

**ANALYSIS OF THE *hgl1* GENE OF *Ustilago maydis***

by

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## ABSTRACT

The cAMP/protein kinase A signal transduction pathway plays an important role in morphogenesis in fungi. Previous work identified a gene encoding a *Ustilago maydis* homologue of the catalytic subunit of protein kinase A, *adr1*, involved in maintaining budding growth and virulence. The filamentous phenotype of an *adr1*<sup>-</sup> mutant was used in a genetic screen for suppressor mutations to identify components of the pathway downstream of protein kinase A. This screen resulted in the recovery of a mutation in *hgl1* (hyphal growth locus 1). *hgl1*<sup>-</sup> mutants display a number of phenotypes including suppression of the filamentous growth of an *adr1*<sup>-</sup> mutant, the production of a yellow pigment, and a failure to complete the sexual phase of the life cycle. There are no similar proteins to Hgl1p in current databases and it is therefore difficult to identify its function by comparison to known genes. To identify a functional role for *hgl1*, additional characterization studies have been conducted including further analysis of the predicted amino acid sequence, phosphorylation experiments, and mutagenesis to isolate suppressors of *hgl1*<sup>-</sup>. The work in this thesis demonstrates that a reversion to the *adr1*<sup>-</sup> phenotype could be obtained through the collection of *hgl1*<sup>-</sup> suppressor mutants. Additional studies demonstrated that Hgl1p serves as a direct target for phosphorylation by *Ustilago maydis* protein kinase A *in vitro*. Analysis of the Hgl1p sequence revealed several interesting features of the protein which will be relevant for future experimentation.

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## LIST OF ABBREVIATIONS

<i>adr1</i>	<u>a</u> romatic <u>d</u> icarboximide <u>r</u> esistance gene, encodes the catalytic subunit of <i>U. maydis</i> PKA
<i>adr::phleo</i>	<i>adr1</i> insertional disruption strain carrying the phleomycin resistance marker
bE/bW	<i>U. maydis</i> regulatory heterodimer formed by the b East and b West proteins
cAMP	<u>c</u> yclic <u>a</u> denosine <u>m</u> onophosphate
<i>FLO8</i>	Flo11p-regulating transcription factor in <i>S. cerevisiae</i> (regulated by Tpk2p).
<i>FLO11</i>	cell surface flocculin protein in <i>S. cerevisiae</i>
GPCR	<u>G</u> -protein <u>C</u> oupled <u>R</u> eceptor
<i>hgl1</i>	<u>h</u> yp <sup>h</sup> al growth <u>l</u> ocus 1 gene
<i>hgl1::nat1</i>	insertional disruption of the <i>hgl1</i> gene with the nourseothricin cassette
MAPK	<u>m</u> itogen <u>a</u> ctivated protein <u>k</u> inase
MAPKK	MAPK <u>k</u> inase
MAPKKK	MAPKK <u>k</u> inase
PEST regions	regions of a protein rich in Proline (P), Glutamate (E), Serine (S), and Threonine (T) residues
PKA	<u>p</u> rotein <u>k</u> inase <u>A</u>
PKI	specific inhibitor of protein kinase A catalytic subunits
bPKA	bovine Protein kinase A
UPKA	<i>U. maydis</i> Protein kinase A
<i>prf1</i>	<i>U. maydis</i> gene encoding the pheromone <u>r</u> esponse <u>f</u> actor
<i>ras1</i>	a gene encoding a small GTP-binding protein
<i>SFL1</i>	transcription factor in <i>S. cerevisiae</i> (regulates flocculation).

<i>TPK2</i>	gene encoding the catalytic subunit of PKA in <i>S. cerevisiae</i>
<i>uac1</i>	<i>U. maydis</i> adenylyl cyclase
<i>ubc1</i>	gene encoding the regulatory subunits of protein kinase A in <i>U. maydis</i>
Ubc::6xHIS	6xHistidine-tagged version of Ubc protein for purification over Nickel columns
URL	<u>u</u> niform <u>r</u> esource <u>l</u> ocator
$\psi$ -BLAST	<u>P</u> osition- <u>S</u> pecific- <u>I</u> terated (PSI = $\psi$ ) BLAST

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## 1. Introduction

### 1.1 Literature review

#### 1.1.1 The dimorphic basidiomycete *Ustilago maydis*

The basidiomycete fungal pathogen *Ustilago maydis* has emerged as a valuable organism for the study of fungal morphogenesis and dimorphic switching. *U. maydis* is an ideal organism for this type of research because of its relatively rapid growth rate, its simple growth requirements, its benign nature towards researchers and its ease of genetic manipulation. Genes for morphogenesis that are identified in *U. maydis* will likely be informative for other fungal systems due to the high levels of conservation among components of signaling pathways, especially among basidiomycete fungi (e.g., *U. maydis* and the human pathogen *Cryptococcus neoformans*).

*U. maydis* requires infection of the monocotyledon *Zea mays* (corn) for completion of the sexual phase of the life cycle (Banuett, 1995; Christensen, 1963). The complex life cycle of *U. maydis* includes saprophytic and parasitic stages, and infection of host tissues is essential for the formation of sexual spores (Figure 1). Saprophytic haploids (N) of compatible mating type are capable of cell fusion to form a filamentous and infectious dikaryon (N+N). Alternatively, under conditions of stress or nutrient starvation, the cells can form chains

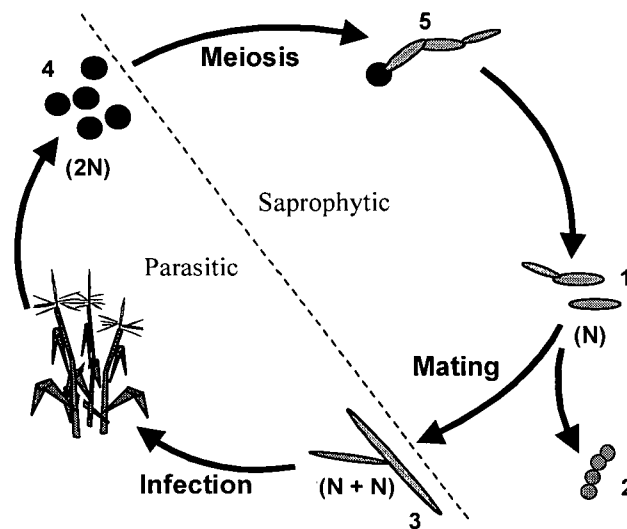


Figure 1. The complex life cycle of *Ustilago maydis*. 1. Haploid budding cells; 2. Chlamydospores; 3. Filamentous, infectious dikaryon; 4. Dormant, diploid teliospores; 5. Germinating teliospores.

of rounded, desiccation-resistant, haploid chlamydospores (Kusch and Schauz, 1989). The parasitic dikaryon establishes itself in the host and initiates tumor formation; this cell type cannot survive outside of the host plant. Following invasion of host tissues and completion of the proliferative stage of infection, the dikaryon undergoes karyogamy (nuclear fusion) and additional morphological changes that result in formation of the highly melanized, dormant, diploid teliospores ( $2N$ ) (Christensen, 1963; Holliday, 1974). The accumulation of teliospores within tumors in the plant produces the hallmark black, sooty ‘smut’ stage of the disease. Completion of the life cycle is signaled by teliospore release from ruptured tumors; these spores subsequently germinate and undergo meiosis to generate haploid sporidia. The ability of *U. maydis* to infect the host plant is tightly correlated with its ability to switch from a budding to a filamentous phenotype – as occurs during formation of the dikaryotic cell type. This switch is

currently known to be controlled by two mechanisms: a mating response pathway and a cAMP/protein kinase A (PKA) pathway.

#### 1.1.1.1 Sexual regulation of the *U. maydis* dimorphic switch

Mating compatibility between haploid cells is determined by possession of non-identical alleles at the *a* and *b* loci. The *a* locus carries genes responsible for the production of pheromones and pheromone receptors (Bolker *et al.*, 1992; Spellig *et al.*, 1994). The products of the *a* locus are necessary for events which occur prior to fusion between haploids, such as recognition of compatible mating types and subsequent conjugation tube extension (Snetselaar *et al.*, 1996; Spellig and Reichmann, 1994). The *b* locus encodes two proteins named bE and bW (b East and b West) that associate with one another and form a novel regulatory protein termed the bE/bW heterodimer (Gillissen *et al.*, 1992). Because bE and bW must originate from different *b* loci for dikaryon formation, conjugation between haploid cells of different mating-type is essential for dikaryon establishment. The bE/bW heterodimer initiates events that result in formation of the dikaryon, and is necessary for maintaining filamentous growth during the pathogenic portion of the life cycle (Gillissen *et al.*, 1992).

The *U. maydis* pheromone response pathway governs gene transcription through a MAP kinase signaling cascade which activates the pheromone response factor Prf1p. This HMG-box transcription factor regulates the transcription of genes at the *a* and *b* mating-type loci. A MAPKK and a MAPK have been identified, and these enzymes are encoded by the genes *fuz7*, and *ubc3*, respectively (Figure 2) (Banuett and Herskowitz, 1996; Mayorga and Gold, 1999). Deletion of either *fuz7* or *ubc3* affects morphological switching and pheromone response-related gene induction. These processes require Prf1p. Epistasis experiments suggest that Fuz7p and Ubc3p may act in different MAP kinase cascades, but additional investigation is still required. *ubc3* mutants do not demonstrate mating assay interactions (filament formation in response to

compatible haploid cells) suggesting that they are compromised for dikaryon formation. However, the pathogenicity of these mutants is not abolished. Prf1p contains MAP kinase phosphorylation sites and binds cis-acting DNA sequences to upregulate pheromone response genes (Hartmann *et al.*, 1996; Urban *et al.*, 1996). Elimination of the MAP kinase target residues in Prf1p eliminates upregulation of pheromone response genes upon mating (Muller *et al.*, 1999). Elevated levels of PKA in a *ubc3* mutant have a detrimental effect on pheromone-induced gene transcription. This result raises the possibility that the MAP kinase and cAMP pathways converge on Prf1p (Muller *et al.*, 1999).

#### 1.1.1.2 Ras1p appears to be involved in filamentous growth in *U. maydis*

Recent work in our laboratory has identified a role for *ras1* in the process of establishing filamentous growth, although it is still unclear how the protein functions in each of the known pathways. A dominant active *ras1* allele (*ras1*<sup>VAL19</sup>) is capable of causing mild filamentation in a high PKA background, but is not epistatic to mutations in the MAP kinase pathway. Cells carrying *fuz7*<sup>-</sup>, *ubc3*<sup>-</sup>, or *prf1*<sup>-</sup> mutations maintain their budding phenotype upon transformation with the dominant active *ras1* allele. Pathogenicity is abolished by deletion of *ras1*, indicating an essential role for this signaling molecule during the infectious cycle (N. Lee, *pers comm.*).

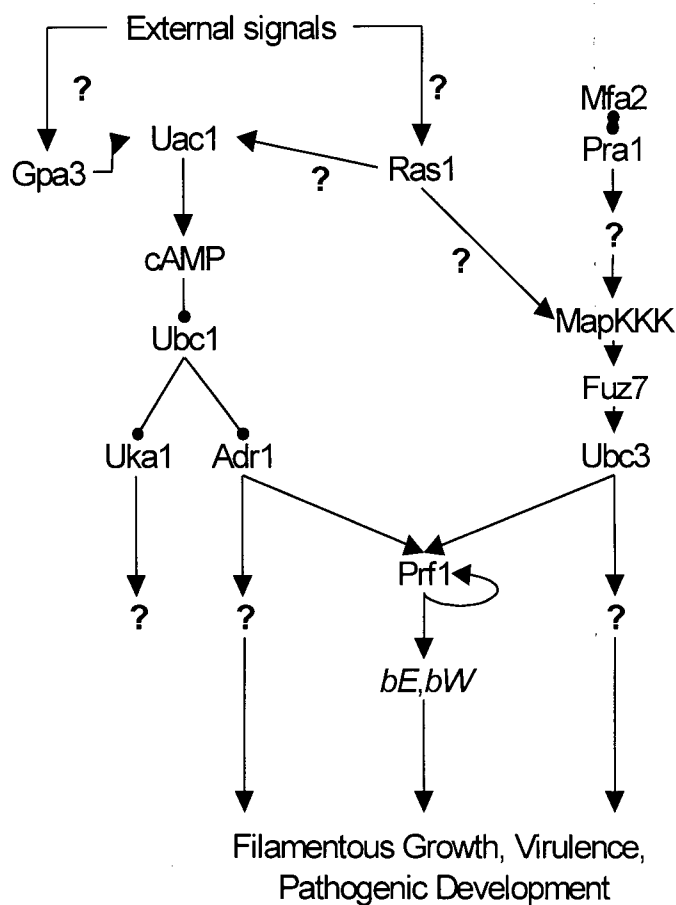


Figure 2. *U. maydis* signaling pathways involved in filamentous growth and virulence. The figure is a combination of models proposed by Dürrenberger *et al.* (1998) and Kahmann *et al.* (2000). Question marks represent areas of uncertainty or areas currently under investigation. The involvement of the Ras-like protein has been established by N. Lee (*pers comm.*) See the accompanying text for gene names.

#### 1.1.1.3 cAMP/PKA signaling participates in *U. maydis* dimorphism

The cAMP/PKA pathway plays key signaling roles in many different fungi. Often PKA signaling has regulatory effects on the development of morphological structures or other events required for virulence. Organisms often have more than one gene encoding the PKA catalytic subunit. For example, the yeast *Saccharomyces cerevisiae* has three genes and the highly homologous subunits were once thought to act redundantly. This has been disproved by recent



work described below. PKA exists as a complex of catalytic and regulatory subunits and these subunits dissociate upon binding of cAMP to the regulatory protein. The regulatory subunit inhibits the protein kinase activity of the catalytic subunits and hypomorphic mutations in the regulatory subunit will increase the activity of PKA. In contrast, mutations in adenylyl cyclase or an activator of adenylyl cyclase (often a G-protein  $\alpha$  subunit or Ras protein) will greatly decrease PKA activity.

The *U. maydis* G-protein  $\alpha$  subunit Gpa3p signals through the cAMP pathway and (among other events) is involved in upregulation of expression of the pheromone-encoding gene *mfal* (Kruger *et al.*, 1998). Strains carrying constitutively active Gpa3p have a yeast-like colony phenotype similar to strains missing the regulatory subunit of PKA. Mutants carrying disrupted versions of *gpa3* display filamentous growth in a haploid background similar to cells deficient in adenylyl cyclase, and are also non-pathogenic upon mating (Kruger *et al.*, 1998). These findings provide further support for the involvement of the PKA pathway in filamentous growth and virulence.

The cAMP-dependent PKA pathway was discovered in *U. maydis* during studies on the connection between cellular morphology and virulence (Barrett *et al.*, 1993). Because the infectious dikaryon is a filamentous cell type, mutants which were either defective, or constitutive, for filamentous growth were identified and investigated. *Ustilago* adenylyl cyclase or *uac1* was identified as a gene necessary for normal budding growth and pathogenicity (Barrett *et al.*, 1993). The filamentous phenotype of adenylyl cyclase mutants can be suppressed by the addition of 6mM exogenous cAMP to culture medium (Gold *et al.*, 1994). Complementation of a *uac1* suppressor mutation by Gold *et al.* (1994) recovered the gene encoding the regulatory subunit of cAMP-dependent protein kinase, or *ubc1* (*Ustilago* *bypass* cyclase). *ubc1* mutants presumably have high PKA activity, grow with a multiple-budding

morphology, and are unable to form the filamentous cell type during laboratory mating assays (Dürrenberger *et al.*, 1998; Gold *et al.*, 1994). Surprisingly, compatible haploids carrying mutations in *ubc1* are still pathogenic (with highly attenuated symptoms) despite their apparent inability to form filaments as haploids (Gold *et al.*, 1994). These findings implicate cAMP pathway signaling in events besides filament formation during pathogenesis. Dürrenberger *et al.* (1998) later identified the *U. maydis* PKA catalytic subunit Adr1p as being essential for pathogenesis.

The gene encoding the catalytic subunit of PKA (*adr1*) was first identified by Orth *et al.* (1994) in a mutant strain demonstrating resistance to the dicarboximide fungicide vinclozolin. Later work by Dürrenberger *et al.* (1998) revealed that strains defective for *adr1* are constitutively filamentous, but (surprisingly) non-pathogenic in the presence of *adr1*<sup>-</sup> compatible mating partners. The filamentous phenotype of *adr1*<sup>-</sup> mutants complicates mating interaction assays, which normally result in filamentous mating colonies when compatible wild-type haploids are co-cultured on culture medium. Therefore it is difficult to determine whether *adr1*<sup>-</sup> mutants are unable to mate, or, alternatively, are compromised during the proliferative stage of infection within the host. Conceivably, compatible *adr1*<sup>-</sup> haploids cannot form the dikaryon, and are therefore unable to form the bE/bW heterodimer. However, diploid strains carrying homozygous *adr1*<sup>-</sup> deletions (which do not require mating interactions to form bE/bW) are also unable to infect plants (Dürrenberger *et al.*, 1998). There are likely other hurdles encountered by *U. maydis* during infection that can not be overcome without functional PKA.

In an effort to identify pathway components downstream of PKA and involved in *U. maydis* morphogenesis, a suppressor mutant was recovered which displayed a budding phenotype in the *adr1*<sup>-</sup> background (Figure 3). Complementation of the mutant recovered *hgl1*,

a previously unidentified gene containing no significant similarity to sequences in existing

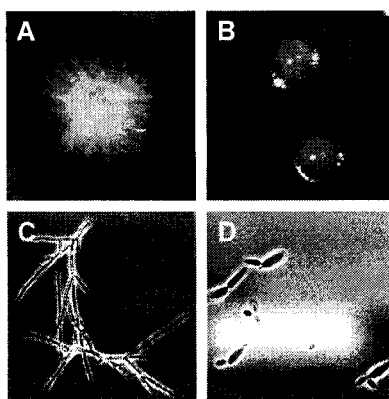


Figure 3. Mutations in *hgl1* suppress the filamentous phenotype of *adr1*<sup>-</sup> mutants. Panel A represents an *adr1*<sup>-</sup> mutant colony at 40x, panel C presents the corresponding filamentous cell type of *adr1*<sup>-</sup> mutant cells. Panel B shows colonies from an *adr1*<sup>-</sup> strain that also carries a mutation in *hgl1*, and panel D represents the corresponding cell type to the *adr1*<sup>-</sup>/*hgl1*<sup>-</sup> colonies of panel B. Photos courtesy Franz Dürrenberger.

databases. Recent work from our laboratory reveals that Hgl1p functions during events after cell fusion between haploids that are necessary for successful teliospore formation (unpublished data). *hgl1*<sup>-</sup> mutants cause normal symptoms in the host such as anthocyanin production and the formation of tumors, but do not form teliospores and as such are compromised in their ability to complete the sexual phase of the life cycle (Figure 4). The involvement of *hgl1* in the mating



Figure 4. The *hgl1* gene affects teliospore formation. Panel A demonstrates ears of corn infected with either compatible wild type haploids (right), or compatible haploids carrying disruptions in the *hgl1* gene. Panel B demonstrates a tissue cross-section from a kernel infected with *hgl1*<sup>-</sup> strains. Panel C represents a tissue cross-section from a kernel infected with compatible wild-type strains of *U. maydis*. Black bars measure 20μm, panels B and C photos courtesy Franz Dürrenberger.

response (i.e., in the sexual phase of the life cycle and mating assays), as well as its involvement in the PKA pathway (as a suppressor of the *adr1*<sup>-</sup> phenotype), suggests that it might act as a mediator between the two pathways.

This thesis presents additional studies on the *hgl1* gene and its product. To understand the potential activity of Hgl1p as a target of PKA, it is useful to review the organization of the

cAMP/PKA pathway in the well characterized yeast *Saccharomyces cerevisiae*. Where appropriate, the following description of signaling in yeast will point out parallels with cAMP signaling in *U. maydis*.

### 1.1.2 Pseudohyphal growth and mating in *Saccharomyces cerevisiae*

As a model organism, the budding yeast *Saccharomyces cerevisiae* illustrates both the complexity and the conservation of components in signal transduction pathways. Morphogenesis in *S. cerevisiae* is controlled by components of the mitogen activated protein kinase pathway (MAP kinase pathway) and the cAMP/PKA pathway. The MAP kinase pathway responds to signals sent through pheromone and environmental (osmotic pressure, ammonium) sensors and regulates cell elongation and invasion (Pan and Heitman, 1999). The cAMP/PKA pathway responds specifically to nutrient conditions in the surrounding environment. Current studies suggest that a convergence of the MAP kinase and cAMP/PKA pathways coordinately regulate the morphological response. As described above there is evidence for similar convergent pathway control of morphogenesis in *U. maydis*. An examination of signaling in *S. cerevisiae* provides a framework for understanding the components expected in other fungi. An evaluation of the MAP kinase and cAMP signaling pathways in *S. cerevisiae* is presented in the following text; links between the two transduction cascades and their similarities to the *Ustilago* system are highlighted where possible.

MAP kinase signaling in *S. cerevisiae* can be stimulated by osmotic stress, pheromones, cell wall damage, and nutrient starvation (Banuett, 1998; Gustin *et al.*, 1998; Herskowitz *et al.*, 1995). Signals from the different stimuli are transduced by conserved components, yet can result in the activation of specific molecules for each pathway that cause the morphological responses. MAP kinase activation during pheromone response and pseudohyphal growth will be presented here. The conservation of components shared between the two pathways suggests

there must be mechanisms to prevent 'crosstalk', or inappropriate signaling through shared effector molecules. The current hypothesis about the sorting of different signals postulates that 'scaffold' proteins associate with the specific components necessary for alternative signaling outcomes (Madden and Snyder, 1998; Madhani and Fink, 1998). The final outcomes of signaling are dictated largely by the specific MAP kinase molecules activated at the end of the cascade, and its target proteins. The outcome of signaling through the MAP kinase pathway for pheromone response is to regulate genes necessary for cell fusion and to arrest the cell cycle in preparation for mating. These combined processes culminate in 'schmoo' formation (Banuett, 1998; Dolan *et al.*, 1989; Errede and Ammerer, 1989; Miyajima *et al.*, 1987; Whiteway *et al.*, 1989).

#### 1.1.2.1 Pheromone-response mediated filamentation in *S. cerevisiae*

The pheromone response MAP kinase pathway is initiated by the binding of pheromones to the pheromone receptors encoded by *STE2/STE3* (Figure 5). The  $\beta\gamma$  subunits of the heterotrimeric G-protein (encoded by *STE4*, and *STE18*) transmit signal from Ste2p and Ste3p to the immediate downstream components Cdc42p, Ste20p and Ste5p (Akada *et al.*, 1996; Hirschman *et al.*, 1997; Leberer *et al.*, 1997b; Madhani and Fink, 1997; Miyajima *et al.*, 1987; Mosch *et al.*, 1996). Pheromone signal is transduced by Ste20p (p21 activated kinase) to the MAP kinase cascade encoded by *STE5* (scaffold protein) *STE11* (MAPKKK), *STE7* (MAPKK) and *FUS3* (MAPK) (Leberer *et al.*, 1997a). The *U. maydis* MAP kinase cascade components Fuz7p (MAPKK) and Ubc3p (MAPK) are homologs of Ste7p and Fus3p respectively. Additional upstream and downstream components will likely be identified in *U. maydis* that are similar to many of the components of the *S. cerevisiae* system. The Ste5p scaffold protein interacts with many cascade proteins bringing separate components of the cascade into close association to transduce signal (Akada *et al.*, 1996).  $G\alpha$  mutations (*gpa1*) resulting in free  $\beta\gamma$

subunit are dependent on Ste20p and Ste5p to constitutively activate the MAP kinase cascade for pheromone response (Hirschman *et al.*, 1997). Cdc42p involvement is not essential for pheromone-initiated signal transduction, but may be relevant for component localization to the site of morphogenesis because Cdc42p interacts with the actin cytoskeleton and other structural proteins (Akada *et al.*, 1996; Leberer *et al.*, 1997b; Leeuw *et al.*, 1995). Syg1p and Akr1p, two

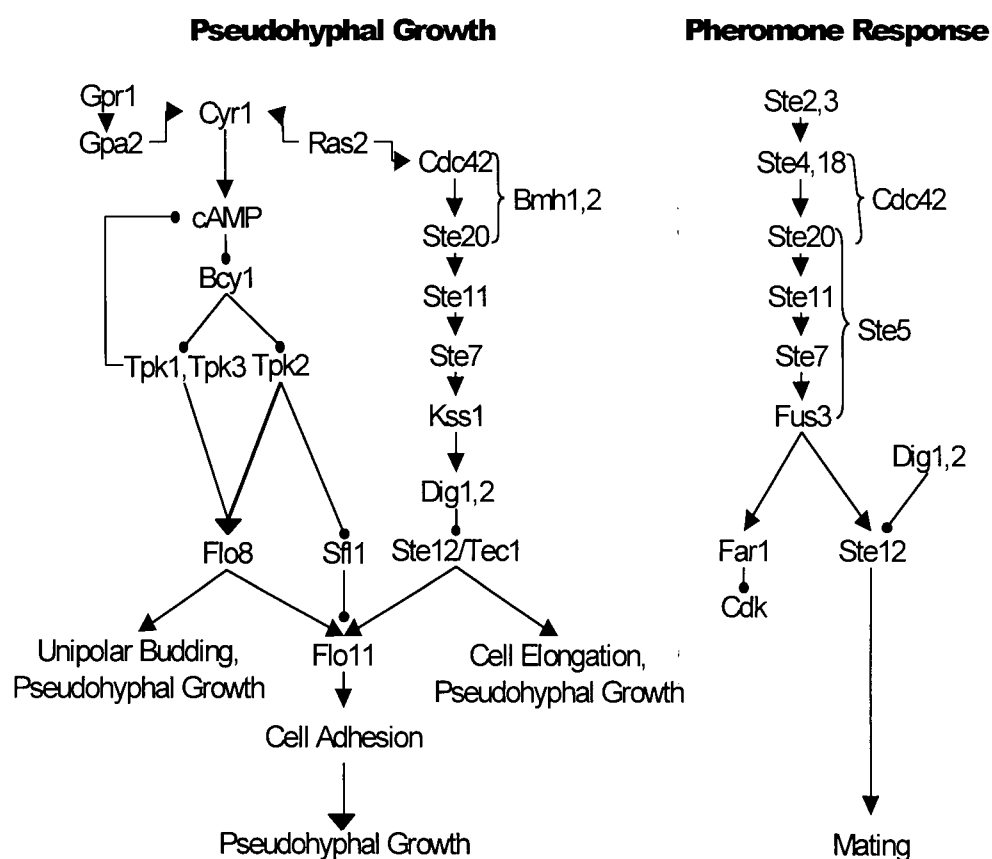


Figure 5. *S. cerevisiae* Signaling Pathways Involved in Pseudohyphal Growth. Compiled from the pathway models presented by Pan and Heitman 1999, Fink *et al.* (1999), Robertson and Fink (1999), Banuett (1998), and Leberer, Thomas and Whiteway (1997). See the accompanying text for definitions of the gene names.

proteins of unknown function in the MAP kinase pathway (not shown), also associate with the G-protein  $\beta\gamma$  subunit. Mutations in Syg1p cause no discernable phenotype, whereas mutations in Ark1p inhibit MAP kinase signaling as well as suppress the *gal* mutant phenotype, indicating Ark1p may also play an additional role in the cAMP pathway (Kao *et al.*, 1996; Pryciak and Hartwell, 1996).

The regulation of transcription factors is a major role of MAP kinase signaling. The Ste11p/Ste7p kinases activate Fus3p, the MAP kinase responsible for activating downstream proteins during the pheromone response. Fus3p phosphorylates the transcription factor Ste12p, which then binds a DNA pheromone response element (PRE) to regulate growth morphology and mating interactions (Elion *et al.*, 1993; Hung *et al.*, 1997; Kronstad *et al.*, 1987). Ste12p can be phosphorylated by both Fus3p and the MAP kinase Kss1p of the pseudohyphal MAP cascade, but only Kss1p can phosphorylate Dig1p and Dig2p, the negative regulators of Ste12p (Cook *et al.*, 1996; Tedford *et al.*, 1997). These subtleties in MAP kinase specificity likely account for some of the differences between the pheromone-induced and pseudohyphal response growth morphologies.

The cyclin-dependent kinase inhibitor Far1p, which is also a target of Fus3p phosphorylation, regulates cell cycle arrest during the mating response. Far1p interacts with the SH3 domain protein Bem1p, and therefore may also function in localizing cascade components (Lew and Reed, 1995). Bem1p independently associates with Ste5p, Ste20p, Cdc24p (not shown), Cdc42p, and actin, and participates in polarized growth during mating to localize signaling components to the shmoo tip (Leeuw *et al.*, 1995; Lyons *et al.*, 1996).

Protein localization studies related to signaling and morphogenesis are starting to be carried out in *U. maydis*, and a kinesin protein (Kin2p) related to Nkin of *N. crassa* has been identified as being required for filamentous growth and virulence (Lehmlier *et al.*, 1997). Kin2



mutants have greatly reduced aerial hyphae in *U. maydis* mating reactions and demonstrate reduced virulence compared to wild-type cells. It is unclear how these motor proteins function during filamentous growth, but they may play a role in localizing necessary components to the site of morphogenesis.

#### 1.1.2.2 The pseudohyphal growth response in *S. cerevisiae*

MAP kinase signaling for the control of pseudohyphal growth in *S. cerevisiae* utilizes many of the same components as the pheromone response pathway, yet is initiated by nitrogen starvation and results in elongated cells capable of invading solid media. The upstream initiators of the MAP kinase cascade during the pseudohyphal growth response are Ras2p, Cdc42p, and Ste20p (Figure 5) (Gimeno *et al.*, 1992; Mosch and Fink, 1997; Mosch *et al.*, 1996; Zhao *et al.*, 1995). The 14-3-3 proteins Bmh1p and Bmh2p interact with Cdc42p and Ste20, and are required for Ras2-dependent pathway activation (Leberer *et al.*, 1997b; Peter *et al.*, 1996). Bmh1p and Bmh2p may also function in the cAMP/PKA pathway to prevent glycogen accumulation (Roberts *et al.*, 1997). Signaling via the MAP kinase cascade for pseudohyphal growth results in the activation of the MAP kinase Kss1p, which ultimately activates the transcription factors Ste12p and Tec1p.

Tec1p forms heterodimers with Ste12p and these regulate *TEC1* expression, cell elongation, and the production of additional proteins such as the cell surface flocculin Flo11p (Baur *et al.*, 1997; Gavrias *et al.*, 1996; Lo and Dranginis, 1998; Madhani and Fink, 1997). Nitrogen availability also regulates *FLO11* expression in that a high abundance of nitrogen results in low *FLO11* transcript levels. Overexpression of *FLO11* can mimic nitrogen starvation conditions during growth on rich media and causes agar invasion by the cells. In contrast, deletion of *FLO11* can eliminate pseudohyphal growth and agar invasion. The complex

promoter region of *FLO11* is also responsive to the transcription factor Flo8p, an effector of the cAMP/PKA pathway; this finding represents an exciting connection between the two signaling pathways that will be discussed below (Rupp *et al.*, 1999).

### 1.1.2.3 cAMP/PKA signaling regulates pseudohyphal growth in *S. cerevisiae*

The *S. cerevisiae* cAMP/PKA pathway has a positive effect on pseudohyphal growth. Cells with elevated PKA activity grow as long chains of budding cells and invade solid media. Additionally, elevated PKA in *S. cerevisiae* results in loss of carbohydrate reserves, a failure to arrest in the G1 phase of the cell cycle, and increased sensitivity to stress (Tokiwa *et al.*, 1994). Conversely, low PKA levels produce unbudded cells arrested in G1; these cells accumulate carbohydrates and demonstrate increased resistance to stress (Thevelein and de Winde, 1999).

The glucose sensitive G-protein coupled receptor (Gpr1p) activates the G-protein  $\alpha$ -subunit Gpa2p to initiate the PKA pathway at the level of adenylyl cyclase (Cyr1p) (Figure 5) (Thevelein and de Winde, 1999; Xue *et al.*, 1998). Gpa2p is a group III G $\alpha$  subunit similar to Gpa3p of *U. maydis*, a relationship identified during phylogenetic analysis of fungal G-proteins by M. Bölker. Other members of this group are also G $\alpha$  subunits that stimulate adenylyl cyclase, such as Gpa2p of *S. pombe* (Bolker, 1998).

*S. cerevisiae* Gpa2p also activates the cAMP pathway in response to nitrogen starvation to cause pseudohyphal growth, and activated forms of Gpa2p initiate pseudohyphal growth in the absence of nitrogen starvation. Ammonium permease (Mep2p) mutants demonstrate a budding growth phenotype instead of pseudohyphal growth and this can be suppressed by cAMP addition or by mutations in the gene encoding the regulatory subunit of PKA (*BCY1*) (Lorenz and Heitman, 1998). Similarly, mutation of *RAS2* to the dominant active *RAS2*<sup>Val19</sup> allele causes cAMP production by adenylyl cyclase (*CYR1*) and activation of PKA (Field *et al.*,

1998; Toda *et al.*, 1985). Constitutive activation of Ras2p can restore pseudohyphal growth in *mep2* mutants, possibly due to Ras2-dependent activation of Cyr1p (Lorenz and Heitman, 1997). *ras* involvement in filamentous growth has been identified in *U. maydis* by Nancy Lee in our laboratory, and might be mediated by components of the PKA and MAP kinase pathways (*pers. comm.*). Overall, the *S. cerevisiae* data indicate that nitrogen sensing and pseudohyphal growth are mediated by the PKA pathway and involve *GPA2* and *RAS2*.

Activation of PKA by mutations in the gene (*BCY1*) encoding the PKA regulatory subunit in *S. cerevisiae* restores pseudohyphal growth in the absence of the transcription factors *STE12* and *TEC1*, implying that there are alternative pathways to MAP kinase activation of filamentous growth (Lorenz and Heitman, 1998; Pan and Heitman, 1999). There are three *S. cerevisiae* PKA catalytic subunits (encoded by *TPK1*, *TPK2*, and *TPK3*), which were at one time assumed to be redundant in function. However, despite their highly conserved C-termini (which encode the catalytic domains), Tpk1p, Tpk2p, and Tpk3p function differently upon activation. The C-terminal regions of the kinases determine target specificity while a function for the N-terminal regions remains unknown; the amino terminal region is not responsible for determining specificity (Pan and Heitman, 1999). Studies have shown that individual homozygous deletions of each *TPK* gene in diploids cause distinct phenotypes, whereas deletion of all three *TPK*'s is lethal. Tpk2p acts as an activator of filamentous growth and Tpk1p and Tpk3p appear to be inhibitory for filament formation. Mutations in *TPK2* are epistatic to mutations in *TPK1* or *TPK3* with regard to filamentation (Pan and Heitman, 1999). *TPK2* transcript levels are not controlled by Tpk1p and Tpk3p, which likely act either upstream or downstream of Tpk2p and may involve a feedback loop inhibiting cAMP production (Pan and Heitman, 1999). Unlike *tpk2* mutants, *tpk1* and *tpk3* mutants are still responsive to ammonium sulfate but exhibit a reduced pseudohyphal phenotype. *U. maydis* has two genes encoding PKA

catalytic subunits, *uka1* and *adr1*. Although *adr1* and *uka1* demonstrate high sequence similarity, the *uka1* allele does not greatly alter cell morphology or virulence when disrupted in the wild type background under the conditions tested (Dürrenberger *et al.*, 1998). The discovery of different functions for the three *S. cerevisiae* PKA genes suggests a role for the *uka1* gene of *U. maydis* may yet be revealed. Perhaps the function of *uka1* will become apparent as the *Ustilago* cAMP and MAP kinase pathways become better understood.

Several targets of Tpk2p have been identified, including the stress regulators Msn2p and Msn4p, the protein kinase Rim15p, the transcription factors Flo8p and Sfl1p, and the daughter cell-specific protein Ash1p. The transcription factors Msn2p and Msn4p are regulated by PKA-dependent phosphorylation, which prevents their nuclear localization (Gorner *et al.*, 1998; Marchler *et al.*, 1993; Smith *et al.*, 1998). Msn2p and Msn4p regulate stress response element (STRE) -controlled genes and are necessary for expression of the *YAK1* protein kinase, which is antagonistic to growth under low PKA conditions (Boy-Marcotte *et al.*, 1999; Martinez-Pastor *et al.*, 1996). The mechanism of Yak1p kinase-dependent growth suppression is not known, but may involve the suppressor of kinase gene, (*SOK2*) discussed later. The protein kinase Rim15p is negatively regulated by PKA phosphorylation, and is required for meiotic and STRE gene expression (Vidan and Mitchell, 1997). This result, combined with *rim15* mutant suppression of a *tpk1/2/3* triple mutant, indicates that some genes required for entry into G0 are inhibitory to growth (Reinders *et al.*, 1998). PKA regulation of Flo8p governs transcription of the gene encoding the cell surface flocculin Flo11p, the cell wall protein essential for pseudohyphal growth and agar invasion. Flo11p also participates in MAP kinase-regulated pseudohyphal growth. The dual regulation of *FLO11* occurs in its promoter region, which has recognition sites for both Flo8p and the Ste12p/Tec1p heterodimer (Rupp *et al.*, 1999). Over-expression of *FLO11* can suppress mutations in the transcription factors *FLO8* and *STE12*, which normally

abolish pseudohyphal growth (Lambrechts *et al.*, 1996; Liu *et al.*, 1996; Lo and Dranginis, 1998). Flo8p contains 5 PKA consensus sequences “[RK] (2)-x- [ST]” and *FLO8* transcript levels are not affected by PKA, suggesting a direct protein-protein interaction is necessary for regulation (Pan and Heitman, 1999). The helix-turn-helix transcription factor Sfl1p also has 5 PKA consensus sequences, and interacts with Tpk2p by yeast two-hybrid analysis (J. Heitman, *pers comm*). Mutations in *SFL1* are epistatic to mutations in *TPK2* and result in increased flocculation, invasive growth, and filamentation (Robertson and Fink, 1998). The daughter-cell specific transcription factor Ash1p acts as a downstream effector of the PKA pathway, and physically interacts with Tpk2p (J. Heitman *pers. comm.*). *ash1* mutants are not capable of growing by the pseudohyphal phenotype and are also incapable of invasive growth. Epistasis analysis supports Ash1p as part of the PKA pathway, and suggests that it is not involved in the MAP kinase pathway (Chandrarapaty and Errede, 1998). As described, the transcription factors Flo8p and Sfl1p each contain 5 PKA target sequences, and demonstrate genetic phenotypes in the PKA pathway of *S. cerevisiae*. By comparison, the Hgl1p protein of *U. maydis* contains 8 PKA target sequences, and demonstrates genetic involvement in processes controlled by the MAP kinase and PKA pathways. Indeed the Hgl1p protein may require phosphorylation by PKA to regulate budding growth in *U. maydis*.

The *SOK2* gene encodes a transcription factor required for *STRE* gene expression that may also act as an effector of cAMP-dependent PKA (Ward *et al.*, 1995). Mutations in *SOK2* produce a pseudohyphal phenotype, suggesting that it may act as a suppressor of filamentation (Ward *et al.*, 1995). The *SOK2* homologue *PHD1* does not induce pseudohyphal growth in diploids when overexpressed in the absence of nitrogen starvation, but is required for the pseudohyphal growth of *sok2* mutant strains. Diploids carrying *PHD1* homozygous deletions exhibit pseudohyphal growth indicating it is not the only effector in this mechanism.

The evolving understanding of regulation of mating and pseudohyphal growth in *S. cerevisiae* indicates that the responses are mediated by complex combinations of different pathways. Convergence of the pathways at succinct points does, however, represent an interesting trend in signal transduction, one that might be similar to signal transduction in *U. maydis*.

## 1.2 Research basis and objectives

Phenotypically, *hgl1* appears to participate in both MAP-kinase and PKA-regulated processes. Identification of the *hgl1* gene as a suppressor of the filamentous growth phenotype of an *adr1*<sup>-</sup> mutant indicates it functions as a part of the cAMP-dependent protein kinase pathway during filamentous growth and virulence. Additionally, involvement of *hgl1* in events governed by mating interactions such as the formation of teliospores near the end of the sexual phase of the life cycle, indicates either that PKA participates in these processes, or that *hgl1* is also involved in processes other than those under PKA control. Studies to examine Hgl1p participation in each of these processes would further develop our understanding of signal transduction and dimorphism in *U. maydis*.

The objective of this study was to investigate Hgl1p involvement in the *U. maydis* dimorphic switch. Three approaches were employed; 1. web-based bioinformatics analysis of the Hgl1p amino acid sequence; 2. PKA phosphorylation of the *hgl1* gene product; 3. Isolation of suppressors of *hgl1*<sup>-</sup> strains to identify genes located downstream of *hgl1* that may function in the dimorphic switch.

The development of web-based molecular biology tools has provided a medium by which gene and protein sequence data can be compared to databases to assist in the prediction of gene function. The premise is that high similarity between sequences may indicate homology and suggest that the proteins perform a similar function. The recovery of novel genes with low overall similarity to the databases presents an interesting situation. That is, the excitement of working on a novel sequence is balanced by the frustration of not finding a clue to the function of the protein. One approach to circumventing low overall similarity to other sequences is to look for conserved motifs (or domains) within the sequence of interest. The predicted amino acid sequence of *hgl1* did not share high similarity to sequences in existing databases under

normal search conditions. When low complexity regions were allowed during BLAST searches, Hgl1p demonstrated a very weak similarity to two sequences, a previously unidentified ORF from the *Candida albicans* genome sequencing project (named con4 2801), and the *S. cerevisiae* cell surface flocculin Flo11p. Comparison of the *S. cerevisiae* and *C. albicans* sequences with one another revealed that the sequences shared a high degree of similarity. Searching domain databases with the Hgl1p amino acid sequence identified several putative functional motifs in the protein, and led to the phosphorylation experiments discussed in this thesis.

The constitutive budding phenotype of strains containing increased levels of PKA (*ubc1<sup>-</sup>* mutants) and the constitutive filamentous phenotype of strains defective for PKA (*adr1<sup>-</sup>* mutants) suggests that PKA activity is responsible for controlling cellular morphology. Increased PKA activity may be responsible for either promoting budding growth, or inhibiting filamentous growth. Adr1p has previously been demonstrated as the catalytic subunit responsible for the majority of PKA activity in *U. maydis* cells (Dürrenberger *et al.*, 1998). An examination of the Hgl1p predicted amino acid sequence suggests there may be as many as 8 PKA phosphorylation sites. Suppression of the *adr1<sup>-</sup>* filamentous phenotype by mutation in *hgl1* suggested that Hgl1p protein was responsible for preventing budding growth in the absence of PKA activity, and may allow budding growth in the presence of high PKA activity. Is Hgl1p a direct target of PKA? To demonstrate an interaction between Hgl1p and PKA, Hgl1p was used in phosphorylation experiments with *U. maydis* and bovine heart PKA.

The cAMP/PKA pathway can be dissected with suppressor mutagenesis to recover genes involved in the morphological change from budding to filamentous growth in *U. maydis* (see Gold *et al.*, 1999 for example). Colony growth morphology is a useful screen for components involved in the pathway, and is effected by mutations in *uac1*, *ubc1*, *adr1* and *hgl1*. To further characterize the cAMP/PKA pathway, a suppressor analysis of filamentous mutants in the



budding *hgl1*<sup>-</sup> background was undertaken. Two mutagenesis experiments were conducted, one in a mutant carrying a single *hgl1*<sup>-</sup> disruption, and the second in an *adr1*<sup>-</sup>*hgl1*<sup>-</sup> double disruption background.

The work in this thesis demonstrates that Hgl1p served as a direct target of *Ustilago maydis* protein kinase A phosphorylation *in vitro*. In addition, suppressor mutants which reverted the *hgl1*<sup>-</sup> phenotype to the *adr1*<sup>-</sup> morphology were obtained, suggesting that Hgl1p was not required for filamentous growth, and may act to inhibit budding. These results also suggest that downstream targets of Adr1p and Hgl1p remain to be identified. Analysis of the Hgl1p amino acid sequence with web-based bioinformatics tools revealed several interesting features of the protein; these will be relevant for future experimentation.

## 2. Materials and methods

### 2.1.1 Strains and media

*U. maydis* strain 001 (*a2b2*) was obtained from R. Holliday (Commonwealth Scientific and Industrial Research Organization, Laboratory of Molecular Biology, Sydney Australia). All *U. maydis* strains were derived from the 001 wild-type strain. Media for growing *U. maydis* cells was PDB or PDA (potato dextrose broth or agar), CM, DCM, DCMS, (complete medium, double complete medium, double complete medium + 0.8 M sorbitol; (Holliday, 1974)). The medium for screening mutants was DCM + 1% activated charcoal (Day and Anagnostakis, 1971). *E. coli* strain DH5 $\alpha$  (Bethesda Research Laboratories) was used for cloning procedures, and was grown in LB (Luria Bertani) medium (Sambrook *et al.*, 1989). *E. coli* strain BL21( $\lambda$ DE3) was used for protein expression experiments.

### 2.1.2 DNA manipulations

Small scale plasmid preparations were accomplished with standard alkaline lysis in SDS as described in Sambrook *et al.* (1989). Large scale plasmid preparations for use in sequencing reactions, protein expression experiments, or transformations of *U. maydis* was prepared with a Qiagen midi kit (Hilden, Germany). Transformation of *U. maydis* were accomplished as described by Wang *et al.* (1989) and disruption of the *hgl1* gene was confirmed by standard genomic southern hybridization analysis (Sambrook *et al.*, 1989). The DNA fragment used for hybridization of the southern blot was isolated from an agarose gel and purified from the agarose with a Gene Clean kit from Bio101 (Vista, CA).

### 2.1.3 Hgl1p sequence analysis

The Hgl1p sequence used for database analysis was the predicted amino acid sequence from the open reading frame determined to be necessary for complementation of a mutant defective in *hgl1* (Figure 6). The sub-cloned coding region for the peptide sequence in figure 6 is capable of reverting an *adr1<sup>-</sup>hgl1<sup>-</sup>* mutant (budding growth phenotype) to the *adr1<sup>-</sup>* filamentous phenotype (data not shown).

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1  MFVATRRPSPPTSSPAVSRSSWVDLPVASAPSSRRGSRVDLSIQTSIANTSVGSDSHAG 60
61  GSHTKLAINQLLTNLSHVSQEARMSSPRSSHSPALNDSPALKRKFEADSDSTAYKVAS 120
121 AHEIHLPTDPVRRASIINLATAAAAVLQSQAGQRRMSNFSDEQKRQRVEQLGSLIEQA 180
181 RKASVTSMSHDMSRHVEAAQQNVAIATVLANHLGLTPQSSAASTPSAGTPAPASPTKPYA 240
241 PSTLAIPQTPPTPRSPLSAAPLTASQISDHQSATQAVAGPSARATNDQAEFSRPKPLVAS 300
301 PPRTPSITLNYSTDSVEPSSSSKLISEPLRASPTSQALFNEAKEAAQTYSRFYRFEKEW 360
361 AQKALELERRRSSIRIDPLFNPNPSPSLPADPNSNSNLNSSPTHNREGTAPISPPQHS 420
421 PESRIMSRRHSPFCDSPVAPSARMSNSGSSGSFSSTHRASSSGLANVLSNFAELIEHRQR 480
481 SCSGLEALAKQAKELPVKRLSQPNPQFRITFGDFSWAKAPPSGTDVNPSAPPLRATPHA 540
541 SRDANTDADRSSVDKHTASASTSTVGVRSTHTLPNAQSTRRVSATAPDSADADANTTA 600
601 TARSTMSIASML 612

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Figure 6. Amino Acid sequence of Hgl1p used in bioinformatics sequence analysis.

### 2.1.4 Web-based algorithms and web-sites used in the analysis of the *hgl1* predicted amino acid sequence

All of the web-based research tools used in the analysis of *hgl1*'s predicted amino acid sequence can be found at one of the following URL's: NCBI, <http://www.ncbi.nlm.nih.gov>; R.E.W. Hancock's links page, <http://cmdr.ubc.ca/bobh/links.htm>; Pedro's biomolecular research tools, [http://www.public.iastates.edu/~pedro/research\\_tools.html](http://www.public.iastates.edu/~pedro/research_tools.html); Stanford Universities domains search algorithms, <http://dna.stanford.edu/projects.html>; TIGR, <http://www.tigr.org>. The sequence in figure 6 was used in searches employing the BLAST, Gapped BLAST and PSI-BLAST algorithms at NCBI and TIGR against all of the available genomic databases as of 10/03/00 (Altschul *et al.*, 1997). In addition to similarity searches based on the complete amino

acid sequence, searches for conserved domains were completed with BLOCKS (Pedro's URL), eMOTIF (Stanford URL), PAT-SCAN (Pedro's URL), Pfam (Hancock URL), PRODOM (Pedro's URL), PROPSEARCH (Pedro's URL), PROSITE (Pedro's URL), ProfileScan (Hancock URL), PRINTS (Hancock URL), SBASE (Pedro's URL), and SMART (Hancock URL) (Bucher and Bairoch, 1994; Henikoff and Henikoff, 1994; Hofmann *et al.*, 1999; Schultz *et al.*, 1998; Wright *et al.*, 1999). For algorithms that filter out low complexity sequences, two searches were completed, the primary search employed the supplied filters while the secondary search was completed with all filters removed. Non-filtered searches increase the sensitivity of the comparisons, but have the limitation of retrieving sequences that do not demonstrate significant complexity to be of relevance.

### 2.2.1 Construction of the Hgl1p expression construct pETHgl

A 1.8 kb PCR fragment encoding the *hgl1* open reading frame was amplified with the primers; HG51, GCGGATCCACCATGTTTCGTCGCTACGCGCA; and HGJ31, GTGCGAAGCTTGCATGCTAGC. The *Bam*HI restriction endonuclease site of HG51 and the *Hind*III site in HGJ31 are underlined. PCR reactions were conducted with pB105 as template (Figure 7) (pB105 contains the coding region of *hgl1*). The 1.8 kb PCR product was digested with *Bam*HI and *Hind*III and inserted into *Bam*HI /*Hind*III digested pET21a (Figure 7) for recombinant expression experiments in *E. coli* strain BL21(λDE3) and in the Promega (Madison WI) TNT<sup>®</sup> wheat germ extract expression system. Successful cloning of the PCR

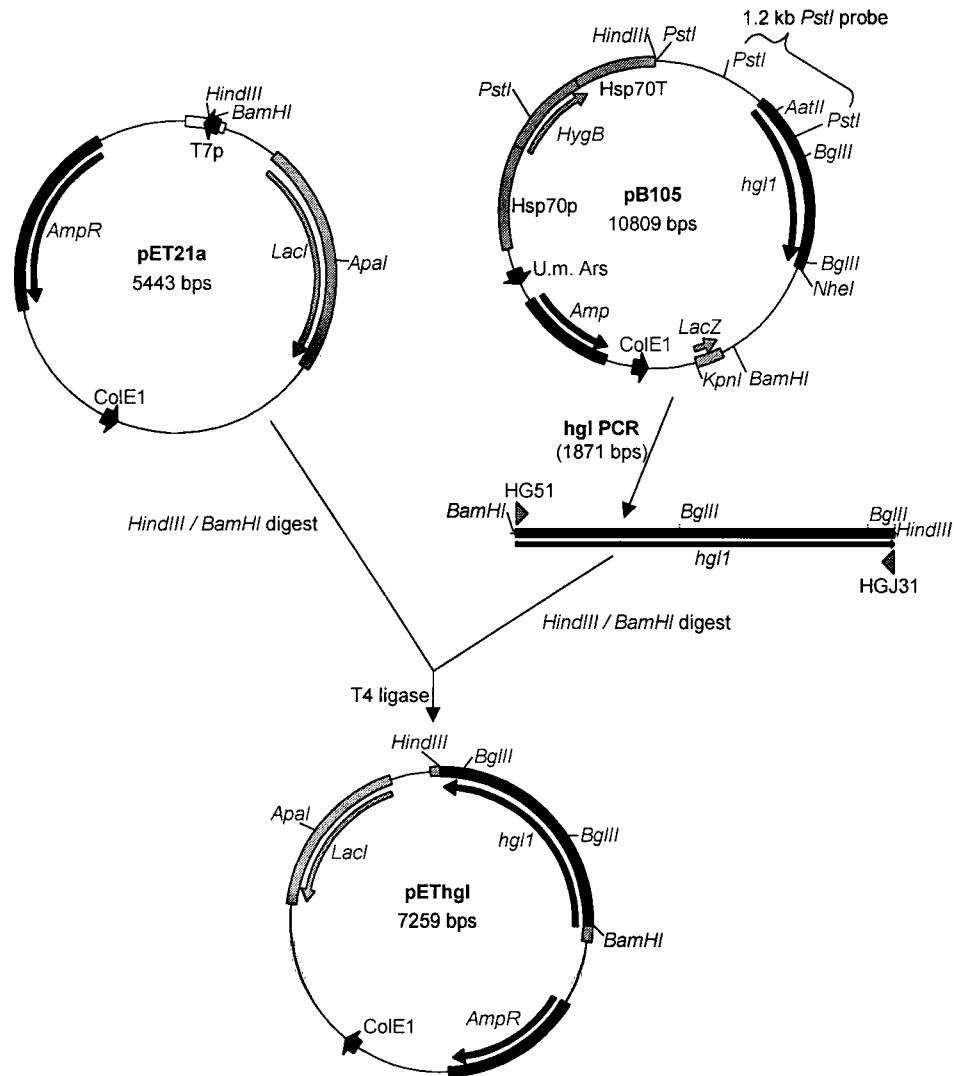


Figure 7. Construction of the plasmid pETHgl. The PCR primers HG51 and HGJ31 were used to amplify the 1.8 kb *hglI* coding region from pB105. This fragment was subsequently directionally cloned into the *HindIII*/*BamHI* sites of pET21a. The 1.2 kb *PstI* fragment of pB105 was used during southern hybridization analysis of *natI* disrupted *hglI*<sup>-</sup> strains.

fragment was confirmed by *HindIII*/*BamHI* and *AatII* digestion of the clones. One plasmid that carried the 1.8 kb fragment in the correct orientation was chosen and analyzed by nucleotide sequencing reactions to ensure proper in-frame ligation of the PCR product into the vector (data not shown). This plasmid was named pETHgl (Figure 7).

### 2.2.2 *In vitro* expression of Hgl1p from the plasmid pETHgl

Hgl1p was produced *in vitro* from pETHgl with a TNT<sup>®</sup> coupled wheat germ extract system according to the manufacturer's instructions (Promega Madison, WI). pETHgl was linearized at the unique *ApaI* site in the *LacI* region of pET21a (Figure 7), precipitated with isopropanol, washed with 70% ethanol in diethylpolycarbonate (DEPC) treated sdH<sub>2</sub>O, and resuspended in DEPC -treated sdH<sub>2</sub>O to a final concentration of 0.5 µg/µl prior to use in the translation reactions. For each set of kinase experiments, a single translation reaction was performed and sub-divided into equal volumes for subsequent treatments. Hgl1p protein was labeled with <sup>35</sup>S-methionine during the translation reaction. A negative control TNT<sup>®</sup> reaction was performed with the pET21a vector; no proteins were produced (data not shown).

### 2.2.3 Production of recombinant Uka1p and Adr1p

Attempts were made to use the TNT<sup>®</sup> transcription / translation system to produce the PKA catalytic subunits Uka1p and Adr1p with Hgl1p for use in Hgl1p phosphorylation experiments. For Promega peptag<sup>®</sup> kinase activity experiments, the plasmids pET21b::adr#31 and pET21b::uka#20 (F. Dürrenberger unpublished) were linearized with *ApaI* and TNT<sup>®</sup> transcribed/translated as per manufacturers instructions. During dual expression experiments with pETHgl and pET21b::adr#31 or pET21b::uka#20, protocols specific for translating two plasmids in the same reaction mix were attempted. Overnight 9% SDS-polyacrylamide gels of the reaction products were electrophoresed at 100 Volts and were removed from the apparatus before drying under vacuum and examination by autoradiography.

#### 2.2.4 Ni-Agarose Ubc::6xHIS affinity purification of PKA from *Ustilago maydis*

*Ustilago* PKA was prepared as described by Dürrenberger *et al.* (1998) with the following modification: kinase was eluted from Ni-Agarose columns with 300 mM imidazole. Kinase activity of the elution fraction was determined visually with the Promega peptag<sup>®</sup> kinase assay for a dilution series of the recovered sample. Equivalent activities of bovine and *Ustilago* protein kinase A catalytic subunits were added based on visual inspection of a peptag<sup>®</sup> kinase assay using 0.04 units of bovine heart protein kinase A. Units of PKA activity describe the number of moles of phosphate transferred per minute to substrate. A 1000 fold excess of PKI (1  $\mu$ M) was used during PKA inhibition experiments to eliminate phosphorylation. 100 units of lambda phosphatase were used for phosphate removal (as per manufacturer's instructions NEB, Mississauga, ON). Kinase reactions were performed as per standard reaction protocols outlined in the Promega kinase assay literature. Control reactions were performed with bovine PKA supplied by the manufacturer.

#### 2.3.1 Disruption of *hglI* with the nourseothricin resistance marker *natI*

Disruption of *hglI* with a nourseothricin (*natI*) resistance marker was accomplished with the 2.0 kb *BglII*-*XbaI* fragment of pUGZ4 inserted into the *BglII* site of pHB101. The *phgl::nat101* was linearized with *HindIII*/*BamHI* and the 5.0 kb fragment was agarose gel purified prior to transformation into *U. maydis* strain 001. The transformation reaction was plated onto DCMS containing 40  $\mu$ g/ml nourseothricin. Colonies demonstrating typical *hglI*<sup>-</sup> phenotypes were screened by southern hybridization analysis using *BssHII* digested genomic DNA preparations. The southern blot was hybridized with the 1.2 kb *PstI* fragment of the plasmid pB105 (Figure 7).

### 2.3.2 Suppressor mutagenesis of strains 3020 and 3034

*U. maydis* strains used for mutagenesis were grown for 24 hours on PDA plates prior to inoculation into 5.0 mls of PDB and incubation overnight at 30°C. The cells in the overnight cultures were recovered by centrifugation and washed once in sdH<sub>2</sub>O before re-suspension in sdH<sub>2</sub>O. The cells were diluted to  $1 \times 10^8$  cells/ml in a final volume of 1 ml and subject to ultra-violet (U.V.) irradiation in an uncovered small petri dish. Exposure time was adjusted to accomplish a 90% reduction in the viable cell count (approximately 2 mins 30 seconds based on plate count assays). Following irradiation and removal from the U.V. light source, the petri-dish lid was replaced and the dish wrapped in aluminum foil for 3 minutes to prevent photo-reactivation (Thoma, 1999). Mutagenized cells were diluted to achieve approximately 10,000 colonies per plate and plated onto DCM + charcoal. Inoculated plates were incubated at 30°C for 2-5 days until colonies appeared. Filamentous mutant colonies were plated on CM to check for phenotype stability before characterization on a variety of laboratory media. Fresh overnight 5.0 ml PDB cultures of the mutants were used to inoculate the solid and liquid medium for the characterization experiments. Aliquots of overnight cultures were frozen at -70°C for long term storage of strains.



### 3. Results

#### 3.1 Sequence analysis of Hgl1p

The amino acid sequence of Hgl1p was subjected to bioinformatics searches to identify similar proteins, or domains of proteins, which may suggest a function for Hgl1p. The analysis was conducted with the predicted amino acid sequence of Hgl1p from figure 6.

##### 3.1.1 Filtered and non-filtered BLAST, PSI BLAST, and BLAST2 with the Hgl1p amino acid sequence

BLAST searches with the complete amino acid sequence of Hgl1p resulted in very low probability matches with low complexity regions of proteins such as the proline rich merozite surface protein, or the serine rich protein mucin (completed 10/03/00, data not shown). These short regions of similarity score an E value of approximately  $10^{-2}$ , and these results were not sufficiently informative to guide further experiments with Hgl1p. E values are an indicator of the likelihood that an alignment has occurred by chance. The lower the E value, the less likely it is that any observed sequence similarity has occurred fortuitously as a result of the database randomly matching the queried sequence. An E value of  $10^{-2}$  suggests that an identified similarity will occur one in one hundred times simply by chance when using the current database and a random string of amino acids. Sequences reported with low probability scores do not likely represent truly homologous alignments.

Non-filtered searches of the NCBI databases using the Hgl1p amino acid sequence independently identified the *C. albicans* contig 4 2801 sequence, and the Flo11p protein of *S. cerevisiae* (Figure 8 Ai, Aii). The *C. albicans* and *S. cerevisiae* sequences used in the comparison were identified in non-filtered BLAST and  $\psi$ -BLAST sequence identity searches.

8 Ai demonstrates the non-filtered BLAST alignment of the *C. albicans* contig 2801 and the predicted amino acid sequence for the Hgl1p protein. Secondary alignments with the Hgl1p peptide sequence can identify alternative alignments with the same sequence which only have a slightly reduced E value. Similarly, the Flo11p alignment also demonstrates similarity to Hgl1p

Figure 8. A. Alignment of the Hgl1p amino acid sequence to the *C. albicans* translated con4 2801 (Ai), and the *S. cerevisiae* flocculin protein Flo11p (Aii). The alignments score E values of  $3 \times 10^{-10}$ , and  $2 \times 10^{-14}$  respectively, but are aligned in 2BLAST using the non-filtered alignment mode and hence represent compositionally biased portions of the proteins. B. Alignments with filters reduces the significance of the apparent matches to levels expected for the alignment of random sequences. Bi, filtered alignment of Hgl1p to translated contig 2801, E = 1.1. Bii, filtered alignment of Hgl1p to Flo11p, E = 4.3.

**Ai.**

```

Hgl1: 50 NTSVGSDDSHAGGSHTKLAINQLLTNLSHVSQEARMSSPRSSHSPALNDSPALKRKFED 109
          N V S G + T      + + S Q      S+ ++ S + N SP
2801: 1 NLEVNQPSTNGATSTGHFFGSPSPSTSTHQQTTPGETSNNVNTKSSSQNSP----- 51

Hgl1: 110 SDSSTAYKVASAHEIHLPTDPVRRASIINLATAAAAVLQSQAGQRRMSNFSDPEQKRQR 169
          S S T+ A+A      P R AS      QS +      + + P
2801: 52 STSPTSTVAAAAATSSSPVASTRPASTSEQKQEETTARQSTSPATTATTSTNTPSPSTS 111

Hgl1: 170 VEQLGSLIEQARKASVTSMHMSR-HVEAAQQNVAIATVLANHLGLTPQSSAASTPSAG 228
          E S Q A+ S + +      Q + + V +      TP ++ S+P+
2801: 112 KETPTSNTAQTSSANNNQSSNTAAPSTSVIQPSTSEVHVQSQQTSTTP-NTPTSSPNT 170

Hgl1: 229 TPAPASPTKPYAPSTL-----AIPQTPTTPRSP----LSAAPLTASQISDHQSAT 274
          T + A+PT AP+T      +P TPTT +P      S AP+T S      +
2801: 171 TTSEAAPTTSAAPTTSAPVTPSTSEVVPNTPTTSEAPNTPTTSEAPVTPSTSEVVPNT 230

Hgl1: 275 QAVAGPSARATNDQAEFSRPKPLVASPPRTPSIT---LNYSTDSVEPSSSSKLISEPLRA 331
          P+ T++      + P + P TP+ +      + +T V P++S++ +      +
2801: 231 TTSKAPNTPTTSE----APATPTTSEAPNTPTTSEAPVPTTSEVVPNTTSGDVAVSTSS 286

Hgl1: 332 SPPTSQALFNEAKEAAQYTSRFYRFEKEWAQKALELERRRSSIRIDPLFNPNSLSPLPA 391
          + T Q      + + T + +      A +      + SS I+      PS S
2801: 287 TSVTEQTTLTSSSTQLPPTTALTQTSTPEASDS----PKPSSTSIE-----TPSTSTFEQ 337

Hgl1: 392 DPNSNSNLNS-----SPTHNRE-GTAPISPQQHSA----PESRIMSRRHSPFCDSFVAP 440
          DP + S++ +      PT E      SP Q S      P + +      ++P +P
2801: 338 DPTTSSVGTPSSEQPQPTTTSELAVTSNSPTQESTSLVEPTTSSLESSNTPT-TPNPSTS 396

Hgl1: 441 SARMSNSGSSGFSSTHRASSSGLANVLSNFAELIEH-----RQRSCSGLEAL 488
          A+ S S S      +T A + L++ ++F+ L+ H      Q S
2801: 397 EAQPSTASQAPPDPTSSAPAPELSSSNADFSNLVLSSETTSLVNPTDSQIDSSSTTDA 456

Hgl1: 489 AKQAKELPVKRLSQPNPQFRTTFGDFSWAK--APPSGTDVNPSPAPPLRATPHASRDANT 546
          QA P      + P      T D + A+ AP S DA S+P +      + + T
2801: 457 VSQATTEPTSE-NTPTAASSVTANDINSAQSSAPTSNADAETASSPV--SEQSLATGSQT 513

Hgl1: 547 DADRSSVDKHDASASTSTVGVRSTHTLPNAQST-----RRVSATAPDSADADANT 598
          D ++      + A+ + ST NA T      S P + A D +T
2801: 514 SLDTTAGASSTASKATAENLSTFSTDGSSNASQTIAETTSNSTDQSVVTPSASASTDVST 573

Hgl1: 599 TAT-ARSTMSIAS 610
          T + S+ S+ S
2801: 574 LPTGESSTSLVS 586

```

**Aii.** Hg11 : 5 TRRPSPTSSPAVSRSSWVLDLPVASAPSSRRGSRVDLSIQTSANTSVGSDSHAGGSHT 64  
 T P PTPSS S+ V P +S S + ++S A S S S  
 Flo11: 310 TTTPVPTPSSSTTESSAPVPTPSSSTTESSAPVTSSTTESSAPVPTPSSSTTESSA 369

Hg11 : 65 KLAINQLLTNLSHVSQEQARMSSPRSSHSPALNDSPALKRKFEADSDSSTAYKVASAEI 124  
 + ++ + S S+ SS +P S + A SST +S+ +  
 Flo11: 370 PVT-----SSTTESSAPVTSSTTESSAPVPTPSSSTTESSAPVTSSTTE--SSAPV 422

Hg11 : 125 HLPTDPVRRASIINLATAAAAVLQSQAGQRRMSNFSDEQKRQRVEQLGSLIEQARKAS 184  
 T A + + T +++A + S S E V S ++ A  
 Flo11: 423 TSSTTESSAPVTSSTTESSAPVTS-----STTESSAPVPTPSSSTTESSAP 472

Hg11 : 185 VTSMSHDMRSRHEAAQONVAIATVLANHLGLTPQSSAASTPSAGTPAPASPTKPYAPSTL 244  
 VTS + + S A + +T ++ +T SS + SA P +S T S+  
 Flo11: 473 VTSSTTESS---SAPVPTPSSSTTESSAPVT--SSTTESSAPVPTPSSST---TESS 524

Hg11 : 245 AIPQTPTTPRSPLSAAPLTASQISDHQSATQAVAGPSARATNDQAE--FSRPKPLVASPP 302  
 A TP++ + S+AP+T+S +S++ V PS+ T + S ++P  
 Flo11: 525 APAPTSSSTTESSAPVTSSTT---ESSAPVPTPSSSTTESSSTPVTSTTESSAPV 581

Hg11 : 303 RTPSITLNYSTDSVEPSSSSKLISEPLRASPTSQALFNEAKEAAQTYSRFYRFEKEWAQ 362  
 TPS + S+ + P+ SS +E A PT + E+ A T S  
 Flo11: 582 PTPSSSTTESSAPVPTPSS--TESSAPAPTSSSTTESSAPVTSS----- 629

Hg11 : 363 KALELERRRSSIRIDPLFNPNSLSPLPADPNSNSNLNssp-----THNREGTAPI--- 413  
 E + + S +P+P +S + +S+P + +AP+  
 Flo11: 630 ---TESSAPVPTPSSSTTESSAPVPTPSSSTTESSAPVPTPSSSTTESSAPVTSS 686

Hg11 : 414 SPQQHSAPESRIMSRHSPFCDSPVAFSARMSNNGSGSFSSTHRASSGLANVLSNFAE 473  
 + + SAP + + S +P + + S++ SST +SS+ + S+ E  
 Flo11: 687 TTESSAPVTSSTTESSAPVPTPSSSTTESSAPVPTPSSSTTESSAPVPTPSSSTTE 746

Hg11 : 474 LIEHRQRSCSGLEALAKQAKELPVKRLSQ-----PNPQFRITTFGDFSWAKAPPSG 523  
 S + + + ++ PV S P P TT + S A P  
 Flo11: 747 -----SSAPVTSSTTESSAPVPTPSSSTTESSAPVPTPSSSTT--ESSAPVPTPS 798

Hg11 : 524 TDAVNPSAPPLRATPHASRDANTDADRSSVDKHDASAS----TSTVGVRSHTLPLNAQS 579  
 + S P+ TP +S + + A S+ T S+S T + + + P + S  
 Flo11: 799 SSTTESSVAPV-PTPSSSNITSSAPSTPFSSSTTESSSVVPTPSSSTTESSAPVSS 857

Hg11 : 580 TRRVSATAPDSDADADANTTATARSTMSIAS 610  
 T S+ AP + +N T++A S++ +S  
 Flo11: 858 TTE-SSVAPVPTPSSSNITSSAPSSIPFSS 887

**Bi.** Hg11: 238 PYAPSTLAIPQTPTTPRSPLSAAPLTASQISDHQSATQAVAGPSARATNDQAEFSRPKPL 297  
 P P+T P TPTT +P++ T S++ S S+ + +Q + L  
 2801: 245 PATPTTSEAPNTPTTSEAPVTP---TTSEVVPTTSTQGDVSTSTSVTEQTTLTSTQL 301

Hg11: 298 VASPPRTPSITLNYSTDSVEPSSSS 322  
 + T + ++DS +PSS+S  
 2801: 302 PPTTALTQTSTFEASDSPKPSSTS 326

**Bii.** Hg11 : 242 STLAIPQTPTTPRSPLSAAPLTASQISDHQSATQAVAGPSARATNDQAE--FSRPKPLVA 299  
 S+ A TP++ + S+AP+T+S +S++ V PS+ T + S +  
 Flo11: 522 SSSAPAPTSSSTTESSAPVTSSTT---ESSAPVPTPSSSTTESSSTPVTSTTESS 578

Hg11 : 300 SPPRTPSITLNYSTDSVEPSSSSKLISEPLRASPTSQALFNEAKEAAQTY 351  
 +P TPS + S+ + P+ SS +E A PT + E+ A T S  
 Flo11: 579 APVPTPSSSTTESSAPVPTPSS--TESSAPAPTSSSTTESSAPVTSS 629

( $E = 2 \times 10^{-14}$ ) due mainly to the compositional bias of the Flo11p protein. Flo11p contained a 600 amino acid, low complexity region identified by the SEG program which consisted mainly of the repeated sequence "PVPTSSSTTSSES" (Schultz *et al.*, 1998). This region of Flo11p demonstrated the highest similarity to Hgl1p, a result similar to the low overall complexity of the con4 2801 sequence. The application of filters to BLAST2 alignments between the Hgl1p and the *Candida* or *S. cerevisiae* sequences reduced the statistical significance of the alignments to E values of 1.1 and 4.3 respectively. Such comparisons did not fall below the probability threshold that the alignment occurred purely by chance, indicating that the non-filtered alignment with Hgl1p should be treated with caution. It was unlikely that Hgl1p is functionally related to the two sequences based on the nature of the similarities which have been identified, however these alignments were the most significant that could be found.

A BLAST alignment between the *Candida* and *Saccharomyces* sequences demonstrates significant similarity between the two sequences. Interestingly, the similarity between contig 2801 and Flo11p was high for both filtered and non-filtered comparisons, perhaps suggesting the comparison data between Hgl1p and each of the others independently may have had significance beyond the E value. In the absence of filters, the E value of the comparison between *C. albicans* con4 2801 and *S. cerevisiae* Flo11p was in the neighborhood of  $4 \times 10^{-66}$ , and even with the filters in place the E value remained at  $5 \times 10^{-8}$  (Figure 9). Comparison of con4 2801 to a second flocculin protein, Flo5p, again identified a high similarity when filters were removed from the program ( $E = 9 \times 10^{-29}$ ), but did not demonstrate the high similarity seen with the Flo11p sequence when filters were re-implemented into the comparison ( $E = 2.3 \times 10^{-2}$ , data not shown). Hgl1p did not share significant similarity to Flo5p in filtered or unfiltered searches. Overall, these results did not provide a reliable clue to the identity of Hgl1p or its

function in *U. maydis*. However, these searches did suggest that the dimorphic pathogen *C. albicans* may have a homologue of the *S. cerevisiae* *FLO11* gene.

```

2801 : 420 LSSSNADFSNLVLHSS--ETTSLVNPNXXXXXXXXXXXXXAVSQATTEPTSENTPTAASSVT 477
      ++SS + S+ + SS E++S P V+ +TTE +S PT +SS T
Flo11: 434 VTSSTTESSAPVTSSTTESSAPVPTPSSSTTESSAPVTSSTTESSAPVPTPSSSTT 493

2801 : 478 ANDINSAQSSAPTSNADAETASSPVSEQSLATGSEQXXXXXXXXXXXXXXXXXENLSTFS 537
      + SSAP +++ E++S+PV T S E+ S
Flo11: 494 ES-----SSAPVTSSTTESSAPV-----PTPSSSTTESSAPAPTSSSTTESSAPV 542

2801 : 538 TDGSSNASQTIAETTSNSTDQSVVTPSASASTDVSTLPTGESSTSLVS 586
      T ++ +S T S+ST +S TP S++T+ S+ P + SS++ S
Flo11: 543 TSSTTESSAPVPTPSSSTTESSSTPVSSTTESSAPVPTPSSSTTES 591

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Figure 9. Filtered alignment of the translated *C. albicans* contig 2801 with the *S. cerevisiae* protein Flo11p. The resulting E value is  $5 \times 10^{-8}$ .

PROPSEARCH with Hgl1p as a query sequence found similarity between Hgl1p and transcriptional regulators of the family involved in nitrogen regulation in filamentous fungi, or to the transcriptional regulators of flocculation (*SFL1* and *FLO8*) in the budding yeast *Saccharomyces cerevisiae*. The PROPSEARCH algorithm looks for compositional similarities between related proteins, and groups them into protein families based on the analysis (Hobohm and Sander, 1995). Examples of the criteria used in the algorithm are: shared amino acid composition, amino acid bulkiness, overall pH, isoelectric point (pI), and average hydrophobicity. PROPSEARCH can identify proteins that share similar amino acid compositions, and may identify a relatedness between two sequences that cannot be identified by traditional sequence alignment algorithms (Hobohm and Sander, 1995). Protein groupings are assessed on a scale that ranks their similarity with each other compared to the identified sequence and an unrelated protein. The comparison is scored as the 'distance' (DIST) between the two sequences; a larger predicted distance between two sequences results in a lower chance

they belong to the same family (reported as a percentage). Results of a PROPSEARCH with the Hgl1p amino acid sequence identified its compositional similarity to a nitrogen regulatory protein family (members identified in bold) (Table 1). Hgl1p scored a distance (DIST) from the nitrogen regulatory proteins of 10.26, or an approximately 80% chance that Hgl1p was from that protein family. The 11.51 and 12.03 DIST's reported between Hgl1p, Sfl1p, and Flo8p suggested there was a 68% chance that Hgl1p was a family member of *S. cerevisiae*

Table 1. Proteins identified by PROPSEARCH using Hgl1p as the amino acid query sequence. Sequences 5,12, and 15 included unidentified orf's and are not presented in the table. ID = identified family member. DIST = relatedness distance from the recovered protein. The length of the reported matching family member is provided in the LEN column. DEFINITION provides a brief description of the protein.

Rank	ID	DIST	LEN	pI	DEFINITION
1	<b>area_aspng</b>	10.26	882	9.78	<b>NITROGEN REGULATORY PROTEIN AREA.</b>
3	<b>area_emeni</b>	10.90	876	8.15	<b>NITROGEN REGULATORY PROTEIN AREA.</b>
4	sfl1_yeast	11.51	766	9.40	FLOCCULATION SUPPRESSION PROTEIN(SFL1).
6	<b>area_pench</b>	11.58	725	7.40	<b>NITROGEN REGULATORY PROTEIN AREA(NRE).</b>
7	<b>area_aspor</b>	11.59	866	7.88	<b>NITROGEN REGULATORY PROTEIN AREA.</b>
8	flo8_yeast	12.03	799	10.12	TRANSCRIPTIONAL ACTIVATOR FLO8(PDH5).
9	<b>weta_emeni</b>	12.20	555	7.91	<b>REGULATORY PROTEIN WETA.</b>
10	htf4_human	12.43	682	7.02	TRANSCRIPTION FACTOR HTF4.
11	stb1_yeast	12.61	459	10.40	STB1 PROTEIN.
13	htf4_rat	12.75	707	6.90	TRANSCRIPTION FACTOR HTF4.
14	<b>area_penro</b>	12.76	860	7.56	<b>NITROGEN REGULATORY PROTEIN AREA.</b>
16	crep_human	12.88	505	7.90	CAMP RESPONSE ELEMENT BINDING PROTEIN.
17	<b>area_fusmo</b>	12.94	970	9.76	<b>NITROGEN REGULATION PROTEIN AREA.</b>
18	<b>nrfa_penur</b>	13.01	865	7.34	<b>NITROGEN REGULATORY PROTEIN NRFA.</b>

transcription factors. A BLAST2 comparison between Hgl1p and AreA of *Aspergillus nidulans*, or the yeast Sfl1p and Flo8p proteins identified by PROPSEARCH did not identify additional similarity. Removal of the filters from the BLAST searches did not increase the sequence similarity.

### 3.1.2 Conserved domains in the Hgl1p amino acid sequence

Given the nature of the similarity between the Con4 2801, Flo11p, and Hgl1p sequences, it was necessary to look for common domains within each protein that might suggest a shared function. SMART analysis of the *Candida* con4 2801, *Saccharomyces* Flo11p, and Hgl1p sequences demonstrated that they did not share an overall domain conservation (data not shown). Flocculin proteins share a similar organization of a signal peptide at the amino terminal end, and seven transmembrane motifs in the carboxyl end of the protein. The *Candida* and *Ustilago* sequences did not share this same domain conservation, suggesting that they did not share functions with the flocculin proteins. The three proteins share portions of the atrophin PRINTS fingerprint, though the E values for the similarities were insignificant for each individual protein (between .05 and 66, data not shown).

Additionally, eMOTIF searches did not identify broad motif similarities between the three proteins, so the Hgl1p sequence was examined individually. The Hgl1p protein had several interesting motifs which might suggest a signaling-molecule function. The eMOTIF database identified both a cdc24 guanine-nucleotide dissociation factor family sign, and a G-protein coupled receptor (GPCR) transmembrane motif (Figure 10). Each of the identified motifs was outside regions proposed to have low complexity in Hgl1p and both occurred with a stringency of approximately  $10^{-5}$ , suggesting they were not false positives. However, the cdc24 GDS family members typically share a second family signature that is absent in the Hgl1p sequence. There were no other indications that Hgl1p was a guanine nucleotide dissociation factor. Similarly, the transmembrane motif of the GPCR was in amino acids 23-32 in the sequence but was the only one identified by eMOTIF, the GPCR family typically share from 2 to 7 additional characteristic motifs which were absent in the Hgl1p sequence (Attwood and Findlay, 1994). Examination of a hydrophobicity plot from the Tmpred search program reveals

two potential transmembrane domains. Of the suggested domains spanning amino acids 134-154 (a length of 21 residues) was the strongly favored transmembrane domain predicted to run from the inside to the outside of the cell (Figure 10). The significance score for such an orientation of this transmembrane domain was 942, and scores of 500 or higher are to be

```

0 MFVATRRPSPTPSSPAVSRSSWVDLPVASAPSSRRGSRVDLSIQTS LAN 50
51 TSVGSDSHAGGSHTKLAINQLLTNLSHVSQEQARMSSPRSSHSPALNDSP 100
101 ALKRKFEADSDSSTAYKVASAHEIHLPTDPVRRAS I INLATAAAAVLQS 150
151 QAGQRRMSNFSDPEQKRQVEQLGSLIEQARKASVTSM SHDMSRHVEAAQ 200
201 QNVAIATVLANHLGLTPQSSAASTPSAGTPAPASPTKPYAPSTLAI PQTP 250
251 TTPRSPLSAAPLTASQISDHQSATQAVAGPSARATNDQAEFSRPKPLVAS 300
301 PPRTPSITLNYSTDSVEPSSSSKLI SEPLRASPTSQALFNEAKEAAQTY 350
351 SRFYRFEKEWAQKALELERRRSSIRIDPLFNP NPSLSPLPADPNSNSNLN 400
401 SSPTHNREGTAPISPQQHSAPESRIMSRRHSPFCDS PVAPSARMSNSGSS 450
451 GSFSSTHRASSSGLANVLSNFAELIEHRQRSCSGLEALAKQAKELPVKRL 500
501 SQPNPQFR TTFGDFSWAKAPPSGTD AVNPSAPPLRATPHASRDANTDADR 550
551 SSVDKHDTASASTSTVGVRSTHTLPNAQSTR RVSATAPDSADADANTTA 600
601 TARSTMSIASML 612

```

Figure 10. Distribution of domains in the predicted amino acid sequence of Hg11p. Sequences in blue identify PKA target sites. High scoring PEST sequences are indicated in bold. Sequences with a single black line below are additional PEST sequences. Regions under dashed lines represent the low complexity regions as determined by the SEG program. Residues 4-11 (underlined in pink) share 100% similarity to a protein tyrosine phosphatase gamma precursor identified by PSI-BLAST. The region from 23-32 under the crimson line identifies the GPCR transmembrane motif, and the region from 164-173 under the blue line represents the GDS cdc24 motif. The sequence from amino acids 134-154 underlined with the yellow line represents the Tmpred identified transmembrane motif.

considered significant (Hofmann and Stoffel, 1993). For example, when a seven transmembrane protein such as Flo11p was scanned with the hydrophobicity program, scores of approximately 1500 were recovered for four putative membrane spanning regions. The



program did not however identify all seven regions, and was incorrect for two of the four predicted domains. The results of such domain-identifying programs should be cautiously applied and used in parallel with experimental evidence.

### 3.2 PKA phosphorylation of Hgl1p

Analysis of the Hgl1p amino acid sequence identified 8 putative PKA phosphorylation sites. To investigate possible interactions between Hgl1p and PKA, *Ustilago maydis* PKA was used to phosphorylate Hgl1p produced *in vitro*.

#### 3.2.1 Purification of *Ustilago maydis* PKA from Ubc::6xHIS chromatographic columns

Attempts were made to produce catalytic subunits of *U. maydis* PKA (Uka1p and Adr1p) in an *in vitro* transcription/translation reaction for use in phosphorylation experiments. Proteins of the expected size for *uka1* and *adr1* gene products were recovered in the TNT<sup>®</sup> system, but these proteins did not demonstrate kinase activity in the peptag<sup>®</sup> kinase assay (Figure 11). The peptag<sup>®</sup> assay quantitates PKA activity by the amount of fluorescently labelled peptag<sup>®</sup> that migrates towards the anode following phosphorylation by kinase. Subsequently, *Ustilago* PKA was recovered from Ubc::6xHIS affinity columns

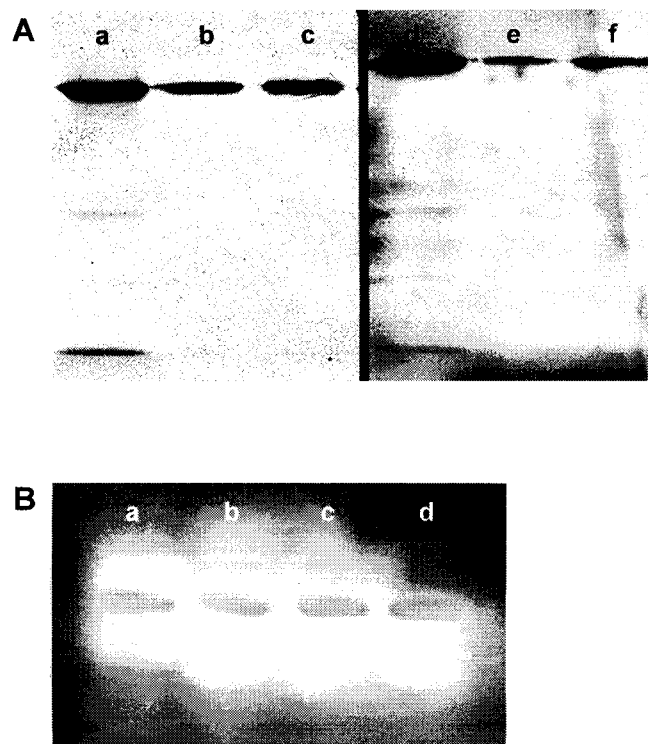


Figure 11. Production of the PKA molecules Uka1p and Adr1p using the TNT wheat germ extract system. Panel A demonstrates the methionine labeled bands produced when the pET21b::adr#31, and pET21b::uka#20 (Franz Dürrenberger, unpublished) plasmids were utilized in TNT<sup>®</sup> transcription/translation reactions and purified over Ni-agarose affinity columns. The autoradiogram is from a single gel that has been cropped to allow direct comparison. Lanes a and d contain Uka1p and Adr1p (respectively) un-separated from the reaction components. Lanes b,c, and e,f demonstrate the recovery of Uka1p and Adr1p from Ni-agarose affinity columns in 300 mM imidazole elution fractions. Panel B represents a Promega peptag<sup>®</sup> kinase assay experiment. Lane a contains control bovine PKA (sample contained low kinase activity); lane b, expressed control luciferase protein (no kinase activity); lane c, TNT<sup>®</sup> produced Uka1p from the elution fraction of lane c in Panel A; lane d, Adr1p from the elution fraction of lane f, panel A recovered from the TNT<sup>®</sup> transcription/translation reaction.

and diluted to achieve an activity equivalent to 0.04 units of bovine heart PKA (figure 12A).

The addition of PKI had an inhibitory effect on the apparent molecular weight increase caused by the *Ustilago* and bovine PKA catalytic subunits, but did not completely eliminate the shift

(Figure 12B). A loss of the apparent molecular weight shift was achieved with the addition of 100 units of lambda phosphatase (Figure 12B).

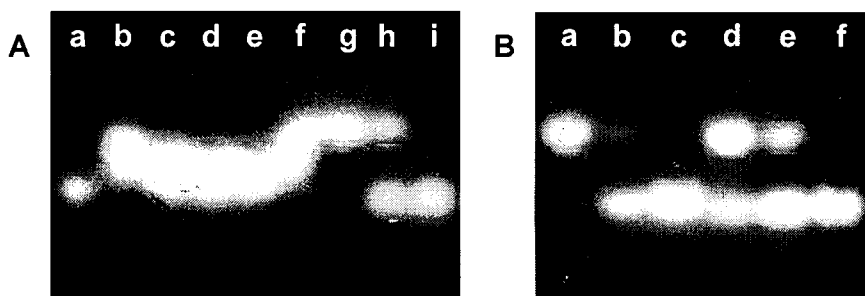


Figure 12. Evaluation of PKA activity and the effects of PKI and phosphatase treatment on the peptag<sup>®</sup> kinase assay. Panel A represents peptag assays with bovine and *Ustilago* PKA. Lane a contains no kinase added. Lanes b-e contain dilutions of bovine PKA as follows: lane b contains  $3.92 \times 10^{-2}$  units; lane c,  $3.92 \times 10^{-3}$  units; lane d,  $3.92 \times 10^{-4}$  units; and lane e  $3.92 \times 10^{-5}$  units. Lanes f-i represent *Ustilago* PKA reactions with: f, undiluted PKA elution fraction; g, 1/10 PKA dilution; h, 1/100 PKA dilution fraction; i, 1/1000 PKA dilution fraction. Panel B represents bovine and *Ustilago* PKA reactions treated with a 1000 fold excess of PKI (1  $\mu$ M) and 100 units lambda phosphatase. Lanes a-c contain: a, bovine PKA; b, bovine PKA + 1  $\mu$ M PKI; c, bovine PKA + 100 units lambda phosphatase. Lanes d-f contain: d, *Ustilago* PKA; e, *Ustilago* PKA + 1  $\mu$ M PKI; f, *Ustilago* PKA + 100 units lambda phosphatase. 1 unit of PKA activity transfers 3.92 pmoles of phosphate to substrate per minute at 37 °C.

### 3.2.2 Expression of Hgl1p from pETHgl

Attempts were made to express Hgl1p in *E.coli* strain BL21( $\lambda$ DE3) for use in PKA phosphorylation assays, several expression conditions were examined but the production of Hgl1p was not successful. For example, protein production was attempted in separate expression cultures incubated at 37°C, 30°C, and 22°C. Each temperature-condition experiment was repeated with varying degrees of aeration, and induction with either 0.5mM or 1mM IPTG. Induced cultures were incubated for 2hrs, 6hrs, or 12hrs for each of the experimental conditions. In all experiments, there was no specific protein of the Hgl1p size observable by either

coomassie brilliant blue staining, or by western blot analysis of both the soluble and insoluble fractions for all of the protein preparations.

The controls for protein expression and expression of Hgl1p fusion proteins included use of the plasmids pHhmyc12 (not shown), pET21b::ubc1#3, pET21b::adr#31 (constructed by F. Dürrenberger), as well as a pGEX plasmid expressing the janus kinase JNK (gift from Rob Gerl), to ensure that induced cultures were producing protein. Control cultures (excluding cultures for Hgl1p) demonstrated high levels of expressed protein. Control cultures (excluding cultures for Hgl1p) demonstrated high levels of expressed protein. Additionally, the HIS-tagged version of the *Ustilago* PKA regulatory subunit used for isolation of *Ustilago* PKA (pET21b::ubc1#3) was purified over a Ni-agarose column and subjected to electrophoresis (SDS-PAGE) to demonstrate recovery of soluble protein from the chromatography column (Figure 13).

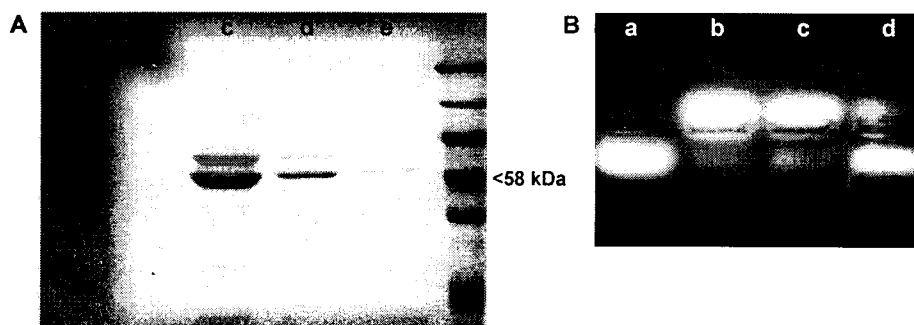


Figure 13. Purification of Ubc1::HIS and PKA from a Ni-Agarose chromatography column. Panel A is a photo of a Coomassie brilliant blue stained SDS-PAGE. Panel A lanes a and b represent flow-through eluate from 20 mM imidazole washes prior to protein elution with 300 mM imidazole. Lanes c, d, and e represent elution fractions from successive washes of the same column with 300 mM imidazole. Panel B, the protein elution fractions from lanes c, d, and e of panel A were tested for kinase activity with the promega peptag assay. Panel B lane a contains a sample from the elution fraction of Panel A lane a. Panel B lanes b, c, and d contain samples from the elution fractions from panel A lanes c, d, and e respectively. The elution fraction from Panel A lane a contained the highest amount of active PKA.

Attempts were made to fuse the Hgl1p protein to the *myc* and hemagglutinin (HA) epitope tags in an effort to recover purified Hgl1p protein. Experiments with the *myc*-epitope tag involved the control protein mycMcm1p (gift from Chris Nelson), which was detectable following SDS-PAGE and western blot analysis with Ab-conjugated chemiluminescence detection procedures (Figure 14). Immunoprecipitations with mAb 9E10 against *c-myc*

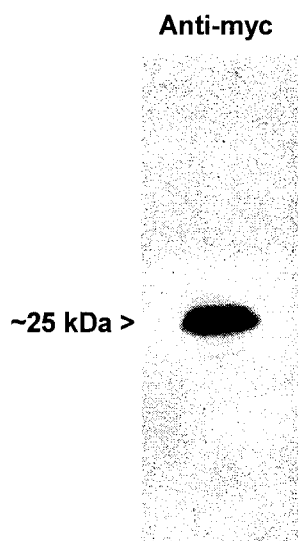


Figure 14. Western hybridization analysis of the myc-tagged protein Mcm1p. Anti-myc mAb 9E10 was used to probe a western blot for the presence of the myc epitope. A band of consistent size with the Mcm1p transcription factor from *S. cerevisiae* (courtesy of Chris Nelson, Ivan Sadowski's lab) was detected as the only myc-tagged protein present on the membrane.

from large (500ml) induced cultures did not detect any Hgl1p protein. Hgl1p may have been expressed at levels too low for detection. Immunoprecipitation was attempted from *U. maydis* cultures carrying the *myc* and HA tagged versions of Hgl1p under control of the native *hgl1* promoter in hopes of recovering protein; these attempts also were unsuccessful. The *hgl::myc*

fusion complemented the original *hgl1* mutant, but the protein was not detectable by the anti-*myc* mAB 9E10.

### 3.2.3 Phosphorylation of Hgl1p with bovine heart and *U. maydis* PKA

Successful phosphorylation of Hgl1p by either *Ustilago* protein kinase A (UPKA) or bovine heart protein kinase A (bPKA) was expected to result in an increased apparent molecular weight during SDS-PAGE (Sadowski *et al.*, 1991). Protein kinase reactions were performed with  $^{35}\text{S}$ -Methionine labeled *in vitro* produced Hgl1p and either UPKA or bPKA. Figure 15 demonstrates that a mobility shift was produced when bPKA (panel A, lane b) or UPKA (panel B, lane b) were used to phosphorylate Hgl1p.

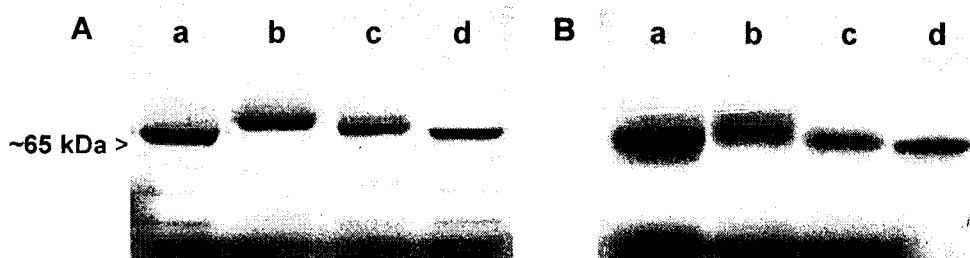


Figure 15. Phosphorylation of Hgl1p by bovine and *U. maydis* protein kinase A. Panel A shows reactions with bovine heart PKA and Panel B shows reactions with *U. maydis* PKA. Lane a of each panel shows the position of  $^{35}\text{S}$ -methionine labeled Hgl1p without the addition of PKA. Lane b of each panel demonstrates the shift produced upon reaction with the catalytic subunits of PKA. Lane c is the reduced shift observed in the presence of the kinase inhibitor PKI, and lane d demonstrates elimination of the mobility shift upon treatment of the kinase reactions with lambda phosphatase (BRL).

*Ustilago* and bovine heart PKA had similar effects on the translated Hgl1p protein product, producing an increased apparent molecular weight shift of the  $^{35}\text{S}$ -methionine labeled

band. The TNT<sup>®</sup> wheat germ extract used to produce Hgl1p did not have protein kinase A activity as determined in the fluorescent peptag<sup>®</sup> kinase assay (Figure 16). Introduction of a

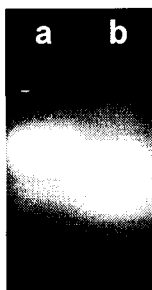


Figure 16. Wheat Germ Extract does not contain intrinsic kinase A activity. Lane a demonstrates migration of the peptag<sup>®</sup> in response to phosphorylation by bovine heart PKA. Lane b contains an amount of wheat germ extract equal to that present in the Hgl1p phosphorylation reactions from figure 15, the charge state of the peptag was not altered, and it migrated towards the cathode.

specific protein kinase A inhibitor (PKI) decreased the shift due to phosphorylation. PKI addition (to 1000 fold excess) did not completely inhibit kinase activity on Hgl1p, a result that was also observed in the peptag<sup>®</sup> assay under the conditions tested (Figure 12). Finally, the addition of lambda phosphatase reduced the shift to the migration level of the control protein. The results of this experiment demonstrated that *in vitro* produced Hgl1p could be phosphorylated by the PKA catalytic subunits from *U. maydis* and bovine heart tissue. This experiment was repeated 8 times and a similar reduction in mobility upon phosphorylation was observed in each experiment.

### 3.3 Suppressor mutagenesis of *hgl1<sup>-</sup>* strains

A suppressor analysis in the strains 3020 (*hgl1::nat1* in 001) and 3034 (*adr1::phleo*, *hgl1::nat1* in 001) was undertaken to determine whether it was possible to isolate mutants

which could suppress the *hglI*<sup>-</sup> colony phenotypes. Mutants were identified (in each of the backgrounds) that recovered a filamentous colony morphology similar to that of *adrI*<sup>-</sup> strains. This indicated that Hgl1p was not essential for filamentous growth, and would support a model that Hgl1p acts as a repressor of budding growth.

### 3.3.1 Disruption of *hglI* with the *natI* cassette

The *hglI* disruption strain 3034 was used for the mutagenesis experiments to identify suppressor mutants. This strain contained the hygromycin resistance marker that was used for disruption of the *hglI* gene and selection for successful integration of the disruption construct. The most reliable cosmid library in our laboratory, for the purpose of complementation, also relied on hygromycin as the basis of selection for successful transformation. Therefore, it was necessary to create an *hglI*<sup>-</sup> strain which did not carry a hygromycin resistance marker. An *hglI*<sup>-</sup> strain which carried a nourseothricin cassette in place of the hygromycin cassette was created. The *HindIII/BamHI* cassette of *phgl::nat* (Figure 17) was used to transform strain 001.



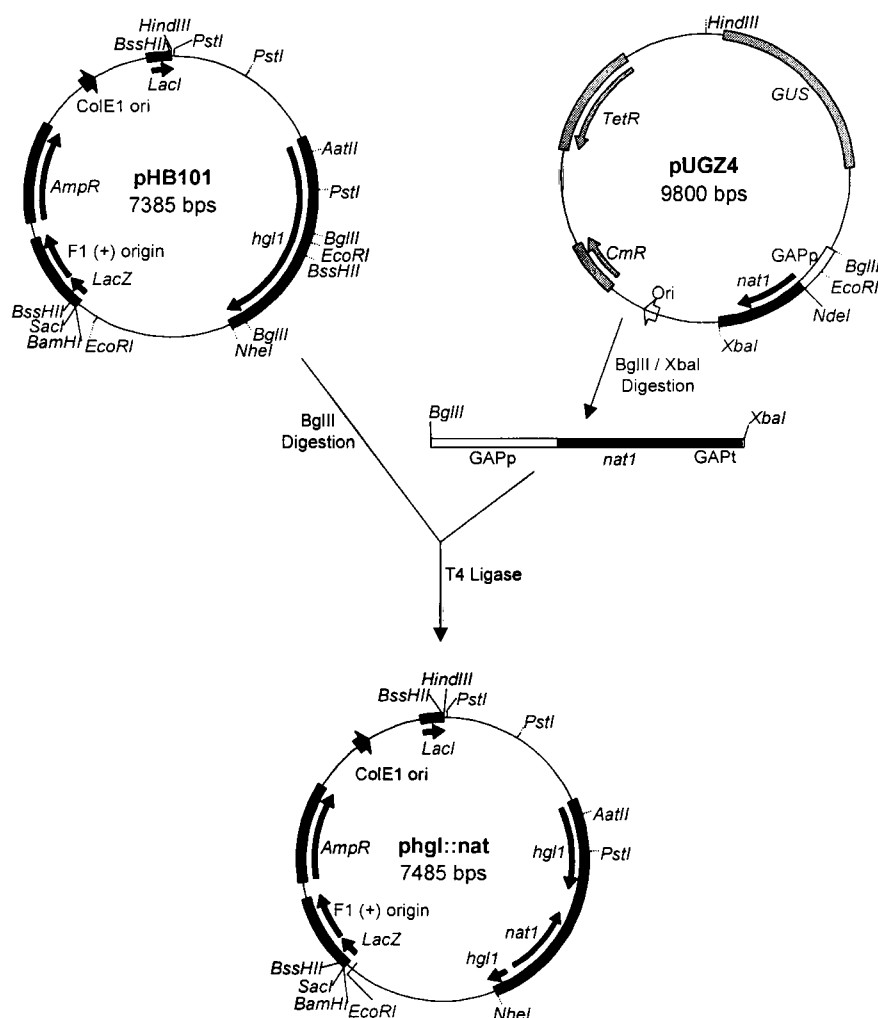


Figure 17. Construction of an allele of *hglI* containing the nourseothricin disruption cassette. The *BglII* fragment of pUGZ4 was gel purified and blunt ended with T4 DNA klenow fragment before ligation into *BglII* digested pHB101. Loss of the 0.9 kb *BglII* fragment from pHB101 eliminates a diagnostic *EcoRI* fragment that was used for identification of successful disruption.

Transformants were chosen for genomic DNA preparation based on their similarity to the original *hglI*<sup>-</sup> phenotypes, e.g., yeast-like colony growth and yellow pigmentation of the media. Successful integration of the *hglI::nat1* construct resulted in a 0.5 kb shift of the 7.0 kb genomic *BssHII* fragment seen in the wild type strain (Figure 18). Strains used for the preparation of genomic DNA in lanes c-g were named “3020 - 3025” and frozen at -70°C for long term storage.

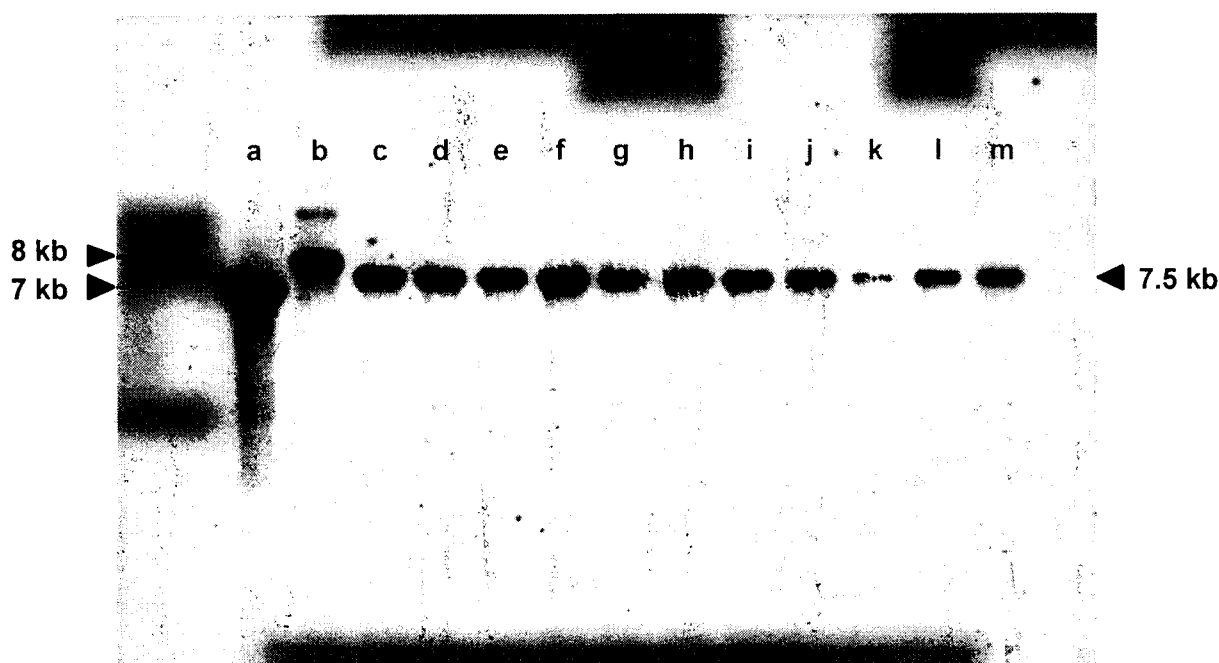


Figure 18. Disruption of *hglI* with the *natI* cassette in the wild type 001 background. Genomic DNA from each strain was digested with *BssHII*, probed with the 1.2 kb *PstI* fragment of pB105 (Figure 17). Successful disruption results in a shift of the wild type 7.0 kb hybridization band to a 7.5 kb band. Lane a contains wild type genomic DNA, b contains genomic DNA of the hygromycin disruption strain 3001 (8.0 kb), while lanes c-m contain genomic DNA from successful integrants of the nourseothricin disruption construct (7.5 kb).

### 3.3.2 Isolation of filamentous suppressor mutants of the budding *hglI*<sup>-</sup> phenotype

A variety of suppressor phenotypes were obtained following mutagenesis of *hglI*<sup>-</sup> strains. Each of the strains (3020 and 3034) was mutagenized in six different experiments. Each experiment involved mutagenesis and plating of approximately  $1.2 \times 10^6$  cells in total. Therefore between the two strain backgrounds, approximately  $1.44 \times 10^7$  mutagenized cells were screened. For the 3020 background, 17 *hglI*<sup>-</sup> suppressor mutants were recovered, and for the 3034 background, 25 *hglI*<sup>-</sup> suppressor mutants were recovered. Phenotypes in each strain background, ranged from a morphology similar to that of the highly filamentous *adrI*<sup>-</sup> strain, to

a weak filamentous phenotype. Similar mutant phenotypes were observed between different isolates from each strain background (3020 and 3034).

Mutant strains were examined for phenotype variability on a variety of common media used for the growth of *U. maydis*. An overview of mutant phenotypes is provided in Table 2, which lists the mutants recovered and their phenotypes on solid media. For simplicity, the filamentous and pigmentation phenotypes were recorded as +, ++, or +++ in order of increasing magnitude. A score of '+' (Figure 19 panel c) indicated that the mutant weakly demonstrates the phenotype of that category, '++' (Figure 19 panel b) indicated a moderate level, and '+++' (Figure 19 panel a) indicated the mutant strongly demonstrates the described phenotype. The degree of colony filamentation was categorized into one of three classes (Figure 19). An example of the pigment produced by the *hglI*<sup>-</sup> strains and the collected suppressor mutants is

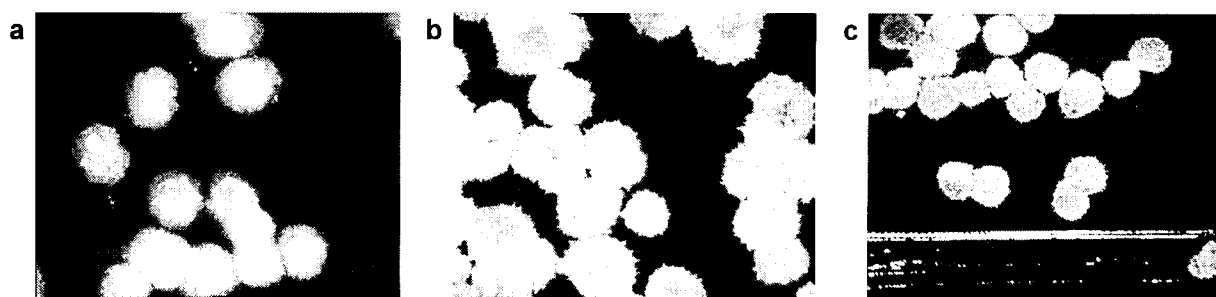


Figure 19. Categories of different *hglI*<sup>-</sup> suppressor mutants. Each of the suppressor mutants described in Table 2 have been assigned as sharing similar phenotypes to the colonies in either panels a, b, or c. Panel a categorizes colonies which have a consistent, thick, covering of short, velvet-like filaments. The body of the colony is difficult to view due to the high degree of filamentation. Panel b categorizes colonies which share longer filaments than those of group a, that are evenly dispersed about the colony surface. The colonies may grow as 'doughnut' shape, a distinct colony body is identifiable. Panel c represents colonies that contain shorter filaments evenly dispersed about the colony surface. The filaments are short, and may appear granular. A distinct colony body is apparent, and the colonies are typically small and 'dome' shaped.

provided in figure 20. When selecting mutants for complementation experiments, the desired phenotypes were maintenance of highly filamentous colony growth and pigmentation on a variety of different media (such as mutant 7.1). This was done in order to minimize the phenotypic variability involved in the complementation experiments.



Figure 20. The pigmentation phenotype of an *hglI*<sup>-</sup> strain. Panel A represents wild type 001 *U. maydis* colonies grown for 48 hours on PDA. Panel B represents strain 3001 (*hglI*<sup>-</sup>) grown for 48 hours on PDA. The pigmentation phenotype of *hglI*<sup>-</sup> strains can be enhanced (more pigment production) or reduced (less pigment production) by U.V. suppressor mutagenesis.

Nutrient abundance played some role in filament growth or colony morphology for several of the mutants. During characterization of the mutants, colony growth was observed on media ranging from the low nutrient MM to the nutrient rich DCM. Generally, as the media contained less nutrients (low glucose, yeast extract, casamino acids), the filamentation phenotype was enhanced. Mutants were recovered which did not respond to changing media composition, indicating that there was more than one additional pathway involved in establishing or regulating filamentous growth. A filamentous mutant which still responded to nutrient levels with an altered colony morphology was likely affected in a component that did

not respond to nutrient availability. Mutants which maintained the same degree of filamentation despite the composition of the growth medium may be mutated for a nutrient sensory-response pathway that participated in filamentous growth. This situation was likely to be mediated by the cAMP/PKA pathway due to the constitutive phenotypes of strains affected for other components of the PKA pathway in *U. maydis*.

Table 2. Growth of suppressor mutants was characterized on a variety of common laboratory media and summarized in table 2. +, ++, and +++ indicate the strength of the phenotype (+ = low, +++ = high). General colony morphology of the mutants is classified as a, b, or c, as shown by the examples in Figure 19. \*colonies display a slow growth phenotype resulting in small colony size.

Mutant	Mutant Growth on Solid Media			
	Degree of filamentation	Pigment production	Nutrient sensitivity	Example colony type
<i>hgl1</i>	-	++	n/a	n/a
5.1	+	+	+++	c
5.2	+	++	++	c
5.3	++	+	+++	b
6.2	++	++	++	b
7.1	+++	+++	+	a
8.1	+++	+	+	a
8.4	+	+	++	c
8.7	+	++	+++	c
9.1	++	+++	+++	b
9.2a	+++	+++	+++	a
9.2b	+++	+++	+++	a
14.6	++	++	+++	b
15.1a	++	++	++	b
8.8	+	+	++	c
26.4.2	++	++	++	b
26.23.4*	++	+	++	b
26.14.1*	++	+	++	b
26.11.1	++	++	++	b
26.19.1	++	+	++	b
26.22.3	++	+	++	b
26.35.3	++	++	++	b
26.24.1	++	+	++	b
26.24.2	++	+	++	b
26.24.3	+	+	++	c

### 3.3.3 Cosmid complementation of suppressor mutations in the 3020 and 3034 strains

Cosmid complementation of one selected mutant for each strain (3020 and 3034) was attempted with a cosmid library in order to recover a gene which acts epistatically to *hgl1*. The complementation experiments were unsuccessful because of unforeseen problems with the selected mutants. Specifically, the original mutant collection in the *adr1<sup>-</sup> hgl1<sup>-</sup>* background appeared to be intrinsically resistant to the fungicide carboxin, the basis of selection for the vector of one of the available genomic libraries. Unsuccessful attempts were made to transform the original filamentous *adr1<sup>-</sup> hgl1<sup>-</sup>* mutants with the *hgl1::nat1* disruption cassette to replace the hygromycin gene (5.0 kb *HindIII/BamHI* fragment of *phgl::nat* from figure 17). As a solution to this problem, *hgl1<sup>-</sup>* suppressor mutants were recovered in a strain carrying the nourseothricin resistance marker (strain 3020). Mutagenesis was repeated with strain 3020 and the hygromycin library was used for complementation experiments. A complementing gene was not identified because of difficulties in obtaining a sufficient number of transformants to screen the library.

## 4. Discussion

### 4.1 Summary of Results

The objectives of this study were to: 1. Conduct a web-based bioinformatics analysis of the Hgl1p amino acid sequence. 2. Conduct PKA phosphorylation experiments involving the *hgl1* gene product. 3. Complete suppressor mutagenesis of *hgl1*<sup>-</sup> strains to identify genes located downstream of *hgl1* in the dimorphic switch.

A bioinformatics analysis revealed several characteristics that pointed towards a signaling function for Hgl1p, but sequence comparisons to the current databases did not identify any significant similarity to the documented sequences. PROPSEARCH suggested that Hgl1p was a regulator of transcription, and was related to a family of proteins that respond to nitrogen availability in other fungi. The Hgl1p predicted polypeptide sequence contained separate motifs common to a tyrosine phosphatase, a guanine nucleotide dissociation factor, and a G-protein coupled receptor molecule. There were 8 putative PKA phosphorylation sites within the sequence, as well as several PEST regions that could be involved in regulating a ubiquitin-mediated turnover of the Hgl1p protein.

Phosphorylation of Hgl1p by PKA was accomplished *in vitro*, and this result suggested that Hgl1p serves as a target for PKA phosphorylation *in vivo*. Phosphorylation was demonstrated as an increase in apparent molecular weight of an <sup>35</sup>S-methionine labeled Hgl1p band during SDS-PAGE. The mobility shift was altered by the addition of the PKA inhibitor PKI, and was eliminated by treatment of the kinase reaction samples with lambda phosphatase. *In vivo* phosphorylation experiments were planned but not completed because of problems with the epitope tagging of Hgl1p.



Suppressor analysis of *hglI*<sup>-</sup> strains revealed that a variety of phenotypes were possible in the *hglI*<sup>-</sup> background, and that *U. maydis* pigment production and filamentation may not be part of the same pathway. Mutants were identified that respond to nutrient availability with an altered phenotype and this result may implicate other signaling pathways in the control of morphogenesis in culture. It was also possible to collect mutants that varied for pigment production. Variability for pigmentation appeared to be independent from filamentation, suggesting that the two processes may not be results of the same pathways. The ability to isolate strains that suppress the *hglI*<sup>-</sup> phenotypes, and mutant identification indicated that Hgl1p was not required for filamentation in culture, and supported the proposed model of Hgl1p as a negative regulator of budding growth.

## 4.2 Discussion of results and future experiments

### 4.2.1 Hgl1p sequence analysis

Primary BLAST comparisons with full length Hgl1p as a query sequence did not reveal significant similarity to sequences presently in any of the databases. Much of the weak overall similarity that was observed was due to the presence of proline, serine or threonine residues and was the result of amino acid compositional bias in the polypeptides. When the filters of low complexity regions are removed from search algorithms, sequences are identified with much lower E values (lower probability of a random alignment). However the apparent similarity between the sequences is due to compositional bias in this situation. The calculation of the true similarity is grossly affected by the compositional bias of the sequence, and may not represent a true alignment of specific amino acids (Boguski *et al.*, 1998). Data from non-filtered searches must be scrutinized prior to predictions about the function of a protein, and can not be relied upon without supporting experimentation.

PROPSEARCH with Hgl1p suggested it was related to a family of transcription factors that regulate nitrogen metabolism in filamentous fungi, and may also be related to other regulators of transcription from *Saccharomyces cerevisiae*. The *AreA* gene of *Aspergillus nidulans* was the most compositionally similar sequence to Hgl1p in the database, with an approximately 80% chance that Hgl1p was from the same family. *AreA*, a GATA binding factor is responsible for upregulating genes required for the utilization of alternative nitrogen sources versus the preferred sources ammonium and glutamine; these compounds repress *AreA*-induced gene expression. Mutations in *AreA* affect the ability of *Aspergillus* to express structural genes necessary for growth on N sources such as nitrate, nitrite, amino alcohols, and

various purines (Wilson and Arst, 1998). Results from PROPSEARCH support the suppressor analysis described here. This work suggested that *hgl1* suppressors involved nutrient sensing mechanisms or pathways. The transcriptional regulators Sfl1p and Flo8p of *S. cerevisiae* are also compositionally similar to Hgl1p, though the probability that the sequences belong to the same family was only 68%. Flo8p is the regulator of transcription for Flo11p; this protein has 5 consensus PKA sites, and is under regulatory control by *S. cerevisiae* PKA (Pan and Heitman, 1999). Phosphorylated Flo8p induces the transcription of *FLO11*. Conversely, the TATA transcription factor Sfl1p is a negative regulator of *FLO11* gene expression. Sfl1p also has 5 consensus PKA target sites and interacts with PKA; Sfl1p represses the transcription of *FLO11* (J. Heitman, *pers comm*). Identification of Hgl1p's similar composition to regulatory transcription factors may imply that Hgl1p is involved in the transcriptional regulation of genes during *U. maydis* morphogenesis. The Hgl1p amino acid sequence did not share any other similarity to the AreAp, Sfl1p, or Flo8p polypeptides that could be identified by a BLAST2 comparison either in the presence or absence of filters.

The conservation of a motif between two proteins places less emphasis on overall sequence similarity, and instead focuses attention on regions of a sequence which may be responsible for protein function. The presence of described motifs may suggest similar functions for regions of a protein irrespective of its overall function. eMOTIF, PRO-DOM, PROSITE, SMART, PAT-SCAN, and BLOCKS represent some of the programs used in the analysis of the Hgl1p domain structure. Not all of the domain databases share the same domain or profile entries, though many are derivatives of the large PROSITE database. Regions identified as having similarity to known structural or functional motifs were examined for their location in the Hgl1p sequence; these sequences were within regions of Hgl1p predicted to have

significant complexity. These regions were also examined for their conservation with other known motifs that commonly co-exist in proteins that share the domains.

eMOTIF searches revealed several putative motifs of interest in Hgl1p (Figure 10). The cdc25 GDS family motif might indicate a role in signaling, and might be supported by the identification of the GPCR motif in the N-terminus of the protein due to their similar roles in G-protein mediated signaling. A signaling role for Hgl1p could be significant given the extensive arrangement of putative phosphorylation sites and possible tyrosine phosphatase motif in the N-terminus (Figure 10). The coupling of kinase and phosphatase sites is a common regulatory structure for signaling molecules (Millward *et al.*, 1999). At present, a definitive function for Hgl1p was not determined from database comparisons. The phenotypes of *hgl1*<sup>-</sup> strains implied that it was involved in both mating and cAMP/PKA filamentation, but it remained unclear how *hgl1* functions in these processes.

The presence of PEST sequences has been demonstrated to affect protein stability in a number of different organisms (Rechsteiner and Rogers, 1996). PEST sequences are regions of a peptide enriched for proline (P), glutamate (E), serine (S), and threonine (T) residues, resulting in hydrophobic properties that increase susceptibility to proteolysis by the ubiquitination pathway. PEST sequences are evaluated based on a scale from +50 to -50. Scores above zero are considered to be probable PEST regions, while scores above 5 are considered to be quite significant (Rechsteiner and Rogers, 1996). The predicted amino acid sequence for the Hgl1p protein contained 4 potential PEST sequences scoring above zero, two of which had a score above 5 (Figure 10). There were two additional PEST regions in the Hgl1p amino acid sequence which scored approximately -6, but were flanked PKA target sequences at one or both ends of the PEST region. Phosphorylation may act coordinately with nearby PEST regions resulting in increased hydrophobicity and susceptibility to proteolysis

(Brown *et al.*, 1995). These results may indicate that Hgl1p has a rapid turnover rate, and may be targeted to the proteolytic pathway in a phosphorylation-dependent manner.

A PROSITE analysis of the Hgl1p amino acid sequence identified eight putative PKA target sequences “[RK](2)-x-[ST]” (Figure 10). Four of the proposed sites resided in the N-terminus, and flanked the possible GPCR transmembrane motif, or the transmembrane motif identified by Tmpred. The identification of *hgl1*<sup>-</sup> as a suppressor of the *adr1*<sup>-</sup> phenotype suggested these phosphorylation sites may be important for Hgl1p function in the presence of PKA. The absence of Hgl1p caused a budding growth phenotype in the presence or absence of PKA, suggesting that it may function as a suppressor of budding growth. Therefore, one proposed model is that phosphorylated Hgl1p may suppress budding growth. This prediction was supported by the *adr1*<sup>-</sup> phenotype, and by the filamentous mutants collected in both the 3020 and 3034 strains. Presumably there was non-phosphorylated Hgl1p present in *adr1*<sup>-</sup> strains which suppressed the budding growth phenotype (ultimately causing a filamentous growth phenotype). Mutation in *hgl1* restored budding growth to *adr1*<sup>-</sup> strains, and also caused budding growth in the wild type background. The demonstration of Hgl1p and Adr1p interactions *in vitro* (Hgl1p phosphorylation experiment) provided additional support for this model.

#### 4.2.2 Phosphorylation of Hgl1p by PKA

Attempts were made to examine the phosphorylation state of Hgl1p in *U. maydis* strains using epitope-tagged versions of Hgl1p. It was not possible to recover purified Hgl1p protein expressed from the *hgl::myc*, or *hgl::HIS* constructs during experiments with *U. maydis* or *E. coli*. It is not clear why Hgl1p protein could not be produced; several expression conditions

were tested and all were unsuccessful. It could be that the product was extremely unstable following expression under native and recombinant conditions, or that there was some unknown mechanism or circumstance preventing its accumulation. It has been documented that proteins rich in arginine codons AGA and AGG are difficult to express in *E. coli* (Baneyx, 1999; Ivanov *et al.*, 1997). An Expasy compositional analysis identified the Hgl1p sequence as arginine rich compared to conventional yeast proteins. Hgl1p does have two such codons (AGA/AGG) early in its coding sequence. However, they do not comprise a significant proportion of the arginine codons present in the polypeptide, likely eliminating this possibility.

The *in vitro* phosphorylation data demonstrated that Hgl1p served as a target for both bovine and *U. maydis* PKA. The PKA-dependent phosphate transfer was partially inhibited by PKI, and phosphate group removal was seen with lambda phosphatase. PKI is a 20 amino acid peptide which specifically and competitively binds the catalytic subunit of PKA to inhibit its kinase activity (Demaille *et al.*, 1977; Scott *et al.*, 1985; Takio *et al.*, 1980). Addition of PKI to the reactions was anticipated to eliminate the mobility shift, however, reactions with Hgl1p and bovine or *Ustilago* PKA in the presence of PKI were not completely inhibited. The PKI partial inhibition result was consistent between the two kinase experiments. Despite the reported stability of PKI and the molar excess added to each experiment (1000 fold), long-term storage and multiple freeze-thaw cycles may have diminished the inhibitory potency or other unknown qualities of the peptide. Both panels in figure 15 show the same trend of increased apparent molecular weight when Hgl1p is phosphorylated by PKA.

While it was demonstrated that Hgl1p served as a target of PKA *in vitro*, it was not possible to imply *in vivo* functional significance based on this result alone. It is interesting, however, that the predicted protein had several phosphorylation sites and served as a suppressor to the *adr1<sup>-</sup>* phenotype. Recent work in *S. cerevisiae* by Pan and Heitman (1999) has addressed

the possibility that the transcription factors Flo8p and Sfl1p serve as targets of the *S. cerevisiae* PKA Tpk2p. Their work is limited to epistasis and yeast two-hybrid analysis, but was conducted under the premise that the 5 PKA sites in Flo8p and Sfl1p may indicate a role in PKA signal transduction. Along the same lines, Hgl1p contained 8 PKA target sites, and by epistasis analysis suppressed the low PKA phenotypes in *U. maydis* (Figure 21). The work presented here further implicated an enzymatic interaction between the Hgl1p and *U. maydis* PKA proteins *in vitro*.

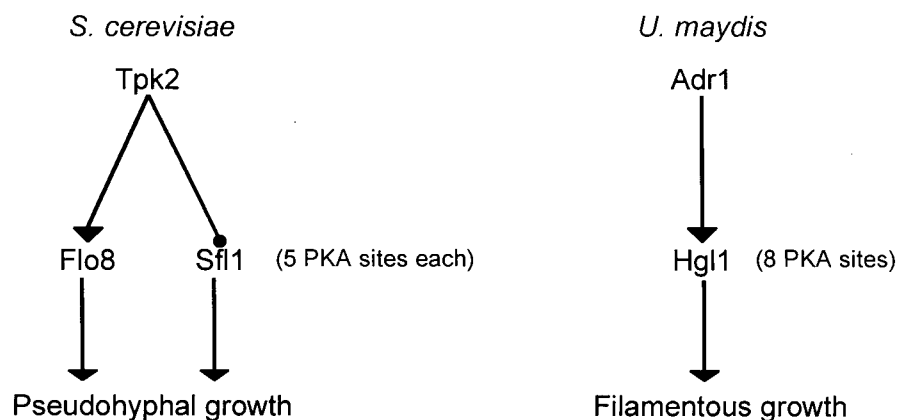


Figure 21. Comparison of the *S. cerevisiae* PKA target proteins Flo8p and Sfl1p with the proposed *U. maydis* PKA target protein Hgl1p. Each of the three proteins (Flo8p, Sfl1p, and Hgl1p) suppresses phenotypes of PKA mutants, and each contains several consensus PKA target sequences.

It will be necessary to conduct experiments which investigate the nature and consequences of Hgl1p phosphorylation *in vivo*. Ideally it would be interesting to recover native Hgl1p from wild-type, *ubc1<sup>-</sup>*, and *adr1<sup>-</sup>* cell types to examine the phosphorylation state of Hgl1p in each of these strains. One would expect wild-type cells to have an intermediary state of Hgl1p phosphorylation dependent on the conditions examined, the *ubc1<sup>-</sup>* strain to have a

hyper-phosphorylated version of Hgl1p, and for the *adr1<sup>-</sup>* cells to have a non-phosphorylated or weakly phosphorylated version of Hgl1p. Dürrenberger *et al.* (1998) identified regulated PKA activity as necessary for pathogenesis and virulence; this may suggest that there is a regulatory function for PKA phosphorylation of Hgl1p that is required *in vivo* for pathogenicity and virulence. A site directed mutagenesis experiment in which each of the kinase target sites individually (and in various combinations) are eliminated could examine the functional importance of phosphorylation. An alternative approach may be to attempt random mutagenesis experiments with *hgl1* to look for mutants which lose the ability to complement the *hgl1<sup>-</sup>* phenotype. It may be possible to recover mutants which only partially complement the existing phenotypes, potentially identifying mutations in PKA phosphorylation sites or other motifs, and providing support for the importance of Hgl1p phosphorylation in the PKA pathway.

#### 4.2.3 Suppressor analysis *hgl1<sup>-</sup>* strains

U.V. induced mutants which either partially, or completely suppressed the *hgl1<sup>-</sup>* budding growth phenotype were isolated. This suggested that there were components of pathways participating in filamentation that were epistatic to *hgl1*. Recovery of filamentous cell types in the *adr1<sup>-</sup> hgl1<sup>-</sup>* background, which strongly resembled *hgl1<sup>-</sup>* suppressor mutants, suggested there were additional factors involved in the *U. maydis* dimorphic switch. Suppression of the budding *hgl1<sup>-</sup>* phenotype in both backgrounds suggested that the mutations recovered were epistatic to *hgl1* and possibly *adr1*. Perhaps additional targets of Adr1p exist which counteract or negatively regulate Hgl1p and result in Hgl1p serving as an activator of filamentous growth (Figure 22). The presence of Adr1p in these mutants might activate these genes or proteins at



the same level as Hgl1p in the pathway to cause a filamentous phenotype. It is not known whether the *hgl1<sup>-</sup>* suppressor mutations also suppress the teliospore defect of the *hgl1<sup>-</sup>* strains.

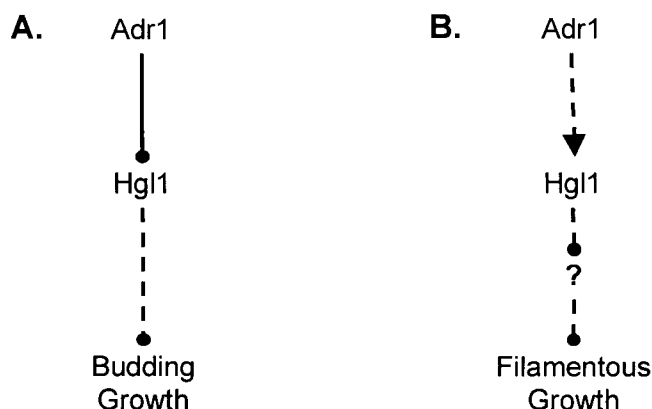


Figure 22. Hgl1p suppressors represent a variety of functions. Dashed lines represent interactions that may involve intermediate components. A. Experiments in this work suggest that Adr1p acts directly on Hgl1p. As a direct target of Adr1p, Hgl1p may serve as a repressor of budding growth. B. If there are intermediate proteins between Adr1p and Hgl1p, then Hgl1p may serve as an inhibitor of some other regulator of filamentous growth. For both models there are likely other proteins downstream of Hgl1p that function to regulate filamentous growth in *U. maydis*.

The isolation of mutations which did not alter their phenotypes on different media suggests nutrient availability may affect filamentous growth. For mutants which do respond to nutrient availability, generally, filamentation became exaggerated as the nutrients were depleted. Similarly, for mutants with increased production of a pigment similar to the *hgl1<sup>-</sup>* yellow pigment, pigment production appeared to increase at lower nutrient levels. It was possible to collect filamentous mutants which had decreased pigment production. A loss of pigment production in a filamentous suppressor mutant suggested that perhaps pigment production was not directly linked to the filamentous growth phenotype. The pigmentation response to nutrient availability appeared common to both the original *hgl1<sup>-</sup>* knockout and the suppressor mutants alike. Perhaps if pigment production was somehow involved in teliospore

formation (deficient in the *hglI*<sup>-</sup> mutants), the increased response to nutrient starvation may implicate nutrient sensing as part of the developmental program. Variability between different suppressor mutants and the amount of filamentation and black pigment production suggested interactions between these two phenotypes, Hgl1p function, and nutrient sensing.

#### 4.2.4 Complementation of *hglI*<sup>-</sup> suppressor mutants

There were several difficulties encountered when selected mutants were used in complementation experiments and these prevented recovery of a complementing gene. The filamentous phenotype of mutant strains made protoplast formation extremely difficult, resulting in a low yield of cells with poor competency. In addition to this, the genomic cosmid libraries used for transformation had low transformation efficiencies. Taken together, the poor competency cells and low transformation efficiency made it unlikely that complementing genes would be identified with this approach. Several rounds of transformation were attempted with several different protoplast preparations for each of the *hglI*<sup>-</sup> disruption backgrounds and all of these trials were unsuccessful. In future work, the insertional mutagenesis approach of restriction enzyme mediated integration (REMI) should be used to isolate suppressor mutants. If these mutants can be obtained, the REMI procedure would facilitate subsequent cloning of the disrupted gene.

#### 4.2.5 Summary of *hglI*<sup>-</sup> phenotypes

The *hglI*<sup>-</sup> gene affects processes leading to filamentous growth in *U. maydis*. A summary of the phenotypes of *hglI*<sup>-</sup> mutants observed in different strain backgrounds supports the model of Hgl1p being a suppressor of budding growth that is inactive in conditions of high PKA activity. Table 3 summarizes the phenotypes of strains defective for components of the

PKA pathway in *U. maydis*, and includes the phenotypes caused by mutations in *hgl1*.

Strain	PKA levels	Hgl1p	Morphology
<b>Wild type</b>	<u>Regulated</u> Adr1	Variable $\oplus$ -Hgl1	Variable morphology
<b><i>ubc1</i><sup>-</sup></b>	<u>High</u> Adr1	$\oplus$ -Hgl1	Budding
<b><i>adr1</i><sup>-</sup></b>	<u>NO</u> Adr1	Hgl1	Filamentous
<b><i>hgl1</i><sup>-</sup></b>	<u>Regulated</u> Adr1	<u>NO</u> Hgl1	Budding
<b><i>adr1 hgl1</i><sup>-</sup></b>	<u>NO</u> Adr1	<u>NO</u> Hgl1	Budding
<b><i>hgl1</i><sup>-</sup> Sup.</b>	+/- Adr1	<u>NO</u> Hgl1	Filamentous

Table 3. Summary of *hgl1* phenotypes. The 'strain' column lists the strains summarized in each of the PKA levels, Hgl1p, and Morphology columns. Column 'PKA levels' describes the state of Adr1, the major catalytic subunit of PKA in *U. maydis*. Column 'Hgl1p' describes the probable state of the Hgl1p protein in the strain,  $\oplus$ -Hgl1p indicates that Hgl1p is likely phosphorylated in the conditions of the background strain. The 'morphology' column presents the overall phenotype of colony growth, the variable morphology of the wild type strain can range from budding to filamentous growth. Row '*hgl1*<sup>-</sup> Sup.' represents *hgl1* suppressors collected in this work.

From table 3, if the Hgl1p protein was in the phosphorylated state in the *ubc1* background, then Hgl1p may be a suppressor of budding growth that was inactivated by PKA. In support of the suppressor of budding growth model, the phenotypes of the *adr1*<sup>-</sup> or *hgl1*<sup>-</sup> strains also suggested that Hgl1p inhibited budding growth. The *ubc1*<sup>-</sup> strain shared similarities with *hgl1*<sup>-</sup> mutants and the presence of Hgl1p in the low PKA background was associated with a filamentous phenotype. This suggested that Hgl1p may have been an activator of filamentous growth in the absence of PKA. The collection of filamentous mutants in *hgl1*<sup>-</sup> strains indicated that Hgl1p was not essential for filamentation, and is not likely an activator of filamentous growth.

### 4.3 Conclusions

Previous studies from our laboratory have identified many components of the cAMP-dependent PKA signal transduction pathway. Many of these components (adenylyl cyclase, and the PKA regulatory and catalytic subunits) are well characterized proteins. This has aided in resolving their function in the *U. maydis* signal transduction pathway to establish filamentous growth. Recovery of a novel *U. maydis* gene (*hgl1*) as a suppressor of the low PKA phenotype prompted experiments to examine how Hgl1p participated during signal transduction and morphogenesis. Sequence analysis of Hgl1p revealed several regions of the polypeptide that suggest it may function as a signaling molecule. In addition to possession of these regions, this thesis demonstrated phosphorylation of Hgl1p by *U. maydis* PKA *in vitro*, providing biochemical evidence for its involvement in the cAMP/PKA signal transduction pathway. Finally, it was possible to suppress the *hgl1*<sup>-</sup> colony and cellular morphologies in culture with mutations obtained by U.V. mutagenesis. Suppression of *hgl1*<sup>-</sup> supported the current model that Hgl1p serves as a repressor of budding growth in culture, and that Hgl1p is not required for filamentous growth in culture. Whether the *hgl1*<sup>-</sup> phenotypes were compromised *in planta* by the suppressor mutants remains to be investigated, but will provide exciting insights and information regarding events occurring during pathogenesis.

## 5. References

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