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Immunity to K1 Killer Toxin: Internal TOK1 Blockade

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Summary

K1 killer strains of *Saccharomyces cerevisiae* harbor RNA viruses that mediate secretion of K1, a protein toxin that kills virus-free cells. Recently, external K1 toxin was shown to directly activate TOK1 channels in the plasma membranes of sensitive yeast cells, leading to excess potassium flux and cell death. Here, a mechanism by which killer cells resist their own toxin is shown: internal toxin inhibits TOK1 channels and suppresses activation by external toxin.

Introduction

Killer strains of *Saccharomyces cerevisiae* carry RNA viruses that direct secretion of peptide toxins lethal to virus-free cells, yet the killer cells themselves are immune to the toxin they release (Boone et al., 1986; Bostian et al., 1980, 1984; Bussey, 1981). This favors environmental dominance of virus-positive cells and is significant in commercial fermentation and microbial pathogenesis (Magliani et al., 1997; Starmer et al., 1992; Wickner, 1996). K1 killer cells contain two double-stranded viral components. The L-A component mediates viral transcription, replication, and encapsulation and M1 encodes a $\delta-\alpha-\gamma-\beta$ preprotoxin. Host enzymes process the toxin precursor and cells release the mature K1 toxin as an α-β heterodimer linked by three disulfide bonds (Bostian et al., 1984; Skipper et al., 1984; Thiele and Leibowitz, 1982). Killing occurs by a two step mechanism: K1 first binds to 1-6-β-D-glucan receptors on the yeast cell wall, a process controlled by the β chain. The α chain then acts at the yeast cell plasma membrane to produce unregulated potassium efflux and cell death (Sturley et al., 1986; Zhu and Bussey, 1991). We recently identified the TOK1 potassium channel as a plasma membrane target for K1 toxin (Ahmed et al., 1999). Toxin activates TOK1 without a requirement for other yeast or viral proteins to produce abnormal ion flux. Overexpression of the *TOK1* gene increases sensitivity to toxin while deletion confers resistance.

How killer cells achieve immunity to the toxin they secrete has remained a mystery. It has long been known that the single open reading frame for the $\delta-\alpha-\gamma-\beta$ preprotoxin is sufficient to confer both the ability to kill and immunity (Bussey, 1991). Further, three findings have indicated that immunity takes place at the plasma membrane. First, killer cells bind toxin normally to their cell walls and remain immune even as cell wall-free spheroplasts (Al-Aidroos and Bussey, 1978). Second, killer resistant (*kre*) target cells commonly show resistance due to diminished cell wall binding of toxin and yield toxin-sensitive spheroplasts (Boone et al., 1990). Third, some *kre* mutants show normal cell wall binding of toxin and produce immune spheroplasts (Schmitt and Compain, 1995). A model for immunity must also account for loss of both killing and immunity in strains carrying mutant toxins with altered α chains (that mediate killing) or γ chains (that affix preprotoxin to secretory vesicle membranes to allow maturation) (Sturley et al., 1986; Zhu and Bussey, 1991). The most complete prior model proposes that incompletely processed toxin travels via the secretory pathway to the external membrane surface where it remains tethered (by γ chain) such that α chain can directly compete with secreted toxin for binding (Boone et al., 1986). However, this proposal does not explain why membrane bound α chain does not damage the cells, or address evidence that immunity proceeds from inside the cell. Thus, strains that process toxin poorly (Wickner and Leibowitz, 1976) or express nonsecreted toxins (such as those lacking the δ-leader chain) (Lolle et al., 1984) accumulate internal toxin (and/or precursors) and do not kill but retain at least partial immunity. Study of TOK1 has now yielded a model for immunity that accounts for all these observations.

We show here that K1 toxin acts directly on TOK1 from inside the cell to suppress the effects of external toxin. Thus, TOK1 is expressed in the plasma membranes of both immune and sensitive cells, but is activated by external toxin only in the sensitive cells. In the absence of external toxin, internal toxin is found to reversibly inhibit TOK1. With external toxin present, internal toxin suppresses activation in a noncompetitive fashion, that is, with no change in the half-maximal dosage for activation. Furthermore, an α chain toxin mutant that does not kill spheroplasts but supports immunity does not activate TOK1 from the outside, but can block the channel from the inside and suppress activation by external wild-type toxin. Thus, K1 toxin acts on TOK1 from opposite sides of the membrane to produce opposing effects that correlate directly with the phenomena of killing and immunity.

Results

TOK1 Channels in Immune Cells Are Not Activated by External K1 Toxin

K1 toxin produces a halo devoid of proliferating cells on lawns of virus-free target cells whether it is secreted from virus-positive cells or isolated and allowed to diffuse from a filter disc (Figure 1A). As previously shown (Ahmed et al., 1999), external toxin applied to virus-free target cells activates TOK1 channels leading to abnormal potassium flux. This is demonstrated in Figure 1B using whole-spheroplast voltage clamp configuration and yeast cells overexpressing TOK1. In contrast, virus-

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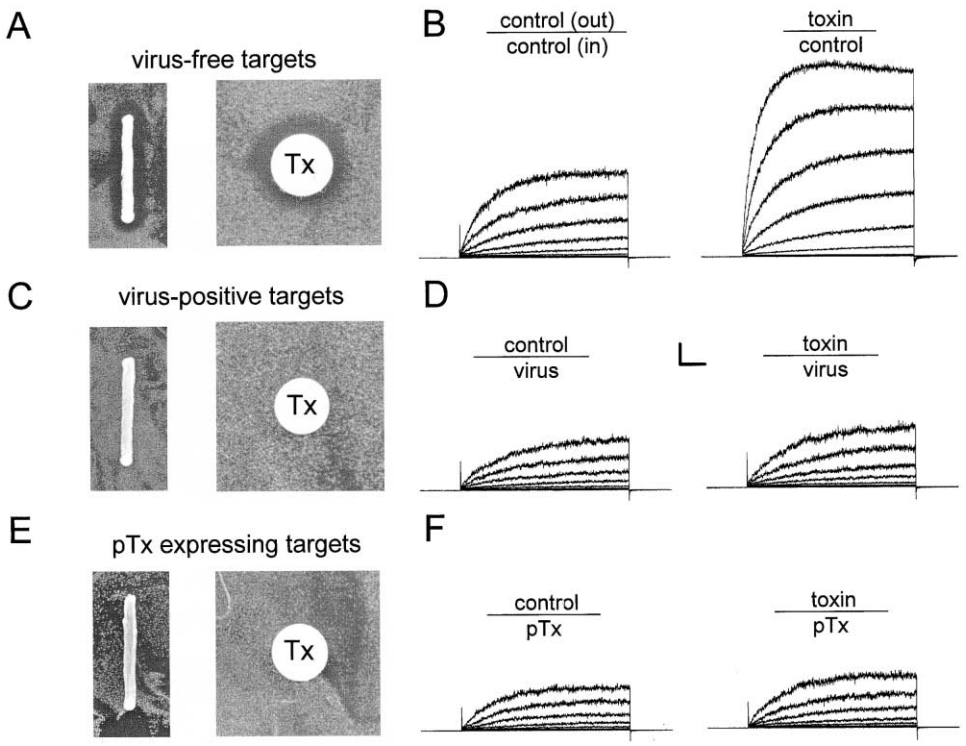

Figure 1. In Immune Yeast Cells, TOK1 Is Not Activated by External K1 Toxin

The K1 gene mediates immunity and inhibits activation of TOK1 channels by external toxin.

(A) Virus-free CY162C cells exposed to a streak of K12 killer cells or a filter disk with 15 μl isolated K1 toxin (~300 nM) on a YPD assay plate. Plates were incubated at room temperature for 48 hr. Killing is seen as a halo in the lawn, resistance the absence of a halo.

(B) Spheroplasts from virus-free CY162 cells expressing pGKH-TOK1 studied in whole-cell configuration with or without ~300 nM isolated toxin in the solution. Voltage protocol: holding voltage −80 mV; 1 s steps from −80 to 60 mV in 10 mV increments; interpulse interval: 1 s.

(C) Virus-positive CY162 cells studied as in (A).

(D) Spheroplasts from virus-positive CY162 expressing pGKH-TOK1 studied as in (B).

(E) Virus-free CY162C cells expressing K1 toxin from pTx as in (A), but on YNB plates.

(F) Spheroplasts from virus-free CY162C cells expressing pGKH-TOK1 and pTx as in (B); scale bars represent 180 pA and 0.1 s.

positive target cells are immune, showing no halo zone (Figure 1C); moreover, TOK1 channels in these cells are unresponsive to externally applied K1 toxin (Figure 1D). Thus, external toxin at ~300 nM increases TOK1 currents in virus-free cells 1.8 ± 0.1-fold but does not enhance the current in virus-positive cells (1.1 ± 0.1, n = 5). Notably, virus-free cells expressing K1 toxin from a plasmid demonstrate both immunity (Figure 1E) and suppression of activation by external toxin (Figure 1F, 1.2 ± 0.1-fold change, n = 3). As expression of the gene for K1 toxin without other viral genes was sufficient to confer immunity and counter activation of TOK1, we sought evidence that the toxin might interact directly with TOK1 from the cytoplasm.

Internal Toxin Blocks TOK1

Excising inside-out patches from virus-free spheroplasts exposes the cytoplasmic face of TOK1 channels to the bath solution. K1 toxin applied in the bath inhibits TOK1 (Figure 2A) in a reversible fashion (Figure 2B, n = 7 patches). The same observation is made when toxin is applied to the inside of TOK1 channels expressed in *Xenopus* oocytes, indicating that TOK1 is the only yeast cell protein required to produce reversible blockade by internal K1 toxin (n = 8 patches); further single channel analysis reveals that inhibition does not alter unitary current magnitude (Figure 2C).

As it is experimentally difficult to expose the inner surface of spheroplasts in stable fashion and TOK1 function in yeast cells and oocytes is similar (Ahmed et al., 1999), the latter were used to assess the attributes of inhibition by internal toxin. A dose-inhibition relationship for internal toxin reveals an inhibition constant (K_i) of ~80 ± 14 nM (n = 5), a Hill coefficient of 1.3 ± 0.1, without significant voltage dependence in the positive range (Figures 2C and 2D).

Previously, we demonstrated that single TOK1 channels open in bursts (periods when the channel moves rapidly and repeatedly between the open state and two short-lived closed states) interrupted by long-lasting closures, sometimes lasting seconds. Here, we demonstrate that in the absence or presence of internal toxin, dwell time histograms again suggest three closed and one open state (Figures 2E and 2F). Internal toxin appears to act primarily to stabilize the long interburst closed state (C_3), increasing its duration over 4-fold (Table 1).

Internal Toxin Suppresses Activation

We next sought to study whether internal toxin in the presence of external toxin could reproduce the behavior

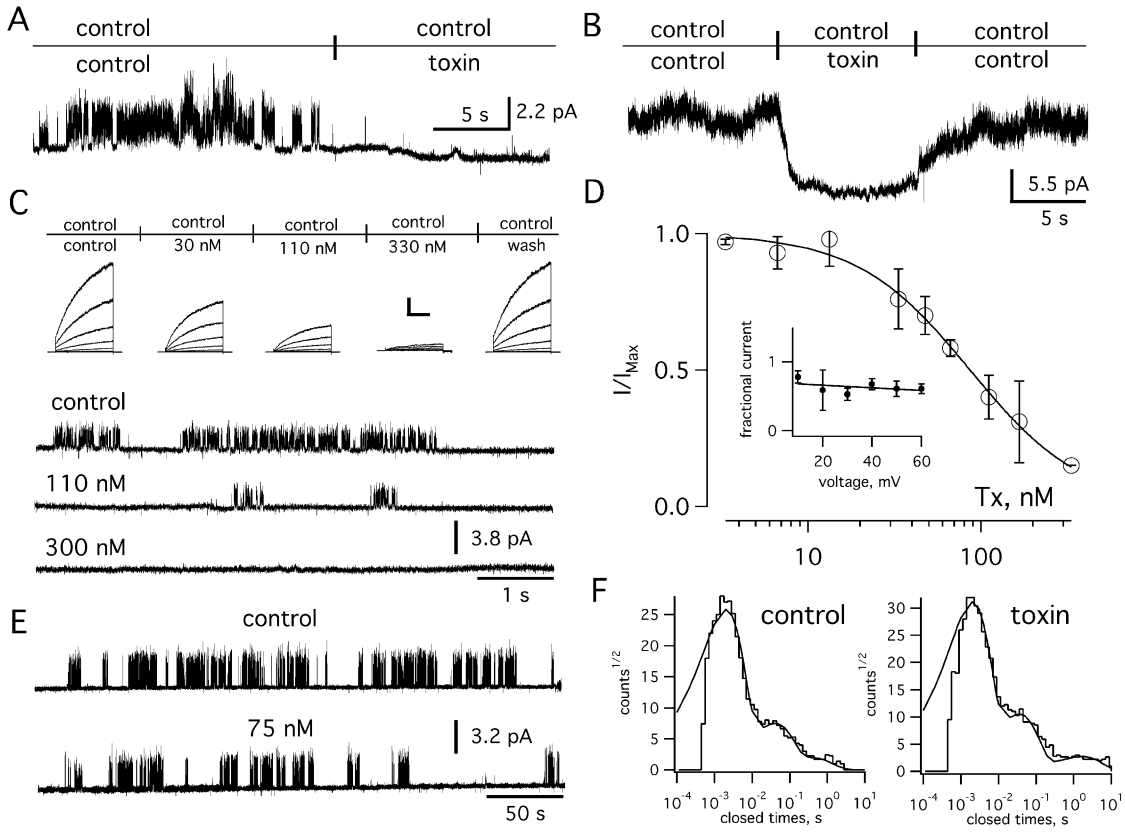

Figure 2. Internal Toxin Inhibits TOK1 Channels by Reverse Stabilization of the Long-Closed State

Internal toxin blocks TOK1 channels in patches excised from yeast cells or *Xenopus laevis* oocytes.

(A) Single TOK1 channel activity recorded in an inside-out patch excised from a virus-free CY162C spheroplast in the absence or presence of ~200 nM toxin as indicated. Holding voltage +80 mV. Data filtered at 0.5 kHz, sampled at 2.5 kHz.

(B) A patch containing several TOK1 channels studied as in (A) with a holding voltage +40 mV. Data filtered at 0.1 kHz, sampled at 0.5 kHz.

(C) TOK1 activity in inside-out patches excised from oocytes with various levels of applied internal K1 toxin as indicated. (Top) A multichannel patch held at −80 mV and studied with 1 s steps from −80 to 60 mV; scale bar indicates 1.5 nA and 0.25 s. (Lower rows) A single TOK1 channel at +60 mV, unitary current was unchanged by toxin application (2.4 ± 0.17 pA). For display, data filtered at 0.5 kHz, sampled at 2.5 kHz.

(D) Dose-response curve for blockade of macroscopic TOK1 currents in oocyte patches (as in [C, top]) fitted to the Hill function, H:

$$H = \frac{1}{1 + ([toxin]/K_{in})^{n_H}}$$

with K_{in} = 87 ± 14 nM and n_H = 1.3 ± 0.1 (n = 5). Inset: voltage dependence of the blockage in the presence of ~75 nM toxin.

(E) Single-channel activity recorded from an inside-out patch excised from an oocyte expressing TOK1 in the absence (upper trace) or presence (lower trace) of ~75 nM killer toxin for kinetic analysis. Holding voltage +80 mV. For display, data were sampled at 0.3 kHz and filtered at 0.1 kHz.

(F) Representative histograms of the closed-state duration in the absence or presence of ~75 nM killer toxin. Values reported in Table 1.

of TOK1 in immune cells. TOK1 channels in virus-free spheroplasts that were first inhibited by internal toxin could reversibly recover activity upon exposure to high levels of external toxin (Figure 3A). This was not associated with changes in the nonvoltage-dependent outward rectification of TOK1 channels (Figure 3A). To quantify the opposing affects of internal and external toxin, spheroplasts were loaded with controlled amounts of internal toxin via the patch pipette and TOK1 activity evaluated with increasing concentrations of external toxin (Figure 3B). Activation by external toxin was found to be half-maximal at ~30 nM in the absence and presence of internal toxin. Conversely, increasing levels of internal toxin progressively limited the maximal re-

Table 1. Kinetic Parameters of TOK1 Channels in the Absence and Presence of Internal K1 Toxin

	τ_o (ms)	τ_{C1} (ms)	τ_{C2} (ms)	τ_{C3} (ms)	O_{burst} (ms)	p_o
Control	0.9 ± 0.5	1.3 ± 0.2	13.3 ± 2.5	383 ± 143	31.1 ± 4.5	0.17 ± 0.07
75 nM Tx	1.1 ± 0.7	1.6 ± 0.3	18.3 ± 3.5	1272 ± 365	26.1 ± 12.7	0.10 ± 0.06

Dwell open (τ_o) and closed times (τ_{C1}, τ_{C2}, τ_{C3}), mean burst duration, and open probability of TOK1 channels held at 80 mV in inside-out patches from oocytes in absence or presence of 75 nM internal toxin. Duration histograms were constructed with 20,000 to 50,000 events. Mean burst duration was calculated according to $[W_{C1}/(1 - W_{C1})][\tau_{C1} + \tau_o] + \tau_o$ where W_{C1} indicates the weight of the fast component in the duration histogram. For analysis, data filtered at 1 kHz and sampled at 5 kHz; all values are means ± SEM for three patches.

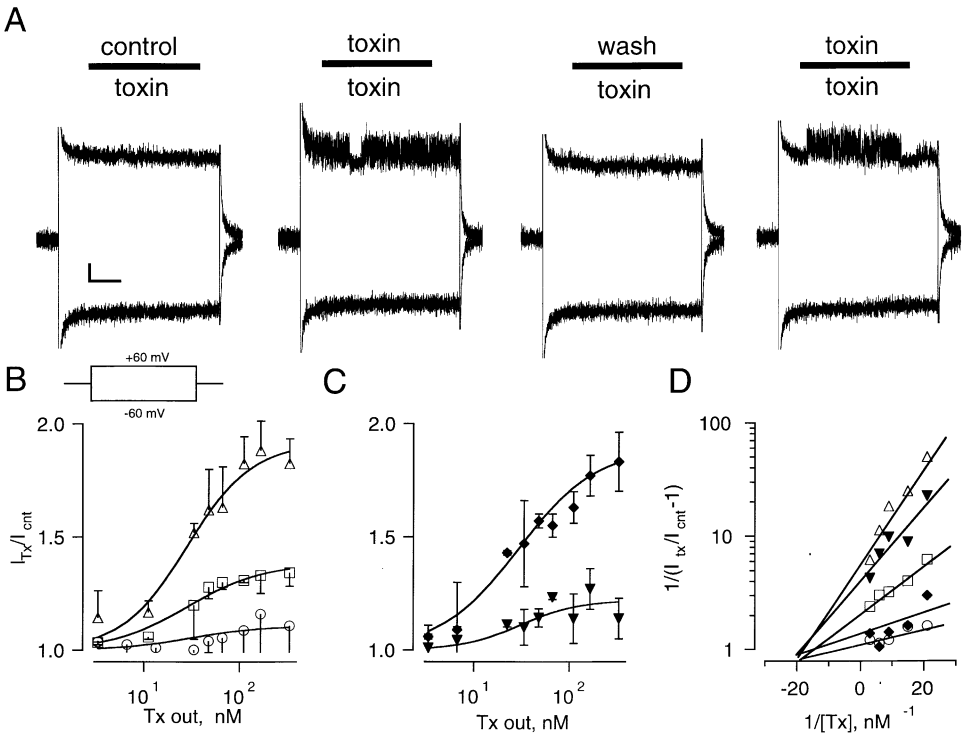

Figure 3. TOK1 Channels in Virus-Positive Cells or Virus-Free, Toxin-Loaded Cells Are Not Activated by External Toxin

(A) Single-channel recording with an outside-out patch from a virus-free CY162C spheroplast expressing pGKH-TOK1 with ~110 nM toxin in the pipette solution, in the absence or presence of ~200 nM killer toxin in the bath, as indicated. Protocol: 1 s steps to +60 and −60 mV with a 1 s interpulse interval. In the absence of toxin, channel activity was observed in 5 of 55 traces. With external toxin, activity was observed in 21 of 55 traces. Scale bars, 2.5 pA, 0.25 s.

(B) Dose-response relationship for external toxin at various levels of internal toxin using virus-free CY162C cells expressing pGKH-TOK1 at +50 mV; cells were dialyzed with ~30 (triangles), ~75 (squares), and ~110 nM toxin (circles) in the pipette and exposed to the indicated levels of external toxin. Data fitted to a modified Hill function, H′:

$$H' = 1 + \frac{A}{1 + (K_{out}/[toxin])^{nH}}$$

and gave: K_{out} = 27 ± 13, n_H = 1.3 ± 0.1 (n = 3), K_{out} = 28 ± 11, n_H = 1.1 ± 0.1 (n = 3), and K_{out} = 30 ± 16, n_H = 1.2 ± 0.3 (n = 5), respectively.

(C) Dose-response relationships for external toxin using virus-positive CY162 cells (triangles) and virus-free CY162C cells (diamonds) in both cases expressing pGKH-TOK1. Data fitted as in (B) and gave: K_{out} = 30 ± 18, n_H = 1.4 ± 0.1 and K_{out} = 28 ± 8, n_H =1.1 ± 0.1 (n = 5), respectively. Native channels in CY162 and CY162C cells showed similar sensitivity: K_{out} = 36 ± 11, n_H = 1.1 ± 0.1 and K_{out} = 30 ± 7, n_H = 1.3 ± 0.1 (n = 5 cells), respectively.

(D) Lineweaver-Burke type double reciprocal plot of the data in (B) and (C) using the same symbols. Extrapolated K_{out} ~40 nM.

sponse to external toxin. Indeed, internal toxin suppressed TOK1 activation by external toxin so that the cells were indistinguishable from those that were naturally resistant; the attributes of virus-positive cells and virus-free cells loaded with ~75 to 110 nM internal K1 toxin were similar (Figures 3B and 3C). A double-reciprocal analysis of the data (Figure 3D) supports the conclusion that varying levels of internal toxin did not alter the concentration of external toxin required to achieve half-maximal activation (based on coincidence of dose-response curves at the X intercept), but did alter its maximal efficacy (curve slope). This is consistent with non-competitive suppression of activation by internal toxin.

ΔTx, a Toxin Mutant that Confers Immunity and Blocks but Does Not Kill or Activate

These results suggest a model for immunity based on opposing actions of toxin on TOK1 that demands the following correlation: a toxin that does not kill yet mediates immunity should be unable to activate TOK1 from the outside, but capable of suppressing activation by external wild-type toxin. A toxin mutant carrying a five residue insertion (NPGLI) in the α chain at position 52 has been reported to confer immunity but not killing (Boone et al., 1986; Zhu and Bussey, 1991). When we produced this toxin variant (ΔTx) and applied it to a lawn of virus-free target cells, it did not produce a halo of growth inhibition (Figure 4A, left panel) as did wild-type toxin (Figure 1A). In keeping with expectations, external ΔTx also did not activate TOK1 channels in virus-free spheroplasts (Figure 4B), even at levels up to ~300 nM (Figure 4C).

Conversely, virus-free cells expressing the mutant toxin from a plasmid (pΔTx) were immune to wild-type toxin (Figure 5A), while cells expressing the empty plasmid vector were killed (not shown). Furthermore, internal ΔTx inhibited TOK1 channels (Figure 5B), showing the same effectiveness as wild-type toxin (Figure 5C). Indeed, internal ΔTx suppressed activation by external

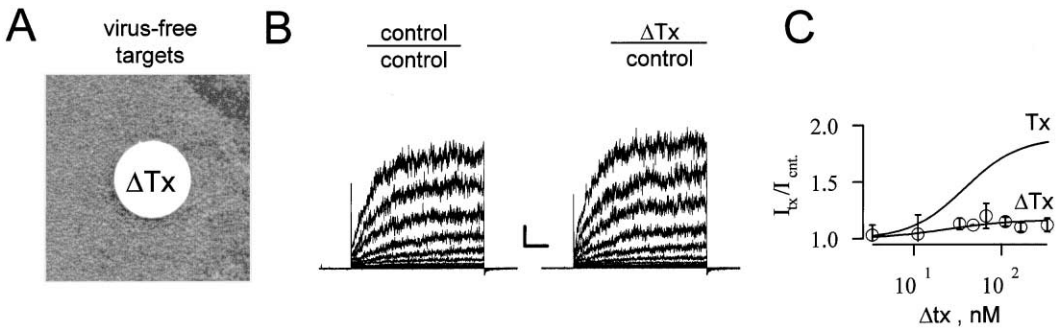

Figure 4. ΔTx Does Not Kill or Activate TOK1

Insertion of five residues (NPGLI) in the α chain produces a mutant toxin that retains the immunity phenotype, but is ineffective in killing and does not activate TOK1 channels.

(A) Virus-free CY162C cells exposed to a filter with 15 μl isolated ΔTx (~300 nM) on a YPD assay plate as in Figure 1A.

(B) Spheroplasts from virus-free CY162 cells expressing pGKH-TOK1 studied in whole-cell configuration in the absence (left) or presence (right) of ~300 nM ΔTx by the protocol in Figure 1B. Scale bars, 200 ms and 100 pA.

(C) Dose-response relationship for external ΔTx using virus-free CY162C cells expressing pGKH-TOK1 at +50 mV. The solid curve is data from Figure 3C for wild-type toxin.

wild-type toxin (Figure 5D) as potently as wild-type toxin (Figure 5E).

Native and Overexpressed Wild-Type and Mutant TOK1 Channels

In this study, both natural and plasmid-encoded TOK1 channels are evaluated, the latter to increase current density and, so, macroscopic measurement precision. CY162 cells are employed because they are amenable to high resistance seal formation (Ahmed et al., 1999) and tolerate overexpression of potassium channels (because they lack function of the potassium transporters TRK1 and TRK2 and, so, require either supplemental bath potassium or a potassium channel to grow [Anderson et al., 1992; Goldstein et al., 1996]). No qualitative differences are apparent between naturally expressed TOK1 in CY162 and plasmid-encoded channels. Thus, current density is depressed to a similar degree when

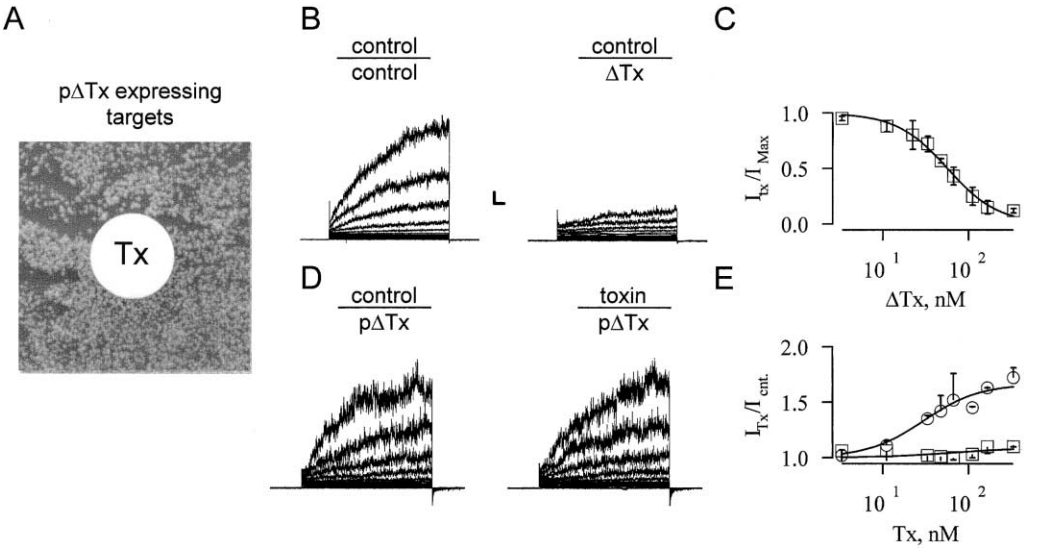

Figure 5. ΔTx Provides Immunity to Virus-Free Cells, Inhibits TOK1 Channels, and Suppresses Activation by External Wild-Type Toxin

(A) Virus-free CY162 cells expressing pΔTx exposed to a filter with 15 μl of isolated wild-type toxin (~300 nM) on a YNB assay plate as in Figure 1A.

(B) Macroscopic TOK1 currents recorded from a giant inside-out patch excised from an oocyte in the absence (left) or presence (right) of ~300 nM internal ΔTx (by the protocol in Figure 1B). Scale bars, 200 ms and 200 pA.

(C) Dose-response curves for internal ΔTx with TOK1 channels expressed in oocytes (as in [B]) calculated at +50 mV. Data were fitted to the Hill function as in Figure 2F, yielding $K_{in} = 56 \pm 12$, $n_H = 1.4 \pm 0.2$ ($n = 5$).

(D) Whole-cell current envelopes recorded from virus-free CY162C pGKH-TOK1 cells dialyzed with ~110 nM ΔTx in the pipette, in the absence (left) or presence (right) of ~300 nM wild-type toxin by the protocol in Figure 1B. Scale bars, 200 ms and 50 pA.

(E) Dose-response relationship for external wild-type toxin at various levels of internal ΔTx using virus-free CY162C cells expressing pGKH-TOK1 at +50 mV as in Figure 3B; cells were dialyzed with ~33 (circles) and ~110 nM ΔTx (squares), respectively. Data fitted to a Hill function H′ as in Figure 3B, and gave: $K_{out} = 38 \pm 13$, $n_H = 1.2 \pm 0.1$ ($n = 3$) and 38 ± 7, $n_H = 1.2 \pm 0.1$ ($n = 5$), respectively.

Table 2. Native and Plasmid-Encoded TOK1 Current Density (pA/pF) and Effect of External K1 Toxin

	Native TOK1		Overexpressed TOK1	
Cell Type (Cells Studied)	Basal	K1	Basal	K1
Virus-free (6)	13.8 ± 4.7	29.0 ± 9.9	218 ± 70	415 ± 133
Virus-positive (7)	5.9 ± 1.6	6.5 ± 1.8	123 ± 40	148 ± 48
Virus-free, pTX (4)	6.3 ± 2.2	6.1 ± 2.1	109 ± 36	120 ± 40
Virus-free, pΔTx (4)	5.9 ± 1.9	6.5 ± 2.1	135 ± 35	128 ± 33

Basal level of current in CY162C (virus-free) and CY162 (virus-positive) cells in the absence and presence of ~300 nM external K1 toxin at 50 mV. Mean current densities (pA/pF, mean spheroplast capacitance was ~2 pF). Unpaired student's t test computed for each group of cells (referred to virus-free) was $0.006 < P < 0.02$. Values are means ± SEM for the number of cells indicated.

virus-free and virus-positive cells are compared (Table 2). Further, external toxin fails to activate either native or plasmid-encoded channels when the cells carried virus or plasmids encoding wild-type or ΔTx K1 toxin (Table 2). Finally, native and plasmid-encoded channels show similar sensitivity to external K1 toxin (Figure 3C) and indistinguishable killing and immunity behaviors in the halo assay (Figure 1).

Our conclusion that dysregulated TOK1 activity is sufficient to kill yeast cells (Ahmed et al., 1999) gains important support from studies showing that galactose-induced overexpression of TOK1 allows survival of *trk1trk2* transport deficient SGY1529 cells in low (but not high) potassium (Fairman et al., 1999), while overactive TOK1 mutants (for example, S330I and V456I channels) kill the cells (Loukin et al., 1997). As noted previously (Ahmed et al., 1999), these mutant channels (studied in oocytes) are reported to show changes in closed-state stability like those we observe when native TOK1 channels in yeast cells are exposed to K1 toxin. Here, we show that the lethal effect of S330I TOK1 channels is not strain, carbon-source, nor killer phenotype specific. Thus, MET25-induced overexpression of wild-type *TOK1* confers growth to CY162 and CY162C cells on plates with 7 mM potassium while induction of S330I TOK1 channels inhibits proliferation of both the virus-positive and virus-free cells (not shown). In contrast to studies with SGY1529 (Fairman et al., 1999; Loukin et al., 1997), CY162 and CY162C cells expressing V456I channels are like those with wild-type TOK1, and overexpression of wild-type TOK1 in the presence of 100 mM potassium does not interfere with growth (Figure 1). Thus, constitutive overexpression of wild-type TOK1 in CY162 and CY162C cells is tolerated (Table 2), whereas induction of S330I channels that are abnormally active due to the mutation, or exposure of wild-type channels to external K1 toxin, blocks proliferation.

Discussion

Killer strains impact yeast cell biology, the commercial spirits industry, and biomedicine. Killer toxins also act on nonyeast cells including bacteria, other molds, and mammalian intestinal epithelia (Magliani et al., 1997; Pettoello-Mantovani et al., 1995). Recently, we showed that external K1 toxin activates TOK1 (Ahmed et al., 1999), the plasma membrane potassium channel of *Saccharomyces cerevisiae* (Ketchum et al., 1995; Zhou et al., 1995) and does not form holes on its own as previously suspected. Channel activation by external toxin was associated with abnormal potassium flux, correlated with cell death, and demonstrated no requirement for other yeast or viral proteins (Ahmed et al., 1999). This report demonstrates that internal K1 toxin blocks TOK1 channels and suppresses activation by external toxin (also without an apparent requirement for other proteins) in a fashion that correlates with yeast cell immunity. Moreover, a K1 mutant that confers immunity but does not kill blocks TOK1 from the inside, but does activate the channel from the outside. These results support a model for killing and immunity of marvelous economy: a single active principle that acts at two counterregulatory sites on one receptor; while secreted K1 activates TOK1 and kills, intracellular toxin suppresses activation and produces resistance.

Single TOK1 channels open in bursts that are interrupted by long-lasting closures (Ahmed et al., 1999; Loukin et al., 1997; Vergani et al., 1998). Previously, we found that external K1 toxin increased the duration and frequency of open bursts without modifying unitary conductance or nonvoltage-dependent outward rectification of the channels (Ahmed et al., 1999). Here, internal toxin is seen to suppress activation in a noncompetitive fashion via stabilization of the long interburst closed state (also without apparent changes in conductance or rectification). That is, internal toxin does not diminish the capacity of external toxin to produce half-maximal current, but limits the maximum achievable current. These opposing effects of toxin from opposite sides of the membrane are seen to correlate directly with expression of killing and immunity.

Like a noncompetitive inhibitor in an enzyme-substrate reaction, internal toxin combines with either free channel or the channel-activator complex at a site other than that occupied by the activator (opposite sides of the membrane) and, once bound, interferes with normal function in a fashion that is not reversed by increasing activator concentration. While it is feasible that the toxin interacts with a single receptor site that is alternatively exposed on opposite sides of the membrane, such a conformational change makes it appropriate to treat the sites as nonequivalent, in keeping with a noncompetitive model.

A model based on opposing effects of K1 toxin from opposite sides of the membrane accounts for previous experimental evidence and reconciles a problem for earlier proposals: toxin mutants whose function suggests immunity proceeds from inside the cell. Supporting the idea that mature toxin is the species that acts from the cytosol, both mature and precursor forms of wild-type

toxin are present at high levels inside killer cells (Bussey et al., 1983; Lolle et al., 1984; Sturley et al., 1986), and toxin mutants that are processed incompletely do not confer wild-type levels of immunity when evaluated quantitatively (Boone et al., 1986). Moreover, there is ample precedent for retrograde transport of mature secreted proteins into the cytoplasm. This pathway is the primary quality control mechanism for elimination of misfolded proteins and is also employed to avoid aggregation of abundant secretory proteins (Kjeldsen et al., 1999; Plemper et al., 1999a). Since it does not require persistent contact with the translocation machinery, the pathway permits bidirectional transport of fully processed proteins (Plemper et al., 1999b). Indeed, the plant peptide ricin (Wesche et al., 1999) and bacterial Shiga toxin (Lord and Roberts, 1998) follow a similar reverse trajectory into the cytosol camouflaged as substrates of the protein degradation machinery. Thus, ricin is transported retrograde through the Golgi apparatus to the endoplasmic reticulum where it is processed to liberate the A-chain that then moves into the cytosol to inactivate ribosomes and suppress protein synthesis. Of particular note, another virally encoded *S. cerevisiae* toxin, K28, has been shown to enter and kill sensitive yeast cells by traveling the secretory pathway in reverse to the cytosol and then moving into the nucleus to block DNA synthesis (Eisfeld et al., 2000). Work is now required to establish the transport trajectory of K1 toxin, to determine if K1 precursors contribute to immunity when cells express a wild-type toxin gene, and to evaluate whether the alternative pathway for killing and immunity revealed by TOK1 deletion (Ahmed et al., 1999) operates in a similar fashion. More fundamentally, the natural role of TOK1 channels (and potassium ions) in yeast cell physiology remains to be elucidated.

Experimental Procedures

Strains and Molecular Biology

Yeast Strains

Studies reported here use the following: (1) M1 virus-positive CY162 cells (*MATa ura3-52 his3Δ200 his4-15 trk1Δ trk2Δ::HIS3*), a gift from R. Gaber (Ko and Gaber, 1991); (2) Virus-free CY162 cells cured of M1 virus by multiple rounds of growth at 40°C on YPD plates (designated CY162C) as per Wickner (1974); (3) M1 virus-positive K12 cells (*MATα ade2-5*); or (4) SGY1529 (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 trk1::HIS3 trk2::TRP1*), a gift of S. Kurtz (Tang et al., 1995). SGY1529 cells were found to be K1 virus positive by halo assay (not shown). CY162 cells were used in this study because they grow well despite TOK1 overexpression (Fairman et al., 1999) and were most amenable to gigohm seal formation for patch clamp analyses among tested strains. Yeast strains were transformed with expression plasmids using 40% PEG 2000, 100 mM lithium acetate, TE with 10 μg boiled salmon sperm carrier DNA, and selection on uracil-deficient plates (Ahmed et al., 1999).

Plasmids

pGKH-K1 has a 900 bp fragment for the K1 gene from pSH6 (Sturley et al., 1986) inserted into pGKH. pGKH-TOK1 has a 2.1 kb fragment containing the TOK1 gene (Ketchum et al., 1995) ligated into pGKH. The wild-type, S330I, or V456I *TOK1* gene was also expressed in a vector supplied by D. Minor containing the Met25 promotor (Minor et al., 1999). TOK1 channel mutations were created by a standard method and confirmed by DNA sequencing (Sesti et al., 2000).

Toxin Production

Toxins were prepared and concentrations estimated as previously described (Ahmed et al., 1999). Briefly, toxin was purified from K12 cells, pGKH-K1, or pGKH-ΔTx by culture, centrifugation, filtration, and ~100-fold concentration and stored at −20°C. Control solutions were prepared boiling the isolated toxin for 30 min.

Killing and Immunity Assays

CY162 and CY162C cells were grown overnight in 3 ml YPD with 100 mM KCl, diluted to OD_{600} = 0.1, and 0.1 ml spread to YPD (or YNB uracil-deficient plates) with 100 mM KCl, 0.03% methylene blue, and 50 mM sodium citrate (pH 4.7). In some experiments, cells were transferred to plates with 7 mM KCl. Plates were incubated at room temperature for 48 hr after a single yeast colony was streaked across the lawn or a filter disk with toxin applied.

Electrophysiology

Spheroplasts

Single colonies were grown overnight in 10 ml YPD media, harvested, and digested 40–50 min at 30°C in 1 ml of a solution containing: 400 U/ml Lyticase, 1 U/ml Chitinase, and 2000 U/ml β-Glucuronidase (Sigma, MO), 1.2 M sorbitol, 50 mM K-phosphate/KOH buffer (pH 7.0), and 40 mM mercaptoethanol. Spheroplasts were enriched by layering the digested cells over a cushion (2 M sorbitol, 1 mM CaCl, 50 mM KH_2PO_4, 40 mM 2-mercaptoethanol [pH 7.0] with KOH), collected free of debris after centrifugation at 500 g for 5 min, and then washed and stored at 4°C in: 250 mM KCl, 10 mM CaCl, 5 mM MgCl, 5 mM HEPES, (pH 7.0) with KOH. As the same results were obtained in perforated-patch configuration and whole-cell mode, the latter was employed routinely as it was more long lasting.

Oocytes

Xenopus laevis oocytes were removed surgically, digested at room temperature 30–60 min in Ringer solution and type 2 collagenase (Worthington Biochemical Co.), and injected with 50 nl containing 10 ng TOK1 cRNA.

Data were recorded with an Axopatch 200B (Axon Instruments, CA), a Quadra 800 Macintosh computer, and Pulse software (Heka, De) at filter and sampling frequencies of 0.5 kHz and 1.5 kHz, respectively. Holding voltage was −80 mV. Voltage protocol for whole-cell current envelopes consisted of 1 s pulses from −80 to 60 mV in 10 mV increments spaced in 1 s interpulse intervals. For dose-response measurements, cells were also stimulated with trains of 11–25 consecutive 1 s voltage jumps from −80 to 50 mV that were averaged online. Solutions for yeast spheroplasts: pipette solution contained, in mM: 175 KCl, 5 MgCl, 10 EGTA, 1 CaCl, 10 HEPES, (pH 7.0) with KOH. Bath solution: 150 KCl, 10 CaCl, 5 MgCl, 10 HEPES, (pH 7.5) with KOH. Solutions for oocytes: pipette and bath solution contained, in mM: 150 KCl, 5 EGTA, 10 HEPES, (pH 7.5) with KOH.

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References

Ahmed, A., Sesti, F., Ilan, N., Shih, T.M., Sturley, S.L., and Goldstein, S.A.N. (1999). A molecular target for viral killer toxin: TOK1 potassium channels. Cell *99*, 283–291.

Al-Aidroos, K., and Bussey, H. (1978). Chromosomal mutants of Saccharomyces cerevisiae affecting the cell wall binding site for killer factor. Can. J. Microbiol. *24*, 228–237.

Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J., and Gaber, R.F. (1992). Functional expression of a probable Arabidopsis thaliana potassium channel in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *89*, 3736–3740.

Boone, C., Bussey, H., Greene, D., Thomas, D.Y., and Vernet, T. (1986). Yeast killer toxin: site-directed mutations implicate the precursor protein as the immunity component. Cell *46*, 105–113.

Boone, C., Sommer, S.S., Hensel, A., and Bussey, H. (1990). Yeast KRE genes provide evidence for a pathway of cell wall beta-glucan assembly. J. Cell Biol. *110*, 1833–1843.

Bostian, K.A., Hopper, J.E., Rogers, D.T., and Tipper, D.J. (1980).

Translational analysis of the killer-associated virus-like particle dsRNA genome of S. cerevisiae: M dsRNA encodes toxin. Cell *19*, 403–414.

Bostian, K.A., Elliott, Q., Bussey, H., Burn, V., Smith, A., and Tipper, D.J. (1984). Sequence of the preprotoxin dsRNA gene of type I killer yeast: multiple processing events produce a two-component toxin. Cell *36*, 741–751.

Bussey, H. (1981). Physiology of killer factor in yeast. Adv. Microb. Physiol. *22*, 93–122.

Bussey, H. (1991). K1 killer toxin, a pore-forming protein from yeast. Mol. Microbiol. *5*, 2339–2343.

Bussey, H., Saville, D., Greene, D., Tipper, D.J., and Bostian, K.A. (1983). Secretion of Saccharomyces cerevisiae killer toxin: processing of the glycosylated precursor. Mol. Cell. Biol. *3*, 1362–1370.

Eisfeld, K., Riffer, F., Mentges, J., and Schmitt, M.J. (2000). Endocytotic uptake and retrograde transport of a virally encoded killer toxin in yeast. Mol. Microbiol. *37*, 926–940.

Fairman, C., Zhou, X.L., and Kung, C. (1999). Potassium uptake through the TOK1 K+ channel in the budding yeast. J. Membr. Biol. *168*, 149–157.

Goldstein, S.A.N., Price, L.A., Rosenthal, D.N., and Pausch, M.H. (1996). ORK1, a potassium-selective leak channel with two pore domains cloned from Drosophila melanogaster by expression in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *93*, 13256–13261.

Ketchum, K.A., Joiner, W.J., Sellers, A.J., Kaczmarek, L.K., and Goldstein, S.A.N. (1995). A new family of outwardly-rectifying potassium channel proteins with two pore domains in tandem. Nature *376*, 690–695.

Kjeldsen, T., Pettersson, A.F., and Hach, M. (1999). The role of leaders in intracellular transport and secretion of the insulin precursor in the yeast Saccharomyces cerevisiae. J. Biotechnol. *75*, 195–208.

Ko, C.H., and Gaber, R.F. (1991). TRK1 and TRK2 encode structurally related K+ transporters in Saccharomyces cerevisiae. Mol. Cell. Biol. *11*, 4266–4273.

Lolle, S., Skipper, N., Bussey, H., and Thomas, D.Y. (1984). The expression of cDNA clones of yeast M1 double-stranded RNA in yeast confers both killer and immunity phenotypes. EMBO J. *3*, 1383–1387.

Lord, J.M., and Roberts, L.M. (1998). Toxin entry: retrograde transport through the secretory pathway. J. Cell Biol. *140*, 733–736.

Loukin, S.H., Vaillant, B., Zhou, X.L., Spalding, E.P., Kung, C., and Saimi, Y. (1997). Random mutagenesis reveals a region important for gating of the yeast K+ channel Ykc1. EMBO J. *16*, 4817–4825.

Magliani, W., Conti, S., Gerloni, M., Bertolotti, D., and Polonelli, L. (1997). Yeast killer systems. Clin. Microbiol. Rev. *10*, 369–400.

Minor, D.L., Jr., Masseling, S.J., Jan, Y.N., and Jan, L.Y. (1999). Transmembrane structure of an inwardly rectifying potassium channel. Cell *96*, 879–891.

Pettoello-Mantovani, M., Nocerino, A., Polonelli, L., Morace, G., Conti, S., Di Martino, L., De Ritis, G., Iafusco, M., and Guandalini, S. (1995). Hansenula anomala killer toxin induces secretion and severe acute injury in the rat intestine. Gastroenterol. *109*, 1900–1906.

Plemper, R.K., Bordallo, J., Deak, P.M., Taxis, C., Hitt, R., and Wolf, D.H. (1999a). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J. Cell Sci. *112*, 4123–4134.

Plemper, R.K., Deak, P.M., Otto, R.T., and Wolf, D.H. (1999b). Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. FEBS Lett. *443*, 241–245.

Schmitt, M.J., and Compain, P. (1995). Killer-toxin-resistant kre12 mutants of Saccharomyces cerevisiae: genetic and biochemical evidence for a secondary K1 membrane receptor. Arch. Microbiol. *164*, 435–443.

Sesti, F., Abbott, G.W., Wei, J., Murray, K.T., Saksena, S., Schwartz, P.J., Priori, S.G., Roden, D.M., George, A.L., Jr., and Goldstein, S.A.N. (2000). A common polymorphism associated with antibiotic-induced cardiac arrhythmia. Proc. Natl. Acad. Sci. USA *97*, 10613–10618.

Skipper, N., Thomas, D.Y., and Lau, P.C. (1984). Cloning and sequencing of the preprotoxin-coding region of the yeast M1 double-stranded RNA. EMBO J. *3*, 107–111.

Starmer, W.T., Ganter, P.F., Aberdeen, V., Lachance, M.-A., and Phaff, H.J. (1992). The ecological role of killer yeasts in natural communities of yeasts. Can. J. Microbiol. *33*, 783–796.

Sturley, S.L., Elliot, Q., LeVitre, J., Tipper, D.J., and Bostian, K.A. (1986). Mapping of functional domains within the Saccharomyces cerevisiae type 1 killer preprotoxin. EMBO J. *5*, 3381–3389.

Tang, W., Ruknudin, A., Yang, W.P., Shaw, S.Y., Knickerbocker, A., and Kurtz, S. (1995). Functional expression of a vertebrate inwardly rectifying K+ channel in yeast. Mol. Biol. Cell *6*, 1231–1240.

Thiele, D.J., and Leibowitz, M.J. (1982). Structural and functional analysis of separated strands of killer double-stranded RNA of yeast. Nucleic Acids Res. *10*, 6903–6918.

Vergani, P., Hamilton, D., Jarvis, S., and Blatt, M.R. (1998). Mutations in the pore regions of the yeast K+ channel YKC1 affect gating by extracellular K+. EMBO J. *17*, 7190–7198.

Wesche, J., Rapak, A., and Olsnes, S. (1999). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. J. Biol. Chem. *274*, 34443–34449.

Wickner, R.B. (1974). "Killer character" of Saccharomyces cerevisiae: curing by growth at elevated temperature. J. Bacteriol. *117*, 1356–1357.

Wickner, R.B. (1996). Prions and RNA viruses of Saccharomyces cerevisiae. Ann. Rev. Genet. *30*, 109–139.

Wickner, R.B., and Leibowitz, M.J. (1976). Two chromosomal genes required for killing expression in killer strains of Saccharomyces cerevisiae. Genetics *82*, 429–442.

Zhou, X.L., Vaillant, B., Loukin, S.H., Kung, C., and Saimi, Y. (1995). YKC1 encodes the depolarization-activated K+ channel in the plasma membrane of yeast. FEBS Lett. *373*, 170–176.

Zhu, H., and Bussey, H. (1991). Mutational analysis of the functional domains of yeast K1 killer toxin. Mol. Cell. Biol. *11*, 175–181.