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ORIGINAL PAPER

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Degradation of *Saccharomyces cerevisiae* Rck2 upon exposure of cells to high levels of zinc is dependent on Pep4

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Abstract In undisturbed cells, the MAPK-activated protein kinase Rck2 of *Saccharomyces cerevisiae* is a stable protein with a turnover time exceeding 60 min. However, we have found that Rck2 is subject to intracellular degradation after exposure of cells to Zn^{2+} concentrations of 5 mM or more. In high-zinc medium, most of the Rck2 pool is degraded within 5 min. This degradation is blocked by inhibiting the vacuolar proteolytic pathway with the protease inhibitor phenyl methyl sulphonyl fluoride or by mutation of the *PEP4* gene. By contrast, blocking the proteasomal pathway with the inhibitor MG132 does not prevent Rck2 degradation upon addition of Zn^{2+}, nor is degradation inhibited in the proteasomal mutations *pre1 pre2*, *cim3*, or *cim5*. The stability of Rck2 is not affected by any of the other stress conditions examined, or by growth rate. Possible mechanisms of the degradation of Rck2 under high zinc conditions, and its physiological significance, are discussed.

Keywords Yeast · Protein kinase · Stress · Protease · Vacuole

Introduction

Zinc is a trace element that is essential for all living cells. It is a component of a large number of metalloproteins, inducing changes in protein conformation and sometimes being required for catalysis. As a consequence, zinc deficiency results in the malfunction of a wide range of cellular components. Although zinc itself is redox-inert, existing only in the Zn^{2+} state under physiological conditions, excess zinc can cause oxidative stress. The mechanisms underlying this effect are not clear, but displacement of redox-active transition metal ions has been suggested as an important contribution. In particular, excessively high zinc concentrations may competetively inhibit the incorporation of iron into the active sites of enzymes, e.g., oxireductases (Martelli and Moulis 2004).

To maintain the cytoplasmic Zn^{2+} concentration within proper limits, the yeast *Saccharomyces cerevisiae* uses the zinc transporters Zrt1 and Zrt2, located in the plasma membrane, to take up zinc ions from the environment when zinc availability is low (Zhao and Eide 1996a, b). To prevent undue zinc import, Zrt1 is degraded in the vacuole when the extracellular Zn^{2+} concentration exceeds 2 mM (Gitan et al. 1998). The vacuole functions as a reservoir, from which zinc can be transferred to the cytoplasm by the transporter Zrt3. The vacuole also serves as the main buffer compartment to protect the cell against Zn^{2+} excess by removing and storing excess zinc from the cytoplasm. The zinc transporters Zrc1 and Cot1 are mainly located in the vacuolar membrane, and are responsible for transporting surplus Zn^{2+} into the vacuolar lumen (MacDiarmid et al. 2000, 2003).

Rck2 belongs to a family of fungal protein kinases that are phosphorylated by stress-activated MAP kinases, such as Hog1 of *S. cerevisiae* (Bilsland-Marchesan et al. 2000; Teige et al. 2001) or Sty1 of *Schizosaccharomyces pombe* (Asp and Sunnerhagen 2003; Sanchez-Piris et al. 2002; Smith et al. 2002). Mutants lacking *RCK2* are sensitive to certain oxidative stress agents, as well as to Cd^{2+} and Zn^{2+} . Overexpression of *RCK2* suppresses the sensitivity of *hog* mutants to *tert*-butyl hydroperoxide (*t*BOOH), Cd^{2+} and Zn^{2+}, whereas overexpression of *RCK2* in a wild-type background makes cells hyperresistant to Zn^{2+} . In a two-hybrid screen, Rck2 was found to interact with a number of membrane-bound metal ion transporters, including Zrc1 (Bilsland et al. 2004).

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In the present study, we investigate the fate of Rck2 under conditions of high-zinc stress. We find that Rck2 is quickly degraded when cells are exposed to external Zn^{2+} concentrations greater than 5 mM, and show that this degradation is dependent on the vacuolar protease Pep4.

Materials and methods

Strains and culture conditions

The strains of *S. cerevisiae* used are listed in Table 1. Cells were grown to mid-log phase in rich medium (YPAD) at 30°C unless indicated otherwise. Temperature-sensitive proteasomal mutants were grown at 25°C and shifted to 37°C for 3 h before protein extraction.

Exposure to stress agents and inhibitors

The chemical to be tested was added directly to the growth medium. Zinc was added as $ZnSO_4$ unless indicated otherwise. MG132 and phenyl methyl sulphonyl fluoride (PMSF) were dissolved in DMSO, and an equal volume of this solvent was added to the control samples. All other chemicals were dissolved in water. Aliquots were withdrawn from cultures at the indicated times thereafter, immediately chilled on ice, and protein was prepared as described below.

Protein preparation, electrophoresis and western blot

Cells were centrifuged and washed once in buffer A (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 0.2%Triton X-100) containing Complete protease inhibitor mix (Roche) supplemented with 1 mM PMSF. Cells were then lysed in the same buffer using a FastPrep 120 apparatus at a speed setting of 4 for 15 s.

For western analysis, aliquots of total protein (20 μg per lane) were fractionated by SDS-PAGE, and blotted onto Hybond P nitrocellulose filters (Amersham), in a semi-dry blotting device (Sigma Aldrich). Polyclonal anti-Rck2 (Ramne et al. 2000) and rat monoclonal anti-tubulin antibodies (Abcam) were used as primary antibodies. Antibody binding was detected with the ECL Western Blotting Analysis System (Amersham), using HRP-linked anti-rat monoclonal IgG (Amersham), or anti–rabbit Ig-POD F_{ab} fragment (Roche) as secondary antibodies.

Two-dimensional gel electrophoresis was done at the SWEGENE Proteomics Facility (Göteborg University).

Results

The level of Rck2 rapidly decreases upon exposure to high concentrations of zinc

We have previously shown that *rck2*Δ mutants are zinc-sensitive, while overexpression of *RCK2* makes cells hyperresistant to Zn^{2+} (Bilsland et al. 2004). We therefore wanted to follow the fate of the Rck2 protein under these circumstances. To our surprise, we found that exposure to levels of Zn^{2+} of 5 mM or higher caused Rck2 to virtually disappear from W303-1A cells within 5 min (Fig. 1a). The same phenomenon was observed in the BY4741 strain background (Fig. 1a), and zinc sulphate and zinc chloride both had the same effect (not shown). Upon prolonged exposure to high zinc levels (up to 1 h), the Rck2 concentration was seen to increase from its lowest level (Fig. 1b).

To determine to what extent this effect of zinc was specific to Rck2, we compared global patterns of protein staining, both by one-dimensional (not shown) and two-dimensional (Fig. 2a) gel electrophoresis. No consistent changes were detectable by either method, ruling out gross changes in a major fraction of abundant proteins.

Table 1 *S. cerevisiae* strains used

Name	Genotype	Source/reference
W303-1A	*MAT*a *ade2-1 leu2-3,112 ura3-1 his3-11 trp1-1a can100*	Stefan Hohmann
WΔ RCK1-L	*MAT*a *rck1::LEU2 ade2-1 leu2-3,112 ura3-1 his3-11 trp1-1a can100*	Dahlkvist and Sunnerhagen (1994)
WΔ RCK2-T	*MAT*a *rck2::TRP1 ade2-1 leu2-3,112 ura3-1 his3-11 trp1-1a can100*	Dahlkvist and Sunnerhagen (1994)
WΔ RCK1,2-LT	*MAT*a *rck1::LEU2rck2::TRP1 ade2-1 leu2-3,112 ura3-1 his3-11 trp1-1a*	Dahlkvist and Sunnerhagen (1994)
BY4741	*MAT*a *his3-*Δ 1 *leu2-Δ0 met15-Δ0 ura3-Δ0*	Brachmann et al. (1998)
Byrck2	*MAT*a *rck2::HIS3MX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
Byerg6	*MAT*a *erg6::kanMX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
Byhog1	*MAT*a *hog1::kanMX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
BYslt2	*MAT*a *slt2::kanMX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
Bytor1	*MAT*a *tor1::kanMX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
Bypkc1	*MAT*α *pkc1::kanMX6/PKC1 his3-Δ1/ his3-Δ1 leu2-Δ0/leu2-Δ 0 met15-Δ0/met15-Δ0 ura3-Δ0/ura3-Δ0*	EUROSCARF
WCG4a	*MAT*a *ura3 leu2-3,112 his3-11,15*	Valérie Goguel
WCG4-11/22a	*MAT*a *pre1-1 pre2-2 ura3 leu2-3,112 his3-11,15*	Valérie Goguel
A364a	*MAT*a *leu2-3,112 ura3-52 trp1-289 his3-Δ200*	Matthias Peter
A364-pep4	*MAT*a *pep4::TRP1 leu2-3,112 ura3-52 trp1-289 his3-Δ200*	Matthias Peter
S288c	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Matthias Peter
S288c cim3-1	*MAT*a *cim3-1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Matthias Peter
S288c cim5-1	*MAT*a *cim5-1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Matthias Peter

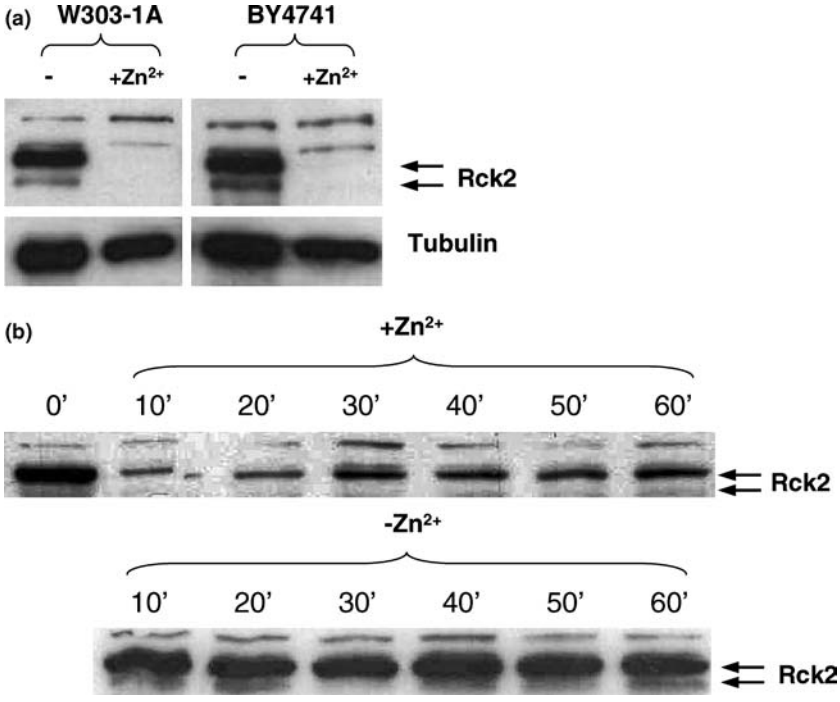


Fig. 1 **a, b** Disappearance of Rck2 after exposure to Zn^{2+}, as revealed by western blotting using anti-Rck2 antibodies. Of the two protein bands labelled as Rck2, the *upper*, major, band represents several unresolved species, some of which are phosphorylated (Bilsland et al. 2004; Bilsland-Marchesan et al. 2000); the *lower* band represents a partial degradation product. **a** Wild-type cells of two different genetic backgrounds. Cells were incubated for 5 min in the presence or absence of 5 mM Zn^{2+}. **b** Time course of recovery of Rck2. At time 0, a culture of wild-type W303-1A cells was split into two halves. One half was further incubated in the presence of 20 mM Zn^{2+} (*top row*), and the other half without added Zn^{2+} (*bottom row*). Cells were harvested at the indicated times thereafter

Moreover, the closely related protein Rck1 (which cross-reacts with the anti-Rck2 antibodies; Bilsland et al. 2004) was not affected by high Zn^{2+} (Fig. 2b). We therefore concluded that the observed effect is specific for Rck2.

Rck2 is a stable protein under undisturbed conditions

The decrease in Rck2 levels upon exposure to excess zinc could be mediated by several mechanisms. We wanted to assess how rapidly this protein turns over, and so measured its half-life by inhibiting its production using the protein synthesis inhibitor cycloheximide and observing the rate of decay of the Rck2 already present in the cell. As seen in Fig. 3, the level of Rck2 remains virtually unchanged over a period of 120 min in the presence of cycloheximide, and so we estimated its half-life under non-stressed conditions to exceed 60 min. This rules out the possibility that the rapid disappearance of Rck2 could occur without a major increase in its degradation rate.

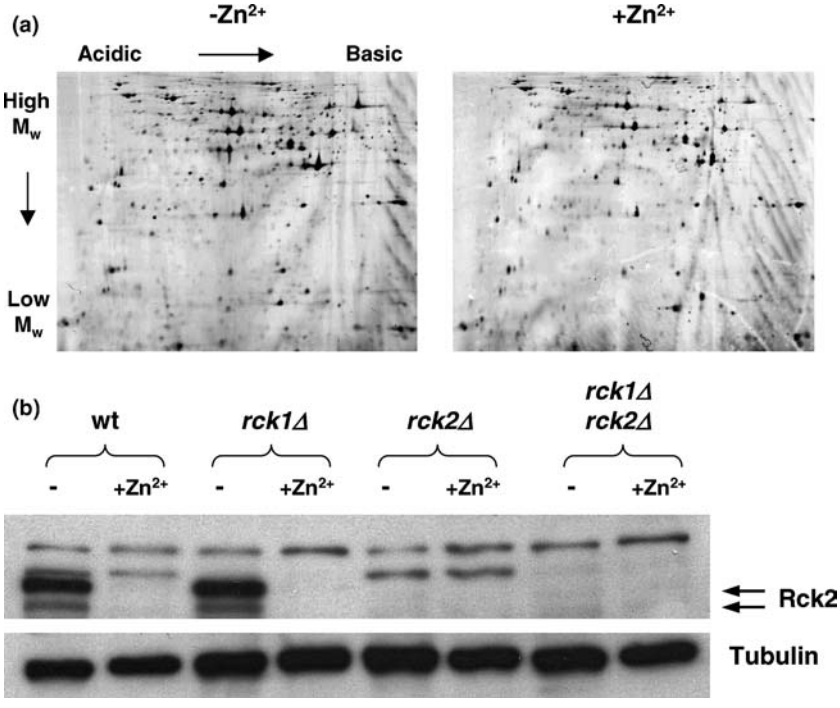


Fig. 2 **a, b** Specificity of zinc-induced Rck2 degradation. **a** Two-dimensional gel electrophoresis of total yeast proteins isolated from cells that had or had not been exposed to 5 mM Zn^{2+}. Gels were stained with SYPRO Ruby (Molecular Probes). **b** Rck1 is not degraded in the presence of high concentrations of zinc. Strains deleted for *RCK1* and/or *RCK2* were exposed to 5 mM Zn^{2+}. After blotting, the filter was probed with anti-Rck2 antiserum

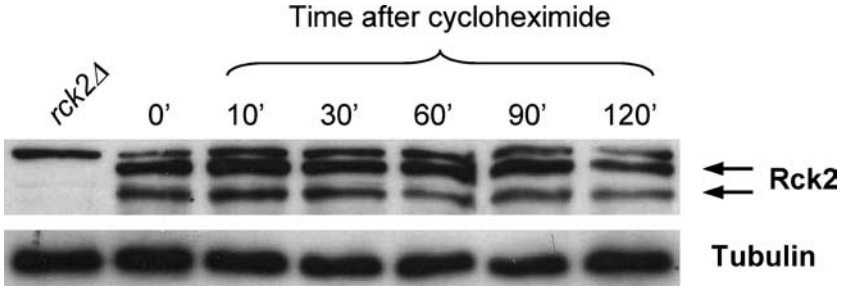


Fig. 3 Rate of turnover rate of Rck2 under undisturbed conditions, as analysed by western blotting. Cycloheximide (100 μg/ml) was added to the wild-type strain W303-1A at time 0 (second lane), and samples were withdrawn from the culture at the indicated times thereafter

Degradation of Rck2 does not occur as a general stress response or because of growth arrest

Mutants that lack the *RCK2* gene are sensitive to several stress conditions besides high Zn^{2+}, including the oxidative stress agents, *t*BOOH and Cd^{2+} (Bilsland et al. 2004). Overexpression of *RCK2* makes cells sensitive to diamide (Bilsland et al. 2004), while expression of the catalytically inactive *rck2*-kd allele causes sensitivity to hyperosmosis (Bilsland-Marchesan et al. 2000) and to *t*BOOH (S. Swaminathan et al. in preparation).

Because of the involvement of Rck2 in this wide range of stress conditions, we wanted to see if these conditions also induce degradation of this protein. We exposed cells to concentrations of stress agents high enough to effectively block cell growth. As seen in Fig. 4a, neither hyperosmotic shock, Cd^{2+}, oxidative stress caused by menadione, diamide or *t*BOOH, cell wall integrity stress due to Calcofluor white, nor inhibition of the TOR pathway by rapamycin, cause degradation of Rck2 in the same time interval. Likewise, neither perturbation of the stress-activated pathways represented by the MAP kinases Hog1 and Slt2 nor of the protein kinase C pathway is able to block Rck2 degradation in the presence of excess zinc (Fig. 4b). Disruption of the rapamycin-

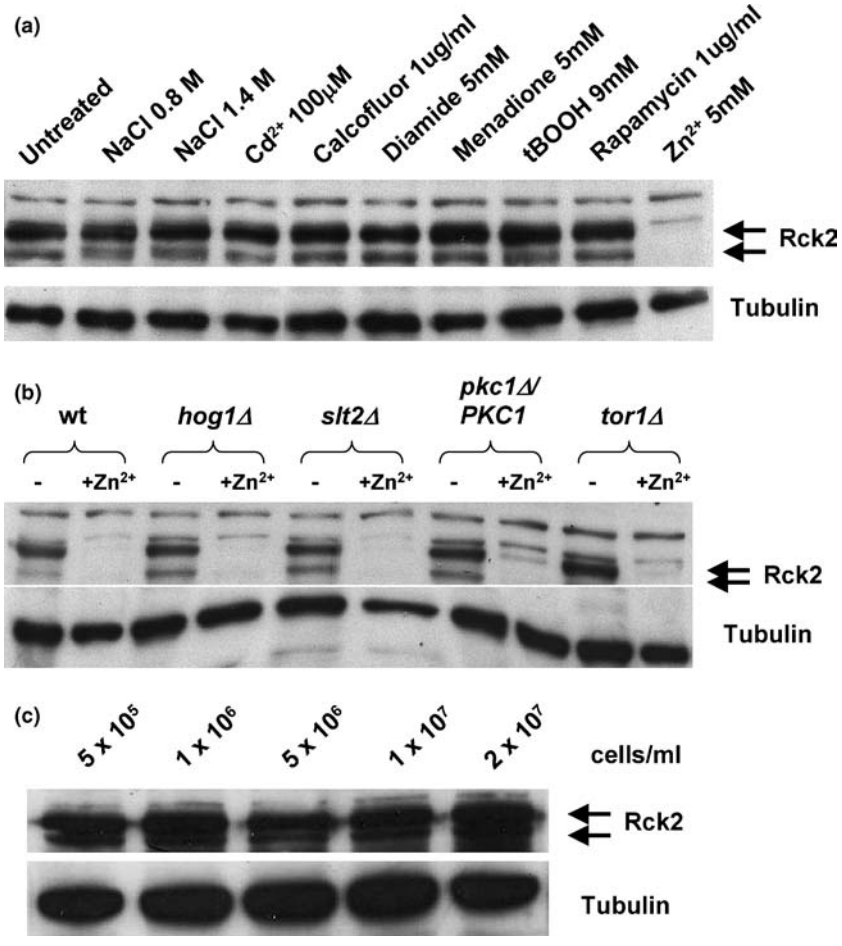


Fig. 4 **a–c** Effects of growth rate and exposure to other stress agents on the stability of Rck2, as assessed by western analysis. **a** The indicated compounds were added to wild-type W303-1A cells in mid-log phase, and the cells were harvested after 5 min. **b** Rck2 degradation in mutants deficient in stress signalling pathways. Wild-type BY4741 and isogenic gene deletion derivatives were exposed to 5 mM Zn^{2+} as above, and harvested after 5 min. Haploid cells were used, except in the case of *pkc1* mutants, where a heterozygous diploid strain was used instead. **c** Dependence on cell density. Wild-type W303-1A was grown to the cell densities indicated, and harvested. Protein extract volumes corresponding to equal numbers of cells were loaded on the gel

sensitive Tor1-containing TORC1 complex (Loewith et al. 2002) also has no effect in this regard (Fig. 4b, right).

Rck2, like mammalian MAPKAP kinases, has a role in post-transcriptional control, including regulation of translation (Mahtani et al. 2001; Smith et al. 2000; Teige et al. 2001; S. Swaminathan et al. in preparation). The translational apparatus is positively controlled by growth rate. Therefore, we also asked if growth phase or rate might influence the level of Rck2, as it does for Cln3, for example (Polymenis and Schmidt 1997). However, our experiments revealed that the amount of Rck2 per cell is not significantly affected by cell density (Fig. 4c).

Fig. 5 a–e Degradation of Rck2 is inhibited by blocking the Pep4-dependent vacuolar proteolytic pathway, but not the proteasomal pathway. Cell extracts were fractionated by SDS-PAGE, and analysed by western blotting. **a** Mutation of *PEP4*. The strains used were A364a (wt) and its isogenic *pep4*Δ derivative. **b** Inhibition of the vacuolar proteolytic pathway with PMSF. Experiments were done in the *erg6*Δ derivative of BY4741; PMSF was added to a final concentration of 1 mM at 2 h prior to the addition of Zn^{2+} . **c** Mutation of *PRE1* and *PRE2*. The strains used were WCG4-a (wt) and its isogenic *pre1-1 pre2-2* derivative. Cells were first grown at 25°C, and shifted to 37°C at 3 h prior to the addition of Zn^{2+} . **d** Inhibition with MG132. Experiments were done in the *erg6*Δ derivative of BY4741; MG132 was added to a final concentration of 0.2 mM. **e** Mutation of *CIM3* and *CIM5*. The strains used were S288c (wt) and its isogenic *cim3-1* and *cim5-1* derivatives. Cells were first grown at 25°C, and shifted to 37°C at 3 h prior to addition of Zn^{2+}

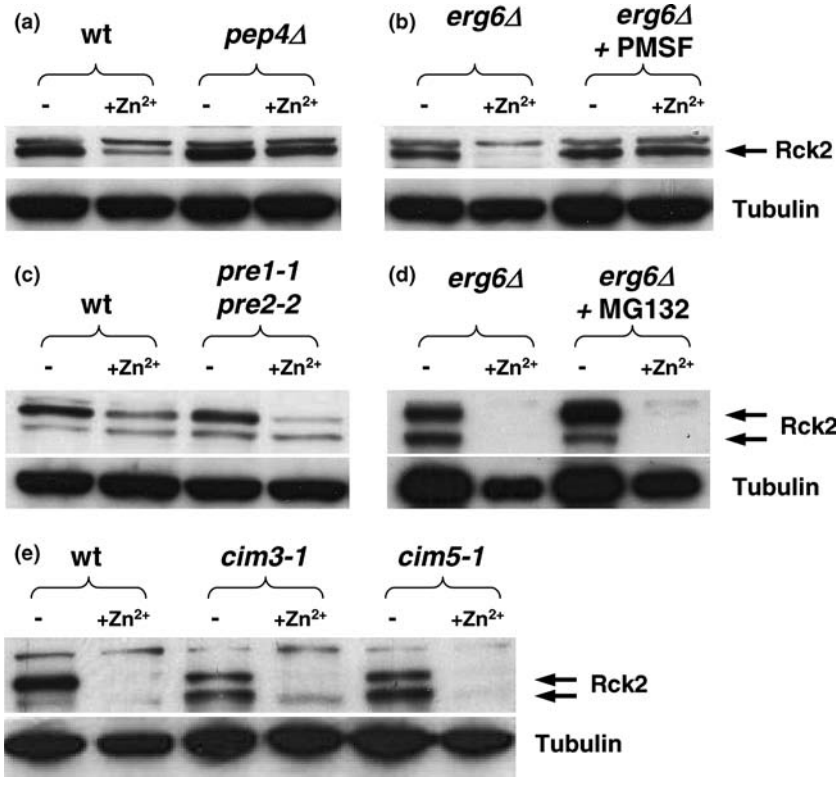
Degradation of Rck2 upon exposure to high levels of zinc is dependent on vacuolar Pep4, but not on the proteasomal, pathway

To identify the pathway(s) responsible for the degradation of Rck2, we used a combination of proteolysis inhibitors and mutants deficient in the major proteolytic pathways. As seen in Fig. 5a and b, blocking the Pep4-dependent vacuolar proteolysis pathway, either by the addition of 1 mM PMSF prior to exposure to Zn^{2+}, or by performing the experiment in a *pep4*Δ background, prevents Rck2 degradation after exposure to 5 mM $ZnSO_4$.

To explore the influence of blocking proteasome function, we exposed temperature-sensitive mutants deficient in the proteasomal degradation pathway to excess zinc. We used *cim3-1* and *cim5-1* mutants, which are defective in AAA-type ATPases of the regulatory proteasome subunit (Ghislain et al. 1993), and the double mutant *pre1-1 pre2-2*, which is defective in *β*-type subunits of the catalytic 20S portion of the proteasome (Heinemeyer et al. 1993). Furthermore, we tested the effect of proteasomal inhibitor MG132 (at 0.2 mM). As can be seen in Fig. 5c–e, none of these mutations or treatments prevented Rck2 degradation upon zinc exposure, indicating that the proteasomal pathway is not the major route for zinc-induced Rck2 degradation.

Discussion

We have shown that exposure of yeast cells to high external concentrations of Zn^{2+} causes Rck2 levels to



drop precipitously, followed by a slow recovery. The rapid disappearance of Rck2 protein after exposure to Zn^{2+} immediately implicates protein degradation as the major factor behind this phenomenon. Since the half-life of Rck2 under normal conditions exceeds 1 h, a down-shift in Rck2 concentration to less than 20% of the initial level within 5 min indicates a drastic destabilisation of the protein. This is corroborated by the finding that Rck2 is stabilised by the protease inhibitor PMSF, and in mutants lacking the protease Pep4. An involvement of other regulatory mechanisms at the transcriptional and post-transcriptional levels has not been excluded, but they cannot make a major contribution to this rapid decline because of their slower mode of action.

In order to be degraded by the vacuolar pathway, Rck2 must be translocated from the cytoplasm to the vacuole. Besides the association between Rck2 and the vacuole membrane-located zinc transporter Zrc1 demonstrated by Bilsland et al. (2004), a physical interaction has also been found between Rck2 and Vps41 (Ho et al. 2002). Vps41 is a GDP/GTP exchange factor with specificity for Rab-type G proteins, is located in the vacuolar membrane, and is implicated in the sorting of proteins to the vacuole (Radisky et al. 1997). It is thus conceivable that at least a portion of the Rck2 pool exists in a complex with proteins near the vacuolar membrane, and that this could explain its exposure to vacuolar proteases following zinc shock. Deletion of *ZRC1* does not affect Rck2 degradation, however, nor is the level of Zrc1 altered under the high-zinc conditions used in this work (data not shown). Considering that cells which overexpress *RCK2* are hyperresistant to high zinc, it is not obvious why Rck2 degradation would be protective under the same conditions. Alternatively, the degradation could be the result of zinc toxicity, possibly mediated by zinc competing with iron for enzyme binding sites. This notion is supported by the fact that high external zinc concentrations induce the iron-responsive Aft1 regulon (Lyons et al. 2004), and that Vps41 is required for iron transport (Radisky et al. 1997). Many conformational changes in proteins are known to be caused by Zn^{2+}, and such extensive changes could make Rck2 susceptible to degradation. Moreover, Rck2 plays a role in the cellular stress response at the translational level (Teige et al. 2001; S. Swaminathan et al. in preparation), and it is conceivable that this response induces large-scale intracellular movements that, in the case of zinc stress, transports Rck2 near the vacuolar membrane, actively or passively.

Among the various classes of proteases of the vacuolar degradation pathway, carboxypeptidases such as Cps1 and Lap4 are activated by metal ions including Zn^{2+}. However, Pep4 itself does not belong to this class of metal ion-activated proteases, nor do most of the proteases that depend on Pep4 for conversion into an active form (Jones 1991). The sole exception is Lap4, the activity of which is reduced in *pep4* mutants (Jones 1991; Klionsky et al. 1992). Besides Pep4, Lap4 is a possible candidate for the protease directly responsible for the degradation of Rck2. However, in a *lap4*Δ mutant, Rck2 degradation is not affected (data not shown). Unequivocal assignments of protein substrates for Lap4 is not possible on the basis of published in vivo data, probably due to the predominance of Pep4 in total vacuolar protease activity, so this result does not rule out a minor involvement of Lap4.

It could be argued that excess zinc might cause intracellular degradation of many proteins. However, we found no indications for this using two-dimensional protein gels. This does not exclude the possibility that selected proteins other than Rck2 are degraded. The specificity of the degradation process is also strongly suggested by that fact that the closely related paralogue Rck1 is not affected by high zinc levels; in agreement with this, Rck1 has not been implicated in resistance to high zinc.

Neither growth arrest nor activation of a general stress response is sufficient to explain the rapid degradation of Rck2. We used concentrations of stress agents that not only induce transcription of stress response genes and other stress responses, but also halt cell growth and division, and result in high cell lethality during prolonged exposure. Growth at elevated temperature (37°C), even for several hours, likewise had no effect on Rck2 levels (Fig. 5c, e). Furthermore, the steady-state level of Rck2 is constant over a wide range of cell densities (Fig. 4c). This demonstrates that the rapid cellular response with respect to Rck2 is specific to conditions of excess Zn^{2+}.

To our knowledge, this is the first reported case where a non-zinc transporter in yeast is degraded upon exposure to excess zinc. Further work is required to elucidate the physiological relevance of this phenomenon. Given the roles of Rck2 in the regulation of translation, as well as its direct interactions with membrane-bound metal ion transporters, its degradation would be expected to have a broad range of effects under these particular conditions of stress.

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