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Review

Fungi pathogenic to humans: molecular bases of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*★

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The frequency of severe systemic fungal diseases has increased in the last few decades. The clinical use of antibacterial drugs, immunosuppressive agents after organ transplantation, cancer chemotherapy, and advances in surgery are associated with increasing risk of fungal infections. Opportunistic pathogens from the genera *Candida* and *Aspergillus* as well as pathogenic fungi from the genus *Cryptococcus* can invade human organism and may lead to mucosal and skin infections or to deep-seated mycoses of almost all inner organs, especially in immunocompromised patients. Nowadays, there are some effective antifungal agents, but, unfortunately, some of the pathogenic species show increasing resistance. The identification of fungal virulence factors and recognition of mechanisms of pathogenesis may lead to development of new efficient antifungal therapies. This review is focused on major virulence factors of the most common fungal pathogens of humans: *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. The adherence to host cells and tissues, secretion of hydrolytic enzymes, phenotypic switching and morphological dimorphism contribute to *C. albicans* virulence. The ability to grow at 37°C, capsule synthesis and melanin formation are important virulence factors of *C. neoformans*. The putative virulence factors of *A. fumigatus* include production of pigments, adhesion molecules present on the cell surface and secretion of hydrolytic enzymes and toxins.

Keywords: pathogenic fungi, virulence factors, aspergillosis, candidiasis, cryptococcosis

INTRODUCTION

In the last decades the problem of severe nosocomial fungal diseases has become more serious, especially in patients with severe immunological impairment. The development of medicine, surgery and transplantology in the last thirty years has caused a dramatic increase in the number of immunocompromised individuals who are more susceptible to fungal infections. Patients with immunological impairment, HIV infection, leukopenia (haematological malignancy patients), after surgery, organ transplantation or cancer therapy are at risk of developing mycoses. Widespread use of broad-spectrum antimicrobial agents, immunosuppressive agents and corticosteroid therapy are also risk factors. The prophylactic use of antifungal therapies is one of the reasons of frequent resistance to antifungal drugs (Perfect & Casadevall, 2006; d'Enfert & Hube, 2007). Among all the fungi only few species are pathogenic to humans. The most frequently diagnosed fungal infections are caused by pathogens from the genera *Candida*, *Cryptococcus* and *Aspergillus* (Richardson, 2005). These fungi are ubiquitous and can be

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Abbreviations: ALS, agglutinin-like sequence; GlcNAc, *N*-acetyl-D-glucosamine; GPI, glycosylphosphatidylinositol; GXM, glucuronoxylomannan; HBMEC, the human brain microvascular endothelial cells; MAP, mitogen-activated protein; PMN, polymorphonuclear neutrophils; RGD, Arg-Gly-Asp adhesion sequence; SAPs, secreted aspartyl proteinases; *SIR2*, Silent mating type Information Regulation-2.

acquired from host surroundings (*Cryptococcus neoformans, Aspergillus fumigatus*) or are components of normal endogenous flora (*Candida albicans*) (Perfect & Casadevall, 2006). The mortality among infected patients is high, even after intensive antifungal treatment, because of patient's immunodeficiency, late diagnosis or fungal drug resistance. Fungi are able to cause a disease and to overwhelm the host defense systems because of possessing several genes and proteins associated with their pathogenicity, called virulence factors (Tomee & Kauffman, 2000). Many of the putative fungal virulence factors have developed naturally during organism evolution and originally acted as a defense against unfavorable environmental conditions, and then, in this way, many of them became important as virulence factors facilitating infection.

CANDIDA ALBICANS

Currently more than two hundred ascomycetous yeasts are included into the genus *Candida*, but only a few species are opportunistic pathogens of humans. Nowadays, *Candida albicans* is thought to be the major fungal pathogen of humans. Severe *Candida* infections are a serious problem, especially in individuals whose immune defense mechanisms have been weakened (Odds *et al*., 2006). *C. albicans* can colonize skin and mucosal surfaces of healthy people and thus occurs commensally in the gastrointestinal tract, oral cavity and vagina, often causing superficial infections (Mavor *et al*., 2005). Moreover, *C. albicans* can enter the bloodstream by direct penetration from the epithelium after tissue damage, or by dissemination from biofilms formed on medical devices introduced into the patient's organism, e.g. catheters, dental implants, endoprostheses, artificial joints or central nervous system shunts (Chandra *et al*., 2001; Mavor *et al*., 2005). Then yeast cells disseminate with the blood flow and infect almost all inner organs, including lungs, kidney, heart, liver, spleen and brain, causing fungaemia and life-threatening septicaemia. Candidiasis may occur as a result of disturbed balance between host immunity and this opportunistic pathogen. This disorder is not only due to the immunological dysfunction of the host, but also to the fungal ability to adapt to new niches, dependent on the expression of infection-associated genes (Brown *et al*., 2007a). These genes and their products contribute to fungal pathogenicity and are described as virulence factors. *C. albicans* virulence factors include, among others, production of different hydrolytic enzymes and adhesins (Chaffin *et al*., 1998). There are also other characteristic properties that influence fungal virulence, for example, the ability to form biofilms on various surfaces, to change morphological form and to switch between various phenotypes (Chaffin *et al*., 1998).

Phenotypic switching

Phenotypic switching is a very important part of fungal adaptability to the changing of environment during invasion of the human organism. The ability to infect many tissues is crucial to a successful attack and dissemination within the host. Occasionally some subpopulations of *C. albicans* cells can change their morphology, cell surface properties, colony appearance, biochemical properties and metabolism to become more virulent and more effective during infection (Odds *et al*., 2006). Colonies change their appearance and assume different shapes, including smooth, rough, fuzzy, wrinkled, fringed or stippled phenotype with a high frequency, approximately one changed colony per $10–10^4$ colonies (Slutsky *et al*., 1985). The molecular basis of this process is still unclear. Probably chromosomal rearrangements and a *SIR2*-like regulation take part in this process (Calderone & Fonzi, 2001). The most popular and well-known example of switched colonies is the white — opaque switching, when a white, oval and smooth colony changes into a grey, rough colony (Slutsky *et al*., 1987). The opaque cells produce aspartyl proteinases 1 and 3 and are less virulent, whereas white cells secrete aspartyl proteinase 2 and are more virulent during systemic infection (Yang, 2003). Phenotypic switching is most likely a signal of large-scale processes involving changes of many molecular and biochemical properties of the pathogen, which are helpful for fungi to survive within the host organism.

Morphological dimorphism

The ability to switch between unicellular yeast cells and filamentous forms called hyphae and pseudohyphae is known as morphological dimorphism. The transition between these different morphological forms in response to diverse stimuli seems to be very important for fungal pathogenicity (Lo *et al*., 1997; Chaffin *et al*., 1998). The morphology can change under a variety of environmental conditions, including response to physiological temperature of 37°C, pH equal to or higher than 7.0, CO_2 concentration of 5.5% or the presence of serum or carbon sources which stimulate hyphal growth (Eckert *et al*., 2007). The production of unicellular forms is stimulated by lower temperatures and more acidic pH, absence of serum and high concentrations of glucose (Whiteway & Bachewich, 2006). Yeast cells are thought to be responsible for dissemination in the environment and finding new hosts, while hyphae are required for tissue damage and invasion.

Both forms are present in biofilms formed on artificial substrates (Chandra *et al*., 2001). Yeast cells have different properties than the mycelial forms: the ultrastructure, biological attributes and composition of the cell wall differ between these forms. Probably both forms, yeast cells and hyphae, are necessary for full virulence, because mutants lacking genes responsible for the production of one or the other are less virulent (Yang, 2003). Despite the fact that the production of filamentous form is well known among the pathogenic fungi, the molecular basis of *C. albicans* morphological dimorphism is still poorly understood. Many genes associated with the formation of different cell shape code for transcription factors and may be also responsible for expression of unknown genes, encoding other virulence factors. Recent studies show that the transcription factor Cph1p, whose phosphorylation is regulated through a mitogen-activated protein (MAP) kinase pathway involving products of genes *CST20*, *HST7* and *CEK1*, and the basic helix-loop-helix transcription factor Efg1p are required to form hyphae during infection (Lo *et al*., 1997). In the Efg1p pathway, the following proteins are involved: homologs of Ras, adenyl cyclase and protein kinase A (TPK2) (Brown & Gow, 1999; Lengeler *et al*., 2000). Other transcription factors, Tup1p and Rbp1p, are negative regulators of filamentation, because mutants lacking these genes exhibit constitutive filamentation (Calderone & Fonzi, 2001; Yang, 2003). Other putative factors may contribute to morphogenesis, but their role and exact function are so far unknown. It is only obvious that co-operation of all the signaling pathways involved in the morphological transition is very important for fungi to infect and survive in the human host.

Adhesion and adhesion molecules

The adherence to the host cells and tissues, as well as the binding of a set of diverse host proteins is essential for *C. albicans* to begin the invasion, followed by dissemination within the human organism. This step is crucial for fungal survival. On the cell wall surface *C. albicans* presents receptors which are responsible for adhesion to epithelial and endothelial cells, serum proteins and extracellular matrix proteins (Chaffin *et al*., 1998). Adhesion to different artificial substrates and formation of biofilm on medical devices is currently a serious problem in medicine, because of the frequent resistance to antifungal agents and increased pathogenicity among the subpopulation of cells forming the biofilm. It has been estimated that in the last few decades microbial infections of humans are strictly correlated with biofilm formation in 65% of cases (Ramage *et al*., 2006). *C. albicans* cells originally present either on the skin, mucosal surfaces or in blood can colonize the surface of medical devices. All known morphological forms, yeast cells, pseudohyphae and hyphae, form a biofilm and they have different properties than those of planktonic or suspended cells (Al-Fattani & Douglas, 2006). The secretion of aspartyl proteinases (SAPs) is higher during biofilm formation (Mendes *et al*., 2007). *C. albicans* cells forming a biofilm are always associated with a matrix composed of polysaccharides containing mannose and glucose residues (Chandra *et al*., 2001). The biofilm matrix production plays a very important role in drug resistance of *C. albicans* biofilms, but development of resistance is rather multifactorial (Al-Fattani & Douglas, 2006). During biofilm formation, *C. albicans* cells express several genes that influence pathogenicity. Products of these genes take part in adhesion (e.g. family of Als proteins), in carbohydrate synthesis, drug resistance (e.g. efflux pumps) and in quorum sensing (Chandra *et al*., 2001).

The ability of *Candida* to invade different environments in the host organism is a result of great flexibility and adaptability of fungi. This phenomenon is in part due to the presence of different adhesins connected with cell surface, which facilitate the first stage of infection. These adhesins include Als proteins family, Hwp1p, Eap1p, Csh1p, and other less known cell surface receptors. The *C. albicans* cell wall is constructed from β-glucans (branched polymers of glucose residues containing β-1,3 and β-1,6 linkages), chitin (unbranched polymers of *N*-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds), mannoproteins, small amounts of other proteins and lipids (Chaffin *et al*., 1998). All known receptors are tightly connected with the fungal cell wall. The family of Als proteins ("agglutinin-like sequence") is one of the well-known examples of *Candida* adhesins. This family includes at least eight *ALS* genes that encode proteins with similar structure, which are connected to the cell surface (Fig. 1). Despite the fact that members of this family have different functions and different sizes, their structures exhibit some common properties. In 2004, Sheppard and coworkers described the structure of the N-terminal fragment of Als proteins. This part contains multiple antiparallel β-sheet domains containing minor α-helical and β-turn components, which indicates that products of *ALS* genes are members of immunoglobulin family (Sheppard *et al*., 2004). Als1p, Als3p and Als5p are believed to be responsible for adherence to collagen, fibronectin, laminin, endothelial and epithelial cells, Als6p binds to collagen and Als9p to laminin. Als4p probably mediates adherence to endothelium, and Als5p is additionally responsible for cell-to-cell aggregation. The role of Als7p is still unknown (Filler *et al*., 2006). A receptor with a molecular mass of approx. 34 kDa, which is present only on the surface of hyphal cells, is called Hwp1p

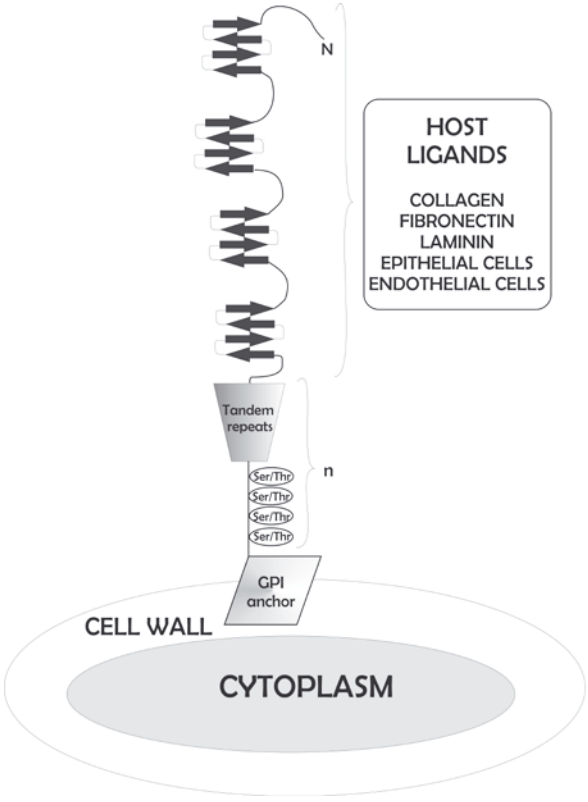

Figure 1. Scheme of Als adhesin structure.
The N-terminal fragment contains a putative signal peptide and a ligand-binding domain, central region is rich in tandem repeats with many serine and threonine residues, as well as consensus sites for glycosylation. The C-terminal part contains a glycosylphosphatidylinositol (GPI) anchorage site. After Filler *et al*. (2006), modified.

(Chaffin *et al*., 1998). The structure of Hwp1p is similar to that characteristic for Als proteins, but unlike those, Hwp1p possess in its N-terminal part a sequence similar to those of small proline-rich proteins of human cells which are substrates for transglutaminases present on the surface of epithelial cells. Therefore, Hwp1p facilitates adhesion to epithelium (Staab *et al*., 1999). Another receptor, Eap1p ("enhanced adhesion to polystyrene 1"), shows homology and structural similarity to Hwp1p, but the host ligands for this protein are still unknown (Filler *et al*., 2006). There are also other receptors of different structure, for example Int1p containing an integrin-like domain and an RGD-binding domain, which bind to different extracellular matrix proteins including fibronectin, entactin, vitronectin, laminin and collagen, and take part in adhesion to platelets and endothelial cells (Chaffin *et al*., 1998; Calderone & Fonzi, 2001; Ruiz-Herrera *et al*., 2006). Although the role of alcohol dehydrogenase, Adh1p, in binding of *C. albicans* cells to fibronectin is still unclear this protein is also thought to have adhesive properties (Filler *et al*., 2006). Host serum proteins, including fibrinogen (Casanova *et al*., 1992), plasminogen (Crowe *et al*., 2003), kininogen (Rapala-Kozik *et al*., 2008) and others can also be bound by *C. albicans* cells through different receptors, from which some are known and partially characterized. A tighter adherence to epithelial and endothelial cells, as well as to extracellular matrix proteins is achieved thanks to increased cell surface hydrophobicity. Singleton *et al*. (2001) have described a novel 38-kDa receptor, Csh1p, which enhances hydrophobicity of *C. albicans* cells, also facilitating specific receptor-ligand interactions. Adhesion to host cells is also dependent on interactions between mannoproteins with lectin-like properties and fucosyl or glucosaminyl glycosides on epithelial cells' surface (Ruiz-Herrera *et al*., 2006). Although many fungal adhesins have been identified, so far little is known about the host receptors involved in adhesion. At present it is obvious that Toll-like receptors 2 and 4 and other receptors present on the surface of human immune cells, such as monocytes, macrophages and dendritic cells, are involved in these interactions. Probably some proteins present on the surface of epithelial or endothelial cells, for example N-cadherin, are important for binding of different morphological forms of *C. albicans* cells, followed by their endocytosis (Phan *et al*., 2005; Filler, 2006). Due to the ability to bind and invade human endothelial cells *C. albicans* can traverse the human blood-brain barrier and cause life-threatening meningitis (Jong *et al*., 2001).

Secreted hydrolytic enzymes

Production and secretion of hydrolytic enzymes, such as proteases, lipases and phospholipases are very important virulence factors. These enzymes play a role in nutrition but also in tissue damage, dissemination within the human organism, iron acquisition and overcoming the host immune system, and strongly contribute to fungal pathogenicity. Many types of secreted hydrolytic enzymes are currently known for *C. albicans*. The activity of phospholipases is very high during tissue invasion, because these enzymes are responsible for hydrolysis of one or more ester linkages of glycerophospholipids, of which the cell membrane is built. *C. albicans* cells isolated from blood produce higher extracellular phospholipase activities than commensal strains (Ibrahim *et al*., 1995). There are four types of secreted phospholipases: A, B, C and D (Calderone & Fonzi, 2001; Yang, 2003), specific towards individual ester bonds in glycerophospholipids (Ghannoum, 2000). Very important for fungal virulence is the activity of phospholipase B (PLB), which has both hydrolase and lysophospholipase-transacylase activities (Ghannoum, 2000; Yang, 2003). Thus, PLB can release fatty acids from a phospholipid and the

remaining fatty acid from a lysophospholipid, and then transfer a free fatty acid to a lysophospholipid and produce phospholipids (Theiss *et al*., 2006). Apart from phospholipases, *C. albicans* can produce at least nine lipases which can hydrolyze ester bonds of mono-, di-, and triacylglycerols (Schaller *et al*., 2005). A well-known group of *C. albicans* secreted hydrolytic enzymes are SAPs (secreted aspartyl proteinases). The family of *SAP* genes includes at least ten different genes *SAP1–SAP10* which encode enzymes with similar functions and character, but different molecular properties, such as molecular mass, isoelectric point and pH for optimal activity (Naglik *et al*., 2003). The expression of the *SAP* genes is regulated at the transcriptional level, and the nascent preproprotein is processed by a signal peptidase in the endoplasmatic reticulum and by a Kex2-like proteinase at the carboxyl-terminal side in the Golgi apparatus (Newport & Agabian, 1997; Yang, 2003). The mature enzymes have molecular masses in the range of 35–48 kDa and two highly conserved regions with reactive aspartic residues, just as other pepsine-like proteinases (Schaller *et al*., 2005). Probably SAPs 1–3 are secreted only by yeast cells and SAPs 4–6 by hyphal forms (Naglik *et al*., 2004; White & Agabian, 1995), whereas both forms produce SAPs 9 and 10, which are connected with fungal cell walls because of possessing a GPI anchorage site (Hube & Naglik, 2001; Albrecht *et al*., 2006). The synthesis and function of SAPs 7 and 8 are still under investigation (Yang, 2003). Many host proteins are hydrolyzed by secreted aspartyl proteinases, including collagen, laminin, fibronectin, mucin, salivary lactoferrin, α_2-macroglobulin, almost all immunoglobulins, the proinflammatory cytokine interleukin-1β, lactoperoxidase, cathepsin D, complement, cystatine A, and precursors of several blood coagulation factors (Fig. 2) (Naglik *et al*., 2004; Schaller *et al*., 2005). The spectrum of optimal pH for SAPs activity is from 2.0 to 7.0, therefore these enzymes may contribute to fungal pathogenesis and developing infections in different sites in the human organism (Naglik *et al*., 2004). Except aspartyl proteinases, *C. albicans* also secrets other proteases: a 60-kDa metallopeptidase and a 50-kDa serine peptidase. The serine peptidase is active in a broad range of pH (5.0–7.2) and hydrolyzes many host substrates including extracellular matrix proteins and serum proteins (dos Santos *et al*., 2006).

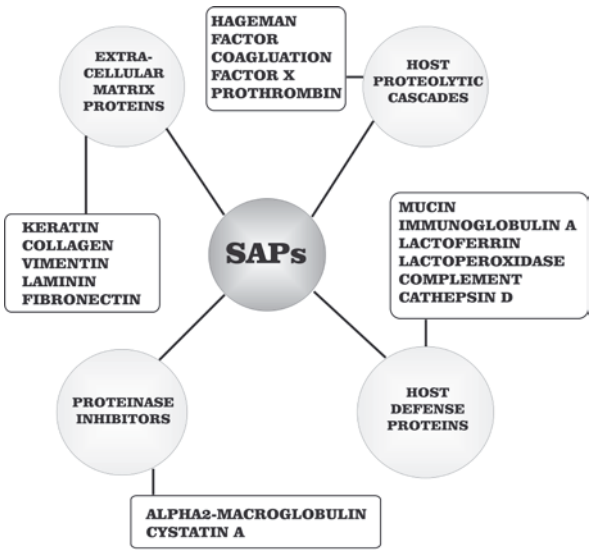

Figure 2. Substrates hydrolyzed by secreted aspartyl proteinases (SAPs) during infection.
Based on data of Naglik *et al*. (2003) and Schaller *et al*. (2005).

Other virulence factors

The ability of pathogenic microorganisms to acquire iron from the environment during infection is another very important virulence factor. The ability to overcome host systems connected with iron transport and accumulation is crucial for the pathogen to survive during invasion of the bloodstream. In *C. albicans* members of Rbt5 family are needed for utilization of hemoglobin and hemin for iron acquisition by the pathogen. Without these proteins the *C. albicans* iron metabolism is severely impaired (Weissmen & Kornitzer, 2004). During infection *Candida* cells are exposed to reactive oxygen species produced by immune cells, hence the organism expresses several virulence factors which help to overcome this host defense mechanism, including catalase, superoxide dismutase and heat shock proteins (Brown *et al*., 2007a). Expression of many virulence factors often depends on environmental conditions, therefore fungi must possess a sensor for environmental changes. Probably calcineurin plays the role of such a sensor. Calcineurin is a highly conserved protein involved in fungal stress responses, composed of two subunits, the A subunit with catalytic activity and the B subunit with a regulatory function (Blankenship *et al*., 2003). The catalytic subunit is encoded by the *CMP1* gene (Bader *et al*., 2003). As a result of calcium influx calmodulin binds to calcineurin A subunit, inhibits the action of the autoinhibitory C-terminal domain of the A subunit, and leads to the formation of the active calcineurin complex, which has a protein phosphatase activity. Afterwards calcineurin may influence the expression of several virulence factors of *C. albicans*. In the case of *C. albicans* it was shown by Blankenship *et al*. (2003) that calcineurin is dispensable for growth at 37°C, germ tube formation, and adherence to the host cells, but is essential for survival in the human serum, so fungal pathogenicity strongly correlates with its activity.

CRYPTOCOCCUS NEOFORMANS

Cryptococcus neoformans is a saprophytic, basidiomycetous, dimorphic organism found worldwide, because its natural habitats are pigeon droppings and contaminated soil. Small-sized basidiospores (1.8 to 3.0 μm) (Buchanan & Murphy, 1998; Lin & Heitman, 2005) can turn into yeast cells, the form preferred at 37°C, or can form dikaryotic hyphae which are favored at 24°C (Whiteway & Bachewich, 2006). The haploid yeast cells are the asexual form of *C. neoformans* found in tissues during fungal infection as well as in standard laboratory media. In contrast, the diploid sexual form, hyphae, which is unstable and transient, is found during culture of *MATα* and *MAT**a*** strains on minimally nutritious media such as V-8 juice agar (Sia *et al.*, 2000; Perfect, 2006). The *α* strains, which are thought to be more virulent than *a* strains, can undergo a true dimorphic transition from haploid yeast cells to haploid hyphal cells producing viable basidiospores (Wickes *et al.*, 1996). Basidiospores or yeast cells may be inhaled by humans, then through the respiratory tract the pathogen can disseminate within the organism causing pulmonary infections, and subsequently, due to the *C. neoformans* predilection for the central nervous system, the life-threatening meningoencephalitis both in immunocompromised and immunocompetent patients. If the infection of the central nervous system is not cured, it is fatal in 100% of cases (Buchanan & Murphy, 1998), and even after treatment the mortality rate is between 10% and 25% (Perfect & Casadevall, 2002). Since the human immune system is in almost all cases able to overcome the intruder, infections can often pass without symptoms, but sometimes even immunocompetent patients can contract a severe disease. In patients after surgery, organ transplantation, with HIV infection or other malignancies, infections are more serious and lead to high mortality (Mitchell & Perfect, 1995; Perfect & Casadevall, 2002). This fungal pathogen is less known than *Candida* species, but nowadays the morbidity and mortality caused by cryptococcosis is a significant problem. In medically advanced countries about 5–10% of patients with AIDS develope cryptococcosis (Subramanian & Mathai, 2005); this infection rate is higher in developing countries and reaches 13–45% (Hakim *et al.*, 2000). Cryptococcocal infection was also documented in 2.8% of solid transplant recipients with a mortality rate near 42% (Husain *et al.*, 2001). The most important virulence factors of *C. neoformans* are: capsule production, melanin synthesis and the ability to grow at 37°C (Perfect, 2006).

Capsule formation

A thick polysaccharide capsule can be obtained by *C. neoformans* during lung infection, in contrast to the natural environment where it is weakly encapsulated. After the invasion, *C. neoformans* can be rehydrated and acquires the capsule composed of glucuronoxylomannan (GXM) (Buchanan & Murphy, 1998) made of unbranched chains of α-1,3-linked mannose units substituted with β-1,2- and β-1,4-xylose and β-1,2-glucuronic acid residues and connected with the fungal cell wall through glucan bridges (Bhattacharjee *et al.*, 1984; Todaro-Luck *et al.*, 1989). α-1,3-Glucan found in the cryptococcal cell wall is very important for these interactions (Reese & Doering, 2003). This structure is similar in composition among different serotypes of *C. neoformans* but differs in antigenicity (McFadden *et al.*, 2007). At least two genes, *CAP59* and *CAP64,* are necessary for capsule formation (Chang & Kwon-Chung, 1994; Chang *et al.*, 1996). The capsule has many important properties and functions; due to its negative charge it shields the pathogen from phagocytosis and killing by neutrophils, monocytes and macrophages. It is also responsible for complement depletion, antibody unresponsiveness and dysregulation of cytokine secretion by monocytes and macrophages, including TNFα, IL-1β and IL-6. The capsule can inhibit the migration of leukocytes from the bloodstream to the inflammation sites (Buchanan & Murphy, 1998; Perfect, 2006). Though the capsule has evolved as a *C. neoformans* defense against adverse environmental conditions such as amoeba attack, it is also useful for invasion of the human organism, facilitating development of infection.

Melanogenesis

The presence of melanin, a grayish, brown or black pigment, in the *C. neoformans* cell wall may be a result of fungal adaptation to environmental changes (Plonka & Grabacka, 2006). It provides protection against ultraviolet radiation, extreme temperatures and other adverse environmental conditions. However, the ability to produce melanin is also one of the important cryptococcal virulence factors, which facilitates fungal escape from lungs and infection of the central nervous system (Perfect, 2006). *C. neoformans* has a simple pathway for melanogenesis, but it must be able to acquire substrates, diphenolic compounds, from the surroundings because it lacks the tyrosinase enzyme (Fig. 3) (Polacheck & Kwon-Chung, 1988; Torres-Guererro & Edman, 1994). The construction and character of the produced pigment depends on the chemical structure of substrates, thus melanins synthesized from *o*-diphenols with hydroxyl groups in the 2,3- or 3,4-positions are usually dark and connected with the cell wall while melanins produced from *p*-diphenols with hydroxyl groups in the 1,4- or 2,5-positions are soluble (Chaskes &

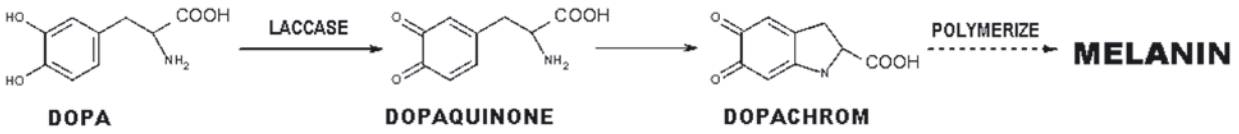

Figure 3. *Cryptococcus neoformans* **melanogenesis pathway.** After Buchanan and Murphy (1998), modified.

Tyndall, 1975). A phenoloxidase called laccase is the crucial cooper-containing enzyme responsible for the conversion of diphenolic compounds to dopaquinone, followed by their polymerization to melanin (Williamson, 1994). Laccase is anchored in the fungal cell wall (Polacheck *et al*., 1982). There are two paralog genes, *CNLAC1* and *CNLAC2,* encoding laccase (Zhu & Williamson, 2004). During central nervous system infection, *C. neoformans* may use neurotransmitters such as dopamine, norepinephrine and epinephrine as substrates for melanin production (Casadevall *et al*., 2000). Some properties of melanin facilitate fungal survival during infection. It protects fungi from reactive oxygen species and plays a role as an antioxidant. It is also responsible for cell wall integrity and negative charge, and may be important in protection against antifungal agents and in binding of different ions (including iron) to the cell surface. Melanin helps *C. neoformans* escape the action of antifungal agents and to abrogate antibody-mediated phagocytosis (Buchanan & Murphy, 1998; Casadevall *et al*., 2000; Perfect, 2006).

Mannitol production

Infection of the central nervous system caused by *C. neoformans* is often associated with production of a large amount of the hexitol D-mannitol by this organism (Liappis *et al.,* 2008). This process may facilitate the development of meningoencephalitis, because mannitol increases the osmolality of the surrounding fluid, thus it may contribute to brain edema, and also prevents oxidative damage to the fungus (Wong *et al.,* 1990). Polymorphonuclear neutrophils (PMN) can kill *C. neoformans* cells by generating toxic oxygen metabolites such as $OH^{\cdot}$ and HOCl, which are thought to be key effector molecules against *C. neoformans*, but production of large amounts of mannitol can protect the fungi from oxidative killing by PMN or by cell-free oxidants (Chaturvedi *et al*., 1996a). Mannitol production is also thought to be helpful for the pathogen to resist other environmental stresses, since Chaturvedi *et al.* (1996b) showed that a *C. neoformans* mutant producing low levels of mannitol was more susceptible to heat stress and osmotic stress. The crucial enzyme for mannitol production by this pathogenic species is mannitol dehydrogenase (Perfect *et al*., 1996).

Adhesion to host cells and crossing the blood-brain barrier

When addressing the problem of *C. neoformans* adhesion to the host cells and proteins it is crucial to recognize all factors which have an influence on fungal pathogenicity, but still the mechanism responsible for adhesion and adhesive structures present on the fungal cell wall, remain unknown. It was proven by Chang *et al*. (2004) that cryptococcal infection of the central nervous system must be preceded by adhesion to the human brain microvascular endothelial cells (HBMEC), followed by transcellular crossing of the blood-brain barrier without disrupting the monolayer integrity. Chang and coworkers showed that there no *C. neoformans* cells were present between HBMEC, so they excluded the possibility of crossing the blood-brain barrier by *C. neoformans via* a paracellular mechanism. However, Olszewski *et al.* (2004) suggested that urease production by *C. neoformans* cells facilitates microcapillaries sequestration and disruption of endothelial cells and, in consequence, crossing the blood-brain barrier *via* a paracellular mechanism. Other hypothetical mechanisms of central nervous system invasion by *C. neoformans* have been put forward. Santangelo *et al*. (2004) and Charlier *et al.* (2009) postulated that *C. neoformans* cells could invade the central nervous system by means of infected immune cells which could carry the pathogen within through the blood-brain barrier (a "Trojan horse" mechanism). Those authors hypothesized that phagocytosed *C. neoformans* cells after delivery into the brain tissues can escape from phagocytes and continue invasion and tissue damage due to the acidification of the environment and activation of extracellular phospholipases. *C. neofromans* has other properties which help in the invasion of the central nervous system. According to Charlier *et al.* (2005), the size of *C. neoformans* cells and the modifications of the fungal capsule during central nervous system invasion suggest the existence of an active mechanism that may be triggered during or after crossing of the blood-brain barrier. *C. neoformans* might induce considerable morphological changes and actin reorganization after adhesion to the HBMEC surface, facilitating engulfment of the fungus by endothelial cells or alteration in tight junctions' permeability (Chen *et al.,* 2003). The mechanism of cryptococcal invasion of the central nervous system

is still unknown and requires further investigation, and so are the fungal properties that facilitate crossing the blood-brain barrier.

Other virulence factors

Probably at some stage of infection *C. neoformans* produces and secretes hydrolytic enzymes, such as proteases and phospholipases, which play a role in nutrition and tissue damage. Cryptococcal extracellular phospholipase exhibits phospholipase B (PLB), lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) activities (Chen *et al.*, 2000). The action of this enzyme can result in destabilization and destruction of the membranes and lung surfactant, cell lysis and release of lipid second messengers (Ghannoum, 2000; Cox *et al.*, 2001). The phospholipase also enhances the adhesion of *C. neoformans* cells to the lung epithelium (Ganendren *et al.*, 2006). The activity of the phospholipase is strongly correlated with cryptococcal virulence, as proved by Cox *et al.* (2001) who constructed *plb1* mutants which were significantly less virulent in animal models and had a growth defect in a macrophage-like cell line. Probably PLB plays an important role in intracellular growth, survival and replication of *C. neoformans* within macrophages (Santangelo *et al.*, 2004). One factor which might be involved in the survival of *C. neoformans* in the presence of macrophages is the production of eicosanoids: prostaglandins and leukotrienes, which can down-regulate macrophage functions (Noverr *et al.*, 2003). Moreover, the phospholipase plays a role not only in the turnover of cryptococcal cell membrane but also in the maintenance of cell wall integrity and therefore fungal survival, particularly during heat stress (Siafakas *et al.*, 2007). It is also known that *C. neoformans* possesses a proteolytic activity, but these findings must be still investigated (Buchanan & Murphy, 1998). Recently, a gene for aspartic proteinase was characterized and the three-dimensional structure of this protein was proposed (Pinti *et al.*, 2007). This protein, called CnAP1, has two homologous domains, each containing an Asp residue in a conserved position (Pinti *et al.*, 2007). The fungal resistance to reactive oxygen species is not only due to the mannitol and melanin production, but also very important is the production of Cu, Zn superoxide dismutase, peroxidases, glutathione peroxidase and glutathione reductase, which play a role in resistance to oxidative and nitrosative species (Brown *et al.*, 2007b).

C. neoformans can also undergo phenotypic switching, when parent smooth colonies are changed into mucoid colonies which are more virulent during lung infection and can modify the immunological host response (Guerrero & Fries, 2008). Another very important virulence factor of *C. neoformans* is its ability to grow in the host's physiological conditions, i.e. at 37°C in an atmosphere of approx. 5% CO_2 and a pH higher than 7.0 (Buchanan & Murphy, 1998). For the fungal survival in the host organism the presence of an intact gene for calcineurin A is essential. Calcineurin A is a phosphoserine-phosphothreonine specific phosphatase encoded by the *CNA1* gene (Odom *et al.*, 1997). After activation by pathways connected with fungal stress response calcineurin can dephosphorylate specific proteins responsible for pathogenicity and for growth within the host organism, but specific targets of this enzyme need still to be investigated. Disruption of the *CNA1* gene can damage the fungal ability to grow at 37°C without affecting the ability to grow at 24°C (Odom *et al.*, 1997).

C. neoformans is also able to acquire iron from its environment during tissue and bloodstream infections. The pathway of signaling of iron presence in the surroundings and iron acquisition is only partially understood. Reductases present at the fungal cell surface can reduce ferric to ferrous iron. This process is followed by transport of the ferrous ions into the cell. This process is mediated by a permease (Cft1) and ferroxidase (Cfo1) complex connected with the plasma membrane (Jung & Kronstad, 2008). The siderophore transporter Sit1, the cell wall protein Cig1 and the mitochondrial proteins Frr1, 3 and 4 also play roles in iron delivery and homeostasis. The Cir1 protein controls the transcription of genes encoding those proteins (Jung & Kronstad, 2008).

ASPERGILLUS FUMIGATUS

Aspergillus fumigatus is an ascomycetous, saprophytic and ubiquitous fungus responsible for moulding. It is found worldwide and its small-sized conidia (2–3 μm) are abundant in the environment (Gniadek & Macura, 2007). Airborne *A. fumigatus* conidia are inhaled by everyone, because their concentration in the air is high, approx. 1–100 conidia per m^3 (Latgé, 2001). It is also well known that *A. fumigatus* conidia are frequently present in food, especially in pepper and tea (Bouakline *et al.*, 2000), in tap water (Warris *et al.*, 2003), at home (Ren *et al.*, 2001) and in the office rooms (Buczyńska *et al.*, 2007). This may be the reason why nosocomial acquired infections and community acquired infections quite often develop in immunocompromised as well as in immunocompetent people (Clancy & Nguyen, 1998). In an immunocompetent person, the efficient immune system is usually able to get rid of these conidia, but sometimes even those people may contract invasive pulmonary aspergillosis (Clancy & Nguyen, 1998). However, especially patients with severely impaired immunity may contract a life-threatening invasive

pulmonary disease, disseminated infections or central nervous system infection (Latgé, 2001; Rhodes & Brakhage, 2006). A high mortality rate, from 60% to 90%, is correlated with late diagnosis and relatively poor knowledge about fungal pathogenicity and virulence factors (Tekaia & Latgé, 2005; Rementeria *et al*., 2005). There are some features thought to be putative virulence factors, including heat tolerance, adhesins, pigment production, toxic metabolites and extracellular enzymes, but most of them evolved as fungal protection against adverse environmental conditions and their role in pathogenicity is often unclear (Alp & Arikan, 2008). The functions and character of the putative virulence factors of *A. fumigatus* are still under investigation; probably there is a substantial co-operation between these properties, many of which are regulated by numerous genes, so it is often impossible to indicate without doubts which of them contribute to the mechanism of pathogenesis.

Melanin production

A. fumigatus conidia are often black or grey due to the presence in the cell wall of a pigment called DHN-melanin, which is synthesized from acetate with participation of enzymatic products of six genes (Fig. 4) (Tsai *et al*., 1999; Latgé, 2001). The melanin functions are protection against ultraviolet radiation, enzymatic lysis, extreme temperatures, as well as against reactive oxygen species during infection (Rementeria *et al*., 2005).

Secreted hydrolytic enzymes

A. fumigatus produces and secretes various hydrolytic enzymes, including serine and aspartic protease, metalloproteinase, dipeptydylpeptidases and phospholipases, which contribute to fungal virulence facilitating lung and other tissue colonization. There is a considerable correlation between phospholipase activity and severity of infection (Alp & Arikan, 2008). The serine proteinase and metalloproteinase have an elastinolytic activity, so lungs being rich in elastin, the *A. fumigatus* ability to degrade this protein is important during pulmonary infections (Hogan *et al*., 1996). The serine proteinase (AFA1p) is a member of subtilisin family and can degrade not only elastin but also collagen, fibrin and fibrinogen. This protein has an extracellular location, but it is also connected with the cell wall (Moutaouakil *et al*., 1993; Tomee & Kauffman, 2000). Apart from the mentioned activities, other hydrolytic enzymes are produced by *A. fumigatus*, including nucleases and phosphatases (Tomee & Kauffman, 2000).

Toxins

Secretion of different kinds of toxins to the environment or during infection within the human organism is one of the characteristic features of *A. fumigatus*. The well-known *A. fumigatus* toxin aflatoxin, which has hepatotoxic and carcinogenic features probably is not produced in the human organism during infection, because its expression is regulated by many genes under complex influence of environmental conditions (OBrian *et al*., 2003; Rementeria *et al*., 2005). The most important toxin with a well-proven *in vivo* activity is gliotoxin. This secondary metabolite from the epipolythiodioxopiperazine family has immunosuppressive properties, because it can inhibit macrophage phagocytosis, T-cell activation and proliferation, and can induce macrophage apoptosis (Hogan *et al*., 1996). Gliotoxin is also responsible for slowing ciliary beating in the respiratory tract and for epithelial layer damage, so the fungal cells cannot be efficiently removed from the host organism (Tomee & Kauffman, 2000). Another toxin secreted *in vivo* is resticotocin, an 18-kDa protein (Aspf1) which cleaves a phosphodiester bond in 28S rRNA of eukaryotic ribosomes (Lamy *et al*., 1991; Hogan *et al*., 1996). *A. fumigatus* also pro-

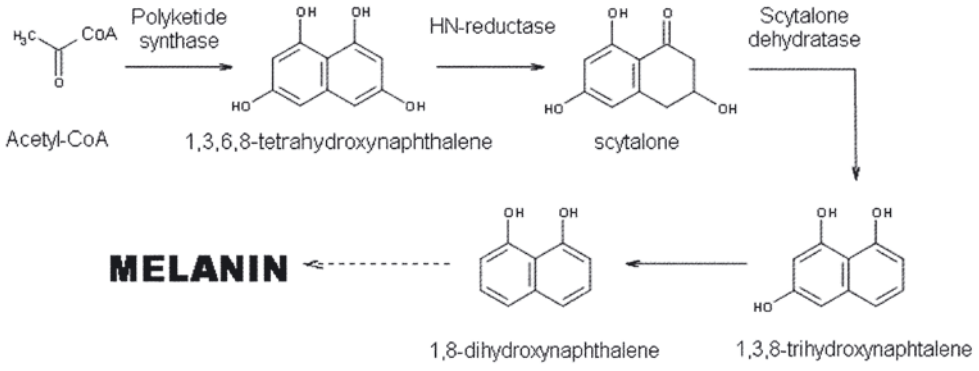

Figure 4. Pathway for melanin synthesis by *Aspergillus fumigatus*.
After Latgé (2001), modified.

duces other immunosuppressive toxins, for example the 14-kDa conidial inhibitory factor and AfD, *A. fumigatus* diffusible product. Pyrogenic, cytotoxic and shock-evoking activities are also characteristic for other endotoxins produced by *A. fumigatus*, including fumitremorgins, fumagilin, fumagatin and helvolic acid (Tomee & Kauffman, 2000).

Other virulence factors

The molecular basis of *A. fumigatus* pathogenicity is associated with its ability to adhere to host tissues and with binding to different host proteins, including laminin, fibrinogen, surfactant A and D, complement, immunoglobulin and fibronectin through different receptors connected with the fungal cell wall, which is built of α, β-1,3-glucan, galactomannan and chitin (Latgé, 2001). The galactomannan can be used as a diagnostic factor, because during infection it can be found in the serum, urine and cerebrospinal fluid (Latgé, 1999; Rementeria *et al*., 2005). Probably laminin and fibrinogen have a common receptor or distinct receptors, but tightly packed on the surface of conidia (Tomee & Kauffman, 2000). *A. fumigatus* conidia are covered with a layer of hydrophobic proteins, called "rodlet layer" built from proteins encoded by at least two genes, *RODA* and *RODB,* responsible for adhesion to albumin and collagen (Latgé, 2001). Protection against reactive oxygen species produced by human immune cells during inflammatory state is also important for developing a fungal infection, therefore *A. fumigatus* has an ability to produce catalases and superoxide dismutases (Latgé, 2001). Three catalases are expressed by *A. fumigatus*. CatA, a homodimeric enzyme composed of two 84.5-kDa subunits, is connected with conidia. The other two catalases, Cat1p and Cat2p, are connected with hyphae and have different structures, because the first one is composed of four 90-kDa subunits, and the second is a monomer. Despite the fact that these enzymes can protect *A. fumigatus* against reactive oxygen species in the environment their role as virulence factors during infection is not clear (Paris *et al*., 2003). Perhaps there are other enzymes with such activity which contribute to the fungal pathogenicity. It has also been proven that *A. fumigatus* superoxide dismutases, containing Mn or Cu and Zn, can efficiently prevent the fungus from oxidative damage (Rementeria *et al*., 2005). The optimal temperature for *A. fumigatus* growth is 37°C, but it is able to grow even at 55°C and to survive temperatures near 75°C (Tekaia & Latgé, 2005). There are some indications that calcineurin catalytic A subunit, encoded by the *CNAA* gene, is very important for *A. fumigatus* growth, tissue invasion and pathogenicity (Steinbach *et al*., 2006).

CONCLUSIONS

The recent progress in medical techniques, transplantology and antimicrobial treatment is unfortunately the reason of the increasing frequency of invasive fungal infections and high mortality rate, even 90%, among patients with disseminated candidiasis, aspergillosis or cryptococcosis (Richardson, 2005). Additionally, the diagnosis is often difficult because of non-specific symptoms and problems in isolation and identification of fungi (Hinrikson *et al*., 2005). At present, the majority of pathogenic fungi are susceptible to conventional antifungal treatment, but an increasing resistance to some antifungal drugs is a new, important problem in medicine. Currently used antifungal drugs belong to one of four groups of different character and mechanism of action (Sanglard & White, 2006). The first group, the polyenes represented by amphotericin B, target ergosterol, a sterol present in the fungal cell membrane, and make pores causing cell death (White *et al*., 1998). Amphotericin B may be used for treating infections caused by *C. albicans*, *C. neoformans* and *A. fumigatus*. The main problem with polyene therapy is that at high and efficient concentrations they are nephrotoxic and must be injected intravenously because of poor solubility (Sanglard & White, 2006).The second group of antifungal drugs is ergosterol biosynthesis inhibitors, which include azoles, morpholines and allylamines. They can inhibit the late pathway of ergosterol biosynthesis and cell division, causing loss of membrane structure and function (White *et al*., 1998). Azoles are the most popular drugs from this group, and they can be divided into two classes: imidazoles, which include ketoconazole and clotrimazole used for superficial infections, and triazoles, which include fluconazole, voriconazole and itraconazole used for systemic infections (Sanglard & White, 2006). Azoles are used for treating candidiasis, but voriconazole is also important for aspergillosis therapy (Macura *et al*., 2000; Pawlik *et al*., 2006), whereas fluconazole is used for cryptococcosis therapy because of its ability to cross the blood-brain barrier (Sanglard & White, 2006). The third group includes inhibitors of nucleic acid synthesis, i.e. 5-flucytosine (Sanglard & White, 2006). The fourth, the newest category of antifungal drugs, includes echinocandins which target glucan synthase. One echinocandin, caspofungin, is currently used for treatment of *Candida* and *Aspergillus* infections; other echinocandins, anidulafungin and micafungin, are in clinical trials (Sanglard & White, 2006). A serious problem in treatment of fungal infections is the resistance to azoles and 5-flucytosine through a mechanism dependent on alternations in the target enzyme and in drug efflux pumps (Es-

pinel-Ingroff, 2008). A way to avoid developing resistance is to use multidrug therapy or different antifungal agents, as well as to limit too frequent and uncontrolled usage of the newest category of drugs (Sanglard & White, 2006). It is also necessary to recognize the mechanism of pathogen–host interactions at the molecular level in order either to prevent the infection or to develop new strategies for therapy and new effective antifungal drugs. Although the knowledge of the fungal pathogenicity and molecular basis of their virulence is already significant, this issue needs further investigation.

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