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Lobe IB of the ATPase Domain of Kar2p/BiP Interacts with Ire1p to Negatively Regulate the Unfolded Protein Response in *Saccharomyces cerevisiae*

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The endoplasmic reticulum HSP70 chaperone BiP/Kar2p is both the sensor for the unfolded protein response (UPR) in the yeast *Saccharomyces cerevisiae* and a target of transcriptional up-regulation by this signaling pathway. In this study, the molecular form of Kar2p that interacts with the Ire1p transmembrane receptor kinase to inhibit UPR signaling was shown to be the substrate-free, ATP-bound conformation. Oligosaccharide shielding experiments localized the binding site for Ire1p to the top of the back face of lobe IB of the Kar2p ATPase domain. The interaction between Kar2p and Ire1p is abolished by substitution of glutamic acid for glutamine 88, a residue on the surface of lobe IB that is likely to be shielded by ectopic oligosaccharide side-chains that also prevented the interaction between the two proteins. Glutamine 88 is conserved significantly throughout the HSP70 chaperone family and others have shown that the NMR resonances of the corresponding glutamine residue in *Thermus thermophilus* DnaK display chemical shift perturbations between the ATP-bound and ADP-bound states and in the presence of a substrate peptide. We conclude that glutamine 88 is part of or close to the Ire1p-binding site displayed on the ATP-bound conformation of Kar2p. Binding of an unfolded polypeptide to the substrate-binding domain of Kar2p could alter the positioning of glutamine 88 and other residues on lobe IB involved in binding Ire1p, releasing Ire1p for activation of UPR signaling.

Keywords: BiP; Kar2p; Ire1p; unfolded protein response; endoplasmic reticulum

Introduction

The endoplasmic reticulum (ER)-resident HSP70 chaperone BiP has many roles in the cell, including translocation of newly synthesized polypeptides across the ER membrane, protein folding in the ER lumen, and sensing the level of unfolded proteins in the ER.[1–4] The role of BiP as a major chaperone of the ER requires that its concentration be modulated in response to the load of unfolded polypeptides present in the ER lumen. In both yeast and mammalian cells, the gene encoding BiP is regulated at the transcriptional level by forms of environmental, chemical or mutational stress that result in the accumulation of unfolded proteins in the ER lumen.[5–10] The transcriptional induction of BiP in response to this accumulation was named the unfolded protein response (UPR).[5] In the yeast *Saccharomyces cerevisiae*, BiP is called Kar2p[6,7] and the transmembrane receptor kinase/endoribonuclease Ire1p is the transducer of the UPR signal.[11,12] Upon ER stress Ire1p undergoes dimerisation and autophosphorylation,[13,14] and the activated endoribonuclease domain processes the mRNA encoding

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Abbreviations used: ER, endoplasmic reticulum; UPR, unfolded protein response; BCSA, bathocuproinedisulphonic acid; 5′-FOA, 5′-fluoro-orotic acid.

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the transcription factor Hac1p,[15,16] facilitating its efficient translation.[17,18] Hac1p then activates the transcription of a wide array of genes that encode proteins involved in a variety of different cellular processes,[19] although major targets include ER-resident chaperones, such as Kar2p, and protein folding catalysts that function to increase the efficiency of protein folding within the ER.

Until recently, the signal that triggers Ire1p to activate the UPR was unknown. Kozutsumi *et al.* were the first to postulate that reduction of the free concentration of mammalian BiP by its sequestration into complexes with unfolded proteins might provide the primary signal for induction of its own synthesis.[5] Evidence supporting this theory was provided by Graham *et al.*[20] and Ng *et al.*,[21] who showed that over-expression of unfolded proteins that do not bind BiP does not elicit a UPR response. Soon after it was demonstrated that failure to retain Kar2p in the ER led to a compensatory up-regulation of its rate of synthesis,[22] whereas over-expression of BiP in ER-stressed mammalian cells,[23] or of Kar2p in ER-stressed yeast cells,[24] inhibited activation of the UPR. Interestingly, over-expression of other ER-resident chaperones or folding catalysts, such as ERp72, PDI[25] or calreticulin[26] does not mitigate UPR signaling.

How yeast Ire1p and its mammalian counterpart IRE1 measure changes in the concentration of Kar2p and BiP has not been elucidated, although Kar2p/BiP and Ire1p/IRE1 were demonstrated to form stable complexes in the absence of ER stress in yeast[27] and mammalian[28] cells. Perturbation of protein folding promotes reversible dissociation of the chaperone from the luminal domain of Ire1p/IRE1, resulting in activation of the sensor by autophosphorylation and initiation of signaling to the nucleus.[27,28] However, these studies provided no information about the molecular form of Kar2p or BiP that interacts with Ire1p or IRE1. Our approach to this question was based on knowledge of how the ATPase cycle of Kar2p regulates the binding and release of unfolded polypeptide substrates.[4] The starting point of our study was therefore to test the hypothesis that under normal growth conditions when the concentration of unfolded polypeptides in the ER is low, Kar2p would accumulate in its substrate-free, ATP-bound form and associate with the Ire1p signal transducer to maintain it in a monomeric, inactive state. When unfolded proteins accumulate in the yeast ER, they would bind to Kar2p (converting it to the ADP-bound form) and sequester it away from Ire1p, allowing the transducer to undergo dimerisation and trans-autophosphorylation and to signal the UPR. This hypothesis leads to the prediction that a Kar2p molecule locked into the ATP-bound state would bind Ire1p, preventing UPR signaling, whereas a Kar2p molecule in the ADP-bound form would not interact with Ire1p. This prediction could be tested using site-directed mutants of Kar2p that are locked in either the ATP-bound or ADP-bound form.

Results

Over-expression of ATP-bound Kar2p mutants mitigates ER stress-induced UPR signaling

Six site-directed mutants of Kar2p[29] have single amino acid substitutions of residues (see Figure 1(a)) which, on the basis of structural and mutagenic analysis of bovine Hsc70,[30–34] are likely to play a role in ATP binding or hydrolysis. Pertinent to this study, X-ray crystallographic studies previously defined the nucleotide bound to the ATPase domains of similar mutants of bovine Hsc70.[33,34] Two of the Kar2p mutants, D14N and D202N, correspond to the Hsc70 D10N and D199N mutants, and are predicted to encode Kar2 proteins blocked in the ATP-bound state. Kar2p E179D, T207V and D209N mutants correspond to the Hsc70 E175Q/S, T204V and D206N mutants and are predicted to encode Kar2 proteins that accumulate in the ADP-bound state. The sixth mutant, G204A, was predicted to bind ATP or ADP poorly if at all, or to bind ADP. This mutant has no counterpart among the bovine Hsc70 mutants. However, inspection of the crystallographic structure of the ATPase domain of wild-type Hsc70 indicated that the replacement of the corresponding glycine (residue 201) by alanine was likely to prevent binding of either ATP or ADP. Consistent with this, substitution of the equivalent glycine (G226) in hamster BiP by aspartic acid was reported to inhibit ATP binding.[35]

The wild-type and mutant Kar2 proteins were expressed in KMY2005 cells using YRpCUP1-based vectors.[6] As expected, cells transformed with the empty vector express very similar levels of the ~80 kDa Kar2 protein from the chromosomal *KAR2* gene before and after incubation in the presence of copper (Figure 1(b), first panel). Cells transformed with YRpCUP1-YB vectors encoding the wild-type or mutant Kar2 proteins expressed very similar amounts of Kar2p at the zero time-point (Figure 1(b)) or after incubation for 3 h in the absence of added copper (not shown), but contained significantly increased levels of Kar2p following incubation for 3 h in the presence of copper as the result of induction of expression of wild-type or mutant Kar2 proteins under control of the *CUP1* promoter (Figure 1(b)). The essentially identical electrophoretic mobilities of the endogenous and exogenous Kar2 proteins indicate that all six ATPase mutants had undergone efficient translocation and signal sequence cleavage in the ER lumen.

Protease digestion of Kar2p results in characteristic nucleotide-dependent patterns of protease protection,[36] providing the opportunity to determine whether the mutant Kar2p proteins exist in conformations corresponding to the ATP-bound or ADP-bound states of the wild-type protein. We chose this approach rather than determining directly which nucleotide was bound to each mutant, because previous studies have demonstrated that amino acid substitutions in mammalian BiP can