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Chromate-induced sulfur starvation and mRNA mistranslation in yeast are linked in a common mechanism of Cr toxicity

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Abstract

Toxicity of the environmental carcinogen chromate is known to involve sulfur starvation and also error-prone mRNA translation. Here we reconcile those facts using the yeast model. We demonstrate that: (i) cysteine and methionine starvation mimic Cr-induced translation errors, (ii) genetic suppression of S starvation suppresses Cr-induced mistranslation, and (iii) mistranslation requires cysteine and methionine biosynthesis. Therefore, Cr-induced S starvation is the cause of mRNA mistranslation. This establishes a single, novel pathway mediating the toxicity of chromate.

Keywords

Chromate toxicity; sulfate transport; frameshift; Sul1; *Saccharomyces cerevisiae*

1. Introduction

Chromate [Cr(VI)] is a highly toxic metal species, linked to cancer among other conditions (Luippold et al., 2003; Urbano et al., 2008). Continuing industrial use of Cr and associated environmental pollution (Salnikow and Zhitkovich, 2008; Wise et al., 2009) reinforces an urgent need to resolve the cellular and molecular mechanism(s) of Cr toxicity, which remains unclear. Chromate is known to induce a range of types of DNA lesion in cells and also provokes protein oxidation (Peterson-Roth et al., 2005; Sumner et al., 2005; Reynolds et al., 2009). A role for protein dysfunction in Cr action was underscored by a recent genome-wide study with the yeast cell model, *Saccharomyces cerevisiae* (Holland et al., 2007). That report revealed that Cr exposure provokes errors in mRNA translation, and that this is a major cause of Cr toxicity. Thus, Cr exhibited synergistic toxicity with a ribosome-targeting drug that is known to act via mistranslation, and manipulation of translational accuracy modulated Cr toxicity. However, the molecular mechanism by which Cr causes mistranslation was not resolved.

A further recent study demonstrated that Cr exposure leads to sulfur starvation in yeast, and that this also has deleterious consequences for growth (Pereira et al., 2008). This starvation arises because chromate inhibits sulfate uptake into cells, and may also compete with intracellular sulfate at the point of metabolism (Pereira et al., 2008; Alexander and Aaseth, 1995). Among other consequences, chromate-induced sulfur starvation is associated with

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depletion of the S-containing amino acids methionine (Met) and, in particular, cysteine (Cys) from cells (Pereira et al., 2008). This was of particular interest to us as amino acid starvation is a well described cause of translation infidelity; altering the competition between cognate and non-cognate aminoacyl-tRNAs at codons, to cause missense, frameshift and nonsense errors (Gallant and Lindsley, 1998; Farabaugh and Bjork, 1999; Sorensen, 2001).

In this short paper, we test the hypothesis that Met and Cys starvation arising during Cr exposure is the cause of Cr-induced mRNA mistranslation. The results showed this to be the case, reconciling these key recent findings to a single Cr toxicity mechanism in the yeast model.

2. Materials and methods

2.1. Strains and plasmids

Saccharomyces cerevisiae BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) was the parental background from which all strains were derived. A Met⁺/Cys⁺ prototrophic 'wild type' strain (*MATa his3Δ1 leu2Δ0 ura3Δ0*) was selected after sporulation of BY4743. A Cys⁻/Met⁻ auxotroph was obtained from the yeast deletion strain collection (Euroscarf, Germany) (*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 cys3::KanMX4*). Strains carrying the *ade2-101* allele were generated as follows. A diploid strain carrying the *ade2-101* allele and expressing a hemagglutinin(HA)-tagged Sul1 protein was obtained from Open Biosystems (Huntsville, AL): *MATa/α ade2-101/ade2-101 HIS3/his3Δ200 leu2Δ98/leu2Δ98 lys2Δ801/lys2Δ801 ura3Δ52/ura3Δ52 trp1Δ1/TRP1 SUL1/HA-SUL1*. This strain was sporulated and a *MATa ade2-101 his3Δ200 leu2Δ98 lys2Δ801 ura3Δ52 HA-SUL1* strain selected, and crossed to *S. cerevisiae* BY4741 (Euroscarf) in order to obtain a strain that did not express HA-tagged Sul1p [confirmed by diagnostic PCR (Longtine et al., 1998)]. This strain was suitable for use in the qualitative nonsense suppression assay (*MATa ade2-101 his3Δ leu2Δ ura3Δ*). Simultaneously, a strain was selected which carried the *met15Δ0* allele (*MATa ade2-101 his3Δ leu2Δ ura3Δ met15Δ0*). The *CYS3* gene was knocked out in the latter using pFA6a-His3MX6 as the template for short flanking homology (SFH)-PCR based disruption (Longtine et al., 1998). This created a Cys⁻/Met⁻ auxotroph expressing *ade2-101* (*MATa ade2-101 his3Δ leu2Δ ura3Δ met15Δ0 cys3::HIS5*). All transformants (Gietz and Woods, 2002) were selected on yeast nitrogen base (YNB) without amino acids (Formedium), supplemented as required (Ausubel et al., 2007). Diagnostic PCR (Longtine et al., 1998) was used to confirm appropriate gene disruption. Where used, colony PCR for diagnosis involved heating a colony in 5 µl water for 5 min at 95°C, before using the entire sample as a template for PCR. A *sul1Δ/sul2Δ* double mutant (*MATa his3Δ1 leu2Δ0 ura3Δ0 sul1::KanMX4 sul2::HphNT1*) was constructed previously (Holland and Avery, 2009). Where indicated, strains were transformed with plasmid pYDL-TY1, which carries a dual-luciferase frameshift reporter construct (Harger and Dinman, 2003), or pDB868, which carries a dual-luciferase missense reporter (Salas-Marco and Bedwell, 2005).

2.2. Growth conditions, mistranslation assays and chromate uptake

Organisms were routinely maintained on YNB agar with appropriate supplements (Khozoie et al., 2009) and prepared for experiments by culturing from single colonies as starter cultures in YNB broth supplemented with Cys, Met or homocysteine where specified. Flasks were incubated with shaking at 120 rev. min⁻¹, 30°C (Bishop et al., 2007). For quantitative determination of mistranslation, experimental cultures of strains carrying one of the mistranslation reporter plasmids (above) were inoculated from overnight starter cultures and pre-incubated as above for 4 h before the addition of CrO₃ from filter-sterilized stock solutions. Cells were pelleted by centrifugation and protein extracts prepared as described previously (Holland et al., 2007). Mistranslation was determined with the Dual Luciferase Assay system

(Promega) exactly as described (Holland et al., 2007). The derived ratios of luminescence attributable to the firefly versus *Renilla* luciferases indicated the level of mistranslation.

For qualitative determination of nonsense codon mistranslation, cells expressing the *ade2-101* allele were cultured to OD₆₀₀~2.0 in YPD broth (Khozaie et al., 2009) before spotting 10-fold serial dilutions on to YNB agar supplemented with Met and Cys as specified. Plates were incubated at 30°C for 4 d before image capture.

Cellular chromate uptake in experimental cultures was determined with inductively coupled plasma mass spectrometry (ICPMS) (Thermo-Fisher Scientific X-Series^{II}), exactly as described previously (Holland and Avery, 2009).

3. Results

3.1. Met or Cys starvation mimics Cr-induced mRNA mistranslation

To test the hypothesis that Met and Cys starvation causes Cr-induced mRNA mistranslation, first we determined whether Met or Cys starvation was sufficient to mimic mistranslation. We measured frameshift error-rate using a reporter construct in which an upstream sequence encoding *Renilla* luciferase is separated from a downstream firefly luciferase by a programmed frameshifting ‘shifty’ sequence (Harger and Dinman, 2003); the firefly gene is out of frame with the *Renilla* gene, so frameshift errors are required to produce functional firefly luciferase. We found that exposure to CrO₃ caused frameshift (Fig. 1A), missense (data not shown) and, previously (Holland et al., 2007), nonsense translation errors. We adopted the frameshift assay for routine measurement of mistranslation during the remainder of this study. In the presence of decreasing concentrations of Met and/or Cys supplied in the medium, even yeast cells which retained Met and Cys biosynthetic capacity exhibited increasing rates of mistranslation (Fig. 1B). The effect was similar to that of increasing Cr(VI) concentration (Fig. 1A). Furthermore, the frameshift rate was elevated in a strain that was unable to synthesize its own Met or Cys (Fig. 1C). Increased mistranslation in the *cys3Δ/met15Δ* strain was evident also from a nonsense suppression assay (Fig. 1D), which is based on read-through of the *ade2-101* UAA stop codon and suppression of the red pigmentation associated with this allele (Liu and Liebman, 1996; Holland et al., 2007). Thus, correct reading of the inserted stop codon leads to accumulation of purine precursors in the vacuole, and a red colony colour. A white colony colour indicates readthrough (mistranslation) of the stop codon, i.e. production of wild type Ade2p. The data substantiated that limitation of Met and Cys mimics the effect of Cr in promoting mRNA mistranslation (Fig. 1D). Note that we have also demonstrated Cr-induced mistranslation with the colour assay (Holland et al., 2007). That evidence counters the possibility that Cr-induced mistranslation detected with the luciferase assay (below) may merely reflect different stabilities of the *Renilla* and firefly proteins in the presence of Cr.

3.2. Chromate induced mistranslation requires the Sul1p/Sul2p transporters

Chromate causes sulfur (and hence Met and Cys) starvation primarily by inhibiting sulfate uptake via the Sul1p and Sul2p transporters in yeast (Pereira et al., 2008). Therefore, we reasoned that if S starvation is the cause of Cr-induced mRNA mistranslation, then this effect of Cr should be suppressed in a *sul1Δ/sul2Δ* double mutant. This was tested in medium lacking Met and Cys, to preclude any Sul1p or Sul2p down-regulation in the wild type control (Pereira et al., 2008). The marked increase in mistranslation rate caused by Cr in the wild type was absent in the *sul1Δ/sul2Δ* double mutant (Fig. 2). We discounted the possibility that this result for the mutant may have been a consequence of decreased Cr uptake [Cr transport in to yeast is partly mediated by Sul1p and Sul2p (Pereira et al., 2008; Holland and Avery, 2009)], as determination of cellular Cr levels revealed that Cr accumulation was no lower in the mutant than in the wild type under the present experimental conditions (Fig. 2, inset) [note that when

we reproduced the experimental conditions described by Pereira *et al.* (2008), including homocysteine addition to the medium, we confirmed their observation that Cr accumulation in the *sul1Δ/sul2Δ* mutant was ~50% of that in the wild type (data not shown)]. It should also be noted that other S acquisition pathways compensate for Sul1p and Sul2p in the double mutant, as the mutant is viable in medium lacking added Met or Cys. This may explain why the mistranslation rate in the mutant is lower than in Cr-exposed wild type cells (Fig. 2), the latter relying primarily on (Cr-inhibitable) Sul1p and Sul2p (Pereira *et al.*, 2008; Holland and Avery, 2009). Collectively, the results supported the hypothesis that it is S starvation mediated via the yeast sulfate transporters that causes Cr-induced mistranslation.

3.3. Chromate induced mistranslation requires Cys/Met biosynthesis

To support the above data, we exploited the Cys and Met auxotrophic strain, *cys3Δ/met15Δ*. For the hypothesis to be correct, Cr-induced mistranslation should be rescued in this mutant as it relies on exogenously-supplied Met and Cys, i.e., Met and Cys in the *cys3Δ/met15Δ* mutant do not arise from biosynthesis, so the levels of these amino acids are not affected by Cr-induced S starvation. Chromate exposure caused increased mRNA mistranslation in the control strain which biosynthesizes Met and Cys, but not in the *cys3Δ/met15Δ* mutant (Fig. 3). This result was reproducible at different external Met and Cys concentrations. The data indicate that the processes of Met and Cys biosynthesis are required for Cr to cause increased translation error-rate. This implicates these targets of Cr-induced S starvation (Pereira *et al.*, 2008) as a cause of mistranslation.

4. Discussion

The evidence presented in this short paper embraces recent observations (Holland *et al.*, 2007; Pereira *et al.*, 2008) in allowing us to crystallize a complete pathway of Cr toxicity with the yeast model, as follows: (i) Chromate competes with sulfate for uptake into cells, causing S starvation and depletion of Met and Cys among other S metabolites (Pereira *et al.*, 2008); (ii) Met and Cys depletion are responsible for elevated mRNA mistranslation that occurs during Cr exposure (present study) [the molecular mechanisms of loss of translation fidelity due to amino acid starvation have been described previously (Gallant *et al.*, 1982; Gallant and Lindsley, 1998; Farabaugh and Bjork, 1999; Sorensen, 2001)] (iii) mRNA mistranslation leads to protein dysfunction including protein aggregation and causes Cr toxicity (Holland *et al.*, 2007). This cements evidence from different labs into a unified model of Cr toxicity. Although the standard assays of translational fidelity used here record only a 2- to 3-fold induction of mistranslation by Cr, threshold effects are well known in metal toxicology (Avery *et al.*, 2004; Smith *et al.*, 2007) and our previous genetic and physiological evidence established that this induced rate of mistranslation is sufficient to cause Cr toxicity (Holland *et al.*, 2007). One challenge that remains is to reconcile this novel protein-centred Cr toxicity mechanism with the previously-established DNA damage caused by Cr in yeast and other organisms (Voitkun *et al.*, 1998; Peterson-Roth *et al.*, 2005; Reynolds *et al.*, 2009). This would help to elucidate how these differing targets may themselves combine to elicit cancer and the other deleterious consequences of chromate toxicity seen in higher organisms.

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Fig. 1.

Met or Cys starvation mimics Cr-induced mRNA mistranslation. (A) *S.cerevisiae* BY4743 transformed with plasmid pYDL-TY1, which carries a dual-luciferase frameshift reporter construct, was cultured in YNB broth with appropriate supplements before treatment for 3 h with the indicated CrO_3 concentrations and subsequent determination of luciferase activities. RLU, relative light units. All values are means $\pm$ SEM from at least three independent determinations. (B) Luciferase activities were from $\text{Met}^+/\text{Cys}^+$ prototrophs transformed with pYDL-TY1 and cultured overnight in YNB supplemented with Met and Cys as indicated. (C) Luciferase activities were from wild type $\text{Met}^+/\text{Cys}^+$ prototrophs and *cys3Δ/met15Δ* auxotrophic cells transformed with pYDL-TY1 and cultured overnight in YNB supplemented

with 22μM Met and 83μM Cys. (D) A wild type Met⁺/Cys⁺ prototroph and a *cys3Δ/met15Δ* auxotrophic strain, both expressing the *ade2-101* allele, were spotted in 10-fold serial dilutions on to YNB agar supplemented with Met and Cys as indicated. Images were captured after 4 d incubation at 30°C.

Fig. 2.

Chromate induced mistranslation requires the Sul1p/Sul2p transporters. Wild type cells (*MAT α his3 Δ 1 leu2 Δ 0 ura3 Δ 0*) and a *sul1 Δ /sul2 Δ* double mutant were transformed with pYDL-TY1 and cultured in YNB broth, before treatment either without (open bars) or with (filled bars) 0.1 mM CrO_3 for 3 h. Total protein was subsequently extracted for luciferase analysis. All values are means $\pm$ SEM from at least three independent determinations. Inset, cellular Cr levels were determined at the indicated intervals during Cr treatment of wild type and *sul1 Δ /sul2 Δ* cells, cultured as above.

Fig. 3.

Chromate induced mistranslation requires Cys/Met biosynthesis. Wild type cells (*MATa his3Δ1 leu2Δ0 ura3Δ0*) and a *cys3Δ/met15Δ* auxotrophic mutant, both transformed with pYDL-TY1, were cultured for 16 h in YNB supplemented with Met and Cys as indicated and in the absence (open bars) or presence (filled bars) of 0.1 mM CrO₃. Total protein was subsequently extracted for luciferase analysis.