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Phylogenetic analysis of fungal heterotrimeric G protein-encoding genes and their expression during dimorphism in *Mucor circinelloides*

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ABSTRACT

In fungi, heterotrimeric G proteins are key regulators of biological processes such as mating, virulence, morphology, among others. *Mucor circinelloides* is a model organism for many biological processes, and its genome contains the largest known repertoire of genes that encode putative heterotrimeric G protein subunits in the fungal kingdom: twelve Gα (McGpa1–12), three Gβ (McGpb1–3), and three Gγ (McGpg1–3). Phylogenetic analysis of fungal Gα showed that they are divided into four distinct groups as reported previously. Fungal Gβ and Gγ are also divided into four phylogenetic groups, and to our understanding this is the first report of a phylogenetic classification for fungal Gβ and Gγ subunits.

Almost all genes that encode putative heterotrimeric G subunits in M. circinelloides are differentially expressed during dimorphic growth, except for McGpg1 (Gγ) that showed very low mRNA levels at all developmental stages. Moreover, several of the subunits are expressed in a similar pattern and at the same level, suggesting that they constitute discrete complexes. For example, McGpb3 (Gβ), and McGpg2 (Gγ), are co-expressed during mycelium growth, and McGpa1, McGpb2, and McGpg2, are co-expressed during yeast development. These findings provide the conceptual framework to study the biological role of these genes during *M. circinelloides* morphogenesis.

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Introduction

Heterotrimeric G proteins are ubiquitous in eukaryotic cells; these proteins play a role in important biological processes such as growth, cell differentiation, pathogenesis, and response to growth factors in fungal, plant, and mammalian cells. These heterotrimers are linked to specific receptors, denominated as G protein-coupled receptors (GPCR) which sense different ligands such as hormones, odor, growth factors, and even photons (Beckerman 2005). After that, the subunits of the heterotrimers activate downstream cellular protein effectors that ultimately elicit changes in cell metabolism (McCudden *et al.* 2005; Entschladen *et al.* 2011).

Heterotrimeric G proteins are formed by Gα, Gβ, and Gγ subunits. Guanine nucleotide binding takes place in the Gα subunit. Binding of guanosine triphosphate (GTP) to the Gα subunit (Gα-GTP) leads to its activation; the heterotrimer dissociates, thereby generating two signaling components: Gα and Gβ-Gγ dimer. Each signaling component is able to interact with different protein effectors, which ultimately trigger a variety of physiological responses. Once the signal is aborted, GTP is hydrolyzed by regulator of G protein signaling (RGS) proteins; these proteins increase the intrinsic guanosine triphosphatase (GTPase) activity of Gα subunits (Hollinger & Hepler 2002). The consequent restoration of the Gα-GDP form promotes its reassociation with the Gβ-Gγ dimer. This guanine nucleotide exchange finally alters the dynamics between heterotrimeric association and the biological response (McCudden *et al.* 2005).

In the majority of organisms studied to date, there are multiple subunits of G proteins; for instance, the *Homo sapiens* genome contains 17 Gα, 5 Gβ, and 12 Gγ subunits (Wettschureck & Offermans 2005); the fruit fly *Drosophila melanogaster* genome contains 11 Gα subunits, 3 Gβ subunits, and 2 Gγ subunits (Ishimoto *et al.* 2005); *Arabidopsis thaliana* genome contains only 1 Gα, 1 Gβ, and 3 Gγ subunits (Thung *et al.* 2012); while most fungal genomes contain 4 Gα, 4 Gβ, and 2 Gγ subunits (Li *et al.* 2007b).

The Ascomycete yeast *Saccharomyces cerevisiae* presents 2 Gα (Gpa1 and Gpa2), 1 Gβ (Ste4p), and 1 Gγ (Ste18p) subunits, which are implicated in pheromone (Nomoto *et al.* 1990) and nutrient responses (Lorenz & Heitman 1997). In the filamentous Ascomycete *Neurospora crassa*, 3 Gα, 1 Gβ, and 1 Gγ subunits, respectively, have been reported; they are involved in reproduction (Krystofova & Borkovich 2005) and growth processes (Turner & Borkovich 1993; Baasiri *et al.* 1997; Kays *et al.* 2000).

The multiplicity of these heterotrimeric subunits in some organisms implies that they may have redundant functions. For example, Gna-1 and Gna-2, two Gα subunits found in *N. crassa*, positively regulate female fertility (Baasiri *et al.* 1997). The Gγ subunits (Gpg1 and Gpg2) are involved in the positive regulation of the pheromone response in the Basidiomycete *Cryptococcus neoformans* (Li *et al.* 2007a). To date, only fungal Gα subunits are phylogenetically characterized, classifying them into four groups (Bölker 1998; Li *et al.* 2007b), however there is no phylogenetic analysis information about of Gβ or Gγ subunits so far.

In the Mucoral *Mucor circinelloides*, 4 Gα subunits have been identified (McGpa1–4) (Meza-Carmen *et al.* 2006), but no Gβ or Gγ subunits have been reported to date. *Mucor* vegetative cells such as yeast and hyphae are produced from spores, this dimorphic process is influenced by certain conditions, for example the presence or absence of oxygen leads to mycelial or yeast growth respectively; in addition yeast cells are favored by presence of fermentable carbon source; as well an increment of intracellular AMPc leads to this morphology. Vegetative cells differentiate into arthrospores when cultures reach the stationary growth phase; aerial structures named sporangiophores are formed in solid media (Orlowski 1991; Lübbehüsen *et al.* 2003). This organism also responds to the presence of light, which stimulates sporulation and carotenogenesis (Lorca-Pascual *et al.* 2004). Furthermore, *M. circinelloides* is an opportunistic pathogen in immunosuppressed patients (Li *et al.* 2011). This fungus is also considered as an alternative source for production of biodiesel, a renewable energy source (Xia *et al.* 2011). Some molecular components involved in *M. circinelloides* differentiation have previously been reported. These include protein kinase A (PKA) isoforms, which are relevant during dimorphism, for example *pkaR4* is essential during hyphal germination (Wolff *et al.* 2002; Ocampo *et al.* 2012). The *cgrA* product, which encodes an E3 ubiquitin ligase involved in regulation of carotenogenesis and decrement of sporulation (Nicolás *et al.* 2008). It has also been demonstrated that *Mcwc-1b* is genetically associated to *crgA*, because its overexpression in a *crgA*Δ strain restores the sporulation defect (Navarro *et al.* 2013). Recently, a regulatory subunit from phosphatase calcineurin *cnbR* has been described to be essential for mycelium development, the mutant in this gene led to monomorphic yeast growth (Lee *et al.* 2013). The consequences of these morphological stages could be linked to functional roles, for example cnbrΔ strain which only grows as yeast cells is less virulent compared to wild-type strain, indicating a morphology-dependent role during virulence.

Although these antecedents indicate that *M. circinelloides* exhibits multiple differentiation processes, many of the mechanisms remain poorly understood at the molecular level. However, it is clear that heterotrimeric G protein subunits participate in morphological changes during fungal phases. This is typified by the activity of Gpa2, which is a Gα a subunit from the Ascomycete *Candida albicans* that positively regulates the yeast–mycelium transition and is involved in virulence (Sánchez-Martínez & Pérez-Martín 2002). Pseudohyphal differentiation induced by nitrogen starvation in *S. cerevisiae* is positively regulated by a Gα subunit, denominated as Gpa2 (Lorenz & Heitman 1997).

In the present study, we performed an *in silico* genome-wide search of the *M. circinelloides* genome database for heterotrimeric G protein subunit identification finding 12 Gα subunits (McGpa1-12), 3 Gβ subunits (McGpb1–3) and 3 Gγ subunits (McGpg1–3). This information was used to perform a phylogenetic and mRNA quantitation analysis of these putative genes during dimorphism of *M. circinelloides*. Our results described for the first time a fungal Gβ and Gγ subunits phylogenetic classification, furthermore the mRNA measurement of all heterotrimeric G protein-encoding genes during dimorphic event, led us to identify specific mRNA accumulation like