Mapping a kingdom-specific functional domain of squalene synthase

Kristin B. Linscott [a,1], Thomas D. Niehaus [b,1,2], Xun Zhuang [b,1], Stephen A. Bell [b,3], Joe Chappell [a,b,*]

[a] Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40506-9983, United States
[b] Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY 40536-0596, United States

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ABSTRACT

Squalene synthase catalyzes the first committed step in sterol biosynthesis and consists of both an amino-terminal catalytic domain and a carboxy-terminal domain tethering the enzyme to the ER membrane. While the overall architecture of this enzyme is identical in eukaryotes, it was previously shown that plant and animal genes cannot complement a squalene synthase knockout mutation in yeast unless the carboxy-terminal domain is swapped for one of fungal origin. This implied a unique component of the fungal carboxy-terminal domain was responsible for the complementation phenotype. To identify this motif, we used Saccharomyces cerevisiae with a squalene synthase knockout mutation, and expressed intact and chimeric squalene synthases originating from fungi, plants, and animals. In contrast to previous observations, all enzymes tested could partially complement the knockout mutation when the genes were weakly expressed. However, when highly expressed, non-fungal squalene synthases could not complement the yeast mutation and instead led to the accumulation of a toxic intermediate(s) as defined by mutations of genes downstream in the ergosterol pathway. Restoration of the complete complementation phenotype was mapped to a 26-amino acid hinge region linking the catalytic and membrane-spanning domains specific to fungal squalene synthases. Over-expression of the C-terminal domain containing a hinge domain from fungi, not from animals or plants, led to growth inhibition of wild-type yeast. Because this hinge region is unique to and highly conserved within each kingdom of life, the data suggests that the hinge domain plays an essential functional role, such as assembly of ergosterol multi-enzyme complexes in fungi.

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1. Introduction

The sterol biosynthetic pathway is a familiar target for the treatment of hypercholesterolemia and pathogenic fungal infections in humans [1, 2]. Reports in recent years document a rise in the incidence of fungal infections in immunocompromised patients [3,4]. Along with increasing resistance to current therapeutics, this has motivated further investigation of the sterol pathway for the development of novel antifungal agents which are both broad spectrum within and specific to the fungal kingdom of life [5].

Squalene synthase (SQS) has garnered attention for its role as the first committed step in the sterol pathway – a gatekeeper determining how carbon flux is distributed between sterols and other essential isoprenoids [6]. This enzyme consists of two recognized domains: an amino(N)-terminal catalytic domain that is highly conserved throughout eukaryotes and a carboxy(C)-terminal domain responsible for tethering the enzyme to the cytosolic face of the endoplasmic reticulum in eukaryotic cells [7–10] (Fig. 1A). The catalytic domain performs two consecutive reactions. Two molecules of farnesyl diphosphate (FPP) are dimerized to form the intermediate presqualene diphosphate (PSPP) and then reductively rearranged using NADPH to form squalene [11,12]. Squalene precedes through the sterol pathway where it is further activated by squalene monooxygenase, then cyclized and subjected to a variety of modifications on its way to forming ergosterol in fungi, cholesterol in mammalian cells, and the three major sterols in plants; stigmasterol, sitosterol and campesterol [13] (Fig. 1B).

The initial discovery and characterization of SQS occurred in the yeast Saccharomyces cerevisiae, followed by cloning of the gene encoding SQS from the fission yeast Schizosaccharomyces pombe, the dimorphic yeast Yarrowia lipolytica, and more recently Granoderma lucidum [7,14–17]. Expression of each of these fungal squalene synthase (ERG9) genes functionally complemented a line of S. cerevisiae with an ERG9 knockout mutation (Δerg9). This was not the case for squalene synthase genes of mammalian or plant origin [7,18,19], which were unable to complement the Δerg9 yeast line. However, Kim et al. recently

Fig. 1. The role of squalene synthase (SQS) in the ergosterol biosynthetic pathway of budding yeast. (A) A homology model of yeast SQS. This enzyme is composed of both a helical amino-terminal catalytic domain and a carboxy-terminal domain which consists of a hinge region and a membrane spanning helix responsible for tethering the enzyme to the cytosolic face of the endoplasmic reticulum. (B) SQS is the first step in the ergosterol biosynthetic pathway. The catalytic domain performs the head-to-head dimerization of two molecules of farnesyl diphosphate (FPP) to form squalene, a 30 carbon isoprenoid oxidized by squalene monooxygenase (Erg1) and cyclized by lanosterol synthase (Erg7). In *S. cerevisiae*, ten additional steps modify the position of methyl groups and double bonds, such as by the C5-desaturase (Erg3), to form ergosterol. Dashed lines indicate multiple steps in the pathway.

reported that three plant squalene synthase genes from *Panax ginseng* were able to complement the ∆*erg9* mutation in yeast, suggesting that there may be exceptions or growth conditions where a non-fungal SQS can restore ergosterol prototrophy [20].

Robinson et al. [7] was the first to investigate the amino acid domain responsible for the complementation phenotype in *SQS*-deficient yeast. These investigators reported that heterologous expression of the human *SQS* gene in yeast resulted in only modest levels of enzyme activity insufficient to complement the ∆*erg9* mutation. In contrast, when the amino-terminal domain of the human gene was fused to the putative membrane spanning domain of the *S. cerevisiae* gene, the full complementation phenotype was restored, leading these authors to conclude that the human *ERG9* gene might somehow be misregulated when expressed in a fungal host.

Then, using radioactive substrate and microsomal preparations made from a ∆*erg9* yeast line expressing various *SQS* genes, Kribii et al. showed that yeast and plant SQS, as well as the corresponding chimeric enzymes, produced equivalent amounts of squalene in yeast [21]. Interestingly, yeast expressing a SQS with a non-fungal C-terminal domain did not produce squalene epoxide or lanosterol. This lead to the suggestion that the C-terminal domain of fungal SQS may be necessary for the enzyme to integrate into a multi-enzyme complex and efficiently deliver squalene to squalene monooxygenase, the next step of the sterol pathway (Fig. 1). This would be consistent with experiments showing that delocalization of sterol biosynthetic enzymes leads to both decreased ergosterol accumulation and cell viability [22].

To better define the functional role of the SQS C-terminal domain in sterol biosynthesis, we have evaluated the ability of SQS enzymes from different kingdoms of life to complement a ∆*erg9* mutation in yeast. Furthermore, we have narrowed down the region responsible for the complementation phenotype to a 26-amino acid hinge domain between the catalytic and membrane spanning domains. We provide evidence that this peptide domain can mediate a kingdom of life specific inhibition of growth, thus validating the domain as a unique structural feature with a novel physiological function and portending a diverse array of technological applications.

2. Materials and methods

See SI Methods for more detailed information.

2.1. Materials

Each *SQS* gene was cloned into the Pesc-Ura vector (Agilent) following the Gal10 promoter [23]. The C-terminal constructs used to assess growth inhibition of wild-type yeast were cloned in duplication into the Pesc-Ura vector, one behind the GAL1 promoter and the second copy behind the GAL10 promoter, to increase ultimate gene expression levels. Chimeric genes were produced using an assembly PCR strategy described by Niehaus et al. [24]. The yeast strain ZX178-08 was previously described [25], and arises from EMS mutagenesis of the parental strain BY4741 and selection for exogenous sterol uptake, followed by complete knockout of the endogenous squalene synthase gene (∆*erg9*) by homologous recombination [25]. Knockout mutations of *ERG1, 7* and *3 genes* were constructed similarly to ∆*erg9* as detailed in the supplementary materials. Yeast were grown on either YPD complete media containing glucose, or synthetic complete media (SC) with appropriate omission of amino acids to select for the corresponding expression vectors and either 2% glucose or 2% galactose. Additionally, YPDE and SCE media are the corresponding media supplemented with exogenous ergosterol.

2.2. Yeast transformation and growth analysis

Yeast strains were transformed using the lithium acetate method [26], and the transformants were selected using SCE glucose media. For spot plate analysis, 2 mL starter cultures representing three independent transformants for each construct were grown to stationary phase in SCE glucose, diluted to OD_{600} equal to 1 and $5\times$ serial dilutions were plated. Growth plates were photographed after 5–7 days of incubation at 28 °C. For growth curve analysis, seed cultures grown to stationary phase in SCE glucose were used to inoculate liquid cultures containing glucose or galactose as the sole carbon source.