KILLER

Toxin-Mediated Cell Cycle Arrest in Yeast: The Killer Phenomenon of *Kluyveromyces lactis*

Progression through the eukaryotic cell cycle requires many diverse processes which have to be regulated in a precise temporal program. Prior to START, a major checkpoint in late G1, *Saccharomyces cerevisiae* cells can exit the mitotic cell cycle in response to either nutrient limitation or mating pheromone. Analysis of the *Kluyveromyces lactis* zymotoxin has shown that it causes a G1 arrest in *S. cerevisiae* cells prior to START, while permitting continued macromolecular biosynthesis. The native toxin is a heterotrimer (αβγ) but toxicity solely resides within its γ subunit. However, activity of holotoxin fully depends on its α and β subunits, presumably by promoting toxin binding and γ subunit import into sensitive yeast cells. The precise mechanism of toxin-mediated G1 arrest and its intracellular target remain unknown. This review will focus on recent molecular approaches to understand the complex nature of the toxin mode of action and to identify components of its response pathway.

KEYWORDS

zymotoxin, cell cycle arrest, chitin, toxin-receptor

The Killer Toxin: Structure and Biogenesis

Native *K. lactis* toxin is a heterotrimer comprised of subunits α, β and γ (M_r 99, 30 and 28 kDA), all of which are encoded by a linear dsDNA killer plasmid (k1) in the producing strain. α and β are products of a single locus (k1ORF2) resulting from proteolytic processing of a common αβ-prepro-precursor by a *K. lactis* Kex2p-like activity, whereas γ represents the processed product of a single gene (k1ORF4) [4, 7]. Endo H digestion and concanavalin A binding demonstrate α to be N-glycosylated (Fig. 1) and structural integrity of holotoxin is maintained by disulphide bonding between β and γ [7]. Holotoxin secretion is targeted by leader peptides on αβ and γ which are subsequently removed. In the absence of αβ production, γ fails to be secreted but accumulates intracellularly. Thus, secretion of the γ subunit is ordinarily coupled to holotoxin assembly [7] (Fig. 1).

Cell Cycle Arrest

The *K. lactis* toxin is acitve against a variety of sensitive yeasts including *Saccharomyces, Kluyveromyces* and *Candida* but not *Schizosaccharomyces* (Fig. 1). Holotoxin arrest causes irreversible growth inhibition at the unbudded stage (G1) of the cell cycle and requires about 10,000 molecules per cell. Toxin-treated cells have a prereplicative (1n) DNA content but are still metabolically active, permitting continued

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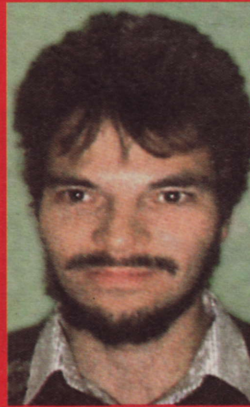

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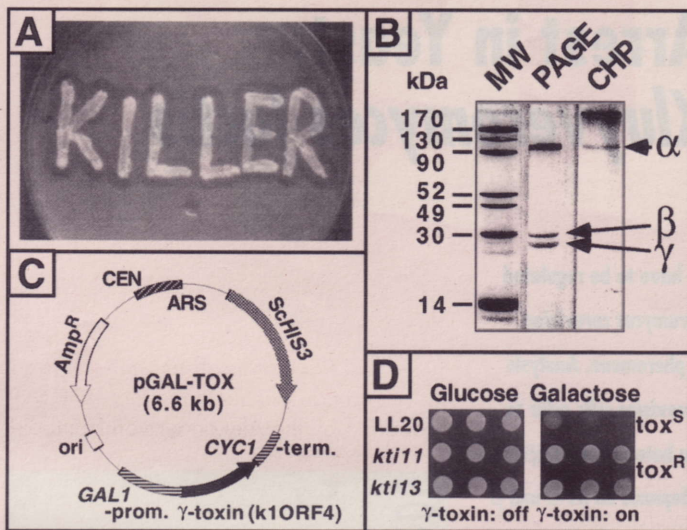

Fig. 1: The *K. lactis* killer system. (A) Bioassay showing growth inhibition of killer strain AWJ137 ("KILLER") against sensitive *S. cerevisiae* strain LL20. (B) SDS-PAGE electrophoretic analysis of holotoxin. Toxin subunits (α, β and γ) are visualized by Coomassie Blue staining (PAGE) in comparison to molecular weight standards (MW). Glycosylation is shown by chromogenic reaction of concanavalin A conjugated horseradish perioxidase (CHP) to electroblotted α subunit. (C) Toxin expression vector, pGAL-TOX. Gene expression is regulated by the *GAL1* promoter and *CYC1* terminator on a centromeric *E. coli/S. cerevisiae* shuttle vector. (D) Growth arrest induced by γ toxin expression. Growth of tox^S strain LL20 is blocked on galactose-medium, whereas mutant strains *kti11* and *kti13* are viable and tox^R.

protein and RNA biosynthesis [2, 7]. Consistently, treated cells do increase in volume similar to START arrests induced by mating pheromone treatment or growth of *cdc28^{ts}* strains at the non-permissive temperature. To execute START, a sufficient level of Cdc28p kinase activity is required, the amount of which is mainly determined by the levels of G1 cylins. Although toxin might act by antagonizing G1 cyclin function, it is noteworthy that neither a hyperacitve *CLN3-1* cyclin allele or overexpression of other G1 cyclins can significantly reduce toxin sensitivity [2].

Isolation of Toxin-Resistant (tox^R) Mutants

Intriguingly, toxicity resides solely within the γ toxin subunit: conditional expression of γ from *GAL* promoters mimics treatment of exotoxin but is fully reversible (Fig. 1) [1]. Based on their ability to grow in the presence of holotoxin, tox^R mutants (*skt, iki* and *kti*) have been isolated independently by three groups [1, 2, 10]. Sensitivity of these mutants to intracellular expression of γ can distinguish toxin binding/uptake (class I) from toxin-target site mutants (class II) [1, 2]. To identify toxin-targets, we exploit a novel screen using a gene knock-out library: yeast transformants which carry a Tn3::*lacZ*::*LEU2* transposon randomly inserted into the genome are screened for tox^R by inducing γ expression on galactose. Candidate clones are then subjected to plasmid rescue in *E. coli* (Fig. 2). In this way, we have identified several tox^R yeast disruptants analysis of which is in progress [6]. Finally, we have commenced yeast two-hybrid screens using γ toxin as bait to isolate interacting partners from genomic and cDNA prey libraries.

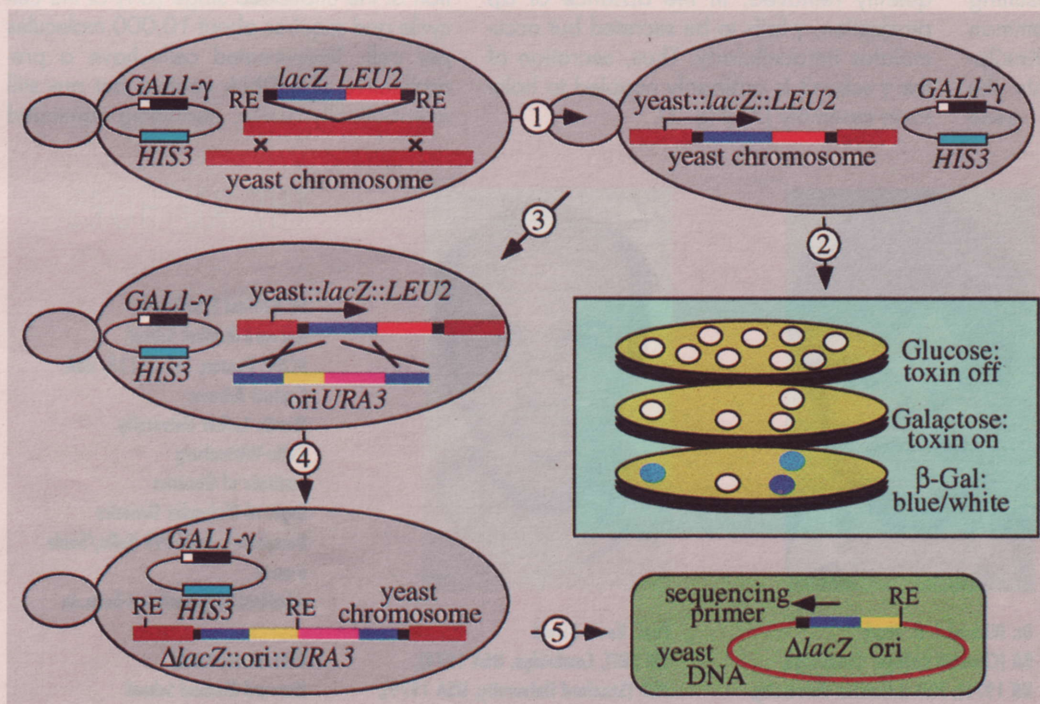

Fig. 2: Gene knock-out screen to identify tox^R genes. (1) A tox^S strain harboring an inducible γ expression vector is transformed with a library pool of randomly constructed yeast gene disruptions carrying a Tn3::*lacZ*::*LEU2* transposon. (2) Glucose-grown, Leu$^+$ disruptants are checked for tox^R by inducing γ expression on galactose and for β-Gal production by color assay with X-gal substrate. (3) Next, yeast candidate clones (tox^R, gal$^+$, β-Gal$^+$) are subjected to *URA3*-mediated disruption of the *lacZ* reporter gene. (4) Genomic DNA of stable lacZΔ knockouts is subjected to restriction enzyme-mediate ligation (RE) followed by (5) plasmid rescue in *E. coli.* Finally, DNA is sequenced using a reverse primer derived from lacZ.

Cell Wall Chitin: A Potential Toxin Receptor

Intracellular expression of the mature γ gene results in biologically active γ toxin whereas exogenously applied γ is not able to inhibit cell growth demonstrating that holotoxin must assist its uptake. As a precondition for γ entry and action, holotoxin is expected to bind to the cell surface. Consistently, the α toxin subunit has exochitinase activity *in vitro* [7]. Additionally, chitin-deficient mutants are fully tox^R and class I mutants define chitin as essential for exotoxin function. *KTI2* is allelic with *CHS3* and *KTI10* corresponds to *CHS6*. *CHS3* codes for the catalytic subunit of chitin synthase III, Chs3p, the *in vivo* activity of which is abolished in *chs6* mutants [5, 9]. Moreover, deletion of *CHS4/SKT5* encoding an activator of Chs3p, renders cells tox^R [10]. The class I *KTI6* gene is non-allelic with *CHS3, 4, 6*. Since its mutation obviously affects toxin uptake, it is possible that it corresponds to *CHS5*, a gene recently shown to function in targeting of Chs3p within chitosomes [2, 5]. Summing up, we propose that primary interaction of exotoxin and sensitive cells is facilitated by binding of α subunit to cell wall chitin which serves as toxin-receptor (Fig. 3).

Genes Involved in the Intracellular Toxin Process

So far five genes (*IKI1, IKI3, KTI12, SIT4* and *SAP155*) have been shown to affect

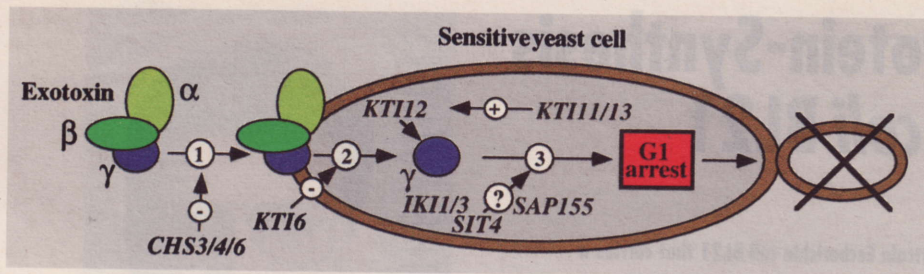

Fig. 3: Working model for the *K. lactis* toxin mode of action in *S. cerevisiae*. As judged from analysis of tox^R genes, the response-pathway can be dissected into three steps: (1) binding of exotoxin to the cell surface, (2) γ toxin uptake into the cell and (3) intracellular communication of γ with target proteins, eventually culminating in a G1 cell cycle arrest. For explanation of the genes involved, see text.

intracellular toxin action [2, 6, 8, 10]. Iki1p is part of an insoluble fraction in cell extracts and Iki3p is predicted to posses a membran-spanning region raising the possibility that an insoluble Iki1p/Iki3p containing compartment is involved in toxin action [10]. Depletion or over-production of Kti12p confers tox^R and so Kti12p may be a potential toxin target: if absent (or mutated), toxin cannot bind while high *KTI12* gene dosage might lead to excess, unbound Kti12p which competes with the Kti12p-toxin complex for a downstream effector [2]. In favour of this, we have observed weak genetic interaction between Kti12p and γ using the yeast two-hybrid system. Class II mutations (*kti11* and *kti13*) can be partially suppressed by high copy *KTI12* suggesting that both genes act upstream of *KTI12* and may limit the Kti12p pool when mutated (Fig. 3). We are presently studying this dependece by cloning the appropriate genes and examining *KTI12* mRNA and protein levels in these mutants. Sap155p associates in a cell cycle-dependent manner with the Sit4p phosphatase which functions in late G1 for progression into S Phase [3, 8]. Sit4p is required for execution of START and *sit4^ts* strains arrest late in G1 prior to START, in part due to the role of Sit4p in expression of G1 cyclin genes [3]. Interestingly, *sit4Δ* strains, which are viable in certain backgrounds, are fully tox^R [6, 8]. Although it is attractive to suppose that the toxin might act to block Sit4p function, we can so far only conclude that toxin-induced G1 arrest requires Sit4p (Fig. 3).

Concluding Remarks

The toxin-response pathway can now be dissected into three steps, namely binding of toxin to the cell surface, uptake of γ subunit into and communication from within the cell (Fig. 3). Binding involves recognition of the toxin-receptor, cell wall chitin, by virtue of the α chitinase function. Whether γ subunit uptake is endocytosis-dependent remains to be elucidated. The presence of as many as ten distinct target-site genes suggests a complex pathway transduces the toxin's inhibitory effect. While some of them may be involved in the expression of targets inhibited by the toxin, a number of proteins could also participate in the process blocked by the toxin. These might act as a biochemical pathway or, alternatively, form a complex containing several components. Preliminary data on Iki1p, Iki3p, Kti12p and Sap155p provide support for the latter idea (Fig. 3). However, more concerted research is needed to analyse the interreleationship between these factors and their role in enabling the toxin to cause a G1 cell cycle arrest.

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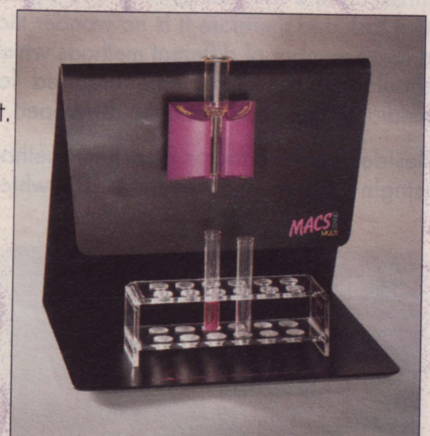

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