

REVIEW

Serine/Threonine Protein Kinases and Phosphatases in Filamentous Fungi

Martin B. Dickman* and Oded Yarden†

*Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68583; and †The Department of Plant Pathology and Microbiology and The Otto Warburg Center for Agricultural Biotechnology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

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Dickman, M. B., and Yarden, O. 1999 Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genetics and Biology* 26, 99-117. Protein phosphorylation and dephosphorylation are one of the central currencies by which living cells perceive and respond to environmental cues. A number of fundamental processes in fungi such as the cell cycle, transcription, and mating have been shown to require protein phosphorylation. The analysis of protein kinases and phosphatases in filamentous fungi is in its infancy; however, it has already become clear that kinases and phosphatases are likely to be important mediators of fungal proliferation and development as well as signal transduction and infection-related morphogenesis. In this review, we describe, summarize, and consider the rapidly expanding field of protein phosphorylation/dephosphorylation in various aspects of filamentous fungal growth and development. © 1999 Academic Press

Index Descriptors: signal transduction; fungal genes; protein phosphorylation; gene regulation; fungal growth; fungal morphogenesis.

All living cells must constantly monitor their internal and external environments for the presence or absence of factors that signal proliferation, differentiation, arrest, or death. Accordingly cells must integrate numerous exogenous signals to control the possible courses of progression or suspension of some or all cellular activities. A primary

means by which this challenge has been addressed is the development of reversible modification of cellular constituents that can serve to integrate external stimuli. In eukaryotes, phosphorylation-dephosphorylation cycles represent a major mechanism for switching cellular pathways in response to changing circumstances, albeit internal developmental cues or external environmental stimuli. Protein kinase-mediated phosphorylation regulates protein function (directly or via transducing the relevant signals) involved in the entire gamut of cellular processes, from metabolic enzymes, through membrane channels and pumps, cytoskeletal proteins, and transcription factors.

The computer-based sequence analysis of the complete *Saccharomyces cerevisiae* genome has yielded 113 genes (of the presumed 6,000), which can be unambiguously identified as protein kinases (Stark, 1996; Hunter and Plowman, 1997). This comprises ~2% of the total number of genes and suggests that at least a similar number (and quite likely more, when considering genome complexity) are present in filamentous fungi. Furthermore, as a significant part of the regulation of protein phosphorylation is based on reversibility, a similar number of protein phosphatases must be present to insure and maintain the full process. Even though a significant percentage of the fungal (and other eukaryotic) genome encodes phosphorylation-related proteins, very little is understood concerning their function, interacting partners, and pathways in which they are involved. While there are clear parallels between *S. cerevisiae* and filamentous fungi with respect to gene structure/function, there is an equal (if not greater) num-

ber of distinct differences. Filamentous fungi harbor a number of unique characteristics, including pathogenic development, secondary metabolism, and virulence, to name but a few, that are not found and cannot be studied in budding yeast. As will be discussed later, these important differences need to be considered when inferring kinase/phosphatase function in *S. cerevisiae* to filamentous fungi (and vice versa).

In contrast to the high number of budding yeast kinases, Stark (1996) has found only 31 yeast sequences that harbor the various protein phosphatase (PP) motifs in the PROSITE database. The difference between the number of kinases and phosphatases identified leads to a basic conclusion regarding substrate specificity, which suggests that in contrast to the highly specific nature of kinases, each phosphatase catalytic subunit is most likely able to interact with more than the single (or few) particular sequence motif(s) recognized by the kinase. Structural diversity (including regulatory subunits and other phosphatase-associated polypeptides), along with temporal and spatial regulation are all possible means of expanding the substrate specificity spectrum of PPs (Cohen, 1997; Faux and Scott, 1996).

Our interest in phosphorylative regulation of cellular processes has led us to probe these enzymes in filamentous fungi with the intention to first identify and eventually link signal transduction cascades, protein modification, and fungal morphogenesis. Functions associated with signaling via phosphorylation in fungi can be broadly grouped in the following manner:

- (1) The response to extracellular signals, e.g. mating factors, hormones, light, physical stimuli, host plant tissue, growth and differentiation factors.
- (2) The regulation of processes which occur discontinuously during the cell cycle such as DNA synthesis and mitosis.
- (3) The response to nutritional and environmental stress.

The purpose of this review is to briefly summarize our current knowledge of the Ser/Thr protein kinases and phosphatases identified in filamentous fungi and to discuss their possible roles in cellular processes on the basis of their structural and functional analyses. In this review we present the currently available information concerning Ser/Thr protein kinases and phosphatases in filamentous fungi. The first part of the review is dedicated to kinases and the latter to phosphatases. As kinases and phosphatases have proven to belong to ever-expanding gene/protein

families, we have seen it fit to also provide an overview of the current classification of these families (presented prior to the detailed descriptions of genes and their corresponding proteins).

The kinases/phosphatases mentioned throughout this review have been, for the most part, grouped on the basis of structural classification and include the cAMP-dependent kinases (PKA), protein kinase type C (PKCs), Mitogen-activated kinases (MAP), and p21-activated kinases (PAK). Additional kinases, which have been analyzed mainly on the basis of their function (e.g., cell cycle-related kinase or the COT1/TB3 kinases) in filamentous fungi follow.

In the second part of the review we provide a similar compilation, describing the protein phosphatases (classification, PP1, PP2A, PP2B, and proteins associated with PPs). We present some of the ways currently used for the analysis of kinases and PPs (combining genetics and pharmacological approaches) and discuss future aspects of the research concerning these proteins.

SER/THR KINASES IN FILAMENTOUS FUNGI

As with other eukaryotes, research on the identification and functional characterization of protein kinases in filamentous fungi has been on the rise. Major efforts have focused on: (i) development and infection related morphogenesis, (ii) cell cycle, and (iii) pathogenicity. The Ser/Thr kinase genes that have been identified and studied in filamentous fungi are listed in Table 1. Studies of the better-characterized fungal protein kinases comprise this part of the review.

Classification

Nomenclature with respect to protein kinase can be confusing since different family members are named either by substrates, regulation, mutant phenotype, or by some arbitrary name or number. We will use the conventional method of classification by the amino acid residue that is phosphorylated. As mentioned, Ser/Thr kinases are the predominant species in filamentous fungi and all eukaryotes in general (e.g., Tyr kinases comprise <0.1% of total phosphorylation events). These enzymes use the gamma phosphate of ATP or GTP to generate phosphate esters, using protein alcohol groups on Ser and/or Thr as acceptors. These kinases are usually modular enzymes,

TABLE 1

Genes Encoding Protein Kinases Isolated from Filamentous Fungi That Have Been Structurally and/or Functionally Analyzed

Kinase	Organism	Gene Designation	Reference
PKA cat subunit	<i>Neurospora crassa</i>	<i>NC2BC</i>	<i>Neurospora</i> data base ^a
PKA cat. subunit	<i>Magnaporthe grisea</i>	<i>cpkc</i>	Mitchell and Dean, 1995
PKA cat. subunit	<i>Colletotrichum trifolii</i>	<i>ct-pkac</i>	Yang and Dickman, 1999 (in press)
PKA cat. subunit	<i>Blastocladiella emersonii</i>	?	de Oliveira <i>et al.</i> , 1994
PKA cat. subunit	<i>Aspergillus niger</i>	<i>pkaC</i>	Bencina <i>et al.</i> , 1997
PKA cat. subunit	<i>Ustilago maydis</i>	<i>uka1</i>	Dürrenberger <i>et al.</i> , 1998
PKA cat subunit	<i>Ustilago maydis</i>	<i>adr1</i>	Dürrenberger <i>et al.</i> , 1998
PKA reg. subunit	<i>Neurospora crassa</i>	<i>mcb</i>	Bruno <i>et al.</i> , 1996
PKA reg. subunit	<i>Colletotrichum trifolii</i>	<i>ct-pkar</i>	Yang and Dickman (in press)
PKA reg. subunit	<i>Blastocladiella emersonii</i>	?	Marquez, <i>et al.</i> , 1992
PKA reg. subunit	<i>Magnaporthe grisea</i>	<i>sumi</i>	Adachi and Hamer GenBank Accession No. AF015753
PKA reg. subunit	<i>Magnaporthe grisea</i>	<i>rpka</i>	Gilbert and Dean GenBank Accession No. AF024633
PKA reg. subunit	<i>Ustilago maydis</i>	<i>ubc1</i>	Gold <i>et al.</i> , 1997
PKC	<i>Neurospora crassa</i>	<i>SC2G1</i>	<i>Neurospora</i> data base
PKC	<i>Aspergillus niger</i>	<i>pkcA</i>	Morawetz <i>et al.</i> , 1996
PKC	<i>Trichoderma reesei</i>	<i>pkc1</i>	Morawetz <i>et al.</i> , 1996
MAP kinase	<i>Magnaporthe grisea</i>	<i>pmk1</i>	Xu and Hamer, 1996
MAP kinase	<i>Fusarium solani</i>	<i>fsmapk</i>	Li <i>et al.</i> , 1997
MAP kinase	<i>Magnaporthe grisea</i>	<i>mps1</i>	Xu <i>et al.</i> , 1998
MAPKK	<i>Neurospora crassa</i>	<i>nrc-1</i>	Kothe and Free, 1998
MEK kinase	<i>Ustilago maydis</i>	<i>fuz7</i>	Banuet and Herskowitz, 1994
Cell cycle kinase	<i>Aspergillus nidulans</i>	<i>nima</i>	Osmani <i>et al.</i> , 1988
CDC2	<i>Aspergillus nidulans</i>	<i>nimx</i>	Osmani <i>et al.</i> , 1994
COT 1	<i>Neurospora crassa</i>	<i>cct 1</i>	Yarden <i>et al.</i> , 1992
COT 1 homolog	<i>Colletotrichum trifolii</i>	<i>tb3</i>	Buhr <i>et al.</i> , 1996
COT 1 homolog	<i>Ustilago maydis</i>	<i>ukc1</i>	Dürrenberger and Kronstad, 1999 (in press)
Histidine kinase	<i>Neurospora crassa</i>	<i>nik-1</i>	Alex <i>et al.</i> , 1996
Histidine kinase	<i>Candida albicans</i>	<i>cos 1</i>	Alex <i>et al.</i> , 1998
Ser/Thr kinase	<i>Neurospora crassa</i>	<i>nrc-2</i>	Kothe and Free, 1998
Ser/Thr kinase	<i>Neurospora crassa</i>	<i>SC3A2</i>	<i>Neurospora</i> data base ^a
Ser/Thr kinase	<i>Colletotrichum lindemuthianum</i>	<i>slk1</i>	Dufresne <i>et al.</i> , 1998
Ser/Thr kinase	<i>Trichoderma reesei</i>	<i>pkt1</i>	Morawetz <i>et al.</i> , 1994
Ca/CAM kinase	<i>Neurospora crassa</i>	<i>SM1H12</i>	<i>Neurospora</i> data base ^a
Lipid kinase	<i>Colletotrichum trifolii</i>	<i>lapk</i>	Yang <i>et al.</i> , submitted
Septation deficient protein kinase	<i>Aspergillus nidulans</i>	<i>sepH</i>	Harris <i>et al.</i> , 1994

^a <http://biology.unm.edu/~ngp/home/html>.

containing a highly conserved catalytic region and a less conserved regulatory domain. These two domains are often bridged by a hinge region or a pseudosubstrate area (e.g., PKC), which serves to keep the enzyme inactive. Sequence alignments have indicated that many Ser/Thr kinases have similar structure, particularly in the catalytic domain. Conserved features have been identified and reveal 12 subdomain regions in the catalytic portion of Ser/Thr kinases. These domains are recognized as being invariant or nearly invariant and thus play essential roles in enzyme structure and function. Other parts of the kinase domain are more variable thus allowing for different mechanisms of control and regulation. This high degree of conservation in protein kinases is found in higher and

lower eukaryotes; a feature that has facilitated the cloning of genes and is in large part responsible for the nearly exponential increase in the number of identified protein kinases. For a more complete discussion of protein kinase sequence classification and phylogeny see Hardie and Hanks (1995). Additional information and updates can be found at <http://bioinfo.weizmann.ac.il/Kinases/> (and similar sites worldwide).

Protein kinases can be divided into at least five groups according to the amino acid residue(s) which they phosphorylate. The first group consists of the so-called two-component histidine kinases. These enzymes are actually aspartate kinases, since the second "acceptor" component is phosphorylated on aspartate, although this process

occurs via a phosphohistidine intermediate in the first (receiver) component. The two components may be separate proteins or separate domains on the same polypeptide. This family was originally thought to be restricted to prokaryotes, but representatives have been found in yeasts (Ota and Varshavsky, 1993; Maeda *et al.*, 1994), *Candida albicans* (Alex *et al.*, 1998), *Neurospora* (Alex *et al.*, 1996; Schumacher *et al.*, 1997), *Colletotrichum* (Dickman, unpublished), as well as in *Arabidopsis* (Chang *et al.*, 1993). A second group consists of the "true" histidine kinase which phosphorylate histidine side chains on their target protein. This activity has been described in mammals, but is currently best characterized in *S. cerevisiae* (Huang *et al.*, 1991). To our knowledge, none have yet been identified in filamentous fungi and it is not clear whether they belong to a distinct gene family. The three remaining groups are the serine/threonine, tyrosine and dual specificity kinases ("MAPKK") which are capable of phosphorylating Ser/Thr and tyrosine side chains. These last three groups form a very large gene family often referred to as the eukaryotic protein kinase superfamily (Hanks and Hunter, 1995).

Analysis of the budding yeast genomic sequence indicates that this organism lacks members of the true protein-tyrosine kinase family. Similarly, no tyrosine kinases have been reported in filamentous fungi, suggesting that the evolution of protein tyrosine kinases may be due to the need for a signaling mechanism for cell-cell communication within a multicellular organism. As the Ser/Thr kinases represent virtually all of the kinases described in filamentous fungi, we will focus our discussion on this family.

Cyclic AMP-dependent protein kinases

cAMP-dependent protein kinase (PKA) was among the first protein kinases to be purified and is among one of the best understood. PKA is also the first protein kinase to have its crystal structure determined (Knighton *et al.*, 1991). PKA has been found in all eukaryotes studied and remains the primary, if not the only, receptor for cAMP.

In its inactive form, the enzyme is a tetramer comprised of two regulatory (R) and two catalytic (C) subunits. Activation is mediated by binding of cAMP to the R subunits which alters the affinity of R for C, thus promoting the dissociation of the complex into a dimer of R subunits and two active monomeric C subunits. The free enzymatically active C subunits then function by phosphorylating target substrate proteins (generally not yet identified; a recurring theme in this area). In mammalian systems, the free C subunit can migrate into the nucleus and activate transcription; however, this has not been

demonstrated to occur (yet) in filamentous fungi. PKAs are characterized by variation in their regulatory subunits, designated RI and RII, which in general can be distinguished by sequence, antigenicity, and affinity for C subunits. In filamentous fungi, the presence of only one type of R subunit (type II) is the rule thus far (Bencina *et al.*, 1997).

Genes encoding the catalytic subunit of PKA have been cloned from a number of filamentous fungi including *Magnaporthe grisea* (Mitchell and Dean, 1995), *Blastocladiella emersonii* (de Oliveira *et al.*, 1994), *Aspergillus niger* (Bencina *et al.*, 1997), *Ustilago maydis* (Dürrenberger *et al.*, 1998), *Colletotrichum trifolii* (Yang and Dickman, in press), and *Neurospora crassa* (*Neurospora* database). Regulatory subunits have been cloned from *Ustilago maydis* (Gold *et al.*, 1997), *N. crassa* (Bruno *et al.*, 1996), *C. trifolii* (Yang and Dickman, in press), and *B. emersonii* (Marquex and Gomes, 1992).

While caution must be exercised in generalizing about specific functions of PKA in filamentous fungi (e.g., no direct substrates of PKA have been identified) potential themes are emerging. In pathogenic fungi (e.g., *M. grisea*, *C. trifolii*, *U. maydis*, and indirectly *C. parasitica*) growth, morphogenesis, and virulence are known to involve functional PKA. In the rice blast pathogen *M. grisea*, PKA was found to be necessary for successful host penetration, as gene disruption of a PKA C subunit resulted in a pronounced delay in appressorium development and, importantly, an inability to penetrate and thus colonize intact rice plants (Mitchell and Dean, 1995; Xu *et al.*, 1997). This defective phenotype is attributable the inability of *M. grisea* to accumulate the high concentrations of glycerol needed to generate the turgor pressure required for mechanical penetration by appressoria (deJong *et al.*, 1997). In *C. trifolii*, targeted inactivation of PKA C subunit also resulted in an inability to penetrate intact host tissue, although appressorial development was comparable to wild-type *C. trifolii* (Yang and Dickman, in press). It is unknown how *C. trifolii* penetrates host tissue, but it is reasonable to hypothesize that PKA may affect pathways involved in the mobilization of carbohydrate reserves to generate the high concentration of glycerol needed to obtain sufficient mechanical pressure. As a considerable degree of identity was found between the PKA-C subunits of these phytopathogenic fungi, including the divergent N-terminus, as well as the fact that both organisms require appressoria for penetration, it is possible that mechanical penetration is regulated by similar PKA-mediated mechanisms.

In *U. maydis*, the causal agent of corn smut, pheromones and their receptors are needed for cell fusion and the formation of the pathogenic filamentous dikaryon. Fungal dimorphism is altered in adenylate cyclase mutants (adenylate cyclase catalyzes the reaction forming cAMP from AMP). Such mutants are constitutively filamentous suggesting that reduced levels of cAMP and low PKA activity induce filamentous growth (Gold *et al.*, 1994). Mutants defective in the R subunit of PKA failed to form symptomatic galls on corn and were defective in cytokinesis (Gold *et al.*, 1997). Recent work has identified two genes encoding C subunits of PKA, *adr1*, and *uka1* (Kronstad, 1997; Dürrenberger *et al.*, 1998). Targeted inactivation of *adr1* resulted in a phenotype similar to the adenylate cyclase mutants, namely constitutive filamentous growth. These haploid mutants were also non-pathogenic. *Uka1* deletion does not affect morphology or pathogenicity. Thus appropriate regulation of *Adr1* activity is essential for infection-related morphogenesis, namely the transition from budding to filamentous growth, and thus for corn smut disease.

While the cloning of the PKA gene(s) from the chestnut blight fungus *C. parasitica* has not been reported, a role for PKA in morphogenesis and virulence is likely. Nuss *et al.* (1996) have studied the effects of hypovirus infection on signaling pathways in *C. parasitica*. In this fungus hypoviral infection results in attenuated virulence, reduced asexual sporulation, female infertility, and altered pigmentation (Nuss, 1996). Two genes encoding guanine nucleotide-binding-proteins (G-proteins), which are known to be key mediators in signal transduction pathways (Neer, 1995), have been cloned; one is similar to an α -subunit of the G_i family (*cpg-1*); the expression of which was nearly undetectable in virus-infected *C. parasitica* (Choi *et al.*, 1995). Further studies, using virus free *C. parasitica* possessing normal levels of CPG-1, showed that when a sense copy of CPG-1 was inserted, cosuppression of CPG-1 occurred, resulting in reduced CPG-1 levels and a similar phenotype as observed with virus infection (altered morphology, reduced virulence, etc.). Interestingly, and perhaps unexpectedly, antisense transformants showed no changes. Thus, reduced G-protein accumulation, effected by virus infection or by transformation, correlated with reduced virulence and altered morphology in *C. parasitica*. In mammalian systems, G_i subunits inhibit adenylate cyclase activity; thus by analogy virus infection and/or cosuppression of CPG-1 would result in increased cAMP levels. Indeed, this was shown to be the case (Chen *et al.*, 1996). Artificially increasing cAMP levels by pharmacologi-

cal reagents also mimicked the effect of viral infection. Taken together, as in mammalian systems, CPG-1 down-regulates cAMP levels (via inhibition of adenylate cyclase). Hypovirus infection suppresses the G-protein levels, resulting in cAMP accumulation and presumably PKA activity leading to the hypovirus-associated phenotypes. PKA is likely to be an important downstream target in this system, although this system illustrates the complexity and cross talk associated with signaling pathways which can complicate the distinction between cause and consequence.

PKAs have also been described in *Neurospora* and *Aspergillus*. When the PKA-C subunit gene of the industrially important filamentous fungus *Aspergillus niger* was overexpressed, growth and development were altered, but transformants phenotypically differed from each other, some sporulating densely while others not sporulating at all (Bencina *et al.*, 1997). In *N. crassa* the R subunit of PKA was identified as the *mcb* gene. The conditional *mcb* mutant of *N. crassa* exhibits a complete loss of growth polarity and mislocalized septa. Cloning and sequencing of the *mcb* gene revealed that it encoded a PKA-R subunit (Bruno *et al.*, 1996). The conclusion was drawn that a PKA pathway regulates growth polarity possibly via an actin dependent mechanism. The fact that the R subunit of *C. trifolii* PKA complemented the *mcb* mutant of *N. crassa* (Yang and Dickman, in press) strongly suggested that the structure and function of genes encoding fungal protein kinases are conserved.

Protein Kinase C

Protein kinase C (PKC), a calcium, phospholipid dependent Ser/Thr kinase, is central to one of the major mammalian signal transduction pathways. PKC is activated upon external stimulation of cells by various ligands including hormones, neurotransmitters, and growth factors (Nishizuka, 1992). These external signals stimulate the hydrolysis of membrane-associated inositol phospholipids generating two second messengers, inositol triphosphate and diacylglycerol (DAG). DAG is an endogenous activator of PKC. Phorbol esters, which are capable of tumor promotion, can substitute for DAG in enzyme activation, but unlike DAG, phorbol esters are not rapidly metabolized and thus are useful for *in vitro* analysis. In mammals, PKC is composed of at least 12 distinct polypeptides or isoforms (Dekker and Parker, 1994). PKC plays a role in differentiation, cell growth, transcription, and disease. Furthermore, there is growing evidence that specific PKC isoforms orchestrate these processes (Jarvis *et al.*, 1996). Moreover, the subcellular distribution of PKC isoforms

changes dramatically when the cell is activated, and specific PKC isoforms have substrate selectivity. Physiologically, DAG-regulated activation of PKC leads to rapid translocation from the cytosol to the membrane. Since PKC can also be found in the nucleus, it can directly phosphorylate nuclear proteins. Thus alterations in PKC activity can have a profound effect on cellular processes. The association of PKC with numerous mammalian developmental and disease related phenomena has created interest in identifying homologs in filamentous fungi.

Two research groups have identified PKC genes in *N. crassa*. Oved and Yarden (1996) utilized an immunodetection approach in order to differentially identify different PKCs in *N. crassa*. Antibodies raised against several different mammalian PKCs (including classical, novel, and atypical isotypes) showed that polypeptides cross-reacting with anti-PKC antibodies were present in *N. crassa*. Putative conventional, as well as novel PKCs were identified. Two of the antibodies tested (anti- ϵ and anti- η isoforms) detected elevated PKC levels during conidial germination, suggesting the involvement of these PKCs in early events of transitional growth. Using degenerate oligonucleotide primers, designed on the basis of a highly conserved amino acid sequence in the PKC catalytic domain, a PKC gene DNA fragment was amplified and a corresponding cDNA clone encoding a novel PKC was identified. Transcripts of this gene were present in dormant spores and increased during spore germination.

Macino and colleagues have presented strong evidence for involvement of PKC in the *N. crassa* blue light signal pathway (Arpaia *et al.*, 1997). Blue light influences development, carotenoid production, and circadian rhythm in *Neurospora*. In screening for compounds which interfere with light-stimulated transcription of the albino-3 gene (*al-3*), specific inhibitors of PKC were found to be effective. *N. crassa* PKC appears to be responsible for the adaptive response of desensitization to light; inhibitors of the enzyme inhibit the decay-phase in the kinetics of light induction of the *al-3* transcript. During conidiation, PKC activity is necessary for the light induction of the *al-3* conidiation-specific transcript. In this phase, PKC inhibitors totally abolish light-induced albino-3 expression, but not conidiation-driven transcription of the gene.

The best characterized example of PKC in filamentous fungi is shown with *Trichoderma reesei* (Morawetz *et al.*, 1996). The protein, which was recently purified (Lendenfeld and Kubicek, 1998) is the first PKC to be isolated from a filamentous fungus. The *T. reesei* kinase has been extensively characterized biochemically and appears to be

a unique member of the PKC family. This PKC (Pkc1p) is stimulated by phospholipids and phorbol esters but is calcium independent. The enzymatic properties of Pkc1p are consistent with those reported for a PKC (PKC1) from *Schizosaccharomyces pombe* (Mazzei *et al.*, 1993), but considerably different from the *S. cerevisiae* (Watanabe *et al.*, 1994) and *C. albicans* PKCs (Paravicini *et al.*, 1996). Several proteins have been proposed to be *in vivo* substrates for Pkc1p (Azzi *et al.*, 1992). A number of proteins in cell extracts from *T. reesei* are specifically phosphorylated by Pkc1p. While none of these potential substrates has been identified, it is interesting to note that one of the more prominent phosphorylated proteins (p85) shares a migration pattern with the myristoylated alanine-rich kinase C substrate (MARCKS), a bona fide mammalian PKC substrate (Aderem, 1992). MARCKS-like proteins have not yet been reported in filamentous fungi or yeast. At present, the function(s) of the *Trichoderma* PKC is unknown.

Mitogen-Activated Protein Kinases

MAP (mitogen activated protein) kinases are groups of Ser/Thr kinases, originally described in mammals, that are activated by a variety of extracellular stimuli, and mediate signal transduction from the cell surface to the nucleus (for reviews see Pelech and Sanghera, 1992; Herskowitz, 1995; Cobb and Goldsmith, 1995; Robinson and Cobb, 1997). In combination with several other signaling pathways, they differentially alter the phosphorylation status of transcription factors. Thus MAP kinases mediate intracellular phosphorylation events linking various extracellular signals to different cellular targets. MAP kinases exist as three kinase-cascade modules, sometimes with different (but functionally equivalent), and thus confusing, names. MAP kinases are highly conserved and have been cloned and characterized in yeast, plants, rat, mouse, man, *Xenopus*, *Drosophila*, and filamentous fungi and are an extremely active area of research.

MAP kinases are intimately associated with numerous aspects of cell growth, development, differentiation, and disease. MAPK is activated by phosphorylation on conserved tyrosine and threonine residues by a unique and dedicated, dual specificity MAPK kinase (MAPKK or MEK). In turn, the MAPKK is activated by phosphorylation on conserved serine and threonine residues by the MAP kinase kinase kinase (MAPKKK or MEKK). MAP kinases become enzymatically activated by phosphorylation in response to various extracellular stimuli and for that reason are also termed ERKs (extracellular signal-

regulated kinases). The primary MEKK in mammalian systems is raf. However, budding yeast lack raf and instead STE11 and BCK1 function as MEKK in this organism (for review see Herskowitz, 1995). MAP kinases exist as inactive dephosphorylated forms in quiescent cells but when cells are treated with mitogens, or in the case of yeast for example, pheromones or osmolarity changes, phosphorylation of MAPK occurs at tyrosine and threonine residues, and kinase activity increases. These phosphorylation sites (TEY) are diagnostic for MAP kinases and are located upstream of the APE sequence in kinase subdomain VIII. This 13 aa sequence is absolutely conserved from yeast to man and is considered the common regulatory phosphorylation sites in MAPKs.

Thus, a canonical MAP kinase module consists of three protein kinases that act sequentially within a pathway: MEKK (a MEK activator), a MEK (a MAP-kinase activator), and the MAP kinase itself. The activated MAPK can translocate to the nucleus where it phosphorylates and thus activates transcription factors. Six major types of MAP kinase cascades have been reported thus far in mammalian cells (there are likely to be more) that respond to different upstream signals. In yeast there are also six identified pathways (Herskowitz, 1995) and five characterized MAP kinases (Hunter and Plowman, 1997) involved with functions such as mating, cell wall integrity, and response to osmolarity changes. In mammals, besides proper growth and development (constitutively active MAPKs can be oncogenic), a number of stresses also trigger such pathways. Currently, an active area of research asks how such a myriad of different environmental stimuli can be funneled through such a small number of common signaling components; in other words, how are specific signals produced? For an interesting experimental examination of this topic in *S. cerevisiae*, see Madhani and Fink (1997) and Madhani *et al.* (1997).

The examples of MAP kinase modules in filamentous fungi are relatively few. However, given the fundamental importance of MAPK pathways in numerous cellular checkpoints, responses and with respect to fungi, mating, morphogenesis virulence, and stress responses, this number is likely to increase in the future. The varying degrees of similarity of these pathways between filamentous fungi and those described in yeasts and mammals is shown in Fig. 1. In studies with *U. maydis*, Banuett and Herskowitz (1994) reported the cloning and characterization of *fuz7*, a MEK dual specificity kinase. *Fuz7* is required for pheromone induced filamentous dikaryon formation essential for growth and tumor formation in maize. Via deletion

analysis, Banuett and Herskowitz proposed that *fuz7* is responsive to *U. maydis* pheromones as well as to plant signals. In the rice blast fungus, *M. grisea*, a MAP kinase (*pmk1*) was isolated by PCR (Xu and Hamer, 1996). PMK1 was confirmed as a functional MAP kinase by complementation of yeast FUS3/KSS1 mutants (defective in MAP kinase). Interestingly, when PMK1 was deleted in *M. grisea*, appressoria did not form. Moreover, when such transformants were inoculated onto wounded tissue obviating the need for appressoria, there was neither lesion formation nor fungal growth, and the fungus could not be recovered from the inoculated plant. PMK1 deletion mutants were indistinguishable from wild type during *in vitro* vegetative growth. Thus, these MAP kinase mutants showed an inability to normally respond thigmotropically for appressorium development and intriguingly, could not infect or colonize in response to putative plant signals during pathogenic growth in the plant. PMK1 appears to act downstream of cAMP production (and PKA), since addition of cAMP initiates but is insufficient to complete appressorium formation. In summary, the *M. grisea* MAPK mutant could not complete infection-related morphogenesis, and were nonpathogenic to the extent that these mutants could not grow in host plant tissue. In both of the above-mentioned examples, it will be of interest to determine interacting partners both upstream and downstream of the MAP kinase.

A MAPKK gene (*nrc-1*) from *N. crassa* was recently isolated by Kothe and Free (1998). Using a novel insertional mutagenesis strategy, transformants were obtained that were defective in normal conidiation. One such mutant lacked normal hyphae and its conidiation capability was impaired. The gene, cloned by plasmid rescue, was found to be a MAPKK with considerable identity to *S. cerevisiae* STE11 and *S. pombe* *byr2*, both of which are MAPKKs and are required for sexual development in their respective organisms. A second gene, *nrc-2*, was also isolated during this screen, and also lacked normal hyphae. This mutant could initiate, but not complete conidial development. *nrc-2* was found to encode a Ser/Thr kinase.

The coupling of different extracellular signals to various physiological responses, mediated by MAP kinase cascades, appears to have evolved from a single prototype protein kinase module, which underwent adaptations to specific requirements of different organisms. A theme that appears to be emerging with respect to fungi is the coupling of cAMP/PKA signaling and MAPK pathways (Kronstad *et al.*, 1998). In addition to the aforementioned relationship between cAMP and the MAPK in *M. grisea*,

MAP KINASE CASCADES

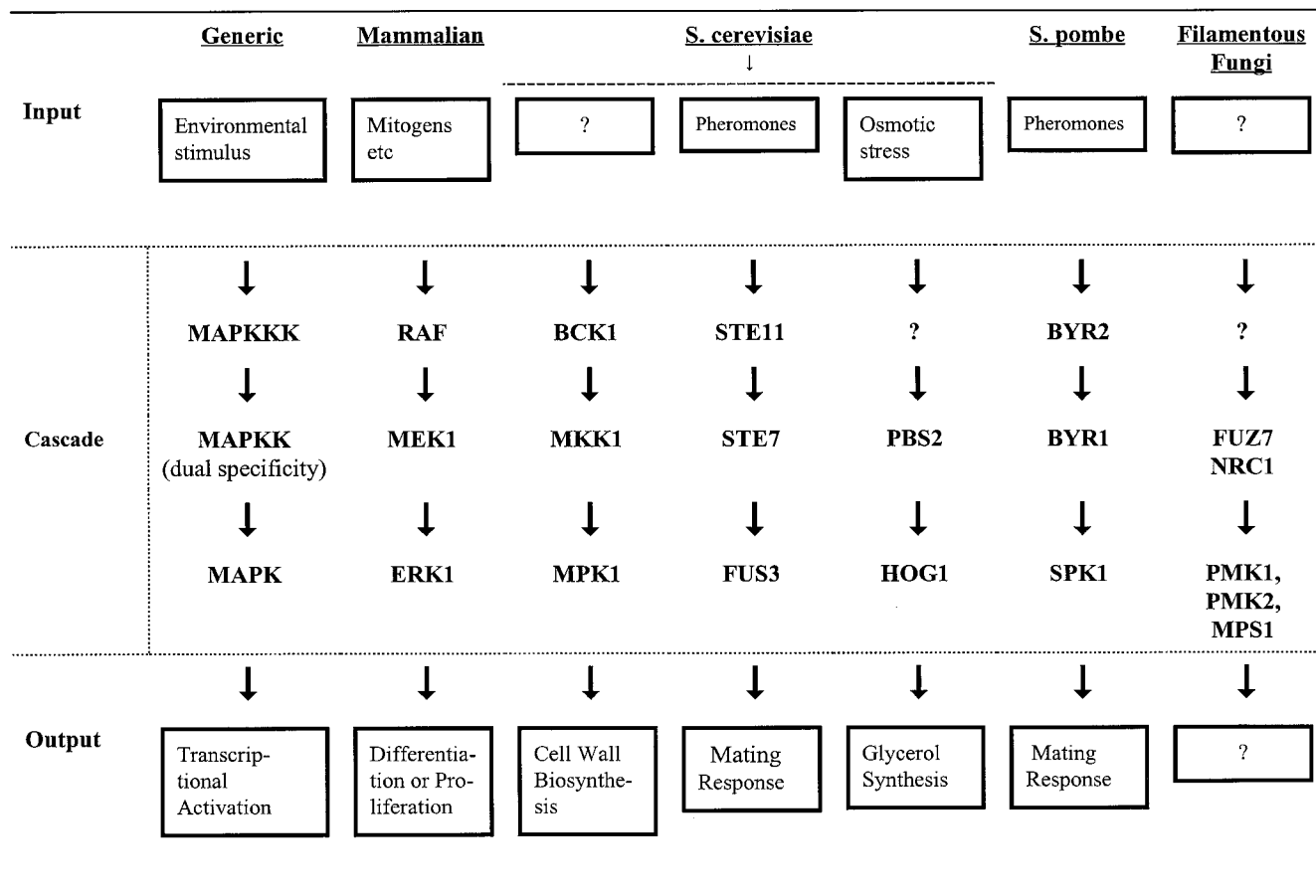


FIG. 1. Comparison of MAP kinase cascades. In mammals and in yeast at least six distinct pathways (and modules) have been described. In filamentous fungi, only individual components of a cascade have been identified. FUZ7 is from *U. maydis*, NRC1 from *N. crassa*, and PMK1,2 and MPS1 are from *M. grisea*.

MPS1, cAMP and MAPK pathways coordinately control a number of the same responses in *S. cerevisiae* ("pseudohyphal growth"), *S. pombe* (pheromone response, stress response), and as suggested by Kronstad *et al.* (1998) in *U. maydis*, the mating response and control of filamentous growth appear to be processes regulated by these signaling pathways. How this MAP-kinase story unfolds in filamentous fungi, particularly with respect to pathogenesis, promises to be exciting.

Other Ser/Thr Kinases

1. PAK. Class I myosins function in cell motility, intracellular vesicle trafficking, and endocytosis. It has been recently established that class I myosins are phos-

phorylated by a member of the p21-activated kinase (PAK) family. PAK phosphorylates a conserved serine or threonine residue in the myosin heavy chain. Phosphorylation at this highly conserved site is required for maximal activation of the actin-activated Mg^{2+} -ATPase activity *in vitro*. May and colleagues have been studying the significance of this phosphorylation by mutating the conserved serine residue of the class I myosin heavy chain gene *myoA* of *A. nidulans* (Yamashita and May, 1998). Mutation to glutamic acid, which mimics phosphorylation and thus activates myosin, results in an accumulation of membranes in growing hyphae. This accumulation of membranes results from activation of endocytosis. In contrast, mutation of the same residue to alanine had no discernible effect on endocytosis. These studies are the first to demonstrate the *in vivo*

significance of a regulatory phosphorylation on a class I myosin. Further, May and colleagues have now cloned the kinase responsible for Ser phosphorylation of MYOA (Greg May, personal communication).

2. Cell cycle kinases. The cell cycle is a highly ordered process that results in the duplication of a cell. How cells coordinate the timing of events in their cell cycles is a fundamental question in biology. A current focus of cell cycle research concerns how these transitions are coordinated to occur at a precise time and in a defined order. Inappropriate or improper regulation of cell cycle progression can have dire consequences for an organism ranging from cancer and programmed cell death to severe developmental aberrations (Hartwell and Kasten, 1994). Cell cycle progression (G_1 , S, G_2 , M, G_1 , etc.) is primarily regulated by two families of proteins, cyclins and cyclin dependent protein kinases (CDKs) (Morgan, 1997). The interaction of specific cyclins with CDK partners results in active protein kinases, phosphorylation of cell cycle specific targets and cell cycle progression. Thus, protein kinases and corresponding phosphatases play an integral part in cell cycle regulation. In yeast and humans, many cyclins and CDKs have been identified and cloned (Morgan, 1997). Far less is known about the cell cycle in filamentous fungi with the notable exception of *A. nidulans*, which is the focus of this section.

An extremely powerful tool in cell cycle studies which is elegantly illustrated in *A. nidulans* (perhaps superceded only by studies performed in *S. pombe* and *S. cerevisiae*) is the generation and complementation of mutants defective in a particular aspect of cell cycle progression. Sophisticated classical and molecular genetic methodologies have been developed for *A. nidulans* and this "requirement" is at least partially responsible for the lack of progress in less genetically developed filamentous fungi.

Since genes involved in the cell cycle are likely to be essential, conditional, temperature-sensitive mutations were generated. This work was initiated in the 1970s by Morris (1976). Cell cycle specific genes were identified from mutations that caused alteration in the mitotic index (the fraction of cells undergoing mitosis in a given sample) of *A. nidulans*. Mutants that resulted in an increase in the mitotic index at the restrictive temperature were designated *bim* for blocked in mitosis (*bimA*, *bimB*, *bimC*, *bimD*, *bimF*, *bimG*). Mutations that caused a decrease in this index at the restrictive temperature were designated *nim* for never in mitosis. The initial *nim* mutations were found to block the cell cycles in S phase (*nimC*, *nimG*, *nimK*, *nimQ*) or G_2 phase (*nimA*, *nimB*, *nimT*, *nimG*,

nimU). Curiously, no G_1 -specific mutants were isolated. The ubiquitous p34^{CDK2} protein kinase was cloned from *A. nidulans* (*nimX*) and was shown to be required for both the G_1 and G_2 phases of the cell cycle (Osmani *et al.*, 1994). In yeast and other eukaryotes, p34^{CDK2} associates with cyclin B during S and G_2 phases and is phosphorylated at threonine and tyrosine resulting in an inactive complex. This phosphorylated cyclin/cdk complex serves as a checkpoint to prevent mitosis from occurring if DNA damage is present. The pathway can be activated by dephosphorylation. This regulatory pathway is also conserved in *A. nidulans*. The Osmani group (Osmani and Ye, 1996; Ye *et al.*, 1997) has shown that during interphase, p34^{CDK2} (NIMX) is associated with cyclin B (NIMG) and is phosphorylated by ANKA, the *A. nidulans* homolog of the fission yeast *wee1* gene (Gould and Nurse, 1989). This complex is activated by dephosphorylation of NIMX by NIMT (a *cdc25*-like protein phosphatase) or inhibited (G_2 arrest) by tyrosine phosphorylation by ANKA (Ye *et al.*, 1997). Whether ANKA turns out to be a true tyrosine kinase or a dual specificity kinase remains to be seen.

The first cell cycle specific gene cloned from *A. nidulans* via mutation/complementation was *nimA* (Osmani *et al.*, 1987). The sequence of *nimA* suggested that this gene was a protein kinase (Osmani *et al.*, 1988) and this has been verified biochemically (Osmani *et al.*, 1991). Conditional inactivation of the NIMA kinase causes a reversible G_2 arrest (Osmani *et al.*, 1988). Overexpression of NIMA in *A. nidulans*, *S. pombe*, *Xenopus*, or human cells (Osmani *et al.*, 1988; O'Connell *et al.*, 1994; Lu and Hunter, 1995), can potentiate mitotic events, including premature mitotic arrest, aberrant spindle formation, chromatin condensation and nuclear envelope breakdown. Expression of dominant negative mutants of *nimA* causes G_2 arrest in *A. nidulans* as well as in humans (Lu and Hunter, 1995). This successful mitosis requires the appropriate regulation of NIMA in *A. nidulans* and possibly other eukaryotes as well.

3. COT1/TB3. Branching frequency in fungal hyphae can be influenced by environmental factors, such as nitrogen or carbon source, or changes in cation presence in the growth medium (Griffin *et al.*, 1974; Trinci, 1994). Interestingly, recent studies of a range of plant-fungal interactions suggest that major nutrient deprivation, carbon and/or nitrogen, in axenic culture may mimic growth conditions at early stages of the infection processes (Talbot *et al.*, 1993; Pieterse *et al.*, 1994; Jellito *et al.*, 1994; van den Ackervecken *et al.*, 1994). In addition to the environmental cues which influence branching frequency, genetic factors involved in hyphal branching have been identified. Hyper-

branching (colonial) mutants of *N. crassa* have been identified. The phenotype of some of these strains is governed by the mutation of genes which are either involved in the transduction of environmental cues or the hierarchy of events triggered by environmental or internal signals. Some of the known colonial mutants of *N. crassa*, in which frequent branching is conditioned by elevated temperatures, have been designated colonial temperature-sensitive mutants (*cot*). Among the *cot* mutants, *cot-1* has been the most extensively studied. Mutations in *cot-1* cause colonial growth (slow growth and excessive hyphal branching) at or above 32°C, but exhibit normal spreading radial growth at or below 25°C (Mitchell and Mitchell, 1954).

The *cot-1* gene has been isolated and, based on the deduced COT1 amino acid sequence, it encodes a Ser/Thr-specific protein kinase (Yarden *et al.*, 1992; Justice *et al.*, 1995). Furthermore, evidence for a linkage between branching and fungal cytoskeleton assembly and function (Plamann *et al.*, 1994) has set the stage for further analysis of the regulation and interaction of components governing the polarity of fungal cell growth.

Other members of the Ser/Thr kinase family that are COT1-related are encoded by the *warts/lats* gene of *Drosophila* and *DM* gene of human. The *warts* gene is required for the control of the extent and direction of cell proliferation as well as for normal morphogenesis (Justice *et al.*, 1995; Xu *et al.*, 1995). Mutations in *DM* cause myotonic dystrophy (Mahadevan *et al.*, 1993). Recently, Verde *et al.* (1998) have shown that the fission yeast gene *orb6*, another member of this kinase family, is required during interphase to maintain cell polarity. Therefore, it is becoming evident that functional expression of such genes is required for normal cell differentiation in different organisms. In *N. crassa*, COT1 kinase is essential, as insertional inactivation of the *cot-1* gene is lethal (Yarden *et al.*, 1992). Recently, Lauter *et al.* (1998) have demonstrated that *cot-1* expression is photoregulated. The *cot-1* gene encodes two transcript species differing in size and the ratio of both transcript species abundance is photoregulated. The origin of the difference between the two species was localized to the 5' end of the *cot-1* transcripts, upstream of the conserved C domains of the Ser/Thr-kinase polypeptide. A single in-frame ATG was identified as a putative initiation codon for the short polypeptide, suggesting that two COT1 isoforms, with different activities may be formed.

Buhr *et al.* (1996) described the isolation and cloning of a Ser/Thr kinase (designated TB3) from *C. trifolii*. The

deduced amino acid sequence of TB3 showed high similarity to COT1. The carboxyl terminal C domains of TB3 and COT1 are highly conserved, but the amino terminal regions are divergent, particularly in the homopolymeric glutamine repeats of TB3. Northern analysis indicated that *tb3* expression was highest 1 h after inducing conidial germination and two hours before germ tubes were observed. *tb3* transcripts returned to constitutive levels by 3 h after inducing germination. Even though some structural divergence was identified between the predicted *cot-1* and *tb3* gene products, *tb3* complemented the *cot-1* mutant of *N. crassa*, demonstrating the functional conservation of this kinase between a pathogenic and a saprophytic fungus (Buhr *et al.*, 1996). A gene similar to *cot-1* and *tb3* has been isolated from *U. maydis*. Disruption of this gene (*ukc1*) resulted in alterations in morphology, pigmentation, and pathogenicity (Dürrenberger and Kronstad, 1998).

PROTEIN PHOSPHATASES IN FILAMENTOUS FUNGI

The biochemical analyses of Ser/Thr protein phosphatases (PPs) in some filamentous fungi has provided evidence for the functional similarity of these enzymes with those studied in higher eukaryotes (Doonan *et al.*, 1991; Higuchi *et al.*, 1991; Szoor *et al.*, 1994; Zapella *et al.*, 1996). The molecular genetic analysis of some PPs has complemented biochemical studies and together, these approaches enable the further structural and functional dissection of these enzymes. A list of the different phosphatases whose genetic basis has been studied in filamentous fungi is presented in Table 2. Based on the limited amount of information available, it is clear that the identification, isolation, and functional analysis of PPs and their interacting polypeptides in filamentous fungi is still in its infancy.

Classification

Protein phosphatases (PPs) play essential regulatory roles in all eukaryotes. As PPs are an integral part of the reversible phosphorylation regulatory machinery, they are key elements in maintaining the balance of many cellular activities. On the basis of substrate specificity, they are classified as Ser/Thr, Tyr, dual specificity, or His PPs. Former classification has grouped Ser/Thr PPs as Type-1 (PP1) and type-2 (PP2A, PP2B, PP2C) phosphatases,

TABLE 2

Genes Encoding Protein Phosphatases Isolated from Filamentous Fungi That Have Been Structurally and/or Functionally Analyzed

Phosphatase	Organism	Gene designation	Reference
PP1	<i>Aspergillus nidulans</i>	<i>bimG</i>	Doonan and Morris, 1989
PP1	<i>Neurospora crassa</i>		Hughes <i>et al.</i> , 1996 Dombardi and Yarden (unpublished)
PP2A	<i>Neurospora crassa</i>	<i>pph-1</i>	Yatzkan and Yarden, 1995, Yatzkan <i>et al.</i> , 1998
PP2A-Reg55	<i>Candida tropicalis</i>	<i>CtCDC55</i>	Rodriguez <i>et al.</i> , 1996
PP2A-RegB	<i>Neurospora crassa</i>	<i>rgb-1</i>	Yatzkan and Yarden, 1999
PP2 B	<i>Neurospora crassa</i>	<i>cna-1</i>	Higuchi <i>et al.</i> , 1991
PP2B	<i>Aspergillus nidulans</i>	<i>CnaA</i>	Prokisch <i>et al.</i> , 1997
PP2B	<i>Cryptococcus neoformans</i>	?	Rasmussen <i>et al.</i> , 1994 Odom <i>et al.</i> , 1997
PPT	<i>Neurospora crassa</i>	<i>ppt-1</i>	Yatzkan and Yarden, 1997
PZL	<i>Neurospora crassa</i>	<i>pzl-1</i>	Szoor <i>et al.</i> , 1998
Tyr-PP	<i>Candida albicans</i>		Csank <i>et al.</i> (Genbank)
Tyr-PP	<i>Aspergillus nidulans</i>	<i>nimT</i>	Oconnell <i>et al.</i> , 1992

members of the latter group have been further subdivided (Shenolikar, 1994). As structural analysis of genes encoding PPs advanced, classification has united PP1, PP2A, and PP2B into one superfamily of enzymes, while PP2C represents a distinct group of phosphatases (Dombradi, 1997). One of the major structural differences between PP2Cs and other Ser/Thr PPs lies in the fact that in contrast to the monomeric PP2C, the other PPs are all composed of several subunits. Most recently, a two family gene classification has been proposed (Cohen, 1997). The two gene families, designated PPP and PPM, are defined by distinct amino acid sequences and crystal structures. The PPP family includes the signature phosphatases PP1, PP2A, PP2B, and PP5 (also known as PPT), while the PPM family comprises the Mg²⁺-dependent protein phosphatases, which include PP2C and pyruvate-dehydrogenase phosphatase. Within the PP1 and PP2A subfamilies, several novel protein phosphatases have been identified that show less than 65% sequence identity with PP1 and PP2A. These novel-type phosphatases also possess properties indicating that they perform cellular functions distinct from PP1 and PP2A (Cohen, 1997).

In addition to the structural features of the catalytic subunits, it is important not to underestimate the involvement of PP regulatory subunits in PP specificity and function. Stark (1996) has listed some 25 yeast proteins associated with phosphatase function and suggests that there are still considerably more PP regulatory polypeptides to be identified. Though in some instances structural conservation between such polypeptides among various organisms may assist in identifying some of these polypeptides in filamentous fungi, the structural diversity of PP regulatory polypeptides is most likely much higher than the catalytic counterparts. Structural features of Ser/Thr PPs, along with the phylogenetic relationships between the various phosphatases and interactions with other proteins and inhibitors, have been recently reviewed (Cohen, 1997; Dombradi, 1997).

To date, two main avenues of research have been the focus of PPs in filamentous fungi: (i) Involvement of PPs in development and morphogenesis and (ii) Cell cycle-related PPs. As studies on PP function advance, it is likely that the linkage between the two foci will be elucidated.

PP1

At the same time that the first PP was isolated from yeasts (Stark, 1996), Doonan and Morris (1989) isolated the *bimG* gene, encoding a type-1 PP, required for completion of mitosis, from *A. nidulans*. The isolation of *bimG* was possibly due to the availability of a conditional lethal temperature-sensitive *A. nidulans* mutant. Recently, Hughes *et al.* (1996) have demonstrated that *bimG* is not an essential gene. Further analysis of the *bimG11* allele (isolated from the temperature-sensitive mutant) showed that a single base pair difference between the two genes, within the 5' splicing site of the second intron, is responsible for impaired splicing of *bimG* in the mutant. This change in splicing leads to the production of a truncated protein comprising an intact N-subdomain and a modified C-terminus. Overexpression of this truncated form of PP1 in a wild-type haploid produces a lethal phenotype, supporting the idea that a toxic protein is produced (Hughes *et al.*, 1996). One suggested explanation for the lethal effect imposed by the production of a truncated version of the *bimG* gene product is the reduction of PP1 activity due to the sequestering of PP1-interacting polypeptides by the truncated protein. Interestingly, a similar case of induced lethality occurred when a truncated version of the *N. crassa cot-1* kinase was introduced into a wild-type strain. There, too, a possible sequestering of the wild-type enzyme activity was suggested (Yarden *et al.*, 1992).

The PP1 catalytic subunit and corresponding gene have been isolated from *N. crassa* (Dombradi and Yarden, unpublished). Based on the biochemical characteristics of the protein and the gene nucleotide sequence, it appears that the fungal PP1 is highly similar to the rabbit muscle PP1, indicating a high level of conservation of PP1 structure during evolution.

As has been shown in yeasts, PP1 functions in a variety of cellular process, which include mitosis, meiosis, cell integrity, morphogenesis, glycogen synthesis, glucose repression, and general amino acid control (Stark, 1996). It would not be surprising that PP1 would also be involved in many processes in filamentous fungi. In fact, mitosis, cell growth, and cell wall formation are several mentioned functions pleiotropically affected by interference with PP1 activity (Borgia, 1992; Doonan and Morris, 1989).

PP2A

The core structure of PP2A consists of a 35-kDa catalytic subunit tightly complexed with a 65-kDa regulatory subunit (also termed A-subunit). The core dimer complexes with a third, variable subunit which controls enzyme activity and specificity (Cohen, 1989; Mayer-Jaekel and Hemmings, 1994). Four types of these variable subunits (also termed B-subunits) are known, ranging in size from 54 to 74 kDa. Recent data suggest that other proteins may associate with the trimeric holoenzymes. The presence of multiple forms of PP2A holoenzyme suggests that they might have various functions *in vivo* (Mayer-Jaekel and Hemmings, 1994). In most organisms studied, at least two isoforms of the PP2A catalytic subunit (PP2Ac) have been identified. Several different holoenzymes, consisting of a catalytic subunit complexed with one or more regulatory subunits of PP2A, have been purified from various organisms. Szoor *et al.* (1995) described the purification of a PP2A catalytic subunit from *N. crassa*. The molecular weight (~34kDa), the sensitivity to various phosphatase inhibitors, and the specific dephosphorylation of the a-subunit of rabbit skeletal muscle phosphorylase kinase, all indicated the conservation of the fungal PP2Ac as compared with PP2Ac subunits from higher eukaryotes. Yatzkan and Yarden (1995) and Yatzkan *et al.* (1998) described the molecular cloning, disruption of the corresponding gene (*pph-1*) and experiments with strains exhibiting reduced PP2Ac activity levels. Failure to obtain viable progeny in which *pph-1* had been inactivated via the repeat-induced point mutations process, and evidence that nuclei harboring a disrupted *pph-1* gene could only be maintained in a heterokaryon, indicated that a functional

pph-1 gene is essential for fungal growth. Furthermore, this was the first report providing evidence that in contrast to yeasts, inactivation of a single PP2Ac-encoding gene results in a lethal phenotype in fungi.

A PP2A B regulatory subunit was recently identified in *N. crassa*. The gene, *rgb-1*, encoding for the PP2A B regulatory subunit is very similar to those found in other organisms (Yatzkan and Yarden, 1999). Strains in which *rgb-1* have been inactivated grew slowly, exhibited abnormal hyphal morphology, were female sterile, and produced abundant amounts of arthroconidia. Microscopic and genetic analyses indicate that *rgb-1* is a regulator of the budding subroutine of the macroconidiation process. In *C. tropicalis*, at least one function of the PP2A regulatory subunit has been identified by complementation experiments. Rodriguez *et al.* (1996) have shown that the *C. tropicalis* CtCDC55 complements the CDC55 PP2A regulatory subunit mutation responsible for cold sensitivity in *S. cerevisiae*. Thus, even though this variable PP2A regulatory subunit (as well as others) has been suggested to play a role in determining substrate specificity, accumulating evidence suggests that this regulatory subunit may well be involved in interactions with several substrates, which are involved in different cellular processes.

PP2B

PP2B, also known as calcineurin, is a highly conserved Ca²⁺/calmodulin-regulated Ser/Thr PP (Kincaid, 1993). The functional enzyme is a heterotrimer composed of a catalytic subunit (calcineurin A, CNA), a regulatory subunit (calcineurin B), and calmodulin. Rasmussen *et al.* (1994) analyzed the calcineurin-encoding *cnaA* gene in *A. nidulans* and demonstrated that it is essential. The *cnaA* mRNA varies in a cell cycle-dependent manner with maximal levels found early in G₁ and considerably before the G₁/S boundary. Analysis of growth-arrested cells following *cnaA* gene disruption revealed that they are blocked early in the cell cycle. Prokisch *et al.* (1997) have shown the effects of suboptimal expression of *N. crassa* calcineurin resulting from inducible antisense expression of the corresponding *cna-1* gene. Antisense induction conditions procured a growth arrest, which indicated an essential function for the *cna-1* gene of *N. crassa*, similarly to that found in *A. nidulans*. Growth arrest was preceded by increased hyphal branching, changes of hyphal morphology and concomitant loss of the distinctive tip-high Ca²⁺-gradient typical for growing wild-type hyphae. Consistently, exposure of growing wild-type mycelium to cyclosporin A (CsA) or FK506 (structurally unrelated inhibitors of calcineurin)

led to a phenotype very similar to that of the *cna-1* antisense-mutants. This demonstrated a specific role of calcineurin in the precise regulation of apical growth, the most common form of fungal cell proliferation. Microscopic inspection of DAPI-stained *A. nidulans* depleted of calcineurin revealed abnormalities in nuclear size and distribution; whereas no similar effects were found in *N. crassa* wild-type hyphae grown on CsA or FK506, or induced *cna-1* antisense-transformants. The effects of calcineurin depletion are not limited to nuclear division and hyphal extension, but have also been shown to impair pathogenicity of the opportunistic pathogen *Cryptococcus neoformans* (Odom *et al.*, 1997a). Odom *et al.* (1997b) have demonstrated that, though viable, *C. neoformans* strains in which the calcineurin-encoding gene had been disrupted had lost their capability to survive *in vitro* conditions that mimic the host environment and were no longer pathogenic in an animal model of Cryptococcal meningitis.

PPT (PP5)

Members of the PPT/PP5 subfamily of phosphatases have been shown to harbor three or four tetratricopeptide repeat (TPR) motifs, arranged in tandem, as a single domain. TPR domains are thought to form scaffold-like structures, providing sights for interaction with other proteins (Cohen, 1997). The possible involvement of this nuclear phosphatase in cellular processes in higher eukaryotes has been reviewed by Cohen (1997). A PPT (designated *ppt-1*) has been identified in *N. crassa*, on the basis of sequence similarity to the *pph-1* (encoding PP2Ac) gene (Yatzkan and Yarden, 1997). Transcript levels of *ppt-1*, encoding a three-repeat TPR motif-containing polypeptide are high in conidia and decrease during germination, suggesting that, as in higher eukaryotes, the PPT is temporally and spatially regulated.

Novel-Type PPs

Similar to PP1, PP2A, and PP2B, the novel-type PP are present in all mammalian tissues examined, though their *in vitro* substrates are unknown (Cohen, 1997). The novel-type phosphatases are grouped as members of the classical PP subfamilies. Thus, PPY, Ppz1, Ppz2, and Ppq1 belong to the PP1 subfamily, while PP4 (originally termed PPX), PP6, PPV 6A, Sit4, Ppe1, and Ppg1 belong to the PP2A subfamily, and PP5/Ppt1 and RdgC are members of the PP5 subfamily (Cohen, 1997). Information concerning novel-type PPs in filamentous fungi is scarce. Based on

studies in yeasts, PPZ phosphatases are part of the network of pathways that regulate cell wall integrity and remodeling during growth (Stark, 1996). In addition, they are involved in salt tolerance. Interestingly, the effect of PPZ on salt tolerance is opposite to that of PP2B, though the effects of the two phosphatases are independent and additive (Posas *et al.*, 1995). In yeasts, novel members of the PP2A family are involved in cell cycle regulation and glycogen metabolism, while the role of novel PP5 subfamily members has yet to be determined. A novel-type PP recently identified in *N. crassa* is the *pzl-1* gene, encoding a PPZ-like subfamily phosphatase (Szoor *et al.*, 1998). The role of this PP (and perhaps, additional PPZ-like PPs) is not known, yet on the basis of the studies in yeasts, it is conceivable that morphological effects as a consequence of interference with PPZ activity may be anticipated.

Assessing Activity/Function of Protein Kinases and Phosphatases

A genetic approach has dominated much of the functional analysis of kinases and phosphatases in filamentous fungi to date, as can be reflected in the majority of cases described in this review. The use of genetics emphasizes some of the advantages of filamentous fungi in analyzing such basic components of the living cell. However, one of the convenient aspects of studying kinases and phosphatases in filamentous fungi is the relative ease in which pharmacological and genetic approaches can be combined and complemented. The next section of the review describes some of the considerations to be taken when using inhibitors and provide some examples for the use of inhibitors for the analysis of protein phosphorylation in fungi.

Inhibitors of enzyme activity are valuable tools for probing kinase/phosphatase involvement in cellular processes. As use of such compounds have inherent limitations due to nonspecific reactions, it is important to bear in mind the properties of these compounds. Especially crucial is whether the inhibitor directly binds to the protein kinase and with what affinity, how specific the inhibitor is for a given kinase, and how permeable the fungal cell is to a given inhibitor? An array of chemically diverse inhibitors, of natural and synthetic origin, some with differential inhibitory effect on different kinases or phosphatases, have been identified. They are now routinely used in biochemical and cellular analyses of kinase/phosphatase function in fungi. Hidaka and Kobayashi (1993) and MacKintosh and Mackintosh (1994) have reviewed some of the structural and functional aspects of kinase and phosphatase inhibitors, respectively. For example, cAMP levels, and thus

PKA activation, can be modulated by increasing its synthesis by activators of adenylate cyclase (e.g., Forskolin, NaF) or by decreasing its degradation by inhibitors phosphodiesterase (e.g., IBMX). Such approaches have been used by a number of researchers studying the cAMP/PKA pathway (e.g., Hoch and Staples, 1984; Lee and Dean, 1993; Ruan *et al.*, 1995; Yang and Dickman, 1997). Though the approach of using pharmacological reagents has inherent limitations, the involvement of PKC in *Neurospora* light regulation is likely (see PKC section). The inhibitors used (calphostin and chelerythrine chloride) are quite specific and diagnostic for the regulatory domains of PKC, unlike many other inhibitors (e.g., staurosporine) which impair catalytic domain function and thus can inhibit many different classes of Ser/Thr kinases. Inhibitors have also been used to study protein phosphatases. For example, Flaishman *et al.* (1995) have studied the effect of phosphatase (as well as kinase) inhibitors on morphogenesis in the plant pathogen *Colletotrichum gloeosporioides* and have shown that exposure to calyculin A induced appressorium formation, whereas application of higher drug concentrations resulted in abnormal fungal cell morphology. By analyzing the effects of inhibitors, they suggest that protein phosphorylation is involved in the mediation of two plant host signals (surface wax and ethylene) within the fungus as part of the infection process.

Larson and Nuss (1994) used two structurally unrelated calcineurin (PP2B) inhibitors (cyclosporin A and FK506) to demonstrate a role for PP2B in maintaining laccase 1 (*lac-1*) transcription under conditions of amino acid sufficiency in *C. parasitica*, the casual agent of chestnut blight. *lac-1* transcription is regulated by two antagonistic (a positive and a negative) regulatory pathways which independently respond to changes in the nutritional environment. Calcineurin blocks the action of the negative regulatory pathway, preventing the repression of *lac-1* transcription (which is mediated by this pathway). Calmodulin (which serves as a regulatory unit of PP2B as well as calmodulin-dependent kinase) inhibitors block the action of both pathways, indicating that calmodulin-dependent enzymes play a key role, along with hypovirus infection, in the regulation of *lac-1* transcription in this fungus (Larson and Nuss, 1994).

CONCLUSIONS AND FUTURE RESEARCH

The study of kinases and phosphatases in filamentous fungi has become an active area of research in biology. This

review provides cumulative evidence for the fact that enzymes involved in phosphorylation play key roles in fungal biology, as they do in other organisms. In addition to the roles protein kinases and phosphatases play in fundamental cellular processes, it is becoming evident that phosphorylation is also part and parcel of the regulation of a variety of unique fungal traits. However, it is also reasonable to assume that the analysis of Ser/Thr kinases and phosphatases in filamentous fungi has implications concerning the understanding of fundamental process in a variety of cell types which occur in organisms well beyond the fifth kingdom.

The pleiotropic involvement of protein kinases and PPs in various cellular processes requires fine-tuning of temporal and spatial regulation of expression. It is clear that transcription and posttranslational regulation play important roles in regulating protein kinase/phosphatase activity. However, it is becoming evident that the involvement of targeting proteins in conferring specificity by localization is yet another key mechanism of regulation. Even though some of these proteins have been identified in filamentous fungi (for examples see Cho-Chung *et al.*, 1995; Faux and Scott, 1996; Stokoe *et al.*, 1994) there have been few functional analyses. Taking into consideration some of the advances made in other systems, we believe functional studies will not only increase but are likely to yield important and novel findings.

Filamentous fungi have been viewed as an experimental compromise between the simplicity of lower unicellular eukaryotes and the complexity of multicellular organisms (Kincaid, 1993). The use of filamentous fungi make it possible to assess effects on phenotype in greater depth and to interpret changes in structures that are important for the elongation and shape of hyphae in ways which may have relevance to cell growth in higher eukaryotic organisms. It has been proposed that although distantly related, the process of hyphal outgrowth and branching may have elements in common with neurite extension in the developing nervous system (Kincaid, 1993). Hyphae contain many of the basic morphological and structural elements found in a neuron, and possess transmembrane ion currents that may play a role in branching. The cellular responses to some immunosuppressive drugs in fungi imply that at least one of the basic components involved in T-cell activation and fungal proliferation (PP2B phosphatase) are common, providing yet another example of the possibility to parallel analyses between what are apparently very diverse biological systems (Prokish *et al.*, 1997; Rassmussen *et al.*, 1994; Tropschug *et al.*, 1989).

The various fungal genomics initiatives currently under way will clearly provide the basis for a change in the methodological approaches used in analysis of the fungal genome and the expression of fungal genes. As these initiatives advance, the available information concerning kinases and phosphatases (in addition to other genes) will certainly soar. Transcription profiling has already been demonstrated in yeasts, providing important clues as to the function of hundreds of yet uncharacterized genes during the sporulation process (Chu *et al.*, 1998). The described analysis also includes new information concerning the protein kinases. On the one hand the results of such analyses provide evidence for the correlation between the pattern in which a gene is expressed and the biological process in which its product participates. *S. cerevisiae* has a well-earned reputation as a paradigm for lower eukaryotes and much has been learned from this fungus particularly with respect to higher eukaryotes. For example, *S. cerevisiae* has been instrumental in furthering our understanding of the eukaryotic cell cycle; in part due to the sophisticated genetics offered by this fungus as well as the high degree of structural and functional relatedness that is present in the cell cycle machinery (e.g., cyclins, cyclin dependent kinases). However, even when analyzed or compared to yeasts, caution must be exercised in correlating the roles such enzymes have in processes occurring in filamentous fungi. For example, the MAPK, *pmk1* of *M. grisea* is highly similar to the *FUS3/KSS1* genes of *S. cerevisiae* when analyzed at the DNA and protein sequence level. The yeast genes are part of a MAPK cascade involved in pheromone signal transduction. In fact the *Magnaporthe* gene, when introduced into yeast strains deleted in these genes, was able to restore the defect in yeast mating. Accordingly it might be assumed that *mpk1* had a similar function in the rice blast fungus, and while of moderate interest, this would be just an extension of the yeast story. However, when *mpk1* was functionally studied in *M. grisea*, Xu and Hamer (1996) found that this gene was essential for both pathogenic development (appressorium formation) and pathogenic growth, traits neither of which can be found in yeast. In a similar fashion Xu *et al.* (1998) found that a second MAPK from *M. grisea*, *mps1*, is required for host penetration. *mps1* has a high degree of similarity (85%) to the yeast *slt2* MAPK, which is involved in cell wall growth in response to stress. While *mps1* mutants are also defective in cell integrity, there are some important differences, including the fact that yeast mutants undergo a high-temperature-sensitive lysis. *M. grisea* mutants, on the

other hand, become osmotically sensitive with age and show specific developmental defects in appressorial penetration, conidiation, and female fertility. Similar examples are evident with PKA. In budding yeast, PKA is involved with regulating growth on nonglucose carbon sources, control of G₁ in the cell cycle, and sporulation. In *U. maydis*, *M. grisea*, and *C. trifolii*, PKA is involved with pathogenicity related development and virulence. Another likely important difference between *S. cerevisiae* and filamentous fungi is the fact that in yeast, ras is involved with the cAMP/PKA pathway. In all other eukaryotes studied, G-proteins serve this role and ras is connected to the MAPK pathway. Though not yet experimentally addressed in filamentous fungi, it will be of interest to determine which pathway ras is coupled with.

Secondary metabolism is another unique and important feature of filamentous fungi. As structural genes and regulation are becoming increasingly understood we believe that involvement of protein phosphorylation in such complex environmentally stimulated and intracellularly balanced processes will be elucidated. The identification of phosphorylation-regulated elements in secondary metabolite biosynthesis is likely to be of broad interest in light of the presence of genes (e.g., sterigmatocystin and aflatoxin gene clusters) involved in secondary metabolism even in fungi which are not regarded as prime producers of secondary metabolites of economic/environmental interest (Keller and Hohn, 1997). While the involvement of kinases/phosphatases in secondary metabolism is presently conjectural, it would perhaps be surprising if this were not the case. We speculate that in the future, regulatory aspects of secondary metabolism via phosphorylation will gain significant attention.

Taking into account the higher complexity of the genomes of filamentous fungi along with the immense diversity of fungal species and their respective life styles, the identification and analysis of many kinases and PP remains to be performed. There is no question concerning the presence and high structural conservation of kinase/PPs among eukaryotic species. The availability of relevant information can, at times, be of assistance in analyzing the fungal proteins. However, in order to elucidate the involvement of phosphorylation in processes, which are essential or unique for the metabolism, growth, development, proliferation, and pathogenicity of filamentous fungi, we must study them in the specific relevant members of the fungal kingdom.

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