Minireview

Fungal virulence studies come of age

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Abstract

Sophisticated molecular biological research has revealed many virulence attributes in at least four pathogenic fungi, but the future study of fungal virulence requires investigators to distinguish between molecules that directly interact with the host, molecules that regulate these, and molecules that are always required for fungal growth and survival, independent of the host.

Molecular studies of virulence in pathogenic fungi reveal a complex interaction between each fungus species and the human host. The fungi that cause invasive disease differ considerably in their inherent pathogenicity, even though few, if any, approach the level of virulence of the best known bacterial and viral pathogens. *Coccidioides immitis, Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis* and dermatophyte fungi are all capable of infecting healthy, immunologically intact individuals; they cause many new cases of community-acquired infection each year. By contrast, species such as *Candida albicans, Aspergillus fumigatus, Rhizopus* species and *Fusarium* species are normally avirulent in healthy people, but can be disseminated to deep tissues and cause fatal infections in patients with suppressed immune function. These infections present with non-specific symptoms, typically fever, and are often difficult to diagnose as a result. The fungus *Cryptococcus neoformans* straddles the boundary between 'true' and 'opportunistic' virulence, since it is known as a cause of community-acquired infection, even though most instances of cryptococcosis arise in an immunologically compromised host.

Pathogenic fungal species differ in their cell morphologies: most exist as filamentous moulds (with hyphae) in the environment, but the most virulent among them often change to a unicellular (yeast or spherule) form when they invade tissues. By contrast, *C. albicans* is an endogenous commensal resident in 15-60% of non-symptomatic individuals, is carried predominantly in the yeast form and commonly exhibits yeast-to-hypha transitions concomitantly with tissue infiltration and infection. Almost all of the fungi that cause fatal diseases gain access to the host via the lungs by inhalation of spores, yet *Candida* species, which account for a high morbidity and mortality in individuals with reduced numbers of neutrophils, become pathogenic only when lowered host defences allow the fungal cells to pass from the gut into the bloodstream. The dermatophytes parasitize epidermal skin, particularly in sites such as the groin and toe webs, which always tend to be moist, and cause long-standing but non-fatal infections at these sites.

The diversity of pathogenic potency, cellular form and route of invasion between fungal species makes it impossible to draw general conclusions about their molecular virulence attributes. In the case of opportunistic fungal pathogens, an understanding of host immune dysfunction is as important as an appreciation of which fungal genes may encode definable attributes of virulence. Perception of the high incidence of fungal diseases, particularly those that threaten life, has led to considerable investment in research into fungal virulence, often based on cutting-edge molecular biological approaches. But the rapid progress made in identifying molecular virulence factors in several fungal pathogens has also raised some caveats and paradoxes that require resolution.

Fungal virulence factors identified by molecular biology

H. capsulatum var. *capsulatum* is a dimorphic fungus whose conidia (spores) are inhaled, germinate in the lungs and convert to a budding yeast form that, uniquely among fungal pathogens, becomes an intracellular parasite that is ingested by pulmonary macrophages but survives and multiplies within phagolysosomes. In order to identify factors that contribute to survival within macrophages, Maresca's group [1] used differential-display reverse-transcriptase-coupled PCR to find genes that were specifically up- or down-regulated during the first hour of the interaction between *H. capsulatum* and macrophages *in vitro*. They detected five mRNAs with increased expression and one with decreased expression in this time period. Only one of these sequences showed similarity to known genes in the databases: it was a homolog of 100-105 kDa transcriptional co-activator proteins in the rat, human and *Caenorhabditis elegans* genomes.

Goldman and colleagues [2] recently showed that a 7.8 kDa calcium-binding protein, secreted only by *H. capsulatum* yeast forms, plays an essential role in ensuring survival of the fungus within macrophages. Disruption of the *CBP1* gene, which encodes this protein, resulted in a mutant strain that was unable to destroy a macrophage-like cell line *in vitro* and showed significantly attenuated virulence in a mouse model of infection [2]. This study also introduced a novel approach to specific gene disruption in *H. capsulatum*, based on using the *URA5* gene as a selectable marker, even though the product of the *URA5* gene is itself essential for intracellular survival and virulence in this fungus [3]. In order to demonstrate the virulence role of the *H. capsulatum* calcium-binding protein (CBP1), therefore, uracil prototrophy had to be restored in the parental strain (by transformation with a ura^+ plasmid) before it could be used as a control in the animal experiments [2]. These experiments highlight the need to distinguish between essential metabolic functions in a fungus, and virulence factors that interact directly with mammalian host cells. The first may be essential for virulence; the second are truly 'virulence factors'. Indeed, Retallack *et al.* [4] have made use of the avirulence of *ura5* auxotrophs to establish *in vivo* expression technology (IVET) for screening other putative *H. capsulatum* virulence genes.

A similar situation exists in virulence factor research of the opportunist fungus *C. albicans*. Gene disruption in this organism is complicated by its state of permanent diploidy. Almost all investigators of its putative virulence factors have used disruption cassettes based on *URA3* or *HIS1* as selectable markers in the absence of other positive selectable markers [5]. Since 1991, at least 40 genes have been identified whose disruption leads to attenuated virulence of the mutant strain when injected intravenously into mice. These genes encode products which range from secreted hydrolytic enzymes through cell-wall polysaccharides and peptides to signal transduction pathway components. *C. albicans ura3* strains are themselves less mouse-virulent than uracil-prototrophic strains, however [6,7], as are many other auxotrophic strains [8]. Although auxotrophic mutations affect the virulence of *C. albicans*, they cannot be described as true virulence factors.

Whereas *H. capsulatum* survives within macrophages after phagocytic ingestion (and is non-capsulate, despite its name), the capsulate yeast *Cryptococcus neoformans* resists phagocytic ingestion in the first place. The initial route of infection with *C. neoformans*, as with *H. capsulatum*, is mainly by inhalation of the fungus. *C. neoformans* is disseminated via the bloodstream, however, which usually results in infection of the meninges and brain, with less involvement of other deep tissues. Formation of a capsule and the production of melanin by means of a phenol oxidase enzyme system have long been recognized as essential virulence attributes in *C. neoformans*, and recent studies have begun to identify individual genes that code for capsule production. Capsule formation serves not only to provide the fungus with a capacity to avoid phagocytosis; it also leads to suppression of cell-mediated and humoral immune responses to the fungus [9]. Gene disruption and complementation studies have shown that at least two genes involved in capsule synthesis, *CAP10* [10] and *CAP64* [11], are essential for virulence. Considerable research, but not yet at the genomic level, has suggested a role for cryptococcal melanin in protection against host antimicrobial peptides [12].

Gene regulation and virulence

Both melanin production and capsule formation in *C. neoformans* appear to be regulated by a homolog of the *Saccharomyces cerevisiae STE12* gene [13]. *STE12* plays a critical role in mating processes in *S. cerevisiae*, but Chang *et al.* [13] consider its role in *C. neoformans* mating regulation as less important than its control of expression of other genes, including those that code for the capsule and for melanin production. Chang and colleagues [13] deserve credit for showing not only that *STE12* is required for virulence in mice, but also that this gene probably regulates other virulence factor genes. They did not simply claim *STE12* itself as a virulence factor. The *C. albicans STE12* homolog, *CPH1*, does not appear to affect virulence in this fungus [14]. A number of regulatory genes have been shown to be required for full virulence of *C. albicans*, however, including *CEK1*, which encodes a MAP kinase [14], *CST20* and *CLA4*, which are related to the *STE20*-encoded protein kinase [15,16], and *HOG1*, which also encodes a MAP kinase [17]. Most of these genes are also known to influence the ability of *C. albicans* yeast cells to form hyphal outshoots, but their influence upon other molecular virulence factors has not yet been examined. Whether it is legitimate to refer to a gene as a virulence determinant when its function is to regulate the expression of host-interactive virulence molecules is an open question. It is likely that most genes involved in central

metabolic pathways may be essential for normal growth *in vitro* and also *in vivo*. Mutants with defects in these genes are likely to be attenuated in virulence, but their inclusion as potential virulence factors seems to be stretching the point. In this respect it may be more useful to consider virulence factors as those genes in which a mutation affects growth only *in vivo*. The *C. albicans FTR1* gene, which encodes a high-affinity iron permease, however, is required for growth in the iron-limiting conditions of the bloodstream and can reasonably be termed a virulence determinant [18].

Virulence phenotypes and the choice of animal model

In all three of the fungal pathogens discussed so far, attenuation of mouse lethality in mutant fungal strains has been the most common approach to elucidating the roles of specific genes in virulence. It is clear, however, that expression of virulence by a fungus can depend on the particular host niche that is being affected. It is therefore possible to miss virulence factors when only one model for virulence is tested. In *C. albicans*, disruption of the *PHR1* (pH-responsive) gene led to attenuated lethality when injected intravenously into mice, whereas disruption of the related *PHR2* gene had no effect on lethality. Conversely, in a rat vaginal *Candida* infection model, the *phr2* mutant was attenuated while the *phr1* mutant showed normal virulence [19]. Similarly, in *C. neoformans*, a mutant with a disrupted *URE1* urease gene showed normal virulence when injected intracisternally into rabbits, but showed attenuated lethality in a mouse infected intravenously [20]. Extensive studies of the family of secreted proteinase enzymes (*SAP* gene products) in *C. albicans* has also indicated differential expression of the individual *SAP* genes according to the stage and type of infection under investigation [21]. Clearly, there is no substitute for thorough investigation of virulence phenotypes in as many models of infection as possible *in vivo* and *ex vivo*, particularly when single-gene disruptants are under scrutiny, if the true role of virulence molecules in the infection process is to be fully determined.

Fungal virulence and the gene-for-gene hypothesis

A. fumigatus is an opportunistic pathogen of the lungs of severely immunocompromised hosts that can sometimes cause disseminated disease. So far, it is the only pathogenic fungus to have been scrutinized for virulence factors by means of signature-tagged and directed mutagenesis [22]. Holden's group [22] created a pool of mutant fungal strains by insertional mutagenesis with recombinogenic DNA molecules, each marked with a unique oligonucleotide sequence. A mixture of up to 96 mutants was injected into an animal host, and mutants disrupted in a gene essential for survival *in vivo* were then identified by their absence among fungi recovered from infected tissues. This approach led to the finding that the gene encoding para-aminobenzoic acid synthetase (*pabaA*) was required for virulence, and that targeted deletion of this gene produced an avirulent variant. Targeted gene disruption experiments revealed that many macromolecules that are thought to serve as virulence factors in other fungi, such as secreted hydrolases, do not contribute significantly to the virulence of *A. fumigatus* [23]. Only the production of blue-green pigment in the fungal conidia has emerged as a convincing virulence factor in this fungus [23,24]. In this study, disruption of the *alb1* gene led both to virulence attenuation and to an immune response in the form of increased complement binding and neutrophil-mediated phagocytosis. Hence the mutation led to increased recognition of the conidia by the immune system.

In plant pathology, the relationship between recognition phenomena and pathogenesis is somewhat contrary to that in medical mycology. In the 'gene-for-gene' model established for plant-pathogen interactions, the loss or mutation of avirulence genes in the fungus leads to escape of pathogen-recognition mechanisms and hence the onset of disease [25]. Hence, mutants can be expected for which virulence is increased. So far, such mutations have not been reported in fungi pathogenic for humans, but we would do well to consider the gene-for gene model should hypervirulent mutations be revealed in future mutagenesis screens.

Studies of virulence at the molecular level have now revealed many factors that contribute to the overall pathogenicity of different fungal types. The only common theme linking the virulence attributes of the four fungi discussed in this review is that no fungus depends upon any single molecule for its virulence. Fungal virulence is a polyvalent, complex process that requires the expression of multiple genes at different stages and different sites of infection. It is important, in future research, that investigators become increasingly self critical. They need to make a clear distinction between those molecules that serve a purely 'housekeeping' function that is equally essential for the fungus *in vitro* and *in vivo*, those that are expressed uniquely in infected tissues and interact directly with the host, and those that regulate the expression of host-interactive virulence molecules. More widespread application of approaches such as IVET, transcript profiling of fungi *in vivo*, differential gene display and signature-tagged mutagenesis are likely to facilitate understanding of fungal virulence at the molecular level.

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