSF	Phenylmethylsulfonylfluoride
SDS	Sodium dodecyl sulfate
STI	Soybean trypsin inhibitor
TA	Transactivating
WT	Wild type

REFERENCES

Amberg, D.C., Basart,E., and Botstein,D. (1995). Defining protein interactions with yeast actin *in vivo*. *Nature Struct. Biol.* **2**, 28-35.

Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin,M. (1994). Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell* **78**, 949-961.

Ballester, R., Marchuk, D., Boguski, M., Saulino, M., Saulino, A., Lechter, R., Wigler,M., and Collins, F. (1990). The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**, 851-859.

Ballester, R., Michaeli, T., Ferguson, K., Xu, H.P-., McCormick, F. and Wigler, M. (1989). Genetic analyisis of a mammalian *GAP* expressed in yeast. *Cell* **59**, 681-686.

Bar-Sagi, D., and Feramisco, J. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by Ras proteins. *Science* **233**, 1061-1068.

Bar-Sagi, D., Rotin D., Batzer, A., Mandiyan, V., and Schlesinger, J. (1993). SH3 domains direct cellular localization of signaling molecules. *Cell* **74**, 83-91.

Barbacid,M. (1987). *ras* genes. *Annu. Rev. Biochem.* **56**, 779-827.

Barry, S.T., and Crichtley, D.R. (1994). the RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both p125FAK and protein tyrosine kinase C-δ to focal adhesions. *J. Cell Sci.* **107**, 2033-2045.

Bauer, F., Urdaci, M., Aigle, M., and Crouzet, M. (1993). Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* **13**, 5070-5084.

Boguski, M.S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366**, 643-654.

Bollag, G. and McCormick, F. (1991). Regulators and effectors of *ras* proteins. *Annu.Rev.Cell Biol.* **7**, 601-632.

Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992). Identification of murine homologs of the Drosophila son of sevenless gene: potential activators of Ras. *Proc. Natl. Acad. Sci. USA* **89**, 6511-6515

Bray, D. (1992) Cell Movements. (Garland Publishing Inc., New York and London)

Broach, J.R., (1991). *RAS* genes in *Saccharomyces cerevisiae*: signal transduction in search of a pathway. *TIG* **6**, 28-33.

Broek, D., Samily, N., Fasano, O., Fujiyama, A., Fuyuhiko, T., Northup, J., and Wigler, M. (1985). Differential activation of yeast adenylyl cyclase by wild-type and mutant RAS proteins. *Cell* **41**, 763-769.

Buday, L., and Downard, J. (1993). Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* **73**, 611-620.

Burridge, K. and Connell, L. (1983). Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin-membrane interaction. *Cell Motil.* **3**, 405-417.

Button, E., Shapland, C., and Lawson. (1995). Actin, its associated proteins and metastasis. *Cell Mot. Cyto.* **30**, 247-251.

Camonis, J.H., Katheline, M., Gondre, B., Garreau, H., Boy-Marcotte, E., and Jacquet, M. (1986). Characterization, cloning and sequence analysis of the *CDC25* gene which controls the cyclic AMP level of *Saccharomyces cerevisiae*. *EMBO J.* **5**, 375-380.

Cao, L.-G., Babcock, G.C., Rubenstein, P.A., and Wang, Y.L. (1992) Effects of profilin and profilactin on actin structure and function in living cells. *J.Cell Biol.* **117**, 1023-1029.

Carlier, M.F., and Pantaloni, D. (1994). Actin assembly in response to extracellular signals: role of capping proteins, thymosin B4 and profilin. *Sem. Cell Biol.* **5**, 183-191.

Carlsson, L., Nyström, L.-E., Sundkvist, I., Markey, F., and Lindberg, U. (1977). Actin polymerization is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* **115**, 465-483.

Carraway, K.L. and Carraway, C.A. (1995). Signaling, mitogenesis, and the cytoskeleton: where the action is. *Bioessays* **17**, 171-175.

Chardin, P., Camonis, J.H., Gale, N.W., Aelst, L.V., Schlessinger, J., Wigler, M.H., Bar-Sagi. D. (1993). Human sos1: a guanine nucleotide exchange factor for ras that binds to GRB2. *Science* **260**, 1338-1343.

Cohen, G.B., Ruibao, R., and Baltimore, D. (1995). Modular binding domains in signal transduction proteins. *Cell* **80**, 237-248.

Colicelli, J., Field, J., Ballester, R., Chester, N., Young, D., and Wigler, M. (1990). Mutational mapping of the RAS-responsive domains of the *Saccharomyces cerevisiae* adenylyl cyclase. *Mol. Cell. Biol.* **10**, 2539-2543.

Dartsch, P.C., Ritter, M., Häussinger, D., and Lang, F. (1994). Cytoskeletal reorganization in NIH3T3 fibroblasts expressing the ras oncogene. *Eur. J. Cell Biol.* **63**, 316-325.

DeFeo-Jones, D., Scolnick, E., Koller, R., and Dhar, R. (1983). *ras*-related gene sequences identified and isolated from *Saccharomyces cerevisiae*. *Nature* **306**, 707-709.

DeFeo-Jones, D., Tatchell, K., Sigal, I.S., Vass, W.C. and Scolnick, E. (1985). Mammalian and yeast ras gene products: Biological function in heterologous systems. *Science* **228**, 179-183.

Downes, C.P., and Carter, A.N. (1991). Phosphoinositide 3-kinase: a new effector in signal transduction? *Cell Sign.* **3**, 501-513.

Downward, J. (1992). Regulation of p21ras by GTPase activating proteins and guanine nucleotide exchange proteins. *Curr. Opin. Gen. Dev.* **2**, 13-18.

Duchesne, M., Schweighoffer, F., Parker, F., Clerc, F., Frobert, Y., Thang, M.N., and Tocqué, B. (1993). Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling. *Science* **259**, 525-528.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H., and Elledge, S. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7**, 555-569.

Fedor-Chaiken, M., Deschenes, R.J., and Broach, J. (1990). *SRV2*, agene required for RAS activation of adenylate cyclase. *Cell* **61**, 329-340.

Feig, L.A., and Cooper G.M. (1988). Inhibition of NIH3T3 cell proliferation by a mutant ras protein with a preferential affinity for GDP. *Mol. Cell. Biol.* **8**, 3235-3243.

Field, J., Nikawa, J.I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Lerner, R., and Wigler, M. (1988). Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell. Biol.* **8**, 2159-2165.

Field, J., Vojtek, A., Ballester, R., Bolger, G., Colicelli, J., Ferguson, K., Gerst, J., Kataoka, T., Michaeli, T., Powers, S., Riggs, M., Rodgers, L., Wieland, I., Wheland, B., and Wigler, M. (1990). Cloning and characterization of *CAP*, the *S.cerevisiae* gene encoding the 70kd adenylyl cyclase-associated protein. *Cell* **61**, 319-327.

Finkel, T., Theriot, J.A., Dise, K.R., Tomaselli, G.F., and Goldschmidt-Clermont, P.J. (1994). Dynamic actin structures stabilized by profilin. *Proc. Natl. Acad. Sci. USA* **91**, 1510-1514.

Freeman, N.L., Zunxuan, C., Horenstein, J., Weber, A., and Field, J. (1995). An actin monomer binding activity localizes to the carboxy-terminal half of the *Saccharomyces cerevisiae* cyclase-associated protein. *J. Biol. Chem.* **270**, 5680-5685

Gerst, J. Ferguson, K., Vojtek, A., Wigler, M., and Field, J. (1991) CAP: a bifunctional component of the *S.cerevisiae* adenylyl cyclase complex. *Mol. Cell. Biol.* **11**, 1248-1257

Gerst, J.E., Ferguson, K., Wigler, M. and Field, J. (1991). CAP is a bifunctional component of the *Saccharomyces cerevisiae* adenylyl cyclase complex. *Mol. Cell. Biol.* **11**, 1248-1257.

Gerst, J.E., Rodgers, L., Riggs, M., and Wigler, M. (1992). *SNC1*, a yeast homolog of the synaptic vesicle-associated protein/synaptobrevin gene family: genetic interactions with the *RAS* and *CAP* genes. *Proc. Natl. Acad. Sci. USA* **89**, 4338-4342.

Gieselmann, R., and Mann, K. (1992). ASP-56, a new actin sequestering protein from pig platelets with homology to CAP, an adenylate cyclase-associated protein from yeast. *FEBS* **298**, 149-153.

Goldschmidt-Clermont, P.J., Furman, M.U., Wachsstock, D., Safer, D., Nachmias, V.T., and Pollard, T.D. (1992). The control of actin nucleotide exchange by thymosin ß4 and profilin. A potential regulatory mechanism for actin polymerization. *Mol. Cell. Biol.* **3**, 1015-1024.

Gross, E., Goldberg, D., and Levitzki, A. (1992). Phosphorylation of *S.cerevisiae* Cdc25 in response to glucose results in its dissociation form Ras. *Nature* **360**, 762-765.

Haarer, B.K., Petzfold, A.S., and Brown, S.S. (1993). Mutational analysis of yeast profilin. *Mol. Cell. Biol.* **13**, 7864-7873.

Haffner, C., Jarchau, T., Reinhard, Hoppe, J., Lohmann, S., Walter, U. (1995). Molecular cloning, structural analysis and functional expression of the proline rich focal adhesion and microfilament-associated protein, VASP. *EMBO J.* **14**, 19-27.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. Rev. Cell Bio.* **10**, 31-54.

Hancock, J.F., Cadwallader, K., Paterson, H., and Marshall, C.J., (1991). A CAAX or CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *EMBO J.* **10**, 4133-4139.

Hawkins, P.T., Eginoa, A., Rong-Guo, Q., Stokoe, D., Cooke, F.T., Walters, R., Wennstrom, S., Claesson-Welsch, L., Evans, T., Symons, M. (1995). PDGF stimulates an increase in GTP-Rac via the activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**, 393-403.

Hill, C.S., and Treisman. R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80**, 199-211.

Jones, S., Viganais, M.-L., and Broach, J.R. (1991). The *CDC25* protein of *Saccharomyces cerevisiae* promotes the exchange of guanine nucleotides bound to Ras. *Mol. Cell. Biol.* **11**, 2641-2646.

Kataoka, T., Broek, D., and Wigler, M. (1985). DNA Sequence and characterization of the *S.cerevisiae* gene encoding adenylate cyclase. *Cell* **43**, 493-505.

Kataoka, T., Powers, S., Cameron,S., Fasano, O., Goldfarb, M., Broach, M., Broach, J., and Wigler, M. (1985). Functional homology of mammalian and yeast ras genes. *Cell* **40**, 19-26.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J. and Wigler, M. (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. *Cell* **37**, 437-445.

Kataoka, Y. Y., Tatsuya, T., Choe, H.R.-, Tanaka, H., Kataoka, T. (1989). Adenylate cyclases in yeast: A comparison from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. **86**, 5693-5697.

Kawamukai, M., Gerst, J., Field, J., Riggs, M., Rodgers, L., Wigler, M. and Young, D. (1992) Genetic and biochemical analysis of the adenylyl cyclase-associated protein, Cap, in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **3**, 167-180.

Kishore, G.M., and Shah, D.M. (1988). Amino acid biosynthesis inhibitors as herbicides. *Annu. Rev. Biochem.* **57**, 627-663.

Koch, C.A. Anderson, D., Moran, M.F., Ellis, C., and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* **259**. 668-674.

Kodaki, T., Woscholski, R., Hallberg, B., Rodriguez-Viciana, P., Downward,J., and Parker, P.J. (1994). The activation of phosphatidyl 3-kinase by Ras. *Curr. Biol.* **4**, 798-806.

Kotani, K., Hara, K., Kotani,K., Yonezowa, K., and Kasuga, M. (1995). Phosphoinostide 3-kinase as an upstream regulator of the small GTP-binding protein Rac in the insulin signaling of membrane ruffling. *Biochem. Biophys. Res. Comm.* **208**, 985-990.

Krauss, R.S., Guadagno, S.N., and Weinstein, I.B. (1992). Novel revertants of *H-ras* oncogene-transformed R6-PKC3 cells. *Mol. Cell. Biol.* **12**, 3117-3129.

Leevers, S.J., Paterson, H.F., and Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* **369**, 411-414.

Lowy, D.R., Johnson, M.R., DeClue, J.E., Cen, H., Zhang, K., Papageorge,A.G., Vass, W.C., Willumsen, B.M., Valentine, M.B., and Look, T.A., (1993). The GTPase Superfamily. *Ciba Foundation Symposium* **176**, 53-67.

Luban, M. and Goff, S. (1995). The yeast two-hybrid system for studying protein-protein interactions. *Curr. Opin. Biotechnol.* **6**, 59-64.

Madden, K., Costigan, C., and Snyder, M. (1992). Cell polarity and morphogenesis in *Saccharomyces cerevisiae*. *Trends Cell Biol.* **2**, 22-29.

Martin, G.A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A.M., and McCormick, F. (1992). GAP domains responsible for p21ras-dependent inhibition of muscarinic atrial K^+ channel currents. *Science* **255**, 192-194.

Matviw,H., Yu,G., and Young, D., (1992). Identification of a human cDNA that is structurally and functionally related to the yeast adenylyl-cyclase associated CAP proteins. *Mol. Cell. Biol.* **11**, 5033-5040.

McCormick, F. (1995). Ras signaling and NF1. *Curr. Opin. Gen. Dev.* **5**, 51-55.

McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L.B. and Pawson, T. (1993). The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. *EMBO J.* **12**, 3073-3081.

Medema, R.H., L.de Laat, W., Marin,G.A., McCormick, F. and Bos, J.L. (1992). GTPase-Activating protein SH2-SH3 domains induce gene expression in a ras-dependent fashion. *Mol. Cell. Biol.* **12**, 3425-3430.

Miller, J.H. (1972). Experiments in Molecular Genetics. (Cold Spring Harbor Laboratory Press, Cold Spring, New York, N.Y.)

Minzer, K.A., and Field, J. (1994). Interactions between adenylyl cyclase, CAP and RAS from Saccharomyces cerevisiae. *Cellul. Signal.* **6**, 681-694.

Mitts, M.R., Grant, D.B., and Heideman, W. (1990). Adenylate cyclase in *Saccharomyces cerevisiae* is a peripheral membrane protein. *Mol. Cell. Biol.* **10**, 3873-3883.

Moodie, S.A., Willumsen, B.M., Weber, M.J., Wolfman, A. (1993). Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658-1661.

Moran, M.F., Polakis, P., McCormick, F., Pawson, T., and Ellis C. (1991). Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21ras GTPase-activating protein. *Mol. Cell. Biol.* **11**, 1804-1812.

Ng, R. and Abelson, J. (1980). Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae. Proc Nat. Acad. Sci. USA* **77**, 3912-3916.

Nobes, C.D. and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Parker, P.J. (1995). PI-3 kinase puts GTP on the rac. *Curr. Biol.* **5**, 577-579.

Pfeuty-D.T. (1985). The coordinate organization of vinculin and of actin filaments during the early stages of fibroblast spreading on a substratum. *Eur. J. Cell Biol.* **36**, 195-200.

Powers, S. (1992). Genetic analysis of *ras* homologs in yeasts. *Sem. Cancer Biol.* **3**, 209-218.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in *S.cerevisiae* encoding proteins with domains homologous to mammalian *ras* proteins. *Cell* **36**, 607-612.

Prendergast, G.C. and Gibbs, J.B., (1993a). Pathways of Ras function: connections to the actin cytoskeleton. *Adv.Cancer Res.* **62**, 19-63.

Prendergast, G.C. and Gibbs,J.B., (1993b). Ras Regulatory Interactions: Novel Targets for anti-cancer Intervention? *BioEssays* **16**, 187-191.

Protopopov, V., Govindan, B., Novick, P., and Gerst, J.E. (1993). Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins fucntion on the late secretory pathway in S.cerevisiae. *Cell* **74**, 1-20.

Qui, R.J., Chen,J., Kim, D., McCormick, F. and Symons,F. (1995) An essential role for Rac in Ras transformation. *Nature* **374**, 457-459.

Quilliam, L.A., Khoshravi-Far, R., Huff, S.Y., and Der, C.J. (1995). Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins. *BioEssays* **17**, 394-404.

Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B.M., and Walter, U. (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *EMBO J.* **14**, 1583-1589.

Ren, R., Mayerm B.J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259**, 1157-1161.

Resnick, R.J., and Racker, E. (1988). Phosphorylation of the RAS2 gene product by protein kinase A inhibits the activation of yeast adenylyl cyclase. *Proc Natl. Acad. Sci. USA*. **85**, 2474-2478.

Ridely, A.J. (1994a). Signal transduction through the GTP-binding proteins Rac and Rho. *J. Cell Sci.* **Supp. 18**, 127-131.

Ridley, A.J. (1994b). Membrane ruffling and signal transduction. *BioEssays* **16**, 321-327.

Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, J., Waterfield, M.D., and Downward., J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**, 527-532.

Rose, M., Novick, P., Thomas, J., Botstein, D., and Fink, G. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromeric-containing shuttle vector. *Gene* **60**, 237-244.

Rose, M.D., Winston, F., and Hieter, P. (1990). Methods in Yeast Genetics (Cold Spring Harbor Laboratory, New York: Cold Spring Harbor Laboratory Press).

Safer, D., and Nachmias, V.T., (1994). Beta-Thymosins as actin-binding peptides. *BioEssays* **16**, 473-478.

Saiki, R., Gelfand, D., Stoffe, S., Schaff, S., Higushi, R., Horn, G., Mullis, K., and Ehrlich, H. (1988). Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* **239**, 487-491.

Sambrook, J., Fritsch, E.F., and Maniatis, T., (1989). Molecular cloning: A Laboratory Manual. Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Schlessinger, J. (1994). SH2/SH3 signaling proteins. *Curr. Opin. Gen. Dev.* **4**, 25-30.

Seger, R., and Krebs, E.G. (1995). The MAPK signaling cascade.*FASEB J.* **9**, 726-735.

Settleman, J., Albright, C.F., Foster, L.C. and Weinberg, R. (1992a). Association between GTPase activators for Rho and Ras families. *Nature* **359**, 153-154.

Settleman,J., Narasimhan, V., Foster, L.C., and Weinberg, R. (1992b). Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from Ras to the nucleus. *Cell* **69**, 539-549.

Simon, M.A., Botwell, D.D.L., Dodson, G.S., Laverty, T.R., and Rubin, G.M. (1991). Ras1 and a putative guanine nucleotide exchange factor performs crucial steps in signaling by the Sevenless tyrosine kinase. *Cell* **67**, 701-716.

Sohn, R.H. and Goldschmidt-Clermont, P.J. (1994). Profilin: At the crossroads of signal transduction and the actin cytoskeleton. *BioEssays* **16**, 465-472.

Sreenath, T.V.L., Brevario, D., Ahmed, N., and Dhar, R. (1988). Two differential protein kinase acitivites phosphorylate RAS2 protein in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm.* **157**, 1182-1189.

Stokoe, D., Macdonald,S.G., Cadwallader, K., Symons, M., and Hancock, J.F. (1994). Activation of Ras as a result of recruitment to the plasma membrane. *Science* **264**, 1463-1467.

Suzuki, N., Tsujino, K., Minato, T., Nishida, Y., Okada, T., and Kataoka, T. (1993). Antibody mimicking the action of RAS proteins on yeast adenylyl cyclase: implications for RAS-effector interaction. *Mol. Cell. Biol.* **13**, 769-774.

Swiston,J., Hibbersty,A., Yu, G., and Young,D. (**submitted**). Differential expression of CAP and CAP2 in adult tissues.

Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi. (1995). Rho as a regulator of the cytoskeleton. *TIBS* **20**, 227-231.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M., Gibbs, J.B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990). *S.cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. *Cell* **60**, 803-807.

Tellam, R.L., Morton, D.J., and Clarke, F.M. (1989). A common theme in the amino acid sequences of actin and many actin binding proteins? *TIBS* **14**, 130-133.

Theriot, J.A., Rosenblatt, J., Portnoy, D.A., Goldschmidt-Clermont, P.J. and Mitchison, T.J. (1994). Involvement of profilin in the actin-based motility of the L. monocytogenes in cells and in cell-free extracts. *Cell* **76**, 505-517.

Thevelein, J.M. (1991). Fermentable sugars and intracellular acidifications as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. *Mol. Microbiol.* **5**, 1301-1307.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Murwitz, M., Krebs, E.G., and Wigler, M. (1987). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cAMP dependent protein kinase in yeast. *Mol. Cell. Biol.* **7**, 1371-1380.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., Wigler, M. (1985). In Yeast, RAS proteins are controlling elements of adenylate cyclase. *Cell* **40**, 27-36.

Trahey, M. and McCormick, F. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238**, 542-545.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R. and O'Conell, P. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type I locus. *Cell* **62**, 187-192.

Vojtek, A., and Cooper, J.A. (1993). Identification of a cDNA encoding mouse *CAP*: a homolog of the yeast adenylyl cyclase associated protein. *J. Cell Sci.* **105**, 777-785.

Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T., Brown, S., and Wigler, M. (1991). Evidence for a functional link between profilin and *CAP* in the yeast *S.cerevisiae*. *Cell* **66**, 497-505.

Wang, J., Suzuki, N., and Kataoka, T. (1992). The 70-kilodalton cyclase-associated protein is not essential for interaction of *Saccharomyces cerevisiae* adenylyl cyclase with RAS proteins. *Mol. Cell. Biol.* **12**, 4937-4945.

Wang,J., Suzuki, N., Nishida, Y., and Kataoka, T. (1993). Analysis of the function of the 70 kilodalton cyclase-associated protein (CAP) by using mutants of yeast adenylyl cyclase defective in CAP binding. *Mol. Cell. Biol.* **13**, 4087-4097.

Welch, M.D., Holtzman, D.A., and Drubin, D.G. (1994). The yeast actin cytoskeleton. *Curr. Opin. Cell Biol.* **6**, 110-119.

Wigler, M., Field, J., Broek, D., Toda, T., Cameron, S., Nikawa, J., Michaeli, T., Colicelli, J., and Ferguson, K. (1988). Studies of RAS Function in the Yeast Saccharomyces cerevisiae. *Cold Spring Harbor Sym. Quant. Biol.* **53**, 649-655.

Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978). Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as a donor. *Cell* **14**, 725-731.

Wu, H. and Parsons, J.T. (1993). Cortactin, an 80/85-kilodlaton $pp60^{src}$ substrate, is a filamentous actin binding protein enriched in the cell cortex. *J. Cell. Biol.* **120**, 1417-1426.

Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990). The catalytic domain of the neurofibromatosis type 1 gene

product stimulates ras GTPase and complements ira mutants of S.cerevisiae. *Cell* **63**, 835-841.

Yatani, A., Okabe, K., Polakis,P., Halenback, R., McCormick, F., and Brown, A.M. (1990). *ras* p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. *Cell* **61**, 769-776.

Yu, G., Swiston, J., and Young, D. (1994). Comparison of human *CAP* and *CAP2*, homologs of the yeast adenylyl cyclase-associated proteins. *J. Cell Sci.* **107**, 1671-1678.

Zelicof, A., Gatica,J., and Gerst, J.E. (1993). Molecular cloning and characterization of a rat homolog of CAP, the adenylyl cyclase-associated protein from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 13448-13453.

Zhang, K., DeClue, J.E., Vass, W.C., Papageorge, A.G., McCormick,F and Lowy, D.R. (1990). Suppression of *c-ras* transformation by GTPase-activating protein. *Nature* **346**, 754-756.