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Low Ctr1p, due to lack of Sco1p results in lowered cisplatin uptake and mediates insensitivity of rho0 yeast to cisplatin

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ABSTRACT

Copper and cisplatin share copper transporter 1 (Ctr1) for cellular import. Copper depletion increases sensitivity of wild type yeast to cisplatin, whereas mitochondrial DNA-deficient rho0 cells are resistant to cisplatin. In the current study, we sought to determine whether copper deprivation modulates sensitivity of rho0 yeast to cisplatin. Yeast cultures grown in low copper medium and exposed to bathocuproine disulfonic acid resulted in significant reduction of intracellular copper. We report here that low copper medium rendered wild type hypersensitive to cisplatin, but failed to sensitize rho0 yeast to cisplatin. Wild type yeast grown in low copper medium exhibited ~2.0 fold enhanced cytotoxicity in survival and colony-forming ability compared to copper adequate wild type cells. The effect of copper restriction on cisplatin sensitivity was associated with upregulation of copper transporter 1 mRNA as well as protein, facilitating enhanced uptake and accumulation of cisplatin. Rho0 yeast also showed increased copper transporter 1 mRNA upon copper restriction, but failed to increase corresponding protein. Loss of synthesis of cytochrome *c* oxidase 1 protein (Sco1) in rho0 cells deregulated copper transporter 1, impaired Pt uptake and lowered cytotoxicity, despite lowered glutathione levels. Sco1Δ mutants exhibited low copper transporter 1, reduced Pt accumulation suggesting that Sco1 mediated regulation of copper transporter 1 is responsible for altered sensitivity to cisplatin. Rho0 cells demonstrated loss of Sco1, resulting in copper deficiency by lowering copper transporter 1 abundance, via mechanism involving increased turnover due to ubiquitination. These findings reveal that a Sco1-dependent mitochondrial signal regulates cellular cisplatin import and cytotoxicity.

1. Introduction

Cisplatin (*cis*-diamine dichloro platinum, CDDP) is a widely used chemotherapeutic agent that induces apoptosis of the tumor cells [1]. Cisplatin loses chloride moieties in aqueous medium and enters the cell through a high affinity copper transporter 1 (Ctr1) [2]. Once inside the cell, the positively charged platinum (Pt) reacts with DNA and forms cross-links and adducts. The formation of DNA adducts induces nucleotide excision repair mechanism, but few Pt-DNA adducts cannot be repaired and accumulation of these adducts correlates with CDDP cytotoxicity [3]. The DNA lesions induced by cisplatin impair DNA replication, induce a G_2 phase cell cycle arrest, inhibit RNA transcription and finally promote cell death through apoptosis [4]. However, many tumor cells can overcome the cytotoxic action of cisplatin by exhibiting decreased drug uptake and accumulation, increased efflux, reduced platination, enhanced DNA repair and defective cell death pathways [5]. These resistance mechanisms contribute to the reduced effectiveness of cisplatin and therefore needs better understanding.

Expression of this Ctr1 transporter is regulated by intracellular copper levels, through metal binding activator 1 (Mac1) and activator of CUP1 expression (Ace1) [6,7]. Yeast also expresses two additional Ctr family members, Ctr2 and Ctr3. Ctr3 is functionally redundant to Ctr1; however, Ctr3 expression in many yeast strains is blocked by a transposition element [8]. Ctr1 transports Cu^+ with a K_m of 1–5 μM [2,9]. After copper is transported across the cell membrane, it is incorporated into copper-requiring proteins via copper-delivery molecules and assembly factors [10–12]. Ctr2 mediates translocation of copper ions across the vacuolar membrane [13]. The *SCO1* (synthesis of cytochrome *c* oxidase) gene of *Saccharomyces cerevisiae* codes for a 30 kDa integral membrane protein of the inner mitochondrial membrane, which is required for post-translational assembly of cytochrome *c* oxidase subunits 1 and 2 (COX I and COX II) [14], and helps in

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sustaining the Ctr1 expression [15]. Ctr1p is rapidly degraded in the absence of Sco1p, and inhibition of the proteasome is able to restore the Ctr1p levels. Sco1p depletion causes copper deficiency by attenuating Ctr1p expression. Ablation of SCO1 expression does not grossly affect CTR1 mRNA levels, suggesting that SCO1 may be required to regulate the abundance of CTR1 post-transcriptionally [15].Our earlier studies clearly demonstrated that cisplatin uptake in wild type yeast can be enhanced by employing bathocuproiene disulfonate (BCS), a copper chelator and that copper deprivation can sensitize the cells to cisplatin-induced cytotoxicity by enhancing cisplatin uptake and accumulation as well as by lowering the antioxidant defense [16,17]. Cisplatin assumes positive charge inside the cell, tending to accumulate in the negatively charged mitochondrial compartment and reacts with nucleophilic sites to form DNA cross-links [18]. In addition, the mitochondrial density also plays an important role in determining the sensitivity of cells to cisplatin, although mitochondria are not a direct target of cisplatin [19]. However, the cytotoxic action of cisplatin was found to be dependent on mitochondrial respiration as well as its functionality. Rho^0 cells (lacking mitochondrial DNA) deficient in respiration are less sensitive and resistant to cisplatin [20,21]. It was found that the Rho0 cells generate relatively low levels of reactive oxygen species, due to the absence of functional respiration. One important feature of platinum resistance in vitro is decreased accumulation, involving decreased uptake and altered endocytic processes [22].

Decreased cytotoxicity of cisplatin has often been correlated to increase in intracellular levels of thiol-containing proteins and antioxidants, that are capable of binding cisplatin and preventing its interaction with DNA [23]. Glutathione is one of the intracellular thiols, which is ubiquitous. Among the total cellular pool, majority of glutathione resides in the cytosol and mitochondria [24]. Reduced glutathione (GSH) is synthesized in the cytosol and transported to the mitochondria [25]. Evidence suggests that intracellular copper binds to GSH, before binding to the chaperones or sequestered by metallothionein (CUP5). Therefore, excess copper can perturb cellular glutathione balance [26]. Considering the fact that glutathione showed more affinity towards copper (II) than platinum (II) [27], it was interesting to study the alterations in GSH (reduced)/GSSG (oxidized) ratio, if any in wild type and rho0 cells with depleted copper status and treated with cisplatin.

It is still not known if the rho0 cells show altered Ctr1 expression and uptake of cisplatin. Thus in the present study, we investigated the Ctr1 expression and cisplatin uptake in wild type and Rho0 yeast cells. We have also determined if copper chelation using BCS can increase Ctr1 expression and thereby modulate cisplatin uptake, accumulation and cytotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Yeast extract, peptone, glucose were obtained from HiMedia (Mumbai, India), unless otherwise mentioned. Bactoagar was obtained from Difco, BD Biosciences, NJ. Bathocuproiene disulfonate (BCS) was obtained from Sigma Chemical Co. (St. Louis, MO). Cisplatin was obtained from Dabur Pharmaceuticals, India. All other chemicals were of analytical grade and were obtained from local sources. Sco1 antibody was from ThermoFisher Scientific (PA5-44581). Ctr1 antibody was from Santa Cruz Biotechnology Inc. (Ctr1 (yP-17):sc-26160).

2.2. Growth conditions of wild type BY4741

The *Saccharomyces cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) strain used in this study was a generous gift from Dr. Anand Bachchawat, IMTECH, Chandigarh, India. The BY4741 strain contains a wild type copy of the *CTR1* gene. BY4741sco1-Δ(MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR037c::kanMX4) was procured from Euroscarf. SD medium without copper (2% glucose, 0.17% yeast nitrogen base without Cu and Fe, 0.5% ammonium sulfate, amino acid, 1 μM $FeCl_3$), but containing 100 μM BCS was used as low copper medium (LCM).Yeast cultures were inoculated in standard yeast extract/peptone/dextrose (YPD) (1% yeast extract, 2% peptone and 2% glucose) or LCM overnight at 30 °C and were seeded from single colonies grown on respective solid media. Effective copper concentration of LCM was < 50 nM compared to the 250 nM in YPD. Growth of experimental cultures was initiated at A_{600nm} = 0.05; the cultures were allowed to grow to A_{600nm} = 1.0 (log phase, 1.5 × 10^7 cells/mL) before use.

Rho0 strain without mitochondrial DNA was generated by growth with ethidium bromide (10 mg/mL) for 3 days in rich medium [YPD: 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose]. Individual colonies were streaked in parallel on YPD and YPEG [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (v/v) ethanol, 3% (v/v) glycerol]. Cells of the rho0 strain grow only on YPD plates [28]. Loss of mitochondrial DNA was confirmed by staining the DNA with 4′,6-diamidino-2-phenylindole.

2.3. Endocytosis-defective Δend3 mutants

To generate endocytosis defective end3Δ mutants, TSY260 (BY4741 end3Δ::natNT2) mutants on wild type and rho0 background, polymerase chain reaction (PCR)-based gene disruption method was used [29]. Disruption cassettes amplified by PCR with pFA6-natNT2 (vector for C-terminal tagging of yeast genes and a nourseothricin selection marker) using oligonucleotide primers END3-S1 (TATTGGAAAGGC CGG TAAA GATAACAGGGATCTCTGAAAACAGCTGAAGCTTCGTACGC TGC), END3-S2 (AAATATTACACATTCATGTACATAAAATTAATTATC GGTGGCATAGGCCACTA GTGGATCTG) were integrated into the gene locus. Gene disruptants were selected on YPD medium containing nourseothricin (clonNAT). DNA transformations were performed using the lithium acetate procedure [30]. Cells were propagated on agar plates with the appropriate SC dropout medium and grown overnight at 30 °C in liquid medium to OD_{600nm} −1.0 (0.5 < OD < 1.5) for experiments.

2.4. CTR-Myc expression

Plasmid 352-CTR-myc carries the CTR1-myc (myelocytomatosis) gene under the control of its native promoter in the 2μ yeast episomal plasmid vector, YEp352, as previously described [2]. To introduce the Myc epitope in the CTR1 coding region, complementary oligonucleotides were generated for a DNA fragment encoding the 10 amino acids of the epitope (EQKLISEEDL) with flanking *Eco*RI sites [31]. The oligonucleotides were annealed and cloned into the *Eco*RI site of the CTR1 sequences contained in the vector pUCEF. Orientation of the inserted epitope and correctness of the reading frame were verified by DNA sequence analysis. The tagged CTR1 was then transferred to the vector YEp352 [32] to create plasmid 352-myc. Cells transformed with plasmid bearing CTR1 under the control of its native promoter were grown in low copper medium supplemented with 10 μM of the copper chelator, BCS.

2.5. Localization of Green Fluorescent Protein (GFP) tagged CTR1

Plasmid p426-GAL1-*CTR1-GFP* is a yeast expression vector with GFP-CTR1 expression from the galactokinase (GAL1) promoter tightly repressed by glucose and strongly induced by galactose. The vector was constructed by amplifying the *CTR1* open reading frame from genomic DNA using *Pfu* Turbo polymerase and the primers CTR1 atgS (5′-GGA CCCGGGATGGAAGGTATGAATATGGGT-3′) and CTR1 tNPH (5′-CGGC TTTTTAAGTGAGTATTGCCGCCGGCGATTGACGTCTTCGAACCC-3′).
The polymerase chain reaction fragment was cloned into the *Sma*I and *Hin*dIII sites on p426-Gal1 [33] and the GFP tag inserted into a *Not*I site