Ph. D. Thesis

Molecular and functional analysis of the *hmgR* gene encoding HMG-CoA reductase of the zygomycete fungus *Rhizomucor miehei*

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2008

Introduction

Members of the zygomycete order Mucorales (*Absidia*, *Cunninghamella*, *Mucor*, *Rhizomucor*, *Rhizopus)* (Zygomycota) have medical, industrial, biotechnological and agricultural importance. Some isolates may cause post-harvest damages in agriculture, others are known as opportunistic pathogens whereas some members are used as producers of extracellular enzymes, organic acids and carotenoids. Based on their specific morphogenesis and sexual processes they are often investigated filamentous fungi. Members of the thermophilic *Rhizomucor* genus (*R. miehei* and *R. pusillus*) are practically important. In immunocompromised patient with leukemia, ketoacidosis, or serious skin injuries they can develop opportunistic fungal infections. On account of their thermophylic nature they can grow fast in humans. The therapy of zygomycoses is still limited because of the intrinsic resistance of these fungi to the majority of the currently used antimycotics. The most effective agents are amphothericin B and a new azole drug, posaconazole. In the cases of the other antifungal agents (like echinocandins, fluconazole, voriconazole or itraconazole) significant effect on pathogen zygomycetes has not been reported.

The enzyme encoded by the *hmgR* gene catalyses the formation of mevalonate, which is a rate limiting step of the acetate/mevalonate (AMV) pathway leading to the isoprenoid synthesis. In eukaryotes, the AMV pathway regulates the biosynthesis of some hormones (gibberellins, steroids and some

mating factors), pigments, carotenoids and important components of the cell membrane, like sterol or ergosterol. The investigation of the genes involved in the biosynthesis of carotenoids has great biotechnological importance. There is currently an increasing interest in fungal sources of carotenoids, which requires the development of novel strategies to improve the production. The progress in the cloning of the carotenogenic genes opens up the possibility of modifying and engineering the carotenoid pathway in fungi. One of these important sources is *Xanthophyllomyces dendrorhous* producer of astaxanthin, a valuable oxygenated β-carotene derivative, which is used in the food, cosmetic and pharmaceutical industry and as feed colour additive (especially to salmons, trout and poultry). Mevalonate pathway also provides prenyl and farnesyl groups necessary for the normal function of many indispensable proteins (e.g. Ras and Rho) of the cell. Blockade of these processes through the inhibition of HMG-CoA reductase decrease the growing rate, disturb the sporangiospore formation and cause morphogenetic changes and apoptosis in fungal cells. Statins are the widely known selective inhibitors of the HMG-CoA reductase. They are commonly used as cholesterol lowering agents in human diseases. The pleiotropic effects of statins in human illnesses are intensively investigated today.

The aim of our study was to isolate and characterize the 3-hydroxy-3-methilglutaryl-coenzyme A reductase (HMG-CoA reductase) encoding gene in the practically important zygomycete fungus *R. miehei*, and to start the identification of the same gene in

the astaxanthin-producing yeast *X. dendrorhous*. In connection with that, the effect of statins as HMG-CoA reductase inhibitors on opportunistic pathogen zygomycetes fungi was investigated. The interaction between amphotericin B and two statins, fluvastatin and rosuvastatin, was also tested *in vitro* in terms of growing inhibition of fungi.

Aims

In the recent years, incidence of zygomycoses has increased on account of the growing number of endangered patients. The therapy provided by newer and available antifungal agents is not sufficient for managing these specific infections. Molecular investigations of Zygomycetes can yield new and better therapy targets; however, because of the peculiarity of this fungal group, their study can be hardly replaced with the research of other fungi. For the development of a more efficient genetic transformation system of these fungi their molecular characterization is also indispensable.

Taking into account all these considerations, the aims of our study were the followings:

- Cloning and identification of the gene encoding the HMG-CoA reductase of *R. miehei*. Starting experiments for the identification of *hmgR* gene of *X. dendrorhous*.
- Characterization of the gene and the corresponding amino acid sequence, additionally, determination of the copy number of the gene in the *R. miehei* genome.

- Transformation of the identified gene into an appropiate zygomycete fungus and investigation of its effect on the host's susceptibility to statins. The purpose of these experiments was to examine the potential applicability of the *hmgR* gene in a new transformation and selection system for zygomycetes fungi.
- Investigation of the biological effect of the selective inhibitors of the enzyme (statins).
- Analysis of the *in vitro* susceptibility of several zygomycetes species to amphotericin B in combination with fluvastatin and rosuvastatin.

Methods

Screening of phage genome library:

- amplification of phages
- plaque-hybridization
- phage DNA purification

Molecular manipulation methods:

- DNA, RNA isolation
- polymerase chain reaction (PCR)
- reverse transcription (RT-PCR)
- Southern-blot
- DNA fragment isolation and molecular cloning
- plasmid transformation and plasmid preparation of *E. coli*

Analysis of sequence data of DNA and protein:

- BLAST
- ClustalW
- contig assembly program, CAP

Transformation of filamentous fungi:

- protoplast preparation

- PEG-mediated transformation
- Susceptibility testing of antifungal agents (*in vitro*)
- Testing of the interaction of antifungal agents (*in vitro*)

Results

Identification and characterization of the hmgR gene

Degenerated PCR primers were designed to the most conserved region of *hmgR* genes, and the resulted PCR fragment was used as homologous probe for screening the lambda Fix II phage library of *R. miehei* genome. The *R. miehei hmgR* gene was identified and cloned into pBluescript vector. The entire gene sequence (4977 bp) was determined. Five introns were found in the *hmg*R gene and a putative 1058 amino acids correspond to the nucleotide sequence. The similarity of *R. miehei hmgR* gene with that of other fungi and eukaryotes was investigated. It was found that four introns of *R. miehei hmgR* are in the same position with all four introns of *Phycomyces blakesleeanus*. Partial sequence (315 bp) of the *X. dendrorhous hmgR* gene encoding the catalytic region (105 aa) of HMG-CoA reductase was cloned and sequence motifs responsible for the substrate binding were identified.

The analysis of the amino acid sequence of the encoded protein of *R. miehei* and the determined partial sequence of *X. dendrorhous* revealed the conserved catalytic domain, which is highly similar to other described HMG-CoA reductases. Motifs playing role in the

NADP and the HMG binding show 100% similarity among fungi. Like in other organisms, the N-terminal region of the proteins shows variability.

Fungal and plant genomes, frequently contain two or more *hmgR* genes which may differ in their expression. Southern-blot analysis revealed that *R. miehei*, similarly to *P. blakesleeanus*, contains only one *hmgR* gene. It was proved by RT-PCR experiments that the isolated *R. miehei hmgR* gene is expressed in the fungus.

Transformation experiments in the zygomycete fungus Mucor circinelloides

The green fluorescent protein gene (*gfp*) under the control of the promoter of the *R. miehei hmgR* gene (642 bps) was successfully transformed into *Mucor circinelloides*. The promoter of *R. miehei hmgR* gene was able to drive the expression of the *gfp* in the transformants, which was proved by fluorescence microscopy.

A vector harbouring the entire *hmgR* gene of *R. miehei* (4656 bps) was also introduced into the zygomycete fungus *M. circinelloides*. The yielded transformant was shown to have better tolerance to fluvastatin, the competitive inhibitor of HMG-CoA reductase. In our experiments the growing of the control strain was inhibited at 4 μg/ml of fluvastatin while the transformant showed 40% of growing compared with its growing in the fluvastatin-free medium. Based on that increased resistance a transformation selection method can be elaborated.

Susceptibility testing of various statins on zygomycetes fungi

Susceptibility to lovastatin of 27 *R. miehei* and *R. pusillus* isolates was investigated. It was demonstrated that in acidic pH (pH 4) or with usage of minimal medium and smaller inoculum size, inhibition of growing triggered by lovastatin is stronger. Based on the significantly higher sensitivity of *R. pusillus* to lovastatin than that of *R. miehei*, a simple and reliable method for species-level differentiation was elaborated. It is supposed that besides the susceptibility to lovastatin, the two *Rhizomucor* species are different in their susceptibility to other antifungal agents also.

In other experiments, susceptibility of 7 opportunistic pathogen species, *R. miehei*, *R. pusillus*, *Absidia corymbifera*, *Mortierella wolfii*, *Syncephalastrum racemosum*, *Rhizopus oryzae*, *Rhizopus microsporus* var. *rhizopodiformis* to various statins was tested. Commercially available statins (lovastatin, simvastatin, rosuvastatin, atorvastatin, fluvastatin, pravastatin) used as cholesterol-lowering agents in human vascular diseases were involved in these experiments. It was found that the susceptibility of the different species is different. The most resistant strain to statins was *M. wolfii*, while the most sensitive were *R. pusillus* and *A. corymbifera* in the *in vitro* susceptibility testing. The order of the inhibition efficiency among the various statins was also different in the isolates. In the background of this phenomenon, besides the differences in the structure and solubility of the statin molecules, there might be the

differences in the cell-structure or other molecular alterations. These differences can also influence the success of the therapy against these fungi with the known antifungal agents.

The potential synergy of fluvastatin and rosuvastatin with amphotericin B was also tested in growth inhibition of opportunistic pathogen zygomycetes fungi. Amphotericin B is one of the most effective antifungal agents against zygomycoses. It was shown that 1 μg/ml amphotericin B caused 75% growth inhibition in most of the examined isolates. The only exception was *Mortierella wolfii,* in case of that the highest concentration of amphotericin B (16 μg/ml) caused 50% inhibition. At the combination of the highest concentration of amphotericin B (16 μg/ml) and the highest concentration of fluvastatin (96 μg/ml), the growth inhibition was more than 75%. Statins used in combination with amphotericin B exerted additive effect on the majority of the examined isolates; in some cases, synergism was also detected.

Summary

In our experiments, the entire *R. miehei hmgR* gene and a partial *hmgR* gene of the *X. dendrorhous* were identified and characterized. Using the isolated gene, two vectors were constructed and successfully transformed into *M. circinelloides* MS12 strain. It was shown that the promoter of *R. miehei hmgR* gene was able to driven the production of functional GFP in *M. circinelloides*. It was also proved that transformation with the entire *hmgR* gene provides higher

resistance to fluvastatin due to the gene dose effect. Based on that result a new transformational selection system can be developed in zygomycetous fungi.

Susceptibility of 27 *R. miehei* and *R. pusillus* isolates to lovastatin was investigated, and effect of the culturing conditions (e.g. pH, composition of the medium and inoculum size) on the susceptibility testing was also examined. Based on these results, a simple method was elaborated for reliable differentiation of the *Rhizomucor* species.

Furthermore, sensitivity of 7 opportunistic pathogen fungi (*R. miehei*, *R. pusillus*, *A. corymbifera*, *M. wolfii*, *S. racemosum*, *Rh. oryzae*, *Rh. microsporus* var. *rhizopodioirmis)* to statins (lovastatin, simvastatin, rosuvastatin, atorvastatin, pravastatin, fluvastatin) presently known as cholesterol lowering agents in medicine was demonstrated. Combinations of fluvastatin and rosuvastatin with amphotericin B had additive effect on fungal growth whereas in some cases, synergism was also observed.

We can conclude that in the increasing number of zygomycoses, usage of statins in combination with other antifungal drugs can provide an effective therapy of these infections. It can also decrease the occurrence of serious side-effects of the currently used antifungal agents and minimize the possibility of the emergence of drug-resistance.

The results summarized in the Ph. D. thesis were published in the following articles:

Lukács, G., Papp, T., Nyilasi, I., Nagy, E., and Vágvölgyi, C. (2004): Evaluation a new medium to differentiate *Rhizomucor* species on the basis of their different sensitivity to lovastatin. J. Clin. Microbiol. 42, 5400-5402.

Lukács G, Linka B, Nyilasi I (2006) *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*: astaxanthin-producing yeasts of biotechnological importance. Acta Alimentaria 5 (1): 99-107.

Lukács, G., Papp, T., Somogyvári, F., Csernetics, Á., Nyilasi I. and Vágvölgyi. C. (2008) Cloning of the *Rhizomucor miehei* 3-hydroxy-3-methylglutharyl coenzyme A reductase gene and its heterologous expression in *Mucor circinelloides. Antonie van Leeuwenhoek. Accepted, in press.*

Patent connected to the experiments of the Ph. D. thesis:

Vágvölgyi Cs., Papp T., Nyilasi I., Pesti M., **Lukács Gy.** (2008) Gombaellenes hatóanyagot és sztatint tartalmazó kombinációs készítmények és alkalmazásuk. Szabadalmi bejelentés. M.Sz.H. P0800305 (2008. május 9.) Szabadalmi Közlöny és Védjegyértesítő 113(7), 241.

Posters summarising the results of the Ph. D. thesis:

Lukács, Gy., Ács, K., Vastag, M., Nyilasi, I., Kasza, Zs. and Vágvölgyi, Cs. (2002) Cloning and partial sequence analysis of the gene encoding 3-hydroxy-3-methilglutharyl coenzyme A reductase of *Rhizomucor miehei*. Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary. Abstracts.

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Nyilasi, I., Ács, K., **Lukács, Gy.**, Papp, T., Kasza, Zs., and Vágvölgyi, Cs. (2003) *Agrobacterium tumefaciens*-mediated transformation of *Mucor circinelloides*. FEMS Microbiol. Letters 222, Suppl.

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