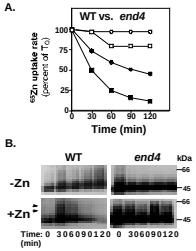
Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter

Raad Gitan and David Eide
Dept. of Nutrional Sciences, University of Missouri-Columbia

Abstract

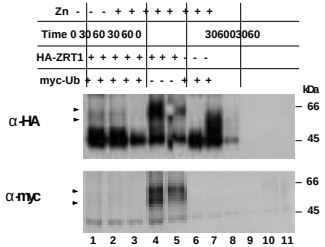
The yeast ZRT1 zinc transporter is regulated by zinc at both transcriptional and post-translational levels. At the post-translational level, zinc inactvates ZRT1 by inducing the removal of the protein from the plasma membrane by endocytosis. The zinc transporter is subsequently degraded in thevacuole. This regulatory system allows for the rapid shutoff of zinc uptake activity in cells exposed to high zinc, thereby preventing overaccumulation of this potentially toxic metal. In this report, we examine the role of ubiquitin conjugation in this process. First, we show that ZRT1 is ubiquitinated shortly after zinc treatment and prior to endocytosis. Second, mutations in various components of the ubiquitin conjugation pathway, specifically the RSP5 ubiquitin-protein ligase and the UBC4 and UBC5 ubiquitin conjugating enzymes, inhibit both ubiquitination and endocytosis. Finally, mutation of a specific lysine residue in ZRT1 blocks both ubiquitination and endocytosis. This critical lysine, K195, is located in a cytoplasmic loop region of the protein and may be the residue to which ubiquitin is attached. These results demonstrate that ubiquitin conjugation is a critical step in the signal transduction pathway that controls the rate of ZRT1 endocytosis in response to zinc.

ZRT1 is modified in response to zinc and prior to endocytosis.



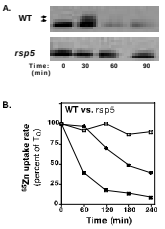
A) WT (squares) and *end4* mutant (circles) cells expressing HA-tagged ZRT1 were grown to exponential phase. The cells were harvested and resuspended in media with 100 µg/ml cyclohexamide and either 2 mM $ZnCl_2$ (+Zn, closed symbols) or 1 mM EDTA (-Zn, open symbols). At the indicated times, cells were harvested and assayed for Zn uptake rate.
B) Total cell homogenates were prepared from cells grown as described in A and analyzed by immunoblotting. Modified forms of *ZRT1* are indicated by arrowheads and the position of molecular-mass markers are shown on the right.

ZRT1 is ubiquitinated in response to zinc



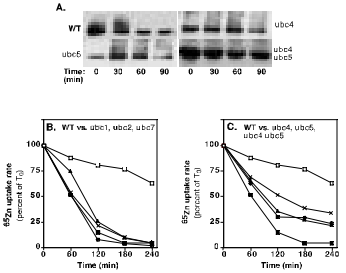
Wild type cells expressing HA-tagged ZRT1 or wild type ZRT1 and either myc-tagged ubiquitin or wild type ubiquitin were harvested at zero time and inoculated into media with 2 mM $ZnCl_2$ (+Zn) or 1 mM EDTA (-Zn) for the indicated times. Cells homogenates were prepared, immunoprecipitated using anti-HA antibodies and analyzed by immunoblotting using anti-HA or anti-myc antibodies. Modified forms of ZRT1 are indicated by arroheads and the position of molecular-mass markers are shown on the right.

Ubiquitination and endocytosis of ZRT1 in response to zinc requires the RSP5 ubiquitin-protein ligase.



A) Wild type and *rsp5* mutant cells expressing HA-tagged ZRT1 were grown to exponential phase. The cells were harvested and resuspended in medium supplemented with 100 mg/ml cyclohexamide and 2 mM $ZnCl_2$. At the indicated times, total cell homogenates were prepared and analyzed by immunoblotting. Modified forms of ZRT1 are indicated by arrowheads.
B) Cell grown as described in A were harvested and assayed for Zn-uptake rate. Filled squares, zinc-treated wild type cells; filled circles, zinc treated *rsp5* cells. Data obtained for wild type cells incubated without zinc are shown for comparison; uptake rates in zinc-limited *rsp5* cells were similar.

UBC4 and UBC5 E2 enzymes are responsible for ubiquitination and endocytosis of ZRT1 in response to zinc.



A) Wild type, *ubc4*, *ubc5* and *ubc4/ubc5* mutant cells expressing epitope-tagged ZRT1 were grown to exponential phase in medium containing 1 mM EDTA. The cells were harvested and resuspended in medium supplemented with 100 µg/ml cyclohexamide and 2 mM $ZnCl_2$. At the indicated times, total cell homogenates were prepared and analyzed by immunoblotting.
B) Cells grown as described for A were harvested and assayed for Zn-uptake rate. Symbols are wild type (filled squares), *ubc1* (filled circles), *ubc2* (filled triangles) and *ubc7* (**X**). Data obtained from wild type cells incubated without zinc are shown for comparison; analysis of uptake rates in zinc-limited *ubc* mutants gave similar results.
C) Conditions are the same as described for B. Wild type (filled squares), *ubc4* (filled circles), *ubc5* (filled triangles), *ubc4/ubc5* (**X**) and wild type incubated without zinc (open squares).

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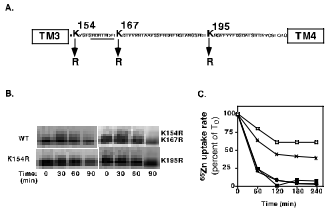
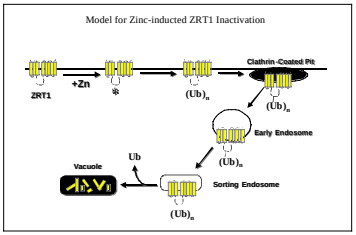
Lys-195 is required for zinc-dependent ubiquitination and endocytosis of ZRT1



A) The amino acid sequence of wild-type and mutant ZRT1 proteins between transmembrane domains 3 and 4. The potential metal binding domain is underlined.
B) Wild type cells expressing wild type or mutant *ZRT1* alleles were grown to exponential phase. The cells were harvested and resuspended in medium supplemented with 100 µg/ml cyclohexamide and 2 mM $ZnCl_2$. At the indicated times, total cell homogenates were prepared and analyzed by immunoblotting.
C) Cells, grown as described for B were harvested and assayed for Zn-uptake rate. Symbols are wild type (filled squares), K154R (filled triangles), K154R/K167R (filled circles) and K195R (**X**). Data obtained from cells expressing the wild type protein incubated with Zn (open squares) are also shown for comarison; analysis of uptake rates in zinc-limited cells expressing *ZRT1* mutant alleles gave similar results.

Summary



Previously, we identified the high-affinity zinc transporter from *S. cerevisiae*, *ZRT1*, and demonstrated that it is rapidly endocytosed in response to zinc. Here we show ZRT1 is ubquitinated prior to endocytosis, probably sending the ZRT1-ubiquitin complex through an endosome-mediated degradation process.

Future Applications

Many other organisms, including plants like *Arabidopsis thaliana*, contain metal transporters similar to ZRT1. For example, the Arabidopsis root-specific iron regulated transporter IRT1 is also post-transcriptionally regulated, perhaps by similar mechanisms. The knowledge we gain from studying these transporters in the model systems *Sacharomyces cerevisiae* and *Arabidopsis thaliana* will help us design plants that can readily take up metals from containminated soils in a process termed phytoremediation. Humans have transporters similar to ZRT1 and IRT1 so ultimately our research will lead to a better understanding of metal ion uptake and homeostasis in humans. Such an understanding is essential for assessing the risk to human health from exposure to ingested metals.