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MINIREVIEW

Fungal Virulence Genes as Targets for Antifungal Chemotherapy

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INTRODUCTION

With an increasing number of immunocompromised hosts and advances in medical technology, there has been a concomitant rise in the number of cases of invasive fungal infections. These infections range from life-threatening invasive mycoses to the merely irritating but generally benign mucocutaneous infections. Ideally, preventive strategies such as the development and administration of effective fungal vaccines will be used for high-risk patients to abrogate the need for antifungal drug treatment in many cases. On the other hand, antifungal agents for prophylactic, empiric, and therapeutic use will likely be the primary focus of pharmaceutical companies and clinicians in the immediate future. The challenge of medical management for mycoses has been partially met by the use of amphotericin B, flucytosine, and a series of azoles for systemic drug treatment. Nevertheless, new antifungal agents with fungicidal activities will be needed for the more effective management of deep-seated fungal infections. There are at least two methods for the identification of new antimicrobial drugs. The first is the classical screening of many classes of synthetic or natural products against a variety of fungi by in vitro susceptibility testing. This method has been very successful for a variety of antimicrobial agents, including antifungal agents. In fact, by its very nature, it screens in vitro growth and does not necessarily select for virulence targets. The second method makes use of molecular biological strategies and the concept of fungal virulence for the development of drug targets. It is now possible to identify molecular targets that are essential to these eukaryotic microorganisms' ability to produce disease. The identification of these unique, important molecular pathobiological functions for the fungus could translate into the use of these molecular targets for the design and development of new antifungal drugs (24). The potential to exploit the molecular pathogenesis of fungi for antifungal agent and vaccine development is now being realized, but an important concept in this field of investigation is the term *virulence*, which is at the center of most research designs. So that investigators can broaden their perspective on this concept and thus identify their goals and interpret their strategies in this new area of research in medical mycology, the first part of this discussion will attempt to define a variety of parameters specific to fungal virulence. The minireview will conclude with a discussion of the actual scientific progress that has been made on the use of virulence for the discovery of molecular antifungal targets.

DEFINITION OF VIRULENCE

A starting point in this discussion is to define virulence. From a simplistic viewpoint, Webster's Dictionary defines virulence as "the relative infectiousness of a microorganism causing disease or ability to overcome the natural defenses of the host." This definition describes the quantitative nature of the concept and allows for the inclusion of particular properties of the parasite. It also identifies the host as a major factor in this interaction, but it gives few specifics. The specific details of virulence have been best developed in bacterial pathogenesis. Unique entry or adherence factors, invasive properties, or toxins and their corresponding genes have been easily recognized as noxious or invasive for the host, and virulence genes have become more easily identified. On the other hand, Steele (72) has made a cogent argument that fungi that cause invasive disease are generally not passed from host to host. Therefore, there has been no selective pressure for pathogenic fungi to develop specific abilities to infect or invade the human host. A fungal infection is simply an accidental encounter in its life cycle, but the fungus must survive in the host to cause disease (72). Therefore, more subtle factors such as the ability to survive and/or grow within the host are essential to the parasite's ability to produce disease. For example, genes controlling stress factors, such as heat shock proteins, may be virulence genes in fungi (36, 43) that are just as important as those associated with more striking phenotypes such as proteinase production, melanin synthesis, or capsule development. However, it is reasonable to exclude from this virulence gene category certain housekeeping genes whose functions are required only for efficient multiplication on nonliving substrates. These types of essential genes and proteins may be simply less interesting to understanding fungal pathobiology. On the other hand, even these generally conserved genes with specific metabolic functions may be exploited for selective antifungal targets or vaccine epitopes. The structures and functions of these essential or housekeeping genes may be different between fungi and mammals, and these genes could be exploited as targets. Careful molecular studies on the use of these genes as targets should be encouraged. It is also important for investigators to examine all mutants prior to animal studies for their growth rates on in vitro substrates. Differences in basic growth rates between mutants and their parental strains could potentially confuse the interpretation of in vivo results of virulence.

Thus, it is important to broaden the concept of virulence to include the unique ability of certain fungi to grow and survive within the host. It should be emphasized that despite approximately 200,000 known species of fungi in the world, only about 270 species are reported to cause disease in humans. Although these numbers may increase as the immunocompromised host pool enlarges, there must be certain common genetic themes of survival within the host for this relatively small group of fungi.

VIRULENCE HYPOTHESIS

It is also likely that the definition of fungal virulence genes should be further broadened. The concept of fungal virulence

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should not be confined to single traits and simplistic mechanisms for these complex eukaryotic pathogens. For example, Cutler (13) has made an excellent attempt of taking into account the genomic variability and the varied virulence properties among fungal strains. He has proposed a virulence-set hypothesis, which states that "virulence traits belong to a set of genes within a given isolate expressing a finite number or subset of traits to make up the composite virulence phenotype of that particular strain. Therefore, a single trait of the set is not sufficient for virulence, and not all genes within the set are necessary, but a critical number of genes must act in concert to cause disease" (13). A further corollary of this more complex concept in pathogenic fungi may be the potential for a certain set of genes to change in importance, depending on the state of the immune status of the host, the inoculum introduced, and the site of infection. If this hypothesis is correct, it suggests that strain variability may make the identification of certain virulence genes complex and variable. The scientific literature supports the fact that wild-type fungal strains do have variable pathogenicities in well-controlled animal models and that these known differences in virulence may be under the control of a series of critical genes. Site-specific gene disruptions are generally single genetic events. If Cutler's virulence-set hypothesis is true, this gene disruption strategy may not reduce the total virulence genes below a critical number, and thus, a virulence phenotype would not be detected, and yet the gene in the sum is important to pathogenesis. Thus, a negative animal test of a virulence gene may not ensure the lack of importance of the gene to the total virulence of a particular fungal strain. This expanded virulence concept warns investigators in molecular pathogenesis that, in their virulence screening, research approaches must be comprehensive but must also be focused on finding certain common themes among infection site-specific genes or identifying a regulatory gene(s) with a wide domain. In practical terms, it will be necessary to (i) check that virulence genes are not strain specific, (ii) examine a potential virulence gene with the possibility that it is a set of interrelated genes in which there is a central control circuit, and (iii) focus on one specific area of infection because the complex interactions within the host at various sites and stages of infection will likely increase the complexity of gene expressions and the composite virulence phenotype. However, it should be emphasized that this hypothesis is not certain and may not apply to all virulence genes. Although it suggests the complexity of some virulence genes, it should not discourage careful investigations into their use as antifungal targets.

SETS OF VIRULENCE GENES

When an approach is made to identify human fungal virulence genes, the design should also consider the impressive body of work on bacterial virulence and the findings on fungal pathogen interactions with plants. For example, compared with saprophytes, bacteria and plant fungal pathogens must have three sets of virulence gene types: (i) those necessary to produce survival or growth in or on the living host, (ii) those necessary to cause disease symptoms, and (iii) host-range determinants (23). The first set of these genes has been identified elegantly in bacteria that cause infections. Bacterial pathogenesis has convincingly shown a variety of virulence genes that sense their environment and that are coordinately regulated to respond. These responses allow the organism to grow and survive in a hostile environment. Genetic mechanisms may be activated by the microorganism's sensing specific molecules such as phenolic compounds or saccharides, as has been shown in phytopathogens (1, 83), or responding to environmental conditions such as iron concentration, temperature, osmolarity, pH, oxygen, CO_2, or calcium (6, 21, 34, 48, 57, 65, 73, 78, 82). It is therefore essential that each investigator design the most appropriate clinical conditions for gene expression(s) and identify the particular site of infection to study.

The use of animal models to reproduce the infection under controlled conditions is essential for the confirmation of virulence. However, an understanding of the pathology of infection is also important. For example, certain virulence genes may be necessary for an intravenous inoculation and the establishment of infection but have no impact on aerosol-produced infections. Understanding of *Cryptococcus neoformans* pathogenesis suggests that at least several sites of infection, the organism potentially expresses different genes, such as those for the initiation of infection in the lung, establishment of dormancy within the lymph node complex, reactivation within the lung, dissemination through the bloodstream and into the central nervous system, and proliferation within the subarachnoid space to produce meningitis (59). Another important consideration when using animal models to detect virulence is the size of the inoculum. Certain genes and their products may be important at low inocula, whereas others may be expressed at a high burden of organisms. Therefore, the identification of important virulence genes expressed with low numbers of fungi and that result in the development of infection may be ideal for vaccines. On the other hand, important virulence genes expressed at high inocula or tissue burdens in the range of 10^5 to 10^7 CFU/g of tissue or fluid may be more useful for antifungal targets. Also, the impact of a gene disruption may simply reduce the ability of infection to reach a certain number of organisms in a host. The outcome to the host resulting from infection with between 10^3 and 10^4 CFU/g of tissue may have little consequence in a pneumococcal infection, but a fungal infection with a reduced number of organisms at the site of infection could tip the balance in favor of the host. Also, careful attention to the storage of fungal strains and the use of clinical isolates should be made. Although changes in the virulence of laboratory-passaged yeast strains may not be as dramatic as they can be for bacterial strains, some fungi will attenuate with in vitro passage, and thus, potential virulence genes could be lost through in vitro storage. In summary, investigators will need to carefully define and control the particular conditions for studying virulence genes in relationship to the complexity of the infection.

The second set of genes consists of genes that produce disease. These genes may be more common than is realized in fungi and may be useful as antifungal targets and in vaccine development. For example, investigators may be able to disrupt through gene replacement a possible virulence gene that will allow the organism to persist in the host but that will have no impact on morbidity or mortality. This particular feature has elegantly been observed with the disrupted *Candida albicans CHS3* gene (8). Animals receiving the disrupted *CH3* gene mutant had prolonged survival compared with the period of survival of those infected with the wild-type strain, but the numbers of *C. albicans* organisms of the two strains in the kidney was similar. In other words, the fungus may survive at the site of infection but will not produce obvious disease in the host. The commensal nature (*Candida* species) and potential for dormancy (*C. neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis*) of some pathogenic fungi would suggest that this type of virulence genes does exist.

The third set of genes consists of those that determine host range specificity (5). For instance, it has been shown that a virulence gene in a *Pseudomonas* species can cross the plant-animal range specificity by its functional necessity for produc-

TABLE 1. Some potential fungal virulence genes that have been approached on a genetic basis

Factor	Genetic crosses[a]	Gene(s)	Gene disruption[b]		Fungus genus	Confirmation[c] in animal studies (nonspecific/specific)	Reference
			Nonspecific	Specific			
Alpha mating locus	Yes	*MAT*α	Yes	No	*Cryptococcus*	Yes/No	40, 54
Amino acid biosynthesis (lysine)	No	—[d]	Yes	No	*Candida*	Yes/No	68
Calcineurin	No	*CnCal-A*	No	Yes	*Cryptococcus*	No/Yes	56
Capsule	Yes	*Cap59*	Yes	Yes	*Cryptococcus*	Yes/Yes	9
Chitin synthesis	No	*CHS2* *CHS3*	Yes	Yes	*Candida*	No/Yes[e]	28
Elastase	No	*AFA/p*	Yes	Yes	*Aspergillus*	Yes/No[f]	31, 69, 75
Fungal transition phase	No	*YP3*	Yes	No	*Histoplasma*	Yes/No	35
Heat shock	No	—	Yes	No	*Histoplasma*	Yes/No	47
Hydrophobin	No	*HYP1*	No	Yes	*Aspergillus*	No/No[f]	58, 77
Infection site-directed	No	Multiple[g]	No	No	*Cryptococcus*	No/No	63
Mannitol	No	—	Yes	No	*Cryptococcus*	Yes/No	10
Melanin	No	—	Yes	No	*Wangiella*	Yes/No	16
Multiple drug resistance	No	*CaMDR1*	No	Yes	*Candida*	No/Yes	4
Myristoylation	No	*NMT*	No	Yes	*Cryptococcus*	No/Yes	45
pH-dependent morphogenesis	No	*PHR1*	No	Yes	*Candida*	No/Yes	25
Phenoloxidase	Yes	*CnLac1*	Yes	No	*Cryptococcus*	Yes/No	42, 62
Porphyrin biosynthesis	No	*Heme3*	Yes	No	*Candida*	Yes/No	37
Proteinases	No	*SARI-7*	Yes	Yes	*Candida*	Yes/No	41, 53, 80
Pseudohyphal growth	Yes	—	Yes	No	*Saccharomyces* and *Candida*	Yes/No	50, 64, 68, 70
Purine metabolism	No	*ADE2*	Yes	Yes	*Cryptococcus* and *Candida*	Yes/Yes	37, 60
Pyrimidine metabolism	No	*URA3*	Yes	Yes	*Candida*	Yes/Yes	37
Temperature	Yes	—	Yes	No	*Saccharomyces* and *Cryptococcus*	Yes/No	49, 50, 62

[a] Use of Mendelian inheritance through sexual crosses.
[b] Nonspecific, mutants produced by random mutagenesis procedures; specific, mutants produced by site-directed mutagenesis through transformation protocols.
[c] Confirmation of virulence to host.
[d] —, no gene identified.
[e] *CHS2* mutant showed no difference; CHS3 mutant was less virulent.
[f] Specific disruptants were found to have no difference in virulence.
[g] Genes cloned but not identified or confirmed.

tion of disease in both hosts (61). A fungus, such as *Fusarium* spp., can cause disease in both plants and humans. Therefore, the principle of host range determinants should be carefully considered in studies of fungal virulence in humans. Much of the work on fungal virulence genes has been and will be performed in carefully controlled animal models. Therefore, it will be necessary to check the importance of identified virulence genes and their disruptions in several animal models, with different inocula, and with different modes of infections to ensure the general importance of the identified genes. It will also be possible to check the identified genes' expression at sites of human infections by using RNA PCR to detect transcripts in human body tissues or fluids. It is important that host range determinants be considered because the identification of mouse- or rabbit-specific fungal virulence genes may have no relevance to the development of antifungal targets in humans.

CONCEPTS IN VIRULENCE GENES

Another concept in fungal virulence genes should be considered in the strategies for their use as antifungal targets. Progress in the molecular biology of some pathogenic fungi has been made, in which specific gene disruptants can be made and their pathobiologies can be examined. The development of a molecular biology foundation, combined with understanding of the biochemistry of potential pathogenic factors (11), allows for specific studies of virulence. Table 1 lists the potential fungal virulence genes that have already been examined on a genetic basis. It is important to consider that specific blocks in biochemical pathways may still allow the organism to survive and cause disease by using redundant or complementary pathways. It may be more useful for antifungal targets to identify a global or central regulatory gene(s) that has an impact on the expression of multiple genes. The concept of a regulatory gene(s) that controls coordinately expressed genes during host invasion is well described in bacterial virulence (21). For example, genes for global control systems or regulons of virulence have been described, including the transcriptional activators such as *toxR* (15, 51), the iron-regulated transcriptional activators of *Vibrio cholerae* (26, 27), the *virF* gene of *Shigella* (66), the *vir* gene controlling phase variation and virulence in *Bordetella pertussis* (66), and the *soxR* gene controlling a superoxide response regulon in *Escherichia coli* (85). Although eukaryotes may have many complex regulatory mechanisms, studies with *Saccharomyces cerevisiae* show that transcriptional regulation is likely a major control point, with certain genes encoding DNA binding proteins that either up-regulate or down-regulate at various *cis*-acting promoter elements (74). Although a regulon has not been characterized in primary fungal pathogens, work with *Saccharomyces* spp. and the *alcR* regulon in *Aspergillus nidulans* (14) provides examples of fungi in which this arrangement occurs. It is reasonable to hypothesize that some potential virulence genes are under a central regulatory gene that controls a circuit of coordinately expressed virulence genes. It is important that this possibility is considered, because discovery of a more central regulatory gene in the circuit could be an ideal antifungal target; its disruption would potentially produce dire consequences for the yeast because of its control of multiple important genes and their virulence phenotypes.

The term *virulence gene(s)* for fungal pathogens should encompass a broad definition that allows investigators to define their goals for the discovery of antifungal targets or vaccine epitopes. With the new tools in molecular biology that are

available, including fungal gene disruptions, preconceived ideas of certain fungal phenotypes associated with virulence, such as elastase for *Aspergillus* spp. (75) and cutinase for *Necator* spp. (71), will likely be proven inaccurate or the phenotypes will be more complex than anticipated. It should be considered that many pathogenic bacteria have been passed through humans for centuries. Thus, certain clonal virulence traits could have been selected during this selection process, and therefore, studies of the virulence of these organisms such as *Vibrio* and *Salmonella* species may have more uniformly identified virulence themes among strains. On the other hand, fungi live within the external environment, and only recently has a new internal environment for their growth within the immunocompromised hosts been created. Present data suggest wide diversity among different fungal species and that there exists within strains a genetic heterogenicity which may have implications regarding the ability to find a single common virulence gene even among different strains. The existence of genetic redundancy in potential virulence genes should also be considered. For instance, proteinases in *Candida* species have been considered possible virulence factors. A series of proteinase genes has been isolated from *Candida* species (53). Careful gene disruptions and genetic analysis of each gene will be necessary to identify and understand a true virulence gene(s) and/or its regulator(s) for these enzymes. Still, despite a broad approach to virulence and the variety of issues outlined, investigators should also be rigorous in their standards for the definitions of virulence. Falkow's (19) cogent argument that there needs to be a fulfilling of the molecular Koch's postulates before assigning a particular property as a contributor to virulence should be applied, as follows: (i) the property is associated with pathogenic organisms, (ii) inactivation of the gene responsible for the property results in decreased virulence, and (iii) restoration of the affected gene is associated with a return to wild-type virulence. If investigators are not rigorous in their experimental designs with the use of these molecular tools, do not have a complete understanding of pathobiology, and do not have a comprehensive and focused definition of virulence, these powerful tools can lead them down nonproductive pathways in the search for drug targets and vaccines. With the exception of several vaccines, it should be emphasized that no directly identified molecular microbial virulence factor for the human host has yet been developed into an antimicrobial agent. On the other hand, the identification of virulence genes and determination of their functions in fungi have great theoretical potential for medical control of these opportunistic pathogens. It is the responsibility of investigators to carefully develop this strategy within this broad concept of fungal virulence.

This discussion of virulence is to make investigators aware of the potential complexities and issues involved in the understanding and use of virulence genes as antifungal targets. It underlines how careful investigators must be in their designs and interpretations. On the other hand, the power of these investigations for the identification of virulence genes as antifungal targets for drugs and vaccines should be emphasized. It allows insights into the mechanism(s) of action and the prediction of toxicities and defines specific areas of chemistry and pharmacology in which drug discovery companies can then mobilize specific resources. With a potential virulence gene target identified and validated in animal models, a series of drug development approaches can then be used. These include directed medicinal chemistry, screening of combinatorial libraries with the generation of biased libraries toward the target, and X-ray crystallography of the actual target structure(s) for the development of specific target-based designs of inhibitors. It will be important to identify and exploit the biochemical or structural features in these eukaryotic microorganisms that are unique compared with those in the mammalian host.

MOLECULAR MODELS FOR FUNGAL VIRULENCE

An excellent recent review of antifungal targets and immunological strategies by Georgopapadakou and Walsh (24) shows the breadth of potential investigations into antifungal drugs and mechanisms. It is interesting that they separated targets into classical and soft targets. The soft targets included virulence genes. These soft targets will be converted to firm targets only through careful identification and validation of these genes. In the last 5 years, there has been significant progress in the genetic and molecular biology foundation for human pathogenic fungi which will allow investigators to identify virulence genes and new potential drug targets. Investigators have generally used model systems to study and test hypotheses. For human pathogenic fungi, several model organisms have begun to be used. For example, the common, diploid pathogens *Candida* species (22, 29, 32, 38) and the generally nonpathogenic but genetically well-developed organism *S. cerevisiae* (49, 50) are proposed as models that can be used to identify virulence characteristics. Among the pathogenic molds and dimorphic fungi, molecular systems have been developed with *Aspergillus fumigatus* (53, 58, 77, 80) and *H. capsulatum* (84) to identify and study virulence genes.

An attractive organism for use as a fungal model for studying molecular pathogenesis and identification of drug and vaccine targets is *C. neoformans*. This yeast has a series of very important features for its development as a model for studying molecular pathogenesis. First, it causes important mycoses with worldwide distributions. These infections have significantly increased in immunocompromised patients during the human immunodeficiency virus pandemic. Although treatments for managing this infection are available, they remain unsatisfactory, since their ability to completely eradicate infection in severely immunosuppressed patients is inconsistent. Second, it is a haploid organism with a known sexual cycle and an organism in which genetic crosses can be performed, and thus, investigators can use the power of recombination through meiosis in genetic studies (39). Third, there is a reasonable understanding of the pathophysiology of cryptococcosis, and excellent animal models of cryptococcosis are available. Proof of virulence and new treatment strategies can be studied and validated with these models prior to studies with humans. Finally, there is the recent development of a molecular biology foundation for this yeast. The genetic analysis of the organism has been described, and a series of genes have been cloned and sequenced (52). Importantly, several methods for DNA transformation have been described and used (18, 79). This technical progress in molecular biology has been substantial and allows for direct and focused investigations into this primary fungal pathogen. It is argued that primary pathogens such as *C. neoformans* and the dimorphic fungi that do not have a human colonization state will possess the most well-developed virulence genes.

On the other hand, the use of well-established fungal model systems such as the *S. cerevisiae* model system will also be extremely helpful in giving guidance to early investigations with these primary pathogens. Although it is important to investigate directly the unique virulence features of each pathogen, the biochemical and molecular pathways in the fungal pathogen may be so poorly understood that the better-characterized fungal species may be needed as a reference. Work with calcineurin A in *C. neoformans* has shown the

unique features of this target in a pathogenic fungus compared with its features in *S. cerevisiae* (56). The results could not have been predicted from studies with *S. cerevisiae*, in which the individual fungi gene for calcineurin A is not essential. This recent molecular work emphasizes both the similarities between fungi and the uniqueness of individual fungi which can be exploited on a molecular level for studies of virulence and antifungal targets. *S. cerevisiae* can also be used effectively to identify a mechanism(s) or a target(s) of an identified antifungal agent. For instance, azoxybacilin, an antifungal product produced by *Bacillus cereus*, was found to act by inhibiting sulfite reductase in *S. cerevisiae* at two steps, transcriptional activation of *MET4* and posttranscriptional regulation in *MET10* expression (2). It is thus reasonable to expect that this type of work can then be applied to studies with fungal pathogens.

STRATEGIES FOR VIRULENCE GENE IDENTIFICATIONS

In the broad but complex definition of virulence reviewed previously, the experimental strategies for determination of virulence genes as molecular targets for antifungal drugs and vaccines can be separated into two groups. The first group of strategies is a direct effort to identify a specific biochemical or structural target and to identify the essential genes in the pathway(s). The second strategy is a more indirect method which sets certain environmental conditions and allows the organism to direct and identify the importance of a certain virulence gene(s) through its differential expression(s) under these in vivo signals. Both strategies are presently being used with the *C. neoformans* model (59).

The first strategy uses biochemical and structural genes known to be unique to fungi in an attempt to specifically and selectively disrupt them and determine their effects on virulence. This directed strategy can be subdivided into investigation of the genes essential for growth both in vitro and in vivo or of the genes necessary for producing only in vivo growth and disease.

The first group represents a series of genes which have been the focus of intense research as antifungal drug targets. The fungal cell membrane target, which has a unique component, ergosterol, as the primary sterol, has successfully been used for antifungal drug development. Disruption of the genes in this sterol pathway generally makes the yeast less virulent (33), and antifungal drugs against this targeted pathway have been particularly successful, including the azoles, the morpholines, and the allylamines. Another important target is the intact fungal cell wall, which is controlled by a certain number of glucan and chitin synthase genes and their products, which are necessary for a fully virulent fungus (7). Several prototype groups of compounds that act against this target, including the echinocandin B congeners and nikkomycins, have already been identified. Areas of higher risk for finding fungal virulence genes that are unique compared with those in mammals include those associated with biochemical (11) or signal transduction pathways. On the other hand, there is precedence for finding unique targets and drugs in basic fungal metabolic and genetic machinery. These drugs include flucytosine for yeast infections and trimethoprim-sulfamethoxazole for pneumocystosis. In fact, even these targets can be related to virulence. For example, flucytosine-resistant *C. albicans* strains have been shown to be less virulent than susceptible strains (20). Several important pathogenic fungal genes in this area have been identified. For example, genes for topoisomerases (59), myristoylation (45), amino acid and folate utilization (76), and transcriptional and translational factors (i.e., elongation factor 3) (12, 55) have been identified in several fungal pathogens. These types of genes stretch even the broad definition of virulence genes since they are simply necessary for growth of the organism and thus are essential for causing disease. However, as these genes are further identified and studied, there will likely be significant differences in structure and function between these fungal genes and their mammalian counterparts which can be exploited in drug development (59).

The second group of genes in the directed strategy represent those which are required specifically for in vivo growth. Progress in the study of this type of fungal virulence gene has also been made in fungal pathogens. Examples include the in vivo importance of the gene encoding phosphoaminoimidazole carboxylase (*ADE2*) in *C. albicans* and *C. neoformans* (37, 60), a capsule gene (*CAP59*) which is essential for capsule production in *C. neoformans* (9), the α mating type locus for *C. neoformans* (40), and the calcineurin A gene for *C. neoformans* (56). These genes are not necessary for in vitro growth under certain conditions, but in vivo they are essential for the production of infection. This direct strategy for identifying virulence genes has great appeal because there is an initial knowledge base for understanding the mechanisms involved. However, it requires careful validation of the gene's importance in vivo and careful exploitation of any differences in the genes between the fungi and the mammalian hosts.

The second strategy for the identification of fungal virulence genes is more indirect in its approach but is potentially very powerful because it allows pathobiology to direct investigations into the importance of genes. The hypothesis to this strategy is simply that environmentally regulated genes and their regulators are essential to the survival of the fungus. The identification of these uniquely expressed genes will thus identify virulence genes which can be used for drug targets or vaccine epitopes.

In this era of genome analysis, a series of methods that can be used to analyze gene expressions by capturing and quantitating cell transcripts under certain conditions have now been developed. These new molecular advances for other systems can now be adapted for use with pathogenic fungi. The first techniques used cDNA libraries from cells as probes for differential hybridization to genomic libraries. These techniques have been used with *H. capsulatum* (36) and *C. neoformans* (63) to identify regulated genes. cDNA library subtraction techniques have further refined this strategy (17), and with the isolation of small amounts of RNA from organisms at the site of infection, a differential PCR display technique may be particularly useful for capturing unique transcripts (44). Recently, two new techniques, serial analysis of gene expression (81) and quantitative monitoring of gene expression patterns with a complementary DNA microassay (67), have also been used, and they could be adapted for pathogenic fungal genes to assess quantitative comparisons of the expressed genes and potentially to identify their importance in vivo. Another system for capturing highly expressed genes at the site of infection is an in vivo expression technology that was developed for bacteria and that is very useful for the identification and validation of virulence genes in bacteria such as *Salmonella* spp. (46). It can be adapted to fungi. In this system in vivo-expressed promoters are identified by their ability to turn on an essential gene in purine metabolism to allow viability in vivo. Since *ADE2* has been shown to be essential in vivo for *C. neoformans* (60), a similar strategy can be used to isolate in vivo regulated promoters and their genes in *C. neoformans*. All of these differentially expressed genes identified by any of the methods described above do not ensure importance. First, these iden-

tified genes will need to be reconfirmed for their actual differential expression by Northern blotting. Second, through site-directed disruption of these genes, mutants will need to be evaluated for the magnitude of their importance on virulence in relevant animal models. Finally, a virulence gene(s) associated with an attenuated phenotype may also be isolated by combining techniques of restriction enzyme-mediated integration to randomly disrupt genes and signature-tagged mutagenesis, which specifically labels each disrupted gene to identify virulence genes by negative selection in vivo (30).

Much of the focus on virulence genes has been on their use as targets for drugs, but they could also be used as vaccine epitopes. In fact, in bacteria the products of virulence genes have been most successfully developed into vaccines. A new exciting technology, expression library immunization, could be used to identify fungal virulence genes through their effects on inducing immunity in the host. Expression library immunization has been used successfully to protect against a mycoplasma infection (3). With this technology, which elevates the creation of nucleotide vaccines to a discovery method, the inoculation of fungal DNA libraries into mice could protect the host against a challenge from the whole organism. In this strategy a library of cDNA from the fungus and under a mammalian promoter is transfected into host tissue, in which fungal proteins can be expressed in the host and the immune system can respond. When a protective library is found, actual protective clones from this library can be identified by the use of sib selection strategy to identify the protective genes producing these epitopes. The proteins encoded by these genes may then be useful in vaccine development, but the genes can also be evaluated for their contribution to the intrinsic virulence of the fungus and its interaction with host immunity.

SUMMARY

Fungal virulence genes have now met the age of molecular pathogenesis. The definition of virulence genes needs to be broad so that it encompasses the focus on molecular antifungal targets and vaccine epitopes. However, in the broad but simple definition of a virulence gene, there will be many complex genetic and host interactions which investigators will need to carefully define. Nevertheless, with the increasing numbers of serious fungal infections produced by old and newly reported organisms, the paucity of present antifungal drugs, and the likelihood of increasing drug resistance, the need for investigations into understanding fungal virulence at the molecular level has never been more important.

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