Generation of Intersectin Homolog Cin1 Isoform-Specific Mutants in Pathogenic Fungus *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen that causes meningoencephalitis in immunocompromised individuals such as those undergoing solid organ transplant, receiving immunosuppressive therapy, or those with HIV/AIDS. This fungus exhibits a propensity for the human central nervous system, thus studies of this neurotropism are an important component of addressing the fungal virulence mechanism. In a previous study, we found that *C. neoformans* contains Cin1, a homolog of human intersectin ITSN1, and that one of the two isoforms, Cin1-S, exhibits a survival advantage in the brain of experimental animals. To test the hypothesis that Cin1 may play a role in fungal neurotropism, mutant strains expressing the Cin1-L isoform are needed. The present study is to create a Cin1-L mutant allele linked to antibiotic resistance and transform the wild type strain to generate Cin1-L mutants expressing only Cin1-L. This newly generated strain will be evaluated in experimental animals, either individually or in combination with the Cin1-S or the wild type strains. A potential *CIN1-L* mutant allele linked to neomycin resistance was obtained, and the majority of the 52 genetic transformants contain the recombinant gene construct and the loss of the wild type *CIN1* gene. This paper discusses the process used to identify this potential mutant. This goal has been one component of the overall research project that has yet to be completed. The potential *CIN1-L* mutant that was identified must be verified before a definitive conclusion can be made regarding the generation of this mutant. This verification will be done by DNA sequencing and Northern blot analysis. Once it has been verified that these transformants contain specific mutants that express only the long isoform of Cin1 (Cin1-L), further research can be conducted using a coinfection study with both the long and short isoforms. This advancement will allow us to finally determine if Cin1 plays a role in the neurotropism of the fungus.

Background

Cryptococcus neoformans is an opportunistic fungal pathogen of global importance. It often targets the central nervous system of immunocompromised patients and causes life- threatening meningoencephalitis, or inflammation of the brain. It is specifically prevalent in third-world populations where there are larger amounts of immunocompromised patients with diseases such as HIV/AIDS. Cryptococcosis affects nearly one million people each year, causing about 600,000 deaths worldwide (1). Due to the lack of effective antifungals and emerging drug resistance, it is very important to identify novel targets for antifungal therapy.

Since the major disease symptom of cryptococcosis is meningoencephalitis, one of the central questions involving cryptococcal pathogenesis is how this mushroom-like fungus crosses the blood-brain barrier upon entering the bloodstream and how it proliferates within the brain. Thus, understanding the mechanism by which *C. neoformans* proliferates within the brain holds the possibility of a new type of effective antifungal therapy. The infection route of *C. neoformans* is illustrated in Fig. 1.

Interestingly, the discovery of cryptococcal intersectin Cin1 has introduced the possibility of using it as a

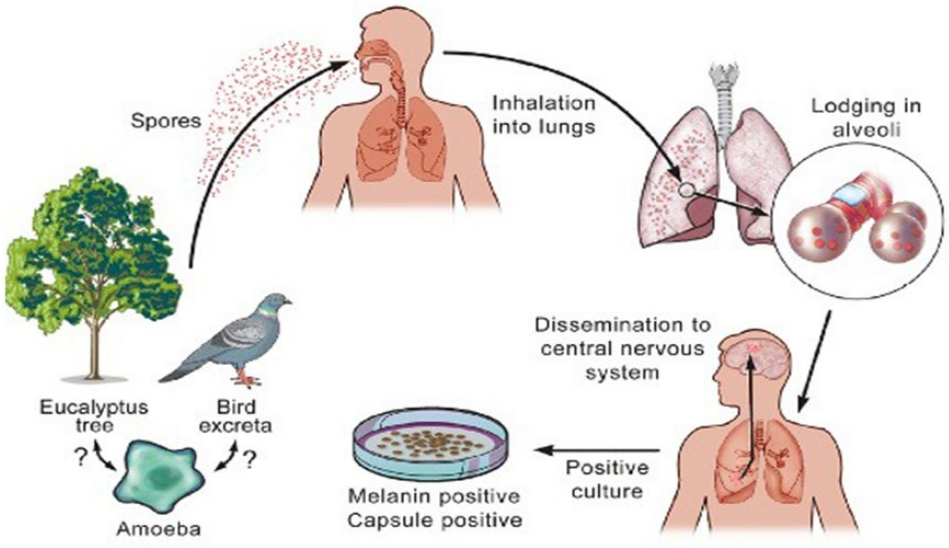

Figure 1: C. neoformans infection route

probe to explore the neurotropism of the fungus. In a previous study for a noncanonical G beta subunit, the cryptococcal intersectin Cin1 was discovered (2). Cin1 shares relatively high homology with the human intersectin-1 protein ITSN1, a cytoplasmic membrane- associated protein that both directly and indirectly coordinates endocytic membrane trafficking with the aid of the actin cytoskeleton (3). As a result of alternate mRNA splicing, ITSN1 appears in both a long isoform, ITSN1-L, and a short isoform, ITSN1-S. These isoforms have been shown to express differently in humans. ITSN1-L is exclusively expressed in the brain, while ITSN1-S is expressed in all human cells. It is thought that the presence of the long isoform only in the brain may be due to the requirement of the massive exocytotic transport accompanying rapid and sustained release of neurotransmitters such as dopamine and endorphins (4).

Similar to human and animal ITSN1, *C. neoformans* Cin1 also exists in two isoforms due to similar alternate mRNA splicing. In an earlier effort to examine whether or not Cin1 plays any role in the neurotropic characteristics of the fungus, the mutant strain expressing the short isoform Cin1-S was obtained through genetic manipulation. This mutant allele was linked to antibiotic Nourseothrycin resistance (5). No specific alteration was found in strains that express only Cin1-S. However, in a co-infection test involving an experimental mouse model of cryptococcosis, Cin1-S was found to exhibit more competitiveness than the wild-type strain in the brains towards the end of disease presentation. This survival advantage was not seen in other tissues, including the kidneys and lungs (Fig. 2). To further examine this unexpected phenomenon, this study aims to obtain the second mutant expressing the long isoform Cin1-L and compare its neurotropism and virulence with those of the wild type and Cin1-S strains.

Materials and Methods

C. neoformans var. *neoformans* strains were used in this study. The wild type strain JEC21 (6) was used as the parental strain for genetic transformation. Standard methods of nucleotide manipulation were performed as described (7) and transformation using the gene gun (Bio-Rad PSD-1000/He Particle Delivery System) were described previously by Perfect et al. (8).

The identification of the Cin1 homolog, as well as its short and long isoforms, presents the question of whether or not the protein plays a role in the neurotropism of the fungus. To examine the likely function, our approach was to generate mutant strains to see how they behave in experimental disease situations. In a previous study, the *cin1* null mutant was unable to express the disease, indicating that the protein is important (5). The *CIN1-S* mutant expressing the Cin1-S isoform was obtained through transformation-mediated genetic recombination. In this case, the C-terminus DH-PH domain of the *CIN1* gene was disrupted with a marker gene for Nourseothricin resistance. Competition tests in animals showed that the short isoform out- competes

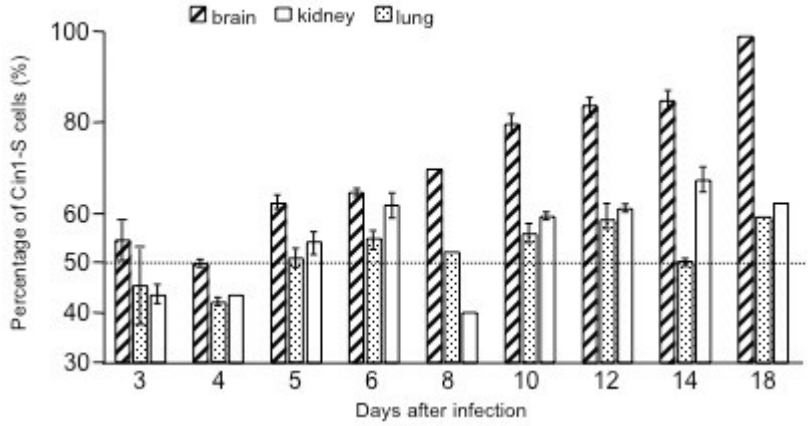

Figure 2: Cin1-S shows a survival and proliferation advantage in the murine CNS during coinfection with the parental wild type strain JEC21. Charts here show the percentage of Cin1-S over JEC21. Balb/c mice were infected with Cin1-S and JEC21 in 1:1 ratio (50% percentage) at the concentration of $1x10^7$ cells per mouse. Mice were sacrificed at predetermined days or when moribund. Brain, lung, kidney were collected, weighted, and homogenized in 1 ml PBS. Following serial dilution and plating, CFUs were obtained on YPD plates. Plates with approximate density (<200 cfu) were replicated to YPD with Nourseothricin. A minimum of 200 colonies was counted for each sampling. Fungal burden was all within the same magnitude ($1.5\text{-}6.7x10^6$/g, except those past day 10 whose brain CFUs were higher at $1\text{-}1.7x10^7$/g). Cin1-S and WT controls were performed and comparable CFUs were obtained (not shown here). Dotted lines represent initial percentage of Cin1-S (50%). Numbers of mice used per group were: 1 (day 8 and 18), 2 (day 4, 6, 10, and 14), and 3 (day 3, 5, and 12).

the wild-type strains in a coinfection. This occurs only in the brain and not in the two other organs studied, the kidney and lung. The fact that the short isoform has a survival advantage over the wild type indicates that the isoform plays a role. By obtaining the long isoform (Cin1-L) and testing it in a coinfection study, we will obtain more clues as to the isoform function versus the neurotropism of the fungus.

Approach

C. neoformans intersectin homolog Cin1 was found to play important roles in intracellular trafficking that underlie important characteristics such as growth, sexual reproduction, presentation of virulence factors such as melanin pigment and polysaccharide capsule formation, and virulence in a mouse model of cryptococcosis (5). Alternate intron splicing of the *CIN1* mRNA resulted in the formation of two isoforms: a 1282 amino acid long Cin1-S and a 2004 amino acid long Cin1-L. To generate the Cin1-L isoform protein, we first changed the first 3' intron boundary sequence CAG to CAA, effectively nullifying intron splicing at the acceptor site of the fourth intron and generating the long protein isoform-producing allele (Fig. 3). This was accomplished by incorporating primers that contain the altered 3' intron boundaries for the long isoform with plasmid constructs containing the full- length *CIN1* gene. This was done through Polymerase Chain Reaction (PCR) amplification and cloning. By changing CAG to CAA, we eliminated the alternate spicing capabilities of RNA, which allowed us to generate the Cin1-L allele. We also needed to link Cin1-L to a selectable marker other than Nourseothrycin resistance. In this case, we used the neomycin resistance gene cassette (*NEO*) available in the field (5). We have incorporated a unique Sma I restriction site for insertion of a neomycin resistance (*NEO*) cassette at the 3' non-coding region of the *CIN1* gene. Since the modification occurs at the 3' terminus of the *CIN1* gene, the partial fragment containing the modified intron splicing site and the *NEO* cassette was obtained eventually through multiple rounds of PCR amplification and gene cloning.

Once the above fragment was obtained, we transformed the *C. neoformans* strain JEC21 with the PCR amplified linear *CIN1-L* allele linked to *NEO* through the use of the gene gun. This system uses high-pressure helium

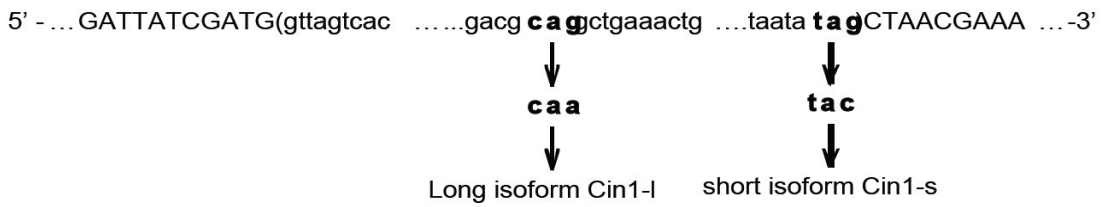

Figure 3: Illustration of Cin1 alternate splicing at Intron #6. Changing the first acceptor site sequence from CAG to CAA eliminates alternate splicing resulting in long isoform (Cin1-L) formation. Changing the second acceptor site sequence from TAG to TAC alters the intron causing the production of short isoform (Cin1-S) only.

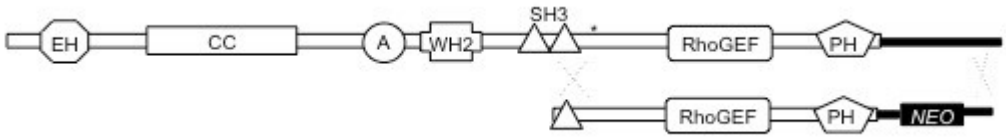

Figure 4: A schematic representation for the Cin1-L mutant generation. The construct was obtained through point mutation, insertion and PCR amplification. Mutant strain screening is now in progress.

and partial vacuum to facilitate the delivery of DNA-coated microcarriers through a macrocarrier sheet to penetrate and transform the fungal cells (9). In this study, we used 1325 psi rupture disks. This method has been proven effective in inducing homologous recombination and gene disruption in this fungus, in comparison to other methods such as polyethylene glycol/calcium carbonate or electroporation (8).

Transformants were first selected based on their resistance to drug (Neomycin) added to the growth medium. Once selected, the candidate transformants were further screened by PCR amplification using DNA primer set PW1753/PW1738, which can distinguish between the wild type *CIN1* Gene and putative transformants with proper recombination. Further verification will be carried out by assays using RT-PCR and Northern blot analysis to identify if these mutant strains indeed express the Cin1-L isoform only.

Results and Discussion

We have used both the 7 kilobase *CIN1-L-NEO* DNA fragment by PCR amplification and the plasmid vector containing the same sized fragment in the initial transformation. Since the open ends of the liberalized DNA that are homologous to the endogenous gene are proven to be more active in undergoing homologous recombination, we paid specific attention to comparing the results of using the two types of DNA fragments. We obtained 52 transformants using the plasmid DNA and 45 using the linearized fragment. We then streaked the putative transformants to a Petri dish with fresh medium and performed the screening with DNA primers designed to amplify the wild type *CIN1* gene. Indeed, we found 32 wild type transformants out of 52 total. In contrast, we found 5 wild type-like transformants out of 45 transformants when using the linearized *CIN1-L* linked to *NEO* DNA fragment in transformation. This is consistent with the understood notion that linear fragments are more capable of inducing the recombination event. As represented in Figure 5, the 3 DNA bands indicate the presence of the wild type *CIN1* gene, while those without bands indicate that the loss of the wild-type gene might be due to integration of the introduced DNA fragment.

We chose to further characterize the transformants from those that were transformed with the linearized *CIN1-L-NEO* fragment. We selected six of this type of transformants for analysis by reverse transcriptase PCR (RT-PCR). For the RT-PCR, primer set PW1078/PW1396 was used. We first isolated the total RNA from these six transformants and synthesized the

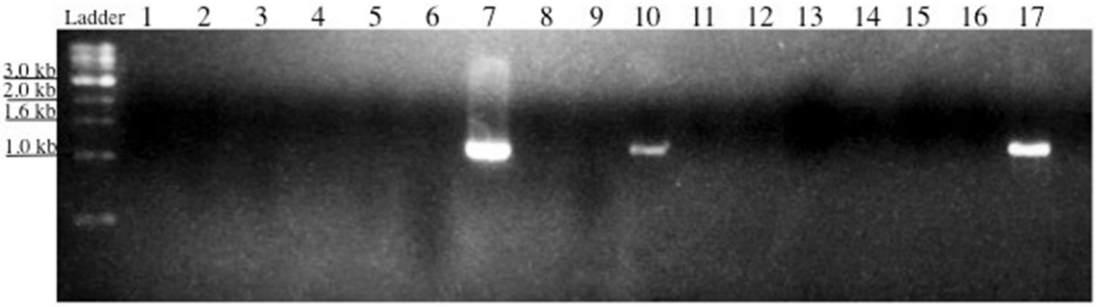

Figure 5: 17 of the 45 transformants represented. Bands represent the wild-type gene. Those that do not contain a band may contain the transformants.

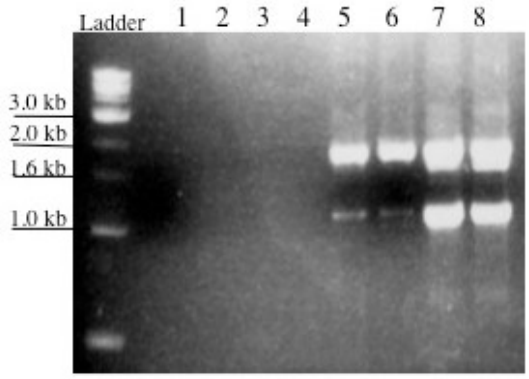

Figure 6: Results from RT-PCR. Lanes with two fragments show the possibility of DNA contamination.

first strand cDNA using the commercially available cDNA synthesis kit. We then performed PCR to examine if there were DNA fragments that contained the expected size following the integration event. Figure 6 showed that: 1) we have the PCR products from transformants #5, 6, 7, and 8; 2) there might be DNA contamination resulting the presence of two fragments in each lane. To confirm such a scenery, we isolated and cloned the two fragments from #5 and #7, respectively, and sequences of these fragments will be verified by DNA sequencing. These fragments are shown in Figure 6.

In summary, we were able to generate the Cin1-L long isoform allele through genetic manipulation, and we were also able to generate *NEO* resistant transformants using the Cin1-L- NEO allele. Further research must be done to verify the mutants. This will be done by DNA sequencing and Northern blot analysis. Once these mutants have been verified, we will be able to perform a coinfection study with both the long and short isoforms in mice to elucidate whether or not Cin1 has a role in neurotropism of the fungus. The results of this following study will determine what further research is done on this fungal strain.

Primer Sequences

PW1078: 5'-AGATCTGCTACCACCATTCGAGTGCGAG-3'
PW1396: 5'-TCTAGATCTCTCCCTCTTATCCATCG-3'
PW1738: 5'-TGGCCGCTGCACCCTTGGAAACC-3'
PW1753: 5'-AAGGGGAGGGGATACTATTGCCC-3'

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