Cysteine residues in a yeast viral A/B toxin crucially control host cell killing via pH-triggered disulfide rearrangements

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ABSTRACT **K28 is a viral A/B protein toxin that intoxicates yeast and fungal cells by endocytosis and retrograde transport to the endoplasmic reticulum (ER). Although toxin translocation into the cytosol occurs on the oxidized α/β heterodimer, the precise mechanism of how the toxin crosses the ER membrane is unknown. Here we identify pH-triggered, toxin-intrinsic thiol rearrangements that crucially control toxin conformation and host cell killing. In the natural habitat and low-pH environment of toxin-secreting killer yeasts, K28 is structurally stable and biologically active as a disulfide-bonded heterodimer, whereas it forms inactive disulfide-bonded oligomers at neutral pH that are caused by activation and thiol deprotonation of β-subunit cysteines. Because such pH increase reflects the pH gradient during compartmental transport within target cells, potential K28 oligomerization in the ER lumen is prevented by protein disulfide isomerase. In addition, we show that pH-triggered thiol rearrangements in K28 can cause the release of cytotoxic α monomers, suggesting a toxin-intrinsic mechanism of disulfide bond reduction and α/β heterodimer dissociation in the cytosol.**

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INTRODUCTION

Traversing biomembranes is a crucial and challenging task for microbial pathogens, viruses, and protein toxins to deliver their toxic or genetic material into the host cell cytosol. Generally, this process includes tight control of spatiotemporal structural changes in a toxin or viral particle to ensure proper timing of membrane passage at a specific subcellular compartment (Inoue *et al.*, 2011; Ravindran and Tsai, 2016). In particular, for viruses, such control is essential to prevent cell damage during host cell entry because viruses critically rely on intact cellular machineries for viral replication and assembly.

With respect to the tight control of protein toxin trafficking in a eukaryotic host, yeast killer toxin K28 is an interesting example. As family member of A/B toxins, K28 is naturally secreted by virus-infected killer strains of *Saccharomyces cerevisiae* (Schmitt and Breinig, 2006) and kills yeasts and fungi by blocking DNA synthesis and arresting cells at the G1/S boundary of the cell cycle (Schmitt *et al.*, 1996). K28-secreting killer cells are immune to their own toxin by an intrinsic mechanism that ensures that the internalized α/β toxin, once it has reached the host cell cytosol, is rapidly complexed with the unprocessed toxin precursor (preprotoxin) and subsequently targeted for ubiquitylation and proteasomal degradation (Breinig *et al.*, 2006). Because self-protection against K28 depends solely on the presence of the toxin precursor in the cytosol, any toxin-sensitive strain can be converted into an immune/resistant strain by cytosolic expression of the unprocessed K28 precursor, defining the cytosol as a crucial compartment for both toxin trafficking and immunity. This also implies that toxin uptake and retrograde transport to the cytosol occur equally in a sensitive target cell and a toxin-secreting killer cell, indicating that it must be tightly controlled in each scenario to prevent cell damage.

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Abbreviations used: βME, β-mercaptoethanol; BSA, bovine serum albumin; DTT, dithiothreitol; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; PDI, protein disulfide isomerase; ppα, preproα; TCEP, tris(2-carboxyethyl) phosphine.

In its biologically active and cytotoxic conformation, K28 consists of two subunits (α and β) covalently linked by a single disulfide that exposes the β-C-terminal HDEL sequence for proper recognition by cellular H/KDEL receptors (Schmitt and Tipper, 1995; Riffer *et al.*, 2002; Becker *et al.*, 2016a,b). Toxicity, therefore, critically depends on both the presence of an HDEL motif at the C-terminus of β and a cellular H/KDEL receptor to ensure endocytic toxin internalization, retrograde transport, and subsequent endoplasmic reticulum (ER)–to-cytosol retrotranslocation (Eisfeld *et al.*, 2000). Correspondingly, numerous essential host cell components of the endocytosis and retrotranslocation machineries have been identified as centrally involved in K28 in vivo toxicity (Eisfeld *et al.*, 2000; Heiligenstein *et al.*, 2006; Carroll *et al.*, 2009). Furthermore, because K28 was shown to enter the cytosol of intoxicated cells from the ER as disulfide-bonded α/β heterodimer, it was concluded that the toxin crosses the ER membrane in an oxidized conformation and only thereafter dissociates into its subunit components (Heiligenstein *et al.*, 2006). This contrasts K28 from A/B toxin family members that exploit cellular ER-associated degradation (ERAD) components to exit the ER and enter the cytosol. In such toxins, disulfide bond reduction occurs either before or during ER-to-cytosol transport and has been assumed to be the signal that induces toxin retrotranslocation from the ER (Majoul *et al.*, 1996; Orlandi, 1997; Rapak *et al.*, 1997; Tsai *et al.*, 2001; Spooner *et al.*, 2004; Suzuki and Schmitt 2015).

In this study, we found that cysteine residues in the toxin's β subunit become reactive at neutral pH and are essential for cell killing but can also cause inactivation of K28 through toxin oligomerization via thiol rearrangements. These cysteines are nonreactive under mildly acidic pH conditions, under which the toxin is naturally produced and secreted by a killer yeast, thereby keeping the toxin in a stable and biologically active heterodimeric conformation. In contrast, once the toxin has entered a target cell by endocytosis, the intracellular pH gradually increases, which would ultimately cause toxin oligomerization and inactivation. We here demonstrate that such pH-induced toxin oligomerization and inactivation is efficiently prevented by the activity of the protein disulfide isomerase (PDI) Pdi1p in the ER lumen. Although the precise function of reactive cysteine thiols in K28 is not fully understood, our data suggest that they mediate release of cytotoxic α monomers in the host cell cytosol, catalyzed by thiol rearrangements in the heterodimeric α/β toxin.

RESULTS

Disulfide bonds in K28 are rearranged by an increase in pH

Because the optimum pH for efficient cell killing by killer toxin K28 is in the range of pH 3.5– 5.8 (Pfeiffer and Radler, 1984; Eisfeld *et al.*, 2000; Riffer *et al.*, 2002; Breinig *et al.*, 2006; Heiligenstein *et al.*, 2006; Carroll *et al.*, 2009), we routinely use pH 4.7 for in vivo toxicity assays and storage of toxin samples for up to 1 yr without activity loss (unpublished data). However, when the toxin is incubated in buffer of pH > 6, its biological activity rapidly decreased (Figure 1A), resulting in a complete and irreversible loss of toxicity (Figure 1B). To gain insight into the molecular mechanism of this pH-dependent inactivation of K28, we analyzed the protein by nonreducing SDS–PAGE and Western blotting (Figure 1C). Toxin produced and stored at pH 4.7 mainly appeared as an α/β heterodimer (21 kDa) consisting of monomeric α and β subunits of similar size (~10 kDa each) and a minor band with an apparent molecular weight of ~45 kDa (Figure 1C, lane 1). In contrast, under more alkaline conditions (pH 8.0), additional toxin-specific signals appeared that indicate formation of K28 trimers, tetramers, and monomers (Figure 1C, lane 3). Because trimers and tetramers completely converted into monomers under reducing conditions, we can conclude that both originated from disulfide-bonded toxin oligomers (Figure 1C, lane 7). By incubating K28 at increasing pH between 5.0 and 9.0, we observed a tendency of oligomer formation in such a way that toxin tetramers appeared at lower pH, followed by K28 trimers and monomers (Figure 1D). Of note, toxin oligomer formation at pH 8.0 was stable and not reversed by a subsequent downshift in pH (Figure 1E). These observations were unexpected because neither reductants nor oxidants had been added during the pH shift (Figure 1, C, lanes 1–3, and D). Oxidation by air could in principle be considered, but the observed formation of both K28 trimers and monomers implies a disulfide bond cleavage reaction that cannot be explained by simple oxidation (Supplemental Figure S1). On the other hand, K28 oligomer and monomer formation could result from toxin-intrinsic disulfide bond rearrangements. Mature and biologically active K28 is an α/β heterodimer that contains three cysteine residues in β that are not part of the interchain α/β disulfide. If one of these cysteines attacked an existing disulfide in the α/β heterodimer, formation of tetramers, trimers, and monomers would be the consequence (Supplemental Figure S1). Because the deprotonated form of a cysteine represents a nucleophilic thiolate anion, whereas a protonated thiol is chemically less reactive (Jensen *et al.*, 2009; Netto *et al.*, 2016), it can be assumed that any cysteine-mediated rearrangement in K28 would be a direct result of pH-driven thiolate anion activation. In support of this, toxin oligomerization and monomer release were efficiently prevented by the addition of thiol-blocking *N*-ethylmaleimide (NEM; Figure 1C, lane 5). Note that to prevent potential changes in the oligomeric states of K28 after sample preparation, NEM was routinely added to the sample buffer in nonreducing SDS–PAGE throughout this study.

β-Subunit cysteines control pH-dependent monomer release

In toxin-secreting killer cells, the K28 precursor (preprotoxin) is post-translationally imported into the ER, where signal peptidase cleavage removes the N-terminal presequence and Pdi1p forms the α/β-connecting disulfide (Riffer *et al.*, 2002; Schmitt and Breinig, 2006). After Kex2p/Kex1p-mediated processing in the Golgi, K28 is finally secreted as biologically active and disulfide-bonded α/β heterodimer with an exposed HDEL motif at the C-terminus of β (Riffer *et al.*, 2002; Schmitt and Breinig, 2006; Figure 2A). In its fully processed and mature form, K28 possesses a single cysteine in α (C56) covalently linked to one of four remaining cysteines in β (C292, 307, 333, 340; Schmitt and Tipper, 1995; Figure 2A). Although C340 has been proposed to form the interchain disulfide in the α/β heterodimer (Riffer *et al.*, 2002), the precise nature of this α/β-connecting disulfide is unknown. To identify this interchain disulfide, we constructed a series of K28 mutant variants containing single cysteine-to-serine mutations in β and analyzed their in vivo effect on K28 processing and α/β toxin secretion (Figure 2B). As positive control, we routinely coexpressed a V5-tagged variant of wild-type K28. Although three of the single cysteine mutants (exception was C292S) showed a significant decrease in secretion of the α/β heterodimer, the effect was most prominent and drastic in the C333S mutant, corresponding to only ~3% of the secretion level of wild-type toxin. From this, it can be concluded that cysteine 333 in β is the most likely candidate to form the disulfide with cysteine 56 in α. Furthermore, the decreased secretion level of the mutant toxins C307S and C340S (~30% of wild type) suggests the importance of these residues in K28 conformation and/or stability. To address this in more detail, we also determined secretion levels for toxin variants containing triple cysteine-to-serine mutations in β (Figure 2C). Because cysteines in these mutants are restricted to just a single residue in each subunit, interchain disulfide

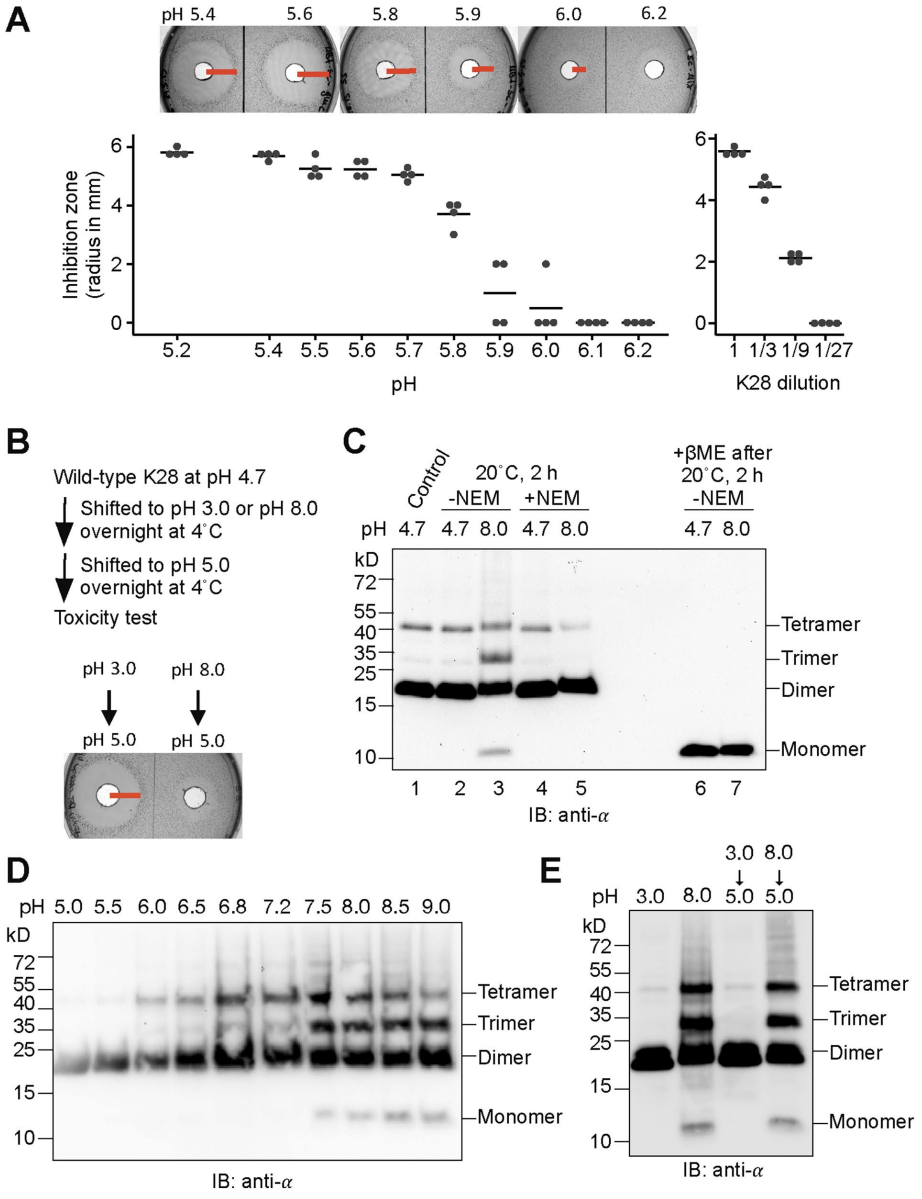

FIGURE 1: Effects of pH on K28 killing activity and conformational rearrangements. (A) In vivo toxicity of K28 after incubation at the indicated pH and 4°C for 20 h. Killing activity was assayed on methylene blue agar (pH 4.7) against *S. cerevisiae* 192.2d. Red bars indicate the radius of growth inhibition caused by the toxin. Results as from four independent experiments (dots) and their respective averages (bars) at the indicated pH. Similar measurements were performed with toxin samples of different dilutions, indicating an exponential dependence between each toxin concentration and the size of the resulting inhibition zone. (B) K28 toxicity after incubation at pH 3.0 or 8.0 and 4°C for 20 h, followed by a shift to pH 5.0 and subsequent incubation for 16 h. (C) SDS–PAGE and Western analysis of K28 incubated at pH 4.7 or 8.0 and 20°C for 2 h in the presence or absence of 50 mM NEM. β-Mercaptoethanol (βME) was added to half of the non-NEM samples. (D) K28 incubated at the indicated pH and 20°C for 2 h; thereafter, the reaction was stopped by the addition of NEM (100 mM), and samples were analyzed by nonreducing SDS–PAGE. (E) As in B, but toxin samples were incubated for 1 h at pH 3.0 or 8.0, shifted to pH 5.0, and incubated for another 3 h. Reactions were stopped and samples analyzed as in D. Immunoblots (IBs) in C–E were probed with anti-α.

bond formation would be essential for α/β heterodimer secretion. As illustrated in Figure 2C, residues C292 and C307 were not capable of efficiently forming a disulfide with C56 in α and therefore resulted in significantly lower secretion levels (<0.5%) than with wild-type toxin (Figure 2C). Although inefficient, mutant variants containing only C333 or C340 in β resulted in α/β heterodimer secretion (~10 and ~6% of wild type, respectively). Assuming C333 as the partner of C56 in the wild-type toxin (Figure 2B), formation of the C56-C340 disulfide in the triple mutant suggests that C56, C333, and C340 may be disposed in close proximity of the structural folding intermediate and/or the mature form of the toxin. The severe decrease in α/β heterodimer secretion seen in the triple mutants further suggests that even cysteines that are not involved in interchain disulfide bonding are important to ensure proper toxin folding and/or stability.

Because cysteines 56 and 333 represent the likeliest candidates that form the α/β-connecting disulfide in vivo, we constructed and characterized a K28 mutant variant lacking all cysteine residues in β except for the cysteine that forms the interchain disulfide with α. In contrast to wild-type K28, this triple mutant (C292S/C307S/C340S) did not form toxin-specific oligomers or monomers at pH 8.2 (Figure 3A), strongly indicating that pH-dependent disulfide bond rearrangements in the α/β heterodimer are mediated by internal cysteines in β rather than by thiols from other toxin molecules. To further confirm the importance of the cysteines in β, we tested in vivo the toxicity of the triple cysteine mutant in an agar diffusion assay and compared it with that of wild-type toxin. Although protein concentration was comparable in both samples and in vivo killing activity was high for the wild-type toxin, the triple-cysteine mutant was completely inactive and incapable of killing cells (Figure 3, B and C). This highlights the importance of cysteines in β for in vivo toxicity.

β-Subunit cysteines control in vivo toxicity

To dissect and mechanistically understand the importance of toxin-intrinsic thiols for host cell killing, we analyzed the effect of the thiol-modifier NEM on the activity of wild-type K28. NEM is a small molecule with a molecular weight of 125.13 g/mol, which specifically interacts in a chemical addition reaction with thiolate anions to form a thioether derivative (Hansen and Winther, 2009). Thus the addition of NEM to active K28 heterodimers should exclusively modify reactive and accessible thiolate anions without affecting toxin-intrinsic disulfides and/or toxin structure. Because the resulting thioether can no longer act as nucleophile to rearrange a disulfide bond, NEM treatment is expected to negatively affect K28 toxicity if thiolates play an important role in biologically active α/β heterodimers (Supplemental Figure S2A). To test this assumption experimentally, we incubated K28 at pH 5.5 for 1 h in the presence of 200 mM NEM, dialyzed it against a buffer of pH 4.7 for NEM removal, and subsequently tested it for in vivo killing activity in an agar diffusion

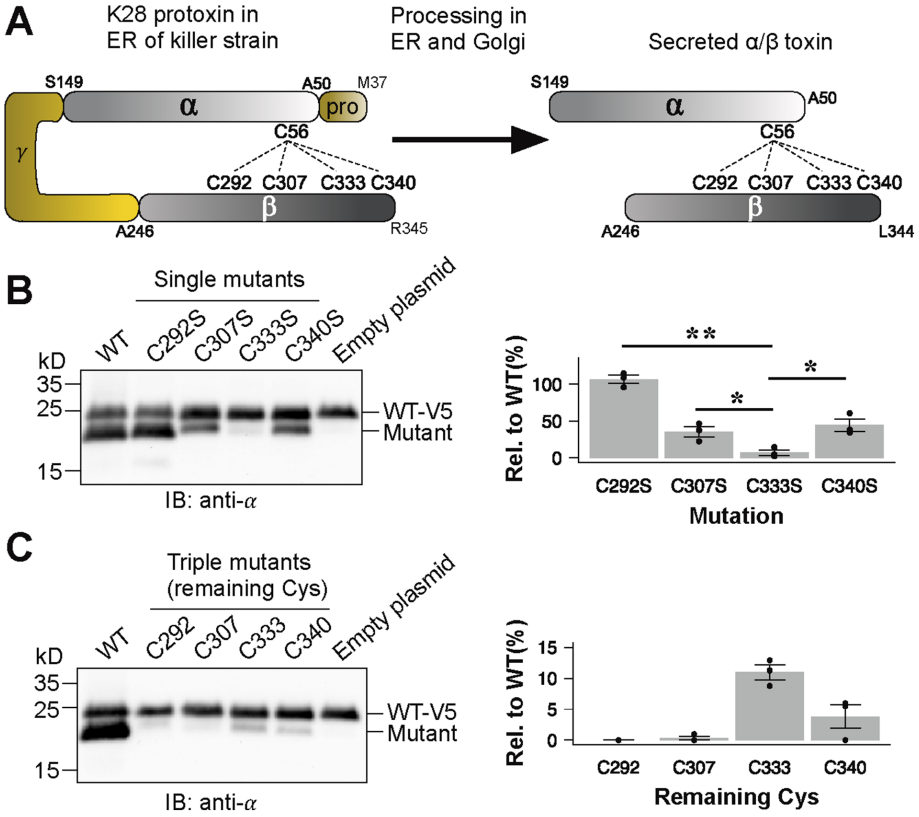

FIGURE 2: Identification of the α/β-connecting disulfide in K28. (A) Position of cysteine residues in the K28 precursor (protoxin) and schematic outline of disulfide bond formation in the ER lumen of a toxin-secreting killer cell. After proteolytic protoxin processing in the Golgi, mature K28 is secreted as an α/β heterodimer in which the single cysteine in α is covalently linked to one of four cysteines in β. (B) Secreted α/β heterodimeric K28 in the cell-free culture supernatant of yeast coexpressing V5-tagged wild-type K28 and one of the indicated single cysteine-to-serine mutant variants. Samples were separated by nonreducing SDS–PAGE and probed with anti-α. K28 signals of the mutant variants were normalized by the positive control (WT-V5); corresponding expression levels relative to the control are shown to the right. Bars indicate the average of independent experiments, dots the value of each experiment ($N = 3$). Error bars indicate SD. *$p < 0.025$; **$p < 0.001$. (C) Secretion level of α/β heterodimeric K28 in the cell-free culture supernatant of yeast coexpressing wild-type K28-V5 and any of the indicated triple cysteine-to-serine mutant variants (as in B).

assay. As illustrated in Figure 4A, NEM caused a complete loss of toxicity, underlining the importance of reduced cysteine residues in K28 for cell killing (Figure 4A and Supplemental Figure S2A). Similarly, toxin treatment with 500 mM dithiothreitol (DTT) under similar conditions (pH 5.5) caused a complete loss of toxicity, although reductive cleavage of α/β heterodimers was incomplete due to weak thiol reactivity at this low pH (Figure 4A and Supplemental Figure S2B). These findings strongly point to the existence of an additional disulfide in K28, besides the subunit-connecting disulfide, which is important for toxicity (Supplemental Figure S2B). Of interest, the α/β heterodimer showed a slightly slower mobility in SDS–PAGE after DTT treatment (Figure 4B), which might reflect changes in its redox state. In contrast, no such effect was observed after treatment with thiol-oxidizing diamide (Figure 4A and Supplemental Figure S2C). Killing activity of all chemically treated toxin samples was also correlated with the ability to form toxin oligomers after upshift to pH 8.0; whereas the diamide-treated toxin responded with oligomer formation under these conditions, no such effect was seen in either NEM- or DTT-treated toxin samples (Figure 4C). Taken together, these results confirm the importance of thiols in the toxin's β subunit.

In contrast to β, the α subunit of K28 contains just a single cysteine (C56), which forms the disulfide with C333 in β, and so α should be affected by neither NEM treatment nor triple-cysteine mutation (C292S/C307S/C340S) in the α/β heterodimer. Within the cytosol, however, monomeric α represents the cytotoxic component that contains a single thiol at position C56, which might be crucial for toxicity. To verify this possibility, we took advantage of the suicidal phenotype after *GAL1*-driven expression of prepro-α (ppα) in the ER lumen (Breinig *et al.*, 2006; Heiligenstein *et al.*, 2006). By comparing the phenotype of yeast transformants after expression of wild-type ppα with that of its cysteine-free mutant variant C56S, we showed that the ppα variants induce a similar growth defect under inducing culture conditions in the presence of galactose (Figure 5), indicating that the cell-killing activity of α does not require a thiol in its subunit.

Pdi1p prevents toxin oligomerization

So far, we have shown that K28 contains thiols that are inactive at pH 4.7 but become reactive at pH 6.0 and higher (Figure 1, A, C, and D). Because the K28-producing killer yeast was originally isolated from grape and subsequent wine fermentation, reflecting environmental conditions that are generally considered mildly acidic (Pfeiffer and Radler, 1982), it can be assumed that mature and secreted K28 is biologically active and stable as α/β heterodimer in the respective pH range between 3.0 and 5.0. However, once the toxin enters a cell through endocytosis, intracellular and compartmental pH gradually increases from 6.0 in endosomes and *trans*-Golgi, through 6.7 in *cis*-Golgi, to 7.2 in ER and cytosol; remarkably, this intracompartmental pH is not affected by changes in the extracellular pH in a wide range between 3.0 and 7.5 (Orij *et al.*, 2009). Considering the in vivo situation of intoxicated cells in which the internalized toxin is transported in a retrograde manner through the secretory pathway before it enters the cytosol (Eisfeld *et al.*, 2000; Heiligenstein *et al.*, 2006; Schmitt and Breinig, 2006), the question arises of how the toxin can retain its activity and conformation during transport through compartments with increasing pH. We therefore focused on ER luminal oxidoreductases, in particular, on Pdi1p, and asked whether this enzyme can prevent toxin-intrinsic subunit rearrangements that are predicted to also occur in the ER of intoxicated cells. From the five PDI family members of oxidoreductases in the yeast ER (Pdi1p, Mpd1p, Mpd2p, Eps1p, and Eug1p), only Pdi1p is essential. However, cell growth of a Δ*pdi1* knockout mutant can be restored by overexpressing either Mpd1p or the a' domain of Pdi1p (Nørgaard *et al.*, 2001: Solovyov *et al.*, 2004). We previously demonstrated that a yeast mutant carrying a chromosomal deletion in all five PDI family genes is viable when Mpd1p is overexpressed and becomes completely resistant against K28 (Heiligenstein *et al.*, 2006). Expression of wild-type Pdi1p in this strain fully restored K28

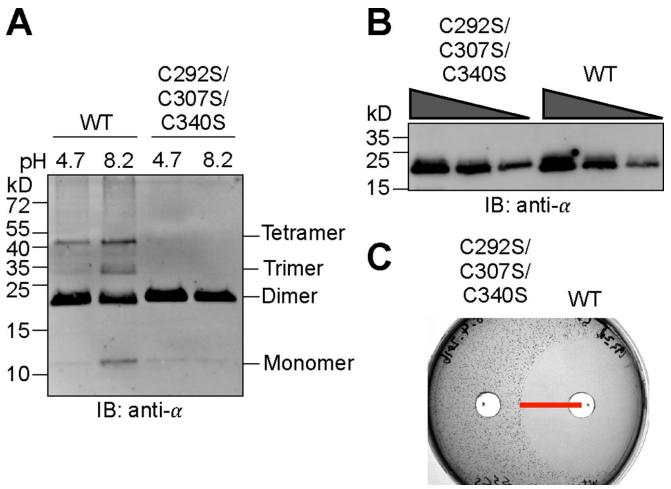

FIGURE 3: β-Subunit cysteines control in vivo toxicity and pH-dependent conformational changes. (A) Wild-type K28 and its triple cysteine-to-serine mutant C292S/C307S/C340S were expressed in *S. cerevisiae* BY4742 and compared with respect to their gel migration behavior after incubation at pH 4.7 and 8.2 and 20°C for 2 h. (B) In each case, 1:3 diluted aliquots from a concentrated cell-free culture supernatant were separated by SDS–PAGE and probed with anti-α. (C) In vivo toxicity of wild-type K28 and its C292S/C307S/C340S mutant variant against *S. cerevisiae* 192.2d. The amounts of wild-type and mutant K28 toxin were approximately the same, as confirmed in B. Samples in A and B were supplemented with 100 mM NEM, separated by nonreducing SDS–PAGE, and probed with anti-α.

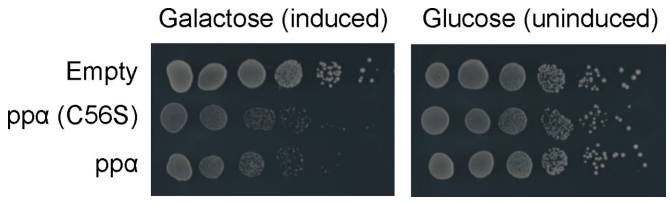

FIGURE 5: Suicidal ppα expression in the ER does not require a cysteine residue in α. *S. cerevisiae* BY4742 was transformed with a plasmid allowing *GAL1*-regulated expression of either wild-type ppα or its cysteine-free mutant variant (C56S). Yeast transformants were cultivated overnight under noninducing conditions in the presence of glucose, 10-fold serial dilutions of each culture were spotted onto agar containing glucose (noninduced) or galactose (induced) as carbon source, and plates were incubated at 30°C for 4 d.

sensitivity and α/β heterodimer translocation from the ER into the cytosol (Heiligenstein *et al.*, 2006), pointing to a central role of Pdi1p in K28 intoxication and in vivo killing. We now observe a similar toxin-resistant phenotype in a strain that carries a single Δ*pdi1* knockout but overexpresses the a' domain of Pdi1p (Figure 6A).

To investigate any effect of Pdi1p on pH-dependent disulfide bond rearrangements in K28, we added purified Pdi1p to the toxin during the pH shift. When added directly from a stock solution, Pdi1p had no effect on the conformational pattern of the toxin, whereas pretreatment of Pdi1p with immobilized tris(2-carboxyethyl) phosphine (TCEP) to shift the oxidoreductase into its reduced conformation effectively suppressed the formation of K28 oligomers (Figure 6B); as expected, no such effects were seen in the negative control protein bovine serum albumin (BSA). These results suggest that reduced Pdi1p can prevent the formation of K28 oligomers that are favored to occur under neutral pH conditions of the ER. However, considering the in vivo situation, Pdi1p is only partially present in a reduced conformation (Xiao *et al.*, 2004). To analyze toxin oligomerization under physiological conditions that mimic the ER environment, we chose a widely accepted condition with a total concentration of 10 mM glutathione (GSX = reduced glutathione [GSH] + 2× oxidized glutathione [GSSG]) at pH 7.2 and a GSH:GSSG ratio of 3:1 (Kim *et al.*, 1998; Delic *et al.*, 2010; Morgan *et al.*, 2013; Niu *et al.*, 2016). When Pdi1p was preincubated under such conditions and added to the toxin during the pH shift, K28 oligomer formation was effectively prevented (Figure 6C). Because K28 retained its α/β heterodimer conformation at high pH in the presence of Pdi1p, we asked whether such a toxin is biologically active. As shown in Figure 6D, K28 treated overnight with Pdi1p at pH 8.0 and subsequently shifted back to pH 5.0 lost its in vivo killing activity completely, indicating that incubation at high pH is likely to cause some irreversible structural/conformational change(s) in the protein that, although not detectable in nonreducing SDS gels, prevent in vivo killing of a target cell.

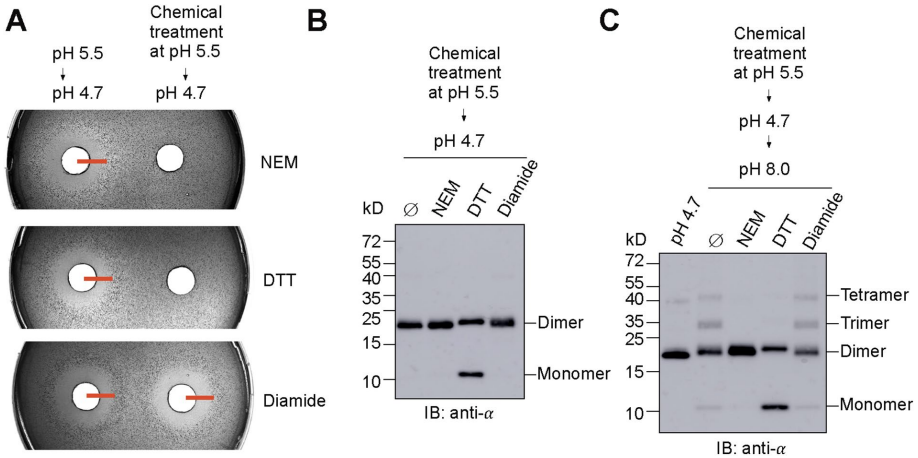

FIGURE 4: Thiol-modifying reagents affect K28 in vivo toxicity. (A) K28 toxicity and subunit profile after incubation at pH 5.5 and 20°C for 1 h in the presence of 200 mM NEM, 500 mM DTT, or 50 mM diamide. Chemicals were removed by dialysis against buffer of pH 4.7 and subsequently analyzed for in vivo toxicity against *S. cerevisiae* 192.2d. (B) Subunit structure of samples used in A was analyzed by nonreducing SDS–PAGE probed with anti-α. (C) Samples used in A were shifted to pH 8.0 and incubated at 20°C for 2 h, followed by nonreducing SDS–PAGE probed with anti-α.

DISCUSSION

Microbial, plant, and viral A/B toxins such as ricin, cholera toxin, botulinum toxin, Shiga toxin, and the yeast viral K28 toxin all use covalent disulfide bonding to connect the respective cytotoxic A/α subunit with the corresponding cell-binding B/β subunit(s) (Sandvig and van Deurs, 2002; Schmitt and Breinig, 2006). In this context, we demonstrate here for K28 that cysteine residues in its β subunit are also crucial for in vivo toxicity because either C/S substitutions in β or treatment with thiol-blocking NEM causes complete loss of K28 toxicity (Figures 3 and 4). In particular, the pronounced sensitivity of K28 to NEM supports that cysteine

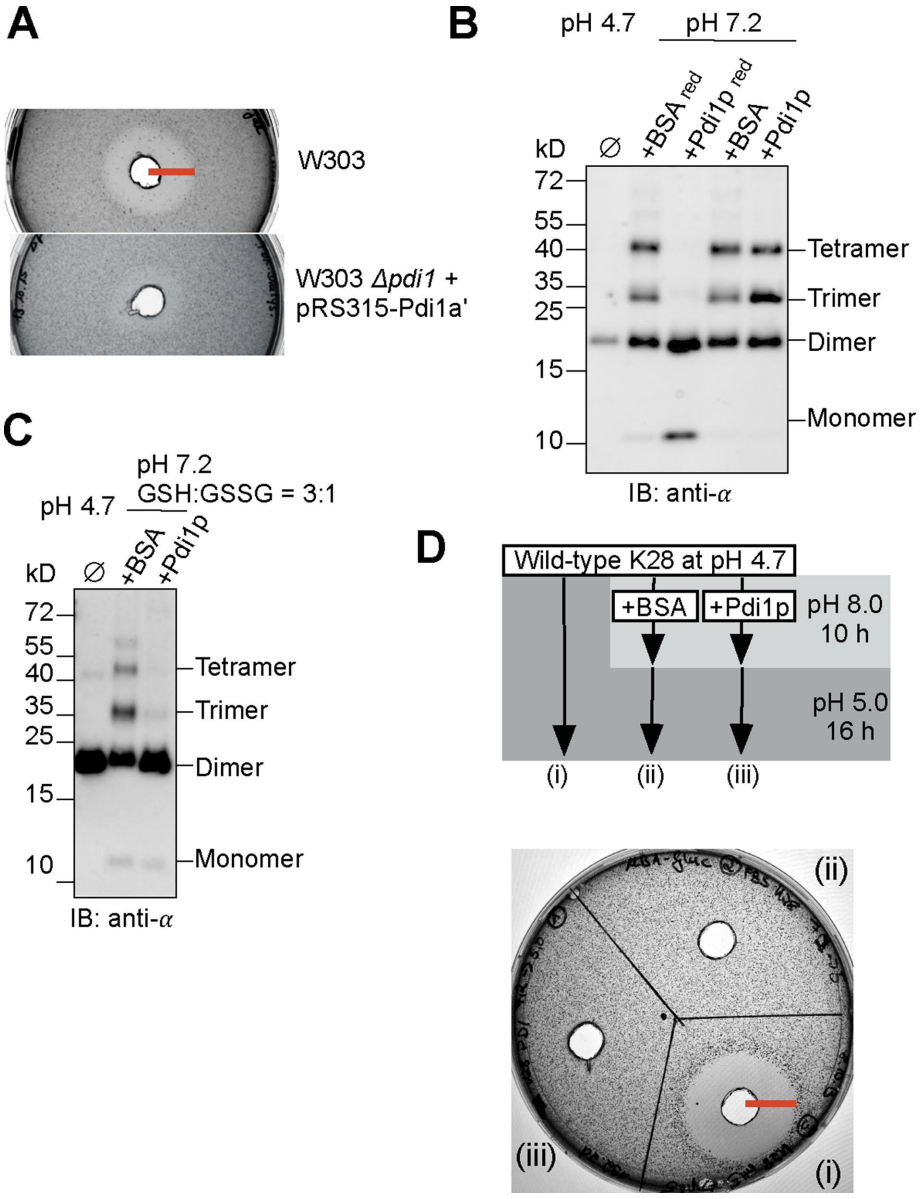

FIGURE 6: Pdi1p suppresses pH-triggered toxin oligomerization. (A) In vivo toxicity of wild-type K28 against *S. cerevisiae* W303 (wild type) and a Δ*pdi1* null mutant kept viable by expression of the a′ domain of Pdi1p. Red bar indicates the radius of growth inhibition caused by the toxin. (B) Pdi1p (5.6 μM) and BSA (5.6 μM) either untreated or reduced by pretreatment with immobilized TCEP for 1 h ($Pdi1p_{red}$, BSA_{red}) were incubated at pH 7.2 and 20°C for 30 min and subsequently analyzed for K28 oligomer formation by SDS–PAGE and immunoblot. (C) Pdi1p and BSA were pretreated in 10 mM glutathione (GSX = [reduced glutathione, GSH] + 2× [oxidized glutathione, GSSG]) at a GSH:GSSG ratio of 3:1 for 1 h at pH 7.2 and subsequently analyzed as in B. (D) Top, schematic outline of the experimental setup of K28 incubated at pH 8.0 and 20°C for 10 h in the presence of 50 μM BSA (ii) or 50 μM Pdi1p (iii) followed by dialysis at pH 5.0 for 16 h. K28 incubated at pH 5.0 and 20°C throughout the experiment served as control (i). All samples were tested for in vivo toxicity against *S. cerevisiae* 192.2d (bottom); red bar indicates the radius of growth inhibition caused by the toxin.

sulfhydryls in the toxin are important for its in vivo killing activity (Figure 4). At higher pH, thiols in proteins tend to be deprotonated and become nucleophilic thiolate anions, which can attack one of the sulfur atoms in a disulfide, resulting in disulfide bond rearrangement (Jensen *et al.*, 2009; Netto *et al.*, 2016). In the case of α/β heterodimeric K28, we observed such disulfide bond rearrangement between and within toxin molecules triggered by an increase in pH (Figure 1). Although K28 remained fully stable as disulfide-bonded α/β heterodimer at lower pH, it converted into tetramers, trimers, and monomers at pH >6. In addition to cysteine C333 in β, which forms the connecting disulfide with C56 in α (Figure 2), the toxin contains three additional cysteines in β (C292, C307, C340), which we showed are required for pH-dependent toxin oligomerization, release of the cytotoxic α monomer, and in vivo killing activity (Figures 1C and 3C).

Although the precise redox state of the α/β heterodimeric toxin and the identity of the attacking cysteine residue have yet to be determined, the present results allow us to propose a first speculative model that highlights the importance of toxin-intrinsic disulfide bond rearrangements: 1) Because β contains four cysteines, from which only a single cysteine is involved in disulfide bond formation with α, the number of reduced cysteines in β can be predicted to be either one or three. 2) Based on the observed effect of DTT at lower pH (Figure 4), at which the toxin is inactivated but a significant amount of toxin molecules remains in the α/β conformation, the existence of an intra-β disulfide must be predicted that is important for in vivo toxicity. Taking this in conjunction with the first point, it is conceivable that the β subunit of biologically active K28 contains a single free cysteine, an intrachain disulfide, and an interchain disulfide that covalently links both subunits in the α/β heterodimer.

Intracellular pH gradients are frequently exploited by pathogens during host cell intoxication. For example, a decrease in pH seems a common factor and possible host cell signal for certain viruses and toxins to sense endosomal localization and subsequently cross a biological membrane, either through conformational changes (as in polio, adeno, and influenza viruses and botulinum, anthrax, and diphtheria toxins) or by proteolytic activation (as in Ebola virus and reoviruses; Ren *et al.*, 1999; Lacy *et al.*, 2004; Marsh and Helenius, 2006; Inoue *et al.*, 2011; Sun *et al.*, 2011; Ravindran and Tsai, 2016). Our present results on K28 add another mechanistic variation, suggesting that the toxin can somehow sense an intracellular pH gradient although it is sensitive to higher pH. Of interest, the toxin-secreting K28 killer yeast was originally isolated from grapes and subsequent must fermentation (Pfeiffer and Radler, 1982), which represents a natural yeast habitat of low pH in the range between 3.4 and 3.7 that perfectly mirrors the optimal condition (pH 3.5) for K28 toxin production and killing activity (Pfeiffer and Radler, 1982). In contrast to this mildly acidic milieu in the extracellular environment, the toxin is exposed to a gradually increasing pH during intracellular transport

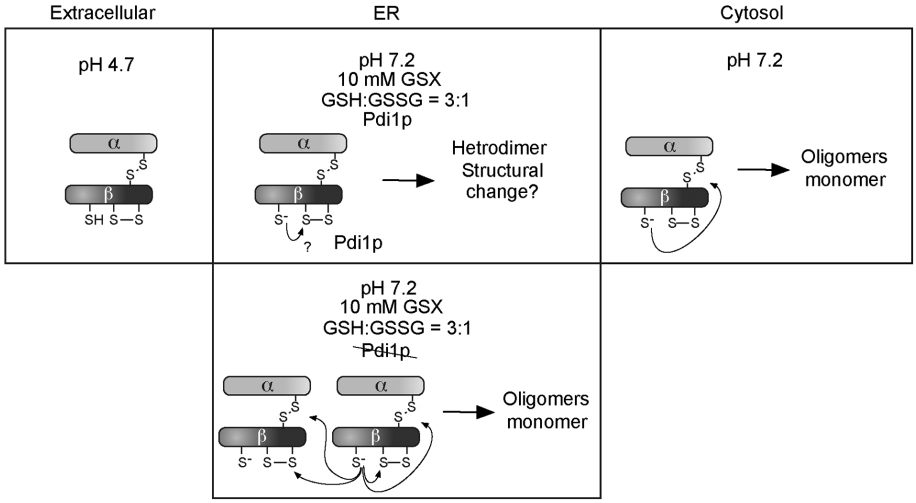

FIGURE 7: Model of pH-triggered, toxin-intrinsic thiol rearrangements on K28 subunit composition and in vivo toxicity. Under mildly acidic pH conditions in the natural environment of a toxin-secreting killer yeast (pH 4.7), K28 is biologically active and structurally stable as a disulfide-bonded α/β heterodimer. During host cell intoxication and intracellular toxin trafficking, K28 faces a continuous intracompartmental increase in pH, which deprotonates sulfhydryls in the β-subunit, subsequently causing the formation of toxin tetramers, trimers, and/or monomers. In vivo and in vitro, these disulfide bond rearrangements are efficiently prevented by the activity of Pdi1p, enabling the α/β heterodimer to exit the ER. In the pH-neutral environment of the cytosol, monomeric α is released from the heterodimer by nucleophilic attack of a reactive thiol in the β-subunit.

through a target cell, which, as we show here, would directly affect its thiol reactivity and ultimately lead to K28 oligomer formation and toxin inactivation. Such a scenario is efficiently prevented in vivo by the activity of ER luminal Pdi1p, which keeps the toxin in its α/β heterodimeric conformation (Figure 6, B and C). Based on the data presented here, it can be assumed that the toxin takes advantage of conformational stability in a low-pH environment and a thiol-activated state at higher pH during intracellular trafficking. We propose a model of host cell intoxication in which the intracellular pH gradient, ranging from mildly acidic conditions in endosomes and the late secretory pathway to neutral pH in the ER, contributes to activate and rearrange thiols in the α/β toxin, which finally generates the driving force to release the cytotoxic α-subunit in the cytosol (Figure 7). Although pH in the ER lumen is similar to the pH in the cytosol, Pdi1p activity is able to keep K28 in its α/β conformation and ensures that the toxin can cross the ER membrane in an oxidized conformation (Heiligenstein *et al.*, 2006). Because we show here that K28 maintained as α/β heterodimer by Pdi1p at pH 7.2 is nontoxic (Figure 6D), we speculate that the conformation of the α/β heterodimer in the ER is different from the α/β conformation in the extracellular environment, even in the presence of Pdi1p. Although the characterization of these structural changes awaits further work, such a mechanism seems unique among A/B toxins and viruses and adds a new repertoire of strategies for host cell penetration.

MATERIALS AND METHODS

Plasmids

Plasmids used in this work are listed in Supplemental Table S1. In vivo recombination was performed as described by Oldenburg *et al.* (1997).

Toxin production

Wild-type K28 toxin was produced from the superkiller strain *S. cerevisiae* MS300c (*MATα ura3-52 leu2 ski2-2 {M28 virus infected}*; Schmitt and Tipper, 1990). Plasmid-driven preprotoxin (pptox) expression of wild-type K28 and its epitope tagged or cysteine-to-serine mutant variants was performed by transforming the wild-type strain *S. cerevisiae* BY4742 (*MATα ura3Δ0 leu2Δ0 his3Δ1, lys2Δ0*) with vectors listed in Supplemental Table S1. For toxin production, yeast cultures (10 l) were grown for 5 d at 20°C in synthetic B-medium (pH 4.7) containing (per liter) 30 g glucose, 1.5 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2$, 40 mg inositol, 20 g malic acid, 0.5 g *tri*-Na citrate, 0.2 mg *p*-aminobenzoic acid, 0.02 mg biotin, 0.02 mg folic acid, 1.0 mg niacin, 1.0 mg pantothenic acid, 1.0 mg pyridoxine hydrochloride, 0.5 mg riboflavin, 0.5 mg thiamine hydrochloride, 2.0 mg H_3BO_3, 2.0 mg $FeCl_3$, 2.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg KI, 1.0 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $LiSO_4 \cdot 2H_2O$, 20 mg adenine, 20 mg uracil, 20 mg L-arginine, 100 mg L-asparagine, 20 mg L-histidine, 30 mg L-isoleucine, 100 mg L-leucine, 30 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 200 mg L-threonine, 20 mg L-tryptophan, 30 mg L-tyrosine, and 150 mg z-valine (Heerde and Radler, 1979). For *GAL1*-driven pptox expression from pYES plasmids, modified B-medium was used in which glucose was substituted by galactose and uracil was omitted. In each case, cells were harvested by centrifugation, and the cell-free culture supernatant was concentrated to a final volume of 100 ml by an ultrafiltration device (Sartorius; Amicon) with YM10 membranes and a molecular mass cutoff of 10 kDa (Schmitt and Radler, 1987). When necessary, the culture supernatant was further concentrated to a final volume of 1 ml by ultrafiltration through a Vivaspin (Sartorius) column, dialyzed against McIlvaine buffer (pH 4.7), and stored at −20°C.

In vivo toxicity/sensitivity assay

Killing activity of K28 samples against the sensitive strain *S. cerevisiae* 192.2d (*MATα ura3 leu2*), as well as toxin sensitivity of a particular yeast strain or transformant, was determined in an agar diffusion assay on methylene blue agar (MBA; pH 4.7) by using an overlay of 10^6 cells per plate (Schmitt *et al.*, 1996). Aliquots (100 µl) of a toxin concentrate were pipetted into wells (10 mm in diameter) cut into the agar, and plates were incubated for up to 5 d at 20°C. In each case, K28 toxicity and toxin sensitivity of cells are expressed by the radius of the resulting zone of growth inhibition around the well. In vivo toxicity of the α subunit and its cysteine-lacking mutant variant (C56S) was tested by the suicidal phenotype after *GAL1*-driven expression of ppα in the ER lumen (Heiligenstein *et al.*, 2006). Yeast cultures were grown overnight under noninducing conditions in the presence of glucose, and 10-fold serial dilutions were plated onto agar containing either galactose (inducing conditions) or glucose (repressing conditions) and incubated for 4 d at 30°C.

K28 subunit profile and oligomeric states

pH- and/or redox-dependent toxin oligomerization and subunit profile were analyzed by SDS–PAGE and immunoblot probed with polyclonal anti-α. Unless otherwise stated, SDS–PAGE was performed under nonreducing conditions. K28 samples were

prepared in Laemmli buffer (2% SDS, 60 mM Tris, pH 6.8, 10% glycerol, 0.0025% bromophenol blue) containing 100 mM NEM, heated for 5 min at 98°C, and subsequently applied onto the SDS gel. After electrophoretic separation, proteins were transferred to a polyvinylidene fluoride membrane by semidry blotting. For colorimetric signal detection, polyclonal anti–α subunit antibodies directed against the C-terminus of the toxin's α subunit and generated in rabbit were visualized by horseradish peroxidase–coupled anti-rabbit immunoglobulin G. After incubation with Western Lightning Plus ECL (PerkinElmer), signals were detected with ChemiDoc XRS (Bio-Rad). Polyclonal anti-α was used at a 1:1000 dilution.

pH dependence of K28 toxin

To study the pH and redox dependence of K28 toxin with respect to its in vivo killing activity and subunit structure, toxin samples were mixed with 1 M Tris/HCl buffer of different pH, final pH was measured with a microelectrode, and samples were subsequently used for in vivo toxicity and Western analysis. Where indicated, pH was shifted by buffer exchange through desalting columns (Zeba Spin 7K MWCO; Thermo Scientific) or by dialysis. In experiments that included redox-active chemicals, reactions were started by exchange buffer containing chemicals through desalting columns. After incubation at 20°C for 1 h, the chemicals were removed through dialysis. Recombinant Pdi1p was purchased from MCLAB. Reduced Pdi1p was prepared by incubation with immobilized TCEP (Thermo Scientific). Detailed procedures for the pH shift experiments and buffer exchanges are depicted in Supplemental Figure S3.

Toxin secretion analysis

Effects of toxin-intrinsic cysteine residues on the secretion level of fully processed α/β heterodimeric K28 were analyzed after cotransformation of *S. cerevisiae* BY4742 with the K28 pptox expression vectors pYX242-K28V5 and pYES-K28Cys/Ser. Cells were precultured overnight at 30°C in SCD medium lacking uracil and leucine (Ura/Leu dropout) containing 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% glucose, 20 mg/l adenine, 20 mg/l L-arginine, 100 mg/l L-asparagine, 20 mg/l L-histidine, 30 mg/l L-isoleucine, 30 mg/l L-lysine, 20 mg/l L-methionine, 50 mg/l L-phenylalanine, 200 mg/l L-threonine, 20 mg/l L-tryptophan, 30 mg/l L-tyrosine, and 150 mg/l L-valine. For induction of K28 pptox expression, cell pellets were washed with water, resuspended in 20 ml of B-medium containing 3% galactose but lacking uracil and leucine (Ura/Leu dropout) to an OD_{600nm} of 1.0, and incubated for another 5 d at 20°C until the culture reached a final OD_{600nm} of ~10. Cells were harvested by centrifugation, and the supernatant was passed through a 0.22-µm filter. Secreted proteins in the cell-free supernatant were precipitated by the addition of 10% trichloroacetic acid (TCA) in the presence of 0.02% sodium deoxycholate. After washing with 5% TCA, protein pellets were solved in 100 µl of Laemmli buffer, and 20-µl aliquots were used for SDS–PAGE, Western analysis, and signal quantification by ImageQuant TL version 8.1 (GE Healthcare Life Science). Statistical analyses were performed by using the software R (www.r-project.org).

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