Dcr2 targets Ire1 and downregulates the unfolded protein response in *Saccharomyces cerevisiae*

Jinbai Guo & Michael Polymenis⁺

Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas, USA

Accumulation of unfolded proteins in the endoplasmic reticulum triggers the unfolded protein response (UPR). How the UPR is downregulated is not well understood. Inositol requirement 1 (Ire1) is an endoplasmic reticulum transmembrane UPR sensor in *Saccharomyces cerevisiae*. When the UPR is triggered, Ire1 is autophosphorylated, on Ser 840 and Ser 841, inducing the cytosolic endonuclease activity of Ire1, thereby initiating the splicing and translational de-repression of *HAC1* mRNA. Homologous to Atf/Creb1 (Hac1) activates UPR transcription. Here, we report that the dose-dependent cell-cycle regulator 2 (Dcr2) phosphatase functionally and physically interacts with Ire1. We identified genetic interactions between *DCR2* and genes, including *IRE1*, which are involved in secretory processes. Overexpression of *DCR2*, but not of a catalytically inactive *DCR2* allele, significantly delays *HAC1* splicing and sensitizes cells to the UPR. Furthermore, Dcr2 physically interacts *in vivo* with Ire1-S840E,S841E, which mimics phosphorylated Ire1, and Dcr2 de-phosphorylates Ire1 *in vitro*. Our results are consistent with de-phosphorylation of Ire1 being a mechanism for antagonizing UPR signalling.

Keywords: UPR; Dcr2; Ire1; Hac1; stress response

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INTRODUCTION

The unfolded protein response (UPR) is triggered when protein synthesis exceeds the protein folding capacity of the cell, and also by acute 'environmental' stresses, such as exposure to tunicamycin or dithiothreitol, which block glycosylation or disulphide bond formation, respectively (Schroder *et al*, 2000; Kaufman *et al*, 2002). In yeast, inositol requirement 1 (Ire1), an endoplasmic reticulum transmembrane protein with its amino-terminal domain located in the lumen of the endoplasmic reticulum, senses unfolded proteins (Patil & Walter, 2001; Rutkowski & Kaufman, 2004). Ire1 has kinase and endonuclease activities, which reside

in distinct cytosolic domains. When Ire1 dimerizes, it is autophosphorylated *in trans*, primarily on Ser 840 and Ser 841, followed by activation of its endonuclease activity towards its sole substrate, the homologous to Atf/Creb1 (*HAC1*) messenger RNA (Patil & Walter, 2001; Niwa *et al*, 2005). If Ser 840 and Ser 841 cannot be phosphorylated, for example, in Ire1-S840A,S841A, UPR signalling is essentially eliminated (Shamu & Walter, 1996).

When the UPR is not induced, the *HAC1^u* transcript is stable in the cytosol but is not translated efficiently because an intron blocks its translation (Patil & Walter, 2001; Rutkowski & Kaufman, 2004). Active Ire1 cleaves *HAC1^u* at two splice sites removing the intron, and the two exons are ligated by transfer RNA ligase, generating *HAC1ⁱ* mRNA (Sidrauski *et al*, 1996; Sidrauski & Walter, 1997). *HAC1ⁱ* is then translated efficiently and Hac1 activates transcription of approximately 300 UPR target genes (Patil & Walter, 2001; Rutkowski & Kaufman, 2004). In addition to regulated *HAC1* mRNA splicing, which is Ire1 dependent, *HAC1* mRNA transcription is also regulated through an Ire1-independent pathway (Leber *et al*, 2004).

The UPR is anti-mitogenic. Constitutive expression of the intron-less *HAC1ⁱ* transcript inhibits cell proliferation in yeast (Kawahara *et al*, 1997). In mammalian cells, the UPR modulates apoptosis and also delays cell-cycle progression (Brewer *et al*, 1999; Brewer & Diehl, 2000). How the UPR is activated is fairly well established, but it is not yet clear how it is downregulated. Given the anti-mitogenic properties of the UPR, a mechanism that turns off the UPR must exist.

We had previously identified the dose-dependent cell-cycle regulator 2 (Dcr2) phosphatase on the basis of its positive role in cell-cycle progression (Pathak *et al*, 2004). In this report, we show that Dcr2 interacts with Ire1 functionally and physically, both *in vitro* and *in vivo*, and that Dcr2 acts antagonistically to the UPR.

RESULTS

Dcr2 antagonizes the unfolded protein response

In a genome-wide screen to identify genetic interactions involving *DCR2* (supplementary information online), we found that *ire1Δ* cells carrying a dominant-negative *DCR2* allele grow poorly (supplementary Fig S1 online). Furthermore, most of the gene products that showed genetic interactions with *DCR2* had some role in transportation (Fig 1). Because defects in transportation trigger the UPR (Patil & Walter, 2001), thereby possibly explaining

Department of Biochemistry & Biophysics, Texas A&M University, 2128 TAMU, College Station, Texas 77843, USA

⁺Corresponding author. Tel: +1 979 458 3259; Fax: +1 979 845 4946;

E-mail: polymenis@tamu.edu

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Fig 1 | Genetic interactions of *DCR2*. Solid lines indicate interactions identified in this study, and dotted lines represent interactions reported previously (Tong *et al.*, 2001). Open reading frames with a role in transportation, on the basis of the annotations of the Saccharomyces Genome Database, are underlined.

our findings, we examined the functional interactions between *DCR2* and *IRE1*. First, we deleted both *DCR2* and *IRE1* and found that *dcr2Δ, ire1Δ* double mutants grow extremely poorly (Fig 2A). We then artificially triggered the UPR, by blocking glycosylation with tunicamycin, and examined cell proliferation (Fig 2B). Under these conditions, loss of *IRE1* is lethal (Fig 2B). It seems that altering the dosage of *DCR2* impairs viability in the presence of tunicamycin, although not to the same extent as *ire1Δ* cells (Fig 2B).

We then examined splicing of *HAC1* mRNA, which provides an unambiguous molecular metric of Ire1 activation (Niwa *et al.*, 2005). If Dcr2 interferes with UPR signalling, the relative levels of the unspliced *HAC1^u* mRNA versus the spliced *HAC1ⁱ* should change in *DCR2* mutants. We monitored *HAC1* splicing by RNA blotting (Fig 2C) and reverse transcription-PCR (RT-PCR; Fig 2D). When we introduced endoplasmic reticulum stress, by the addition of tunicamycin for 30 min, splicing of *HAC1* was inhibited in cells overexpressing *DCR2* (Fig 2C,D), but not the catalytically inactive *DCR2^{DN}* allele (Fig 2D). After prolonged incubation (2 h) with tunicamycin, *HAC1^u* mRNA was eventually spliced in cells overexpressing *DCR2* (Fig 2C). We confirmed UPR induction by monitoring the mRNA levels of *karyogamy 2* (*KAR2*), a UPR-responsive gene (Fig 2C). Our observations suggest that the Dcr2 phosphatase antagonizes the UPR at a step preceding *HAC1^u* mRNA splicing.

Dcr2 de-phosphorylates Ire1

We examined whether Ire1 interacts physically with Dcr2. Ire1, when activated, is autophosphorylated at Ser 840 and Ser 841 (Shamu & Walter, 1996). We constructed strains carrying as their sole *IRE1* copy *IRE1-S840E,S841E* or *IRE1-S840A,S841A* mutant alleles, which mimic or abolish phosphorylation, respectively. The viability of cells carrying either of these alleles is decreased in conditions that trigger the UPR, especially for *IRE1-S840A,S841A* cells (Fig 3A), suggesting that reversible phosphorylation of Ire1 is important for proper UPR signalling.

We then examined whether Dcr2 interacts physically *in vivo* with mutant or wild-type Ire1 (Fig 3B). For this experiment, *DCR2* was epitope tagged at its carboxyl terminus with the 'TAG' epitope (6 × His, haemagglutinin, protein A) and it was expressed from a galactose-inducible promoter (Gelperin *et al.*, 2005). *IRE1* and the mutant *IRE1* alleles were expressed as C-terminal fusions with the 'TAP' epitope (calmodulin-binding protein, protein A) from its

Fig 2 | Unfolded protein response signalling is sensitized in *DCR2* mutants. (A) Synthetic growth defects of the indicated strains (all in the BY4741 background) were evaluated by spotting tenfold serial dilutions of the cultures on standard (YPD) medium. The plates were incubated at 30 °C and photographed after 2 days. (B) Growth of the indicated strains (all in the BY4741 background) was evaluated on solid galactose medium, containing tunicamycin or DMSO alone. The plates were incubated at 30 °C and photographed after 4 days. (C) Steady-state levels of *HAC1* and *KAR2* RNA are shown on an RNA blot. Each lane was loaded with total RNA prepared from the indicated strains (all in the BY4741 background). The ethidium bromide (EtBr)-stained gel is shown at the bottom, to indicate loading. (D) The indicated strains (all in the BY4743 background) were grown in galactose-containing medium in the presence of tunicamycin or DMSO alone, for 30 min. Cells marked as *P_{GAL}-DCR2^{DN}* or *P_{GAL}-DCR2* were carrying the corresponding plasmids, but were otherwise wild type. RNA was extracted and transcription-PCR products with *HAC1*-specific primers were resolved by agarose gel electrophoresis and visualized with EtBr. WT, wild type.

chromosomal location (Ghaemmighami *et al.*, 2003). From these cells, we used cobalt ion beads to precipitate Dcr2 (through the 6 × His epitope), and we noticed that Ire1-S840E,S841E co-precipitated with Dcr2 (Fig 3B). We did not observe interactions between Dcr2 and wild-type Ire1 or Ire1-S840A,S841A,

Fig 3 | Dcr2 physically interacts with Ire1 *in vivo*. (A) Growth of the indicated strains carrying Ser 840 and Ser 841 substitutions (all in the BY4741 background) was evaluated on solid glucose medium. All the *IRE1* alleles were tagged at their carboxyl terminus with the tandem affinity purification (TAP) epitope. The plates were incubated at 30 °C and photographed after 2 days. (B) Whole-cell extracts (WCE) from the indicated strains were subjected to SDS-polyacrylamide gel electrophoresis either directly (right) or after Dcr2 was precipitated with cobalt ion beads (left). For immunoblotting (IB), we used the peroxidase-anti-peroxidase (PAP) reagent, which recognizes the protein A (PrA)-tagged Ire1 and Dcr2.

suggesting that the glutamic acid (E) substitutions allow stable (and detectable) interactions with Dcr2. These results indicate that Dcr2 interacts physically with Ire1 *in vivo*, and that this interaction is dependent on the phosphorylation status of Ire1 at Ser 840 and Ser 841.

We also examined whether recombinant Dcr2 from bacteria can de-phosphorylate recombinant Ire1. For this experiment, we expressed in bacteria the cytosolic portion of Ire1 (Ire1*), which was purified and autophosphorylated (Papa *et al*, 2003). In the presence of recombinant Dcr2, Ire1* was de-phosphorylated, but not when phosphatase activity was blocked (Fig 4A). Complete de-phosphorylation of Ire1 by Dcr2 would not be expected because some phosphates might be on sites not targeted by Dcr2. Our results are consistent with the idea that Ire1 is a substrate for Dcr2.

DISCUSSION

Here, we present experiments that link the Dcr2 phosphatase with Ire1 and the UPR. We had originally identified *DCR2* for its promitogenic properties when it is overexpressed (Pathak *et al*, 2004). This could perhaps be accounted for by Dcr2 acting as an Ire1 phosphatase, as endoplasmic reticulum stress arrests the cell cycle and the UPR is sensitive to nutrients (Schroder *et al*, 2000; Kaufman *et al*, 2002) and ATP levels (Papa *et al*, 2003). However, if Dcr2 acted solely on Ire1 to affect cell proliferation, cells lacking both *IRE1* and *DCR2* should proliferate as fast as those lacking

Fig 4 | Dcr2 de-phosphorylates Ire1 *in vitro*. (A) Recombinant autophosphorylated Ire1* was incubated with recombinant Dcr2 (the ×1 and ×10 designations refer to the relative amount of Dcr2 added to the reactions) or λ-protein phosphatase (λ-PPase), in the presence (+) or absence (-) of phosphatase inhibitor (PPase inh.) cocktails (cocktail I alone, or I+II), as indicated. The reactions were analysed by SDS-polyacrylamide gel electrophoresis and blotting, followed by phosphoprotein-specific detection (top), immunoblotting with a 6 × His antibody (middle) or immunoblotting with the peroxidase-anti-peroxidase reagent (bottom). The relative amounts of phosphorylated Ire1* detected are shown on the chart below; they were calculated from the relevant band intensities. (B) A schematic model of the role of Dcr2 as an unfolded protein response (UPR) antagonist, acting on Ire1 and before *HAC1^u* splicing. ER, endoplasmic reticulum.

IRE1 or *DCR2* alone (Fig 2A). It is likely that Dcr2 also has an impact on overall cell proliferation independent of Ire1 (Pathak *et al*, 2004; R. Pathak and M.P., unpublished data).

How important is the role of Dcr2 in downregulating UPR signalling? Given the functional interactions between *DCR2* and genes involved in transportation in general (Fig 1)—and *IRE1* in particular (Fig 2)—as well as the physical interactions between Dcr2 and Ire1 (Figs 3,4), it is reasonable to conclude that Dcr2 has a significant role in the UPR. This role of Dcr2 is probably shared with other phosphatases. Although loss of Dcr2 alone only weakly sensitizes cells to endoplasmic reticulum stress (Fig 2B; supplementary Fig S2A online), the combined loss of Dcr2 and protein phosphatase type 2C (Ptc1) leads to markedly reduced viability (supplementary Fig S2B online). Another phosphatase, Ptc2, was also previously shown to de-phosphorylate Ire1 *in vitro*

(Welihinda *et al*, 1998), and overexpression of *PTC2* down-regulated the UPR (Welihinda *et al*, 1998). It is important to note, however, that Ptc2 and Ire1 have not been shown to associate *in vivo* and genetic interactions of *PTC2* with *IRE1* or any other gene involved in secretion and/or transportation have not been reported. Finally, although Dcr2 levels are not affected by the UPR, Dcr2 seems to be post-translationally modified when the UPR is triggered (supplementary Fig S3 online), indicating some kind of feedback control. Overall, it is clear that de-phosphorylation of Ire1 provides a mechanism for turning off the UPR after it is activated (Fig 4B). Other mechanisms that downregulate the UPR in yeast must certainly exist, especially because Ire1-independent pathways also contribute to UPR signalling (Schroder *et al*, 2003; Leber *et al*, 2004).

METHODS

Strains and DNAs. Cell cultivation, media and yeast molecular biology techniques were performed as described by Kaiser *et al* (1994). Induction of the UPR was triggered by the addition of tunicamycin (0.5 µg/ml from a 1 mg/ml stock in DMSO). For yeast transformations in a 96-well plate format, we followed the protocol of the *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/transprot.html).

All the strains were BY474 derivatives in the S228C genetic background (*his3Δ leu2Δ met15Δ ura3Δ*). The homozygous diploid yeast deletion panel (in the BY4743 background) was purchased from Research Genetics (Invitrogen Corp., Carlsbad, CA, USA). Detailed information on strain and plasmid construction is given in supplementary information online.

RNA and protein techniques. RNA was purified using reagents from Qiagen (Valencia, CA, USA), according to their instructions. RNA blotting was performed as described previously (Polymenis & Schmidt, 1997), using *HAC1* and *KAR2* probes generated by PCR, which were labelled using non-radioactive reagents from Pierce (Rockford, IL, USA), according to their instructions. For RT-PCR, we used reagents from Stratagene (La Jolla, CA, USA), and a pair of primers corresponding to sequences flanking the *HAC1* RNA splice sites (at position +461 in the *HAC1* open reading frame and at position +1,112 in the *HAC1* 3'-untranslated region). For protein extract preparation from yeast, we followed methods published previously (Han *et al*, 2005), except that the extraction buffer contained 0.9% NP-40 to facilitate Ire1 extraction from membranes. For precipitation through the 6 × His epitope, we used TALON cobalt ion beads from BD Biosciences (San José, CA, USA), according to their instructions. For immunoblotting, we used procedures and reagents described previously (Han *et al*, 2005). For the experiment shown in Fig 4A, recombinant proteins were purified through their epitope tags. Ire1* was autophosphorylated *in vitro*, followed by phosphatase assays, as described previously (Welihinda *et al*, 1998). The phosphatase inhibitor cocktails were from Sigma (St Louis, MO, USA). For phosphoprotein detection, we used non-radioactive reagents from Pierce, according to their instructions.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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