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Activation of the unfolded protein response in *Pichia pastoris* requires splicing of a *HAC1* mRNA intron and retention of the C-terminal tail of Hac1p

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ABSTRACT

We have shown that the unfolded protein response (UPR) in *Pichia pastoris* requires splicing of a non-conventional intron in the *HAC1*^{u} mRNA in common with other eukaryotes. *P. pastoris* is a favoured yeast expression host for secreted production of heterologous proteins and the regulation of the UPR in *P. pastoris* may hold the key to its effective folding and secretion of proteins. We have also shown that the C-terminal region of the Hac1p from *P. pastoris* is required for functionality. Although the C-terminal regions of Hac1p from both *S. cerevisiae* and *P. pastoris* are rich in phenylalanine residues, the *P. pastoris* Hac1p lacks a C-terminal serine that is known to be important in the efficient functionality of Hac1p from *S. cerevisiae*.

1. Introduction

Proteins that are targeted for secretion by eukaryotes are folded within the lumen of the ER. Folding is assisted by ER-resident chaperones and foldases and the process can present a bottleneck particularly for heterologous proteins that, by definition, have not co-evolved with the folding environment within the ER. The accumulation of unfolded proteins in the ER is sensed and leads to initiation of the unfolded protein response (UPR) which then initiates homeostatic mechanisms that include the induced expression of chaperones and foldases, and the ER-associated degradation (ERAD) of proteins by the proteasome. In the yeast *Saccharomyces cerevisiae*, nearly 400 genes are transcriptionally affected by the UPR [1] and a similar proportion of genes has also been reported to be affected by the UPR in other yeasts and filamentous fungi [2,3]. Attempts to overcome this secretion bottleneck by overexpression of individual chaperones and foldases, or Hac1p, in yeasts, including *P. pastoris*, and filamentous fungi have only been successful in some cases [4].

S. cerevisiae has been the major model organism for study of the UPR in fungi. In this organism Ire1p, a trans-membrane kinase and endoribonuclease residing in the ER membrane and its lumenal amino terminal domain, is essential for the detection of unfolded proteins. Ire1p is therefore the primary signal transducer [5,6] from unfolded protein to the nucleus that leads to the UPR. The mechanism of detection is hypothesized to be either direct sensing of unfolded proteins or via association/dissociation with the molecular chaperone BiP (Kar2p) [7,8]. The cytoplasmic domain of Ire1p has site-specific endoribonuclease activity and is activated by oligomerisation and autophosphorylation of Ire1p [9,10]. Ire1p is then able to splice its only known substrate, *HAC1*^{u} (un-induced) mRNA, which is then religated without the intron to yield a translationally-competent *HAC1*^{i} (induced) mRNA [11,12]. The structures of the yeast and human lumenal [7,13] and cytosolic domains [9,14] of Ire1p have been solved and molecular mechanisms have then been proposed for binding of unfolded proteins in the ER lumen, oligomerisation of Ire1p and activation of the cytosolic kinase and ribonuclease domains. Following activation Hac1p is translocated to the nucleus where it activates target gene transcription via a specific upstream sequence termed the unfolded protein response element (UPRE) [15].

The role of Hac1p and the presence of a non-spliceosomal intron in *HAC1*^{u} mRNA have been identified in all eukaryotes investigated. However, despite this conservation, the intron size varies from species to species, being 252bp in *S. cerevisiae* [16,17], and 20bp in the filamentous fungi *A. niger, A. nidulans* and *T. reesei* [18,19]. Several regulatory mechanisms have been described to control Hac1p levels. In *S. cerevisiae*, base-pairing between the *HAC1* mRNA intron and a region of the 5′UTR stalls translation [20] and efficient

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loading of *HAC1* mRNA to ribosomes was shown to be dependent upon splicing of *HAC1*^{u} mRNA to form *HAC1*^{i} mRNA [21]. In the filamentous fungi *A. niger, A. nidulans* and *T. reesei*, and in *Candida albicans*, activation of the UPR is further controlled and depends not only on the removal of the unconventional intron from *HAC1*^{u} mRNA but also on truncation of the 5′UTR of this mRNA [18,22,23]. In addition to splicing of the *HAC1* mRNA, *S. cerevisiae* has been shown to contain a second ER-to-nucleus signalling pathway, which increases *HAC1* mRNA production in the absence of Ire1p, although the exact mechanism remains unknown [24].

The availability of genome sequences of *P. pastoris* [25,26] has enabled an exploration of the genes involved in the UPR in that yeast. In contrast to other eukaryotes, it was reported that the *HAC1* mRNA was constitutively spliced and, therefore, the UPR was constitutively active [27]. Induction of the UPR in *P. pastoris* occurred under ER stress conditions by transcriptional regulation of *HAC1* rather than by splicing of the *HAC1* mRNA [27]. That report was therefore interesting because it showed that the UPR in *P. pastoris* may function differently to other species. Here, however, we report that *HAC1* mRNA intron splicing can, at least under some conditions, provide regulation in *P. pastoris*. Furthermore we provide further insight into the mechanism of induction of the UPR in *P. pastoris* by demonstrating the importance of the C-terminus of Hac1p for activation of the UPR.

2. Materials and methods

2.1. Chemicals

All restriction enzymes and Phusion polymerase were purchased from New England Biolabs Ltd (Hitchin, UK). Synthetic oligonucleotides and chemicals were purchased from Sigma–Aldrich (Gillingham, UK).

2.2. Strains and plasmids

All plasmids were propagated in the *E. coli* strain DH5α (Invitrogen, Paisley, UK). pPICZA and the *P. pastoris* strain GS115 were purchased from Invitrogen. Sequences for the cloning of the *P. pastoris HAC1* gene were obtained from the genome sequence [27]. Cloning of the *HAC1* gene into pPICZA was performed using *EcoRI* and *NotI* sites All clones were confirmed by sequencing. The Δ*HAC1* strain of *P. pastoris* was generated by inserting the *HIS4* gene from pPIC9 (Invitrogen) into the *HAC1* gene locus via homologous recombination. Sequences for the flanking regions of *HAC1* were obtained from the Integrated Genomics website (http://www.integratedgenomics.com/pichia.html) and oligonucleotides were designed to amplify 500bp upstream and downstream of the gene locus. The *HIS4* gene was then amplified by PCR from pPIC9. These products were then fused via overlapping PCR to create a gene disruption cassette. This cassette was then transformed into *P. pastoris* following the electroporation methodology described within the Invitrogen *P. pastoris* manual. Integration was confirmed via PCR of genomic DNA from positive colonies. The strains used are listed in Supplementary Table 1 and primers in Supplementary Table 2. Extractions of genomic DNA [28] and RNA [29] from *P. pastoris* were achieved using published protocols. Truncated versions of Hac1p were constructed using sequences for the spliced form of *HAC1*.

2.3. Culture conditions

All % compositions of media used are % (w/v). YEPD (1% yeast extract, 2% peptone, 2% glucose). YEPDZ (1% yeast extract, 2% peptone, 2% glucose, 100 μg/ml zeocin). Buffered Minimal Glycerol (BMG) (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol). Buffered Minimal Methanol (BMM) (As BMM but 0.5% methanol in place of glycerol). SD (standard dropout) (0.67% YNB, 2% glucose, adenine 0.02 g/l, arginine 0.1 g/l, histidine 0.02 g/l, leucine 0.1 g/l, lysine 0.03 g/l, 0.02 g/l, uracil 0.02 g/l). SDU (standard dropout medium uracil) (as SD but lacking uracil). SDH (standard dropout medium histidine) (as SD but lacking histidine).

2.4. Activation of the UPR by treatment with DTT and tunicamycin

P. pastoris cells were grown in 50 ml liquid cultures (in 250 ml flasks) at 30 °C with shaking at 200 rpm to mid-exponential phase (judged from growth curves based on absorbance) in YEPD or appropriate SD. Dithiothreitol (5–15 mM final concentration) was then added with an equivalent volume of water added to control cultures. The cultures were incubated as described above for 1hr before the cells were harvested and RNA isolated.

2.5. qRT-PCR

qRT-PCR was carried out on cDNA synthesized from total mRNA using Superscript III (Invitrogen). Primer and probe sets were designed using Primer Express 3 (Applied Biosystems) and synthesized at Sigma–Aldrich. Probes were synthesized with TAMRA and FAM probes for use in the Taqman method of qRT-PCR. All samples were compared to the internal standard actin and relative quantities were calculated in relation to this control. Standard curves were constructed from pooled cDNA samples. The reaction was carried out using Applied Biosystems fast-universal master mix and the reactions were designed using the Applied Biosystems 7500 fast real-time PCR software.

3. Results and discussion

The *HAC1* gene of *P. pastoris* is 1.2 kb in length [27]. Sequence alignment between the *S. cerevisiae* Hac1p and the predicted protein encoded by the *P. pastoris HAC1* gene indicated homology at the DNA-binding domain in the N-terminal region of the protein (Fig. 1A) with the typical leucine-zipper domain downstream. In addition, analysis [27] of the *HAC1* gene from *P. pastoris* for predicted functional elements of the *HAC1* gene of *S. cerevisiae* revealed that the promoter region contains a potential unfolded protein response element (UPRE) [15] 133 b upstream from the ATG, a proposed 3′UTR *HAC1* mRNA recruitment element described for *S. cerevisiae* [30] and 2 splice sites at positions 852 and 1174 bases. These splice sites are predicted to be the locations for removal of a non-conventional intron of 322 nucleotides resulting in a *Pichia HAC1* coding mRNA of 915 bases for translation to Hac1p of 305 amino acids. This *HAC1* gene was confirmed as an activator of the UPR by measuring *KAR2* up-regulation in a strain where the *HAC1* gene was over-expressed [27]. Here, we further characterised this gene by creating a *HAC1* knockout strain of *P. pastoris* (PpHAC1ko) via insertion of the *HIS4* gene into the *HAC1* locus. In addition, the 915 base *HAC1*^{i} from *P. pastoris* cDNA was cloned into the pPICZA plasmid and transformed the PpHAC1ko strain to create the reconstituted PpHAC1(r) strain.

The *HAC1* knockout strain of *S. cerevisiae* was characterised as being an inositol auxotroph, due to the involvement of the UPR in lipid biogenesis [31], so the ability of *P. pastoris* wild-type (WT), PpHAC1ko and PpHAC1(r) strains to grow on inositol-deficient media (Fig. 1B) was assessed. The WT and PpHAC1(r) strains were able to grow on inositol-deficient media, whereas the PpHAC1ko strain was unable to grow. These results indicate that the *HAC1* gene is affecting pathways that are involved in the UPR and

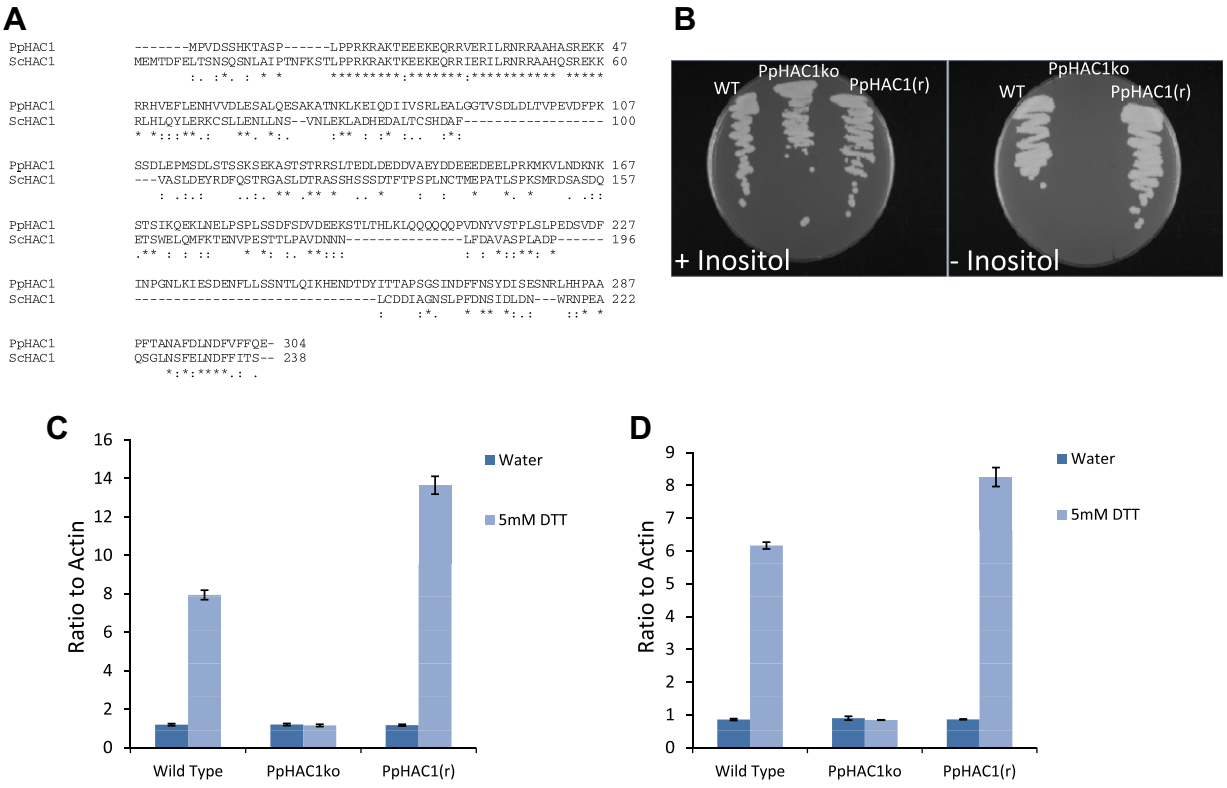

Fig. 1. (A) Alignment of the predicted protein sequences of Hac1p from *S. cerevisiae* and *P. pastoris*. (B) Growth of the PpHAC1ko, WT and PpHAC1(r) strains on ±inositol media. (C) mRNA levels of *KAR2* from the PpHAC1ko, WT and PpHAC1(r) strains treated with either water or 5 mM DTT. Bars represent the mean of three independent experiments and the error bars are the standard deviation. (D) As C but with *PDI1* transcript levels measured.

corroborate the conclusion that this gene indeed encodes a functional Hac1p [27]. To confirm that the identified *HAC1* gene was having a direct effect on activation of the UPR the transcript levels of *KAR2* and *PDI1*, both of which contain putative UPREs in their promoter regions, were measured in response to a challenge with 5 mM DTT in the WT, PpHAC1ko and PpHAC1(r) strains (Fig. 1C and D). Treatment of the WT and PpHAC1(r) strains with 5 mM DTT led to an increase in the levels of *KAR2* (8–15 fold) (Fig. 1C) and *PDI1* (6–9 fold) (Fig. 1D), consistent with activation of the UPR. However, no change in the transcript levels from *KAR2* or *PDI1* were observed in PpHAC1ko under the same conditions confirming the functionality of *HAC1* in the UPR in *P. pastoris* [27].

HAC1 mRNA splicing was investigated in *P. pastoris* by treating the WT *P. pastoris* strain with 0, 5, 10 and 15 mM of DTT and assessing the cDNA for the spliced and un-spliced forms of *HACI* using PCR (Fig. 2A). The data show that no *HAC1* spliced cDNA product was detectable in the absence of DTT. However, exposure to DTT led to detection of both a spliced and un-spliced form of *HAC1* cDNA, and the identities of the forms were confirmed by size and sequencing. Furthermore, the relative amount of spliced *HAC1* increased with increasing DTT concentrations (Fig. 2A). The levels of transcript from *PDI1* and *KAR2* were raised upon exposure to DTT and were highest in cells exposed to the highest level of DTT (Fig. 2B and C). These data show, in contrast to a previous report [27], that *HAC1* does indeed undergo a splicing reaction, at least under the conditions examined. Furthermore, we constructed a Δ IRE1 strain of *P. pastoris* and showed that the splicing of *HAC1* mRNA was dependent on the presence of *IRE1*, as was the induction under ER stress conditions of both *PpPDI1* and *PpKAR2* (Supplementary Figure 1). The *HAC1* promoter region contains a UPRE element, suggesting that the *HAC1* gene is able to regulate its own expression as in *S. cerevisiae* [32], and a raised level of *HAC1* transcript (data not shown) after exposure to DTT was

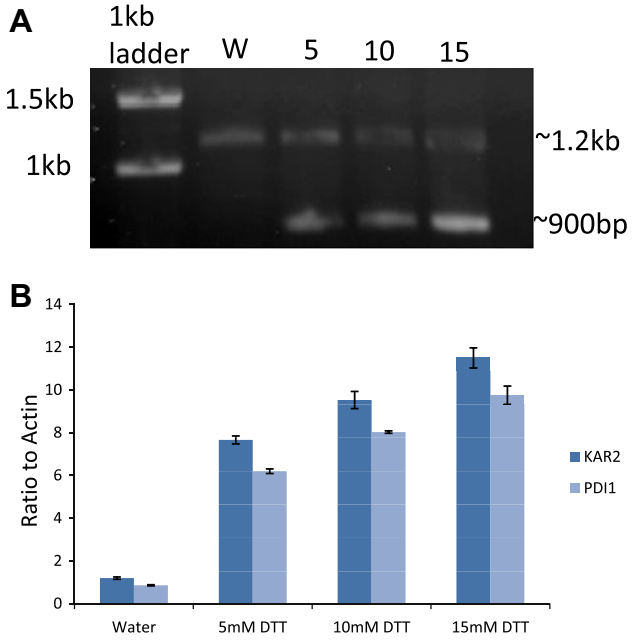

Fig. 2. (A) The splicing of *P. pastoris HAC1* mRNA in response to varying concentrations of DTT. (B) The levels of *KAR2* and *PDI1* mRNA in response to treatment with 5, 10 and 15 mM DTT. Bars represent the mean of three independent experiments and the error bars are the standard deviation.

observed. Exposure of *P. pastoris* to DTT was previously examined for genome-wide transcriptional effects and, while there were similarities to *S. cerevisiae*, differences between the two yeast species were highlighted [33]. In conclusion, similar to the mechanisms

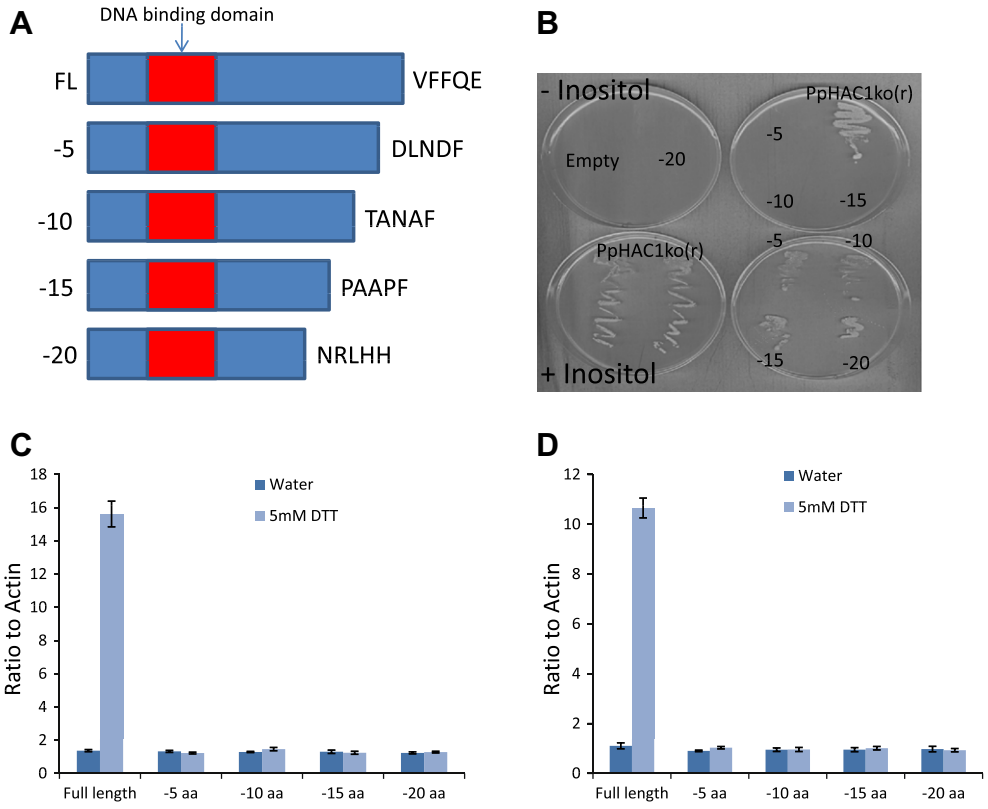

Fig. 3. (A) Schematic diagram indicating the truncations of the constructed Hac1p variants (truncations were constructed using the spliced version of *HAC1*). The five terminal amino acids are shown for comparison. (B) Growth of the PpHAC1ko transformed with full length, −5, −10, −15 and −20 amino acid truncations of Hac1p on ±inositol media. (C) mRNA levels of *KAR2* from the PpHAC1ko transformed with −5, −10, −15 or −20 amino acid truncations of Hac1p treated with either water or 5 mM DTT. Bars represent the mean of three independent experiments and the error bars are the standard deviation. (D) As C but with *PDI1* transcript levels measured.

observed in other organisms, the primary response in *P. pastoris* to ER stress induced by DTT is splicing of the *HAC1* intron to activate the UPR.

The ability of *S. cerevisiae* Hac1p to activate the UPR is due to removal of the intron within *HAC1u* mRNA because that event not only removes a translational block but also produces a new C-terminal amino acid sequence resulting from translation of *HAC1*^{i} mRNA. It is also possible that phosphorylation of the terminal serine (Ser238) is important for activation of the protein [34]. No terminal Ser is predicted in Hac1p from *P. pastoris* (Fig. 1A) and, indeed, the Hac1p sequences from *S. cerevisiae* and *P. pastoris* appear to have diverged significantly. In order to assess whether the C-terminal region of Hac1p from *P. pastoris* was also important for functionality, truncated versions of Hac1p in *P. pastoris* were compared (Fig. 3A). The truncated proteins were constructed sequentially with −5, −10, −15 and −20 amino acids deducted from the full length protein (Fig. 3A). Each construct was expressed from the pPICZA vector and transformed into the PpHAC1ko strain to ascertain if it was able to rescue UPR activation. In all cases the truncated constructs were unable to restore inositol prototrophy or restore UPR activation (Fig. 3B–D). This indicates that the last five amino acid residues of Hac1p are essential for activation of the UPR. The terminal amino acid of the *P. pastoris* Hac1p is glutamic acid and acidic residues in the Hac1p terminal region have been shown to be important for functionality in *S. cerevisiae* [34]. The terminal amino acid sequences of Hac1p in both *S. cerevisiae* and *P. pastoris* are rich in phenylalanine residues (Fig. 1) but the absence of a serine in the *P. pastoris* Hac1p is a difference. It was previously shown by sequence alignments that other fungal Hac1 proteins also lack a C-terminal serine and they need not contain phenylalanine either [27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.02.036.

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