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Directed Evolution of Sphinganine Hydroxylase for the Production of Sphingosine in Yeast

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Sphingolipids are ubiquitous and essential components of eucaryotic cells, and have many effects on cells including inhibition or stimulation of growth. Ceramide, sphingosine and sphngosine-1-phosphate are major sphingolipid metabolites, which are involved as bio-effectors and second messengers in growth of mammalian cells. Sphingosine, a main long chain base of mammals has attracted considerable attentions after finding of activity as a central component of signaling messengers in mammals. However, compared to the situation for ceramide and sphingosine-1-phosphate, very few studies have been directed toward characterizing the cellular functions of sphingosine. Most of functional studies about sphingosine as a potent elicitor of apoptosis were focused on exogeneous sphingosine. Therefore, the molecular mechanism of intracellular actions of sphingosine remains unclear in comparison with extracellular sphingosine.

To study the physiological effects of intracellular sphingosine, and to produce sphingosine, which is rarely present in yeast, we constructed sphingosine producing *Saccharomyces cerevisiae* by directed evolution of sphinganine hydroxylase to produce sphingosine from sphinganine, directly. Until now the conversion of sphinganine hydroxylase activity to sphinganine desaturase by rational protein design is very difficult, because only three sphingoid base hydroxylases from *S. cerevisiae*, *Arabidopsis thaliana* and *Pichia. ciferrii* were identified and sphingoid base desaturase genes are not cloned from any organisms, moreover the structural information is not known. Recently, dihydroceramide desaturase genes were identified from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and *Candida albicans* by bioinformatics approach. These enzymes showed detectable level of sphingoid base desaturase activity in *S. cerevisiae* but most of sphinganine were not affected by expression of these enzymes. Therefore, these enzymes have higher substrate specificity for dihydroceramide rather than sphinganine. We aligned these desaturases with those three sphinganine hydroxylases using clustal W program. The dihydroceramide desaturases share only limited similarity with any other proteins characterized by histidine box motifs and the spaces between histidine clusters are considerably various.

The ease with which a fatty acid Δ12-desaturase can be converted into a C-12-hydroxylase and vice

versa by exchanging just a few amino acids shows how similar the active sites of desaturases and hydroxylases. Two amino acid residues have been identified in the *A. thaliana* fatty acid Δ12-desaturase that, when exchanged with different amino acids, were sufficient to convert the desaturase into a bifunctional desaturase/hydroxylase. However, inspection of amino acid alignments indicates that neither of the equivalent residues in the desaturase family is likely to be important in determining the desaturation/ hydroxylation ratio. Instead, when comparing the amino acid sequences of murine desaturase and bifunctional deasturase/hydroxylase, three single amino acid differences in the vicinity of the histidine box motif are striking. This indicates that rather than particular amino acid side chains, the overall architecture of the active site might determine the desaturation/hydroxylation ratio. Directed evolution has emerged in just a few years as one of the most effective approaches to adapting biocatalysts to the industrial requirement. The critical phase of any directed evolution experiment is deciding how to search for variants with desired properties. For most practical problems, this search is both time-consuming and expensive. Therefore, strong and efficient screening or selection system is prerequisite for directed evolution of target proteins.

Contrary to *S. cerevisiae* sphingoid base kinases (*LCB4, LCB5*), which have broad substrate specificity, human sphingosine kinase has higher substrate specificity for sphigosine than sphinganine. Therefore, human sphingosine kinase was applied to distinguish the structural difference of sphinganine, phytosphingosine, and sphingosine. It was identified that the level of sphinganine-1-P and phytosphingosine-1-P increases to eight and five fold in heat stressed yeast cells. *S. cerevisiae* unable to break down long chain base phosphates, due to deletion of sphinganine-1-phosphate lyase gene (*DPL1*) and sphinganine-1-phosphate phosphatase gene (*LCB3*), shows a dramatically enhanced survival upon severe heat shock. Although the effects of sphingosine and sphingosine-1-P in yeast were not previously examined, we expected that sphingosine-1-P could induce heat tolerance like the sphinganine-1-P in yeast. Extrapolating from the results and assume described above, a novel screening system was devised and applied for the evolution of sphinganine hydroxylase to sphinganine desaturase (sphingosine synthase) (Figure 1). Human sphingosine kinase gene (*hSPHK1a*) was expressed under the control of *GAL10* promoter in *S. cerevisiae* B4 strain (*lcb4*::*Tc, lcb5*::*Tc, sur2*::*Tc, leu, ura, his*) which accumulate sphinganine to construct B4K strain (*lcb4*::*Tc, lcb5*::*Tc, sur2*::*Tc, hSPHK1a, ura, his*). To lower the heat tolerance of host cells caused by sphinganine-1-P, which was formed by human sphingosine kinase, *LCB3* gene encoding *S. cerevisiae* sphingoid base 1-phosphate phosphatase was over-expressed. The over-expression of *LCB3* gene reduced the increased level of sphinganine 1-P to basal level, and the heat tolerance of the host cell severely decreased. We upgraded this screening system by adapting another screening step.

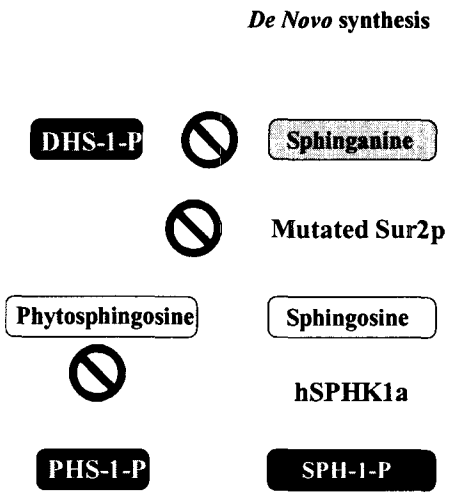

Figure 1. Screening system for the evolution of sphinganine hydroxylase to sphinganine desaturase.

It has been reported that extracellular sphingosine activate calcineurin dependant Ca^{2+} signaling in *S. cerevisiae*. *PMC1* is final target gene induced by influx of extracellular Ca^{2+}. To use promoter of *PMC1* gene as reporter for screening of sphingosine producing cells, G418 resistance gene was integrated into the downstream of *PMC1* promoter in B4KL strain to construct B4KLP strain. Finally, *S. cerevisiae* B4KLP strain (*lcb4::Tc, lcb5::Tc, sur2::Tc, LCB3, hSPHK1a, PMC-G418, ura, his*) was used for directed evolution of *SUR2* gene as host cells.

We applied *in vivo* recombination techniques for construction of mutant library. Recent reports indicated that plasmid repair is extremely efficient process and that recombination between overlapping as short as 30 bp can be readily achieved and it has been also reported that to achieve optimum directed evolution, protein expression bias should be minimized. Library construction using *in vivo* recombination in *S. cerevisiae* has many advantages over the traditional methods, such that it bypasses the need for extensive purification, ligation, modification and *E. coli* transformation techniques and it has no expression bias because it need not to use *E. coli* transformation.

Several point mutations (4-5 mutations/kb) were introduced into the *SUR2* gene by error prone PCR and this mutant amplicon was linked to C-terminal fragment of *URA3* to reduce number of false transformants. This mutant amplicon was co-transformed to the *S. cerevisiae* B4KLP strain with *Bam*HI/*Stu*I digested fragment of YGsSU, which has C-terminal truncated *URA3* gene as selection marker. We expected that if the mutated *SUR2* gene product has sphinganine desaturase activity, sphinganine would be converted to sphingosine and subsequently, phosphorylated to sphingosine-1-P by human sphingosine kinase, pre-expressed in *S. cerevisiae* B4KLP strain and the produced sphingosine-1-P would enhance the heat

tolerance of host cell and induce the expression of G418 resistance gene. After induction of human sphingosine kinase, we selected colonies that acquiring heat tolerance and G418 resistance simultaneously. Finally, we selected two evolvants, of which sphingosine is increased to five and ten times relative wild type. We identified two amino acid changes, (H180Q and I272N) which occurred in the vicinity of histidine cluster motif of these variants. These amino acid changes are probably one of important determinant, which contribute the product specificity of sphinganine desaturase. Until now, most of proteins applied for directed evolution are restricted to enzymes that contain easily screenable characters such as thermostability, antibiotics resistance, hollow, and color/fluorescence. Therefore, product specificity change, (production of different products form the same substrate) is more difficult because of screening problems.

To the best of our knowledge, this study is the first report on the production of sphingosine in *S. cerevisiae* by directed evolution of sphinganine hydroxylase to sphinganine desaturase using indirect screening system.