The Unfolded Protein Response Transducer Ire1p Contains a Nuclear Localization Sequence Recognized by Multiple β Importins

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INTRODUCTION

Within the lumen of the endoplasmic reticulum (ER), a variety of resident ER proteins assist newly translocated nascent polypeptides to fold into their correct tertiary and quaternary structures (Stevens and Argon, 1999). These resident proteins include molecular chaperones that recognize and stabilize partially folded intermediates during polypeptide folding and assembly, as well as enzymes that catalyze rate-determining steps in folding, such as protein disulfide isomerase and peptidyl prolyl isomerases. Under normal growth conditions these chaperones and folding catalysts are synthesized constitutively and abundantly. However, their rates of synthesis can be increased significantly by the accumulation of mutant proteins in the ER or by a variety of stress conditions whose common denominator is thought to be the accumulation in the ER of unfolded polypeptides (Kozutsumi et al., 1988; Mori et al., 1992). This “unfolded protein response” (UPR) operates in yeast and higher eukaryotes to regulate the levels of ER chaperones and protein folding catalysts (for review, see Ma and Hendershot, 2001; Patil and Walter, 2001; Kaufman, 2002; Ron, 2002). Microarray analysis of yeast cells demonstrated that the UPR also activates genes encoding a variety of other proteins involved in diverse processes such as translocation, glycosylation and degradation of secretory proteins, lipid/inositol metabolism, cell wall biogenesis, vesicle trafficking/transport, and vacuolar protein sorting (Travers et al., 2000). Conserved elements (UPREs) are present in the promoter regions of many UPR-regulated yeast genes (Mori et al., 1992, 1998; Patil et al., 2004). Thus, an intracellular sensing system monitors events in the lumen of the yeast ER and transduces signals across the ER membrane and into the nucleus to activate the transcription of UPRE-controlled genes.

In Saccharomyces cerevisiae, the ER-to-nucleus (ERN) signal transduction pathway contains two unique components, the Ire1p/Ern1p transmembrane protein and the bZIP Hac1p transcription factor that binds UPREs. These components are not essential for vegetative growth, but they are absolutely necessary for survival under conditions that cause UPR stress (Cox et al., 1993; Mori et al., 1993). Ire1p contains in one molecule three of the essential components of the UPR pathway: the lumenal sensor, the mechanism for transducing the signal across the ER membrane, and the mecha-
Table 1. List of yeast strains and plasmids

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The references cited are as follows: 1. Mori et al. (1996); 2. Kawahara et al. (1997); 3. Winston et al. (1995); 4. Loeb et al. (1996); 5. Koepp et al. (1996); 6. Wong et al. (1997); 7. Seedorf and Silver (1997); 8. Ferrigno et al. (1998); 9. Aitchison et al. (1996); 10. Seedorf et al. (1999); 11. Morehouse et al. (1999); 12. Mori et al. (1993); 13. Niedenthal et al. (1996). Note that ern1Δ = ire1Δ.
the permissive temperature (23°C) before incubation under nonpermissive conditions (37°C). All plasmid manipulations were carried out using standard protocols (Sambrook et al., 1989). The sequences of oligonucleotides used in this study are available upon request. DNA sequence analysis was used to confirm the accuracy of introduced mutations.

Mutations in ERN1/IRE1 encoding alterations in the classical nuclear localization signal (cNLS) were generated by oligonucleotide-mediated site-directed mutagenesis (Kunkel, 1985). Restriction fragments encompassing the mutated sequences were inserted into both the pRE11EM and pERE1C, plasmids, replacing the corresponding wild-type sequence. Plasmids capable of expressing GFP-Ire1p fusion proteins under the control of the MET25 promoter were constructed as follows: Nucleotides encoding the C-terminal region of Ire1p (residues 556–1115) were amplified by polymerase chain reaction (PCR) using oligonucleotides V51 and V52 as primers and pERE1C3 as the template. The primers also added terminal SpeI and XhoI restriction sites that were used to clone the amplified fragments into the SpeI and XhoI sites of pGF-N-FUS to generate pGFIL. Plasmid pGFIL(K644T, R645T) was created from pGFIL using the complementary mutagenic oligonucleotides V53 and V54 and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmid pGFIL(K644T, R645T) was transformed into E. coli DH5α and used to clone the amplified fragments into pGF-N-FUS to give rise to pGF IL (see sequences in Figures 4A and 9).

Expression of GST Fusion Proteins

Expression and purification of murine (m-PTAC58/Rch1 and PTAC97) or yeast (KAP123) importin α and β subunits fused to the GST protein were performed as described previously (Lam et al., 1999). Human Ran was also expressed as a GST fusion protein (Hu and Jans, 1999) and then GST-free Ran was prepared by thrombin cleavage and loaded with nucleotides as described by Chi et al. (1996). All purified proteins were dialyzed against storage buffer (20 mM HEPES, pH 7.3, 100 mM KCl, and 2 mM dithiothreitol (DTT)) and kept frozen at −80°C until use.

Enzyme-linked Immunosorbent Assay (ELISA)-based Importin Binding Assay

Binding of GST-importin fusion proteins to Ire1p peptides was quantitated using an ELISA assay (Hu and Jans, 1999). The amount of bound importin was determined using an antibody directed against the GST moiety of the importin fusion protein (goat anti-GST antibody; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Biosensor-based Importin Binding Assay

Biosensor analyses were performed using an optical biosensor (BIAcore 2000; Biacore, Uppsala, Sweden). Aliquots of peptide-linked resin (25 μl of packed resin suspended in 50 μl of 0.1 M Tris-HCl, pH 7.0, 1 M NaCl, and 1 mM EDTA) (EQ buffer) were incubated for 1 h on ice with 50-μl aliquots of yeast cell extracts containing importin β-GFP fusion proteins, prepared as described by Seedorf et al. (1999). The unbound fraction was separated from resin by centrifugation in a Microfuge, and the resin was then washed three times with EQ buffer containing 0.5% Triton X-100 before bound importin-GFP proteins were eluted from the resin with 0.1 M Tris-HCl, pH 8.3, 1 mM EDTA, and 20 mM DTT. In some experiments, lysates were incubated in the presence of 1 mM guanosine 5′-O-(3-thiotriphosphate) (GTPγS) or GDP (Sigma-Aldrich, St. Louis, MO) before the peptide resin was added.

Cell Extracts and Immunoblotting

Protein extracts from yeast cells were made using EZ buffer [60 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 5% (vol/vol) β-mercaptoethanol] and quantitated using the Bradford protein assay kit (Bio-Rad, Hercules, CA). Volumes of extract containing equal amounts of total protein (~150–200 μg) were boiled in 5X SDS sample buffer for 10 min and then loaded onto 8 or 12% acrylamide gels for PAGE. For immunoblot analysis, proteins were transferred onto nitrocellulose (0.45-μm Protran; Whatman Schleicher and Schuell, Dassel, Germany) via wet transfer in the Mini Trans-Blot Cell (Bio-Rad) and blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Detection of Ire1p was performed using a polyclonal antibody directed against residues 32–259 of Ire1p/Ern1p (Mori et al., 1993), and GFP fusion proteins were detected using polyclonal anti-GFP antibodies (Seedorf et al., 1999). Subsequently, the membranes were probed with monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a gift from Trevor Lithgow, University of Melbourne, Melbourne, Australia) as a control for protein loading. The proteins were detected using ECL reagents (Pierce Chemical, Rockford, IL, or Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

β-Galactosidase Assay

β-Galactosidase activity was assayed by the crude extract method of Kaiser et al. (1994). Protein concentrations were determined using the DC Protein Assay kit (Bio-Rad).

HAC1 mRNA Splicing

Total cellular RNA from intact yeast cells was isolated as described by Ausbel et al. (1995). Total RNA (5 μg) was denatured using glyoxal and separated by electrophoresis on a 1.4% agarose gel. Separated RNA species were transferred onto nitrocellulose membrane (Nylon N+; GE Healthcare) and probed with random primer 32P-labeled HAC1 PCR products. Autoradiography was performed either at room temperature or ~70°C. Experimental details are reported in the figure legends.

Yeast Fluorescence Microscopy

Yeast cells expressing GFP fusion proteins were prepared for visualization as follows: log-phase cultures (OD600 ~0.6–0.8) were grown at 30°C in synthetic medium in the absence of methionine and uracil to induce expression from the MET25 promoter of pGF-N-FUS vector. When the expressed fusion proteins contained the iodination domain (Spencer et al., 1997), the cell-permeant dimerizer AP20187 (Ariad Pharmaceuticals) was added to the medium at a final concentration of 2 μM, and incubation was continued for 2 h. Cells were fixed by addition of a 1:10 volume of a 33% stock solution of formaldehyde directly to the medium and incubating for at least 30 min. Harvested cells were washed twice with 0.1 M potassium phosphate, pH 7.5, followed by two washes with 1X phosphate-buffered saline (PBS), and then resuspended in PBS (200 μl per 10 mm cover slip) and smeared evenly on cover slips coated with poly-L-lysine and allowed to dry. The coverslips were then inverted onto a 10-μl drop of Mowiol containing 5 ng/μl 4,6-diamidino-2-phenylindole (DAPI) on a glass slide. Fluorescence...
microscopy was carried out using either an Axioplan 2 imaging system (Carl Zeiss, Jena, Germany) and picture analysis using the Axioplan 3.0 software (Carl Zeiss), or an Olympus D70 inverted microscope with picture analysis using Openlab software (Improvision, Lexington, MA). When desirable, fluorescence images were deconvoluted using Velocity software 3.7.0. Data sets for two channels were captured using GFP and DAPI filters. Exposure for the green channel was for 300 ms, whereas for the blue DAPI channel was for 150 ms. Iterative deconvolution microscopy was performed on Z-stacks of 61 images with a step size of 0.2 μm. Significance was determined using 99.5% confidence limit or 25 iterations.

RESULTS

The Linker Region of Ire1p Contains a Nuclear Localization Sequence

The kinase and endonuclease domains of Ire1p do not contain any clusters of basic residues that might act as NLSs. However, as noted previously (Mori et al., 1993) and shown in Figure 1A, the 117-residue linker region (residues 556–672) contains a highly basic sequence (residues 642–659, underlined) that includes a stretch of six residues (642KKKRKR647) that closely resemble the paradigmatic cNLS of SV40 large T antigen (127KKKRKV132; Dingwall and Laskey, 1991), as well as six additional lysine and arginine residues just downstream from this sequence.

To determine whether the Ire1p linker sequence contains a functional NLS that could direct the nuclear localization of the GFP reporter, we constructed various plasmids expressing GFP–Ire1p fusion proteins, under the control of the MET25 promoter. The GFP moiety was fused either to the entire C-terminal domain (GFIC; Figure 1A, ii) or to the linker region (GFIL; Figure 1A, iii). The parental plasmid that expresses unfused GFP was used as the negative control. The pGFP, pGFIC, or pGFIL plasmids were introduced into RG11907 yeast cells, and the expression levels of GFP and GFP fusion proteins were analyzed by immunoblotting with an anti-GFP antibody (Figure 1B), whereas their intracellular localizations were analyzed by fluorescence microscopy (Figure 1C). Cells expressing unfused GFP displayed an even distribution of fluorescence (Figure 1C, first column, top), with the exception of a dark patch that does not colocalize with the nucleus (shown by DAPI staining, middle), but probably corresponds to the vacuole. The attachment of the entire C-terminal domain or just the linker sequence caused accumulation of GFP in the nucleus (Figure 1C, second and third columns). We concluded that the C-terminal domain of Ire1p contains a functional NLS that is likely to involve basic amino acids located between residues 642 and 659.

The potential cNLS in the basic sequence contains three overlapping versions of the cNLS consensus motif (K.R/K.x.R/K, Fontes et al., 2000). To determine whether one or more of these motifs is essential for nuclear targeting, a mutant version of pGFP-Ire1L was constructed in which threonine residues were substituted for K644 and R645 in the encoded fusion protein. These two amino acid substitutions interrupted all three versions of the cNLS motif and correspond to mutations that individually abolish or diminish the activity of the T-ag cNLS (Kalderon et al., 1984). Cells expressing the GFP–Ire1L(K644T,R645T) fusion protein (Figure 1B, see immunoblot in right-hand panel) showed greatly reduced nuclear fluorescence, which occurred at a level only slightly higher than that in the surrounding cytoplasm (Figure 1C, right-hand column). We concluded that residues K644 and R645 are important but not absolutely essential for nuclear accumulation of the fusion protein.

Mutation of the Potential cNLS in Ire1p Inhibits the UPR

To test whether the overlapping cNLS motifs in the Ire1p linker region are required for UPR signaling, site-directed mutagen-
the anti-N1 Ire1p-specific primary antibody. (B) KMY1115 resolved by SDS-PAGE and analyzed by immunoblotting by using total proteins (measured by Bradford DC assay; Bio-Rad) were

Figure 2. UPR signaling in cells expressing Ire1 proteins with mutant cNLS. (A) Wild-type and mutant Ire1p/Ern1p proteins were expressed in KMY2115 ern1Δ yeast cells from multicopy (2μ) pERN1-EM vectors (Mori et al., 1993). Mid-log cultures were incubated for 3 h at 32°C in the presence of tunicamycin (Tu; 5 μg/ml final). Proteins were then extracted, and equal amounts of total proteins (measured by Bradford DC assay; Bio-Rad) were resolved by SDS-PAGE and analyzed by immunoblotting by using the anti-N1 Ire1p-specific primary antibody. (B) KMY1115 ern1Δ yeast cells containing wild-type or mutant Ire1 proteins expressed from single copy (CEN) pERN1-BC vectors (Mori et al., 1993) were grown to mid-logarithmic phase and then incubated at 30°C for 3 h in the presence (closed bars) or absence (open bars) of Tu (5 μg/ml final). Protein extracts were prepared and assayed for β-galactosidase activity by using a UPRE-controlled lacZ reporter gene (Mori et al., 1992). The results are presented as mean ± SD, based on duplicate determinations with three independent transformants and are normalized to 100% of the activity obtained with wild-type Ire1p.

Although the single K644T substitution had little or no effect on signal-

The Ire1p NLS Is an Extended Basic Sequence

Plasmids were constructed that encode GFP fused to Ire1p residues 642–659 (GFP-Ire1B), or mutant versions of this basic sequence in which lysine and arginine residues that lie downstream of R645 for maximal UPR signaling or that deletion of all six residues abolishes signaling as the result of misfolding of the mutant protein. However, the data are not compatible with some or all of residues 642–647 constituting a cNLS that is essential for the function of Ire1p because the double substitu-

A

B

C

Figure 3. Point mutations of basic residues in the Ire1p linker sequence inhibit nuclear localization. (A) Residues mutated within the Ire1p linker sequence in GFP-Ire1Δ fusion proteins are underlined. (B) Equal amounts of protein extracts prepared from yeast cells expressing GFP or GFP-Ire1Δ fusion proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-GFP and anti-GAPDH antibodies. (C) Single colonies of RGI1907 yeast cells freshly transformed with plasmids encoding GFP or GFP-Ire1Δ fusion proteins were grown to an OD600 of 0.6 in SC-URA-MET medium before examination of the cells by fluorescence microscopy.

tions (see above; Figure 1C). ERN signaling was essentially absent in cells expressing the ΔNLS mutant, suggesting ei-

tions observed in the nuclear targeting activity of the GFP-Ire1Δ protein containing the same amino acid substitu-

Figure 3. Point mutations of basic residues in the Ire1p linker sequence inhibit nuclear localization. (A) Residues mutated within the Ire1p linker sequence in GFP-Ire1Δ fusion proteins are underlined. (B) Equal amounts of protein extracts prepared from yeast cells expressing GFP or GFP-Ire1Δ fusion proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-GFP and anti-GAPDH antibodies. (C) Single colonies of RGI1907 yeast cells freshly transformed with plasmids encoding GFP or GFP-Ire1Δ fusion proteins were grown to an OD600 of 0.6 in SC-URA-MET medium before examination of the cells by fluorescence microscopy.
Components of the Ran Cycle Are Required for HAC1 mRNA Splicing and for Import of Ire1p NLS-containing Proteins

To determine whether components of the nuclear localization apparatus are essential for the early events of UPR signaling, we tested whether mutations in proteins involved in the Ran cycle required for the orientation of importin-mediated nuclear transport (reviewed by Görlich and Kutay, 1999) would affect ER stress-induced HAC1 mRNA splicing. We assayed HAC1 splicing rather than the induction of UPRE-controlled genes because the overall UPR signaling pathway requires two additional nuclear transport steps (export of HAC1 mRNA and import of the translated Hac1p transcription factor) that also might be affected in these mutants. Significant levels of stress-induced HAC1 mRNA splicing were observed at 23 and 37°C in wild-type yeast cells (strain PSY580; Figure 4A, first panel). Similar levels of splicing occurred at the permissive temperature of 23°C in cells carrying temperature-sensitive (ts) mutations in the genes encoding RanGTPase itself (gsp1Δ expressing gsp1-1 strain PSY961), RanGAP (prp20-1 strain PSY868), and RanGEF (prp20-1 strain PSY713). However, after incubation at the nonpermissive temperature of 37°C, HAC1 mRNA splicing was significantly reduced in gsp1-1 and prp20-1 cells.

To test whether the nuclear targeting activity of the Ire1p NLS is dependent on a functional Ran cycle, the pGFP-Ire1C, pGFP-Ire1Δ (K646A,R647A) and pGFP-Ire1A (K646A,R647A,A648G) plasmids were introduced into PSY580 and PSY868 (rna1-1) cells. The expression levels (Figure 4B) and intracellular localizations of these proteins in the parental PSY580 cells had little or no effect on the expression levels of the GFP fusion proteins (Figure 4B), and it did not alter the cellular localization of these proteins in the parental PSY580 cells. However, in rna1-1 cells the GFP fusion protein was predominantly localized to the nucleus after incubation at 23°C, but it was redistributed to the cytoplasm after incubation at 37°C. By contrast, the largely cytoplasmic localizations of unfused GFP and the GFP-Ire1C fusion protein were maintained at 23°C, whereas the second aliquot was shifted to 37°C. After incubation for 4 h, the cells were examined by fluorescence microscopy.
not affected by the change in temperature. Essentially identical results were obtained when these experiments were repeated in PSY713 (prp20-1) cells (data not shown). Together, these results indicate that a functional Ran cycle is required for the nuclear targeting activity of the Ire1p NLS and for HAC1 mRNA splicing by Ire1p.

Membrane-anchored Ire1p Sequences Are Targeted to the Nuclear Membrane

Ire1p is normally present in yeast cells at such low levels (Mori et al., 1993) that the wild-type protein cannot be visualized by immunocytochemistry. A perinuclear and peripheral ER localization pattern has been reported for an Ire1p–GFP fusion protein expressed under the control of the constitutive TEF promoter (Kals et al., 2005). However, it is possible that this localization does not reflect the normal situation, because evidence from studies on the transport of membrane protein precursors targeted to the inner nuclear membrane suggests that this fusion protein could not be imported into the nucleus because the addition of GFP at the C terminus of Ire1p increases the size of the C-terminal domain beyond that (~67 kDa) compatible with transport through the lateral channels of nuclear pore complexes (Holmer and Worman, 2001; Wu et al., 2002).

Membrane-anchored forms of Ire1p in which the majority of the N-terminal receptor domain of Ire1p is replaced by bZIP dimerization sequences activate UPR signaling (Liu et al., 2000), indicating that the Ire1p C-terminal domains of such constructs are targeted to the intracellular location(s) required for splicing of HAC1 mRNA. We designed a plasmid capable of expressing a membrane-anchored form of a GFP–Ire1 fusion protein in which the N-terminal receptor domain is replaced by GFP and an Fv domain, which facilitates dimerization upon addition of the cell permeant organic molecule AP20187 (Spencer et al., 1993; Clackson et al., 1998). In this construct, which was based upon the pGFP-N-FUS based vector, DNA sequences encoding the Ire1p ER targeting sequence, GFP and Fv were fused upstream of the IRE1 sequence encoding the transmembrane, linker, kinase, and endoribonuclease domains (Figure 5A). A parallel construct contained the K646A,R647A substitutions within the NLS in the linker sequence. These pISGFFVITMC and pISGFFVITMC(K646A,R647A) plasmids were introduced into RG11907 cells, and after addition of the AP20187 dimerization agent, the expression levels of the fusion proteins were measured by immunoblotting (Figure 5B), whereas their intracellular localization was analyzed by confocal microscopy (Figure 5C). Confocal images made under our standard conditions displayed faint perinuclear fluorescence in cells expressing the GFFVITMC fusion protein (Figure 5C, first column). This pattern was not apparent in cells expressing the GFFVITMC(K646A,R647A) protein (Figure 5C, second column). Deconvolution of images of cells expressing GFFVITMC (Figure 5C, third column) revealed a distinct pattern of perinuclear fluorescence that is very similar to that observed for a variety of GFP-fused nuclear pore and inner nuclear membrane proteins localized to the nuclear periphery of yeast cells (Huh et al., 2003). The fluorescence pattern for GFFVITMC particularly resembles that reported by Murthi and Hopper (2005) for Trm1-II, which normally resides as a peripherally associated protein of the yeast inner nuclear membrane. No reticular or cortical fluorescence characteristic of ER localization (Huh et al., 2003) was observed. Deconvolution of images of cells expressing GFFVITMC(K646A,R647A) (Figure 5C, fourth column) confirmed the absence of perinuclear fluorescence.
indicating that the NLS mutations prevent targeting of the fusion protein to the nuclear membrane. Instead, a reticular pattern is observed. Although the “classic” ER pattern includes brighter perinuclear and cortical fluorescence, the localization data presented for a large collection of ER proteins by Huh et al. (2003) shows that patterns of generalized reticular fluorescence consistent with relatively even distribution of the fusion protein throughout the membrane system of the ER are not unusual, particularly for less abundant proteins. We therefore conclude that the membrane-anchored form of Ire1p is targeted to the inner nuclear membrane by an NLS-dependent process.

The Basic Sequence in the Ire1p Linker Domain Is Recognized with High Affinity by Importin β

To characterize the Ire1p NLS by using in vitro techniques, we synthesized a peptide containing the cNLS sequence and the adjacent basic residues for use in an ELISA-based importin binding assay (Hu and Jans, 1999). During synthesis of this peptide (ENLS-1, residues 642–660 of Ire1p), we also obtained two N-terminally truncated early termination peptides (ENLS-2; residues 646–660, and ENLS-3; residues 648–660). The three peptides (see sequences in Figure 6A) were purified and tested for recognition by the yeast importins Kap60p/Srp1p (the sole importin in S. cerevisiae) and Kap95p/Rsl1p (importin β), which in vivo form the importin α/β heterodimer involved in nuclear import of cNLS-containing substrates (see Figure 6B for a representative experiment and Table 2 for pooled data).

The Kap60p/Kap95p heterodimer bound ENLS-1 with very high affinity (1.7 nM). Unexpectedly, Kap95p and Kap60p alone bound ENLS-1 with affinities (1.6 and 2.2 nM, respectively) very similar to that of the heterodimer, although Kap60p exhibited somewhat lower maximal binding. These data imply that ENLS-1 contains recognition sites for both importin α and importin β. ENLS-2 was also bound by the heterodimer and by the two individual importins with similarly high affinities (albeit in each case with reduced B_{max}), indicating that the first four amino acids of the putative cNLS (642KKKR645, absent in ENLS-2) are dispensable for in vitro binding by the importins and suggesting that importin α may recognize a second minimal cNLS motif (646KRGSRGGKKGRKSRI660) present toward the C terminus of both peptides. ENLS-3 was recognized with greatly reduced affinity by both importins, indicating that residues K646 and R647 are part of the minimal NLS sequence recognized by both yeast Kap95p and murine importin β1, i.e., ENLS-3 (646KRGSRGGKKGRKSRI660). The ENLS-1 peptide was bound only weakly by importin α2, indicating that the putative cNLS, 642KKKR647, does not represent a high-affinity binding site for importin α2, despite its close similarity to the T antigen cNLS (see above). Importin α2 is also unable to bind the alternative cNLS, present within ENLS-2, that is recognized by Kap60p. Thus, the yeast and murine importin α proteins differ significantly in their ability to recognize the Ire1p NLS.

![Figure 6. Binding of yeast and murine importins to synthetic Ire1p NLS peptides as determined using an ELISA-based binding assay. (A) Sequences of synthetic Ire1p NLS peptides. Lowercase letters represent residues additional to the Ire1p linker sequence (upercase letters). The three peptides were tested for recognition by yeast importins (Kap60p/α and Kap95p/β) (B) or murine importins (Imp α2 and β1) (C). A standard ELISA-based binding assay was performed (see Materials and Methods). The K_{D} values are shown on each graph below the peptide name. Results shown are from a single typical experiment, performed in triplicate, with pooled data shown in Table 2.](5316)
Curve fit. The apparent association (data fitted as shown in Figure 6. The results for the apparent dissociation constants \( K \) of RAN on the interaction of the Ire1p NLS with importin were reversed by RANGTP but not by RAN GDP (for review, see Görlich and Kutay, 1999). We therefore investigated the effect of RAN on the interaction of the Ire1p NLS with importin β1. As shown in Figure 7, RANGTPγS significantly decreased the binding of ENLS-1 (or a control NLS peptide, PTHrP 67–94) to a GST–importin β fusion protein, whereas RANGDP consistently had little or no effect on peptide binding.

**Kinetic Analysis of Binding of Yeast and Murine Importins to the Ire1p NLS**

We used a BIAcore biosensor to characterize the kinetic parameters of binding of the yeast and murine importins to the Ire1p NLS(s). ENLS-1 peptides were linked to the biosensor chip via their N-terminal cysteine residues, and binding data generated by passing increasing concentrations of the GST-importin fusion proteins over the immobilized peptides. As shown by ELISA, yeast Kap60p and Kap95p and murine importin β1 all showed significant binding, whereas murine importin α2 bound ENLS-1 very poorly, if at all (Figure 8 and Table 2). The affinity of Kap95p for the NLS peptide (\( K_D = 0.52 \text{ nM} \)) was 20- to 25-fold greater than that of Kap60p (12.5 nM) or murine importin β1 (11.8 nM). The association rates of the two yeast importins were comparable (\( k_a = 5.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) but also dissociated at an extremely fast rate (330 \( \times 10^4 \text{ M}^{-1} \text{s}^{-1} \)). Murine importin β1 bound more rapidly (28.3 \( \times 10^4 \text{ M}^{-1} \text{s}^{-1} \)) but also dissociated at an extremely fast rate (330 \( \times 10^4 \text{ M}^{-1} \text{s}^{-1} \)). Kap95p displayed the most stable binding, with an apparent dissociation rate (\( k_d = 2.8 \times 10^{-5} \text{ s}^{-1} \)), significantly lower than that displayed by Kap60p (25 \( \times 10^{-5} \text{ s}^{-1} \)). These data suggest that within yeast cells, binding of Ire1p to Kap95p should be favored over binding to Kap60p and that importin β-dependent nuclear import should predominate in vivo.

**Redundancy of Importin Binding to the Ire1p NLS**

To determine whether Kap60p or Kap95p is essential for the function of Ire1p in UPR signaling in vivo in yeast cells, we

| Table 2. Binding parameters for the interaction of importins with synthetic Ire1p NLS peptides |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | Yeast importin binding parameter |                  |                  |
|                                  | Kap60p/Kap95p    | Kap95p          | Kap60p          |
| A                                 |                  |                  |                  |
| Peptide                          | \( K_D \) (nM)   | \( B_{\text{max}} \) (%) | \( K_D \) (nM)   | \( B_{\text{max}} \) (%) | \( K_D \) (nM)   | \( B_{\text{max}} \) (%) |
| ENLS-1 cgKKRKRG . . . IgY         | 1.7 ± 0.3 (5)    | 100             | 1.6 ± 0.1 (4)   | 92 ± 4             | 2.2 ± 0.5 (4)   | 66 ± 4             |
| ENLS-2 KRG . . . IgY              | 2.5 ± 0.6       | 79 ± 3.2        | 3.2 ± 1.1       | 66 ± 5             | 5.0 ± 1.1       | 36 ± 2             |
| ENLS-3 G . . . IgY                | 83 ± 47 (2)     | 41 ± 13         | 113 ± 26 (2)   | 30 ± 4             | 126 ± 40 (3)   | 17 ± 6             |
| B                                 |                  |                  |                  |
| Peptide                          | \( k_a \times 10^{-4} \) (M\(^{-1}\) s\(^{-1}\)) | \( \chi^2 \) | \( k_d \times 10^5 \) (s\(^{-1}\)) | \( \chi^2 \) | \( K_D \) (nM)    |
| Kap60p                           | 2.0             | 0.045           | 25             | 0.049             | 12.5            |
| Kap95p                           | 5.4             | 0.039           | 2.8            | 0.122             | 0.52            |
| Impo2                            | --              | --              | --             | --                | --              |
| Impβ1                            | 28.3            | 0.025           | 330            | 0.043             | 11.8            |
| Kap123p                          | 4.0             | 0.052           | 4.35           | 0.058             | 1.1             |

Importin binding parameters were determined using an ELISA-based binding assay as described in Materials and Methods from experimental data fitted as shown in Figure 6. The results for the apparent dissociation constants (\( K_D \)) representing the concentration of importin at which the level of binding is half-maximal) and the maximal level of importin bound (\( B_{\text{max}} \), normalized relative to that obtained for ENLS-1 when both α and β importins are added) are shown as the means ± SE (n in parentheses), where n is not indicated, the SE is determined from the curve fit. The apparent association (\( k_a \)) and dissociation rate constants (\( k_d \)) derived from the biosensor analysis of the interaction between immobilized ENLS-1 peptide and importins α2, β1, Kap60p, Kap95p, and Kap123p (Figure 8) were calculated from regions of the sensograms where 1:1 Langmuirian interactions seemed to be operative. The apparent dissociation constant (\( K_D \)) is the concentration of importin at which the level of binding is half-maximal. The binding of importin α2 was too low to perform kinetic analysis. The accuracy of the fit between experimental data and fitted curves (\( \chi^2 \)) was estimated by chi-square analysis (Catimel et al., 1997; Nice and Catimel, 1999).
tested the capacity of yeast cell mutants that are conditionally deficient in these importins to support ER stress-mediated HAC1 mRNA splicing. We observed little or no difference in the extent of stress-induced HAC1 mRNA splicing at permissive or nonpermissive temperatures in wild-type yeast cells (strain PSY580) and cells carrying ts mutations in Kap60p (srp1-31 strain PSY688) or Kap95p (rsl1-4 strain PSY1103) (Figure 9A, 1-3), even though these strains display defects in nuclear import in vivo at nonpermissive temperatures (Loeb et al., 1995; Koepp et al., 1996). We also analyzed HAC1 mRNA splicing in a number of other strains bearing ts mutations or deletions in the genes encoding several other importin family members involved in nuclear import in yeast (Kap104p, Kap108p/Sxm1p, Kap121p/Pse1p, Kap123p/Yrb4p, or Nmd5p, see Table 1 for strain descriptions and references). Again, no defects in splicing were observed (Figure 9A; data not shown). The results described previously (Figure 4) for cells carrying ts mutations in the genes encoding RanGTPase, RanGAP, and RanGEF indicated that nuclear targeting by the Ire1p NLS involves Ran cycle-dependent, importin-mediated nuclear transport, suggesting that the lack of a requirement for any individual importin protein is due to redundant binding of two or more importins to Ire1p. Because our binding data suggested that Kap60p would play a minor role in vivo, we surmised that more than one importin β family member might recognize the Ire1p NLS. To test this hypothesis, we first analyzed the binding of the ENLS-1 peptide to a GST–Kap123 fusion protein using both the ELISA assay and the BIAcore biosensor. We observed (Figure 9, B and C) that the Kap123 protein exhibited high affinity for the Ire1p NLS, with binding and kinetic constants very similar to those previously observed for Kap95p (Table 2). To determine whether the Ire1p NLS is recognized by additional members of the importin β family, protein extracts were prepared from seven yeast strains each expressing a different importin β family member that is fused at its C terminus to a bright derivative of GFP (Morehouse et al., 1999; Seedorf et al., 1999). Their individual levels of expression were verified by immunoblotting by using the anti-GFP antibody (Figure 10A). The data shown in Figure 10B demonstrate that all seven of these importin β–GFP fusion proteins could be removed from the extracts by incubation with ENLS-4 peptide (see Figure 10 legend for sequence), which had been linked via its N-terminal cysteine residues to thiopropyl-Sepharose 6B. The fusion proteins did not bind to peptide-free resin (see Figure 10B, top row, second panel for Kap95p-GFP; data not shown for the other
A Consensus Sequence Recognized by Multiple Importin β Family Members?

The capacity of Ire1p to interact with multiple importin β proteins is reminiscent of the interaction of ribosomal proteins and histones with two or more importin β family members (Schlenstedt et al., 1997; Rout et al., 1997; Jäkel and Görlich, 1998; Claussen et al., 1999; Muhlhauser et al., 2001; Mosammaparast et al., 2002). Significantly, the combined NLS we have defined for Ire1p displays a high degree of similarity to the highly basic sequences present within the β importin binding (BIB) domains defined for the yeast L25 and human rpL23a ribosomal proteins and to the short BIB NLSs defined for Xenopus and human rpL5 and for human histones H3 and H4 (Table 3A). The NLS of Ire1p also resembles closely the NLSs defined for eight additional non-ribosomal proteins known to translocate into the nucleus in an importin β-specific manner (Table 3B). Alignment of all these sequences yielded a “BIB consensus” sequence (Table 3C). When we interrogated the mammalian and yeast protein sequence databases with this consensus sequence, a very significant proportion of the proteins identified were additional ribosomal subunits (see Table 3D). Although many of the other sequences identified as containing the consensus were hypothetical ORFs, the set included a number of nonribosomal proteins confirmed to have a nuclear localization (Table 3E) as well as a large variety of other proteins that function in the nucleus, including transcription factors, polymerases, and mRNA processing enzymes (data not shown).

DISCUSSION

The starting point for this work was a puzzling topological issue associated with Ire1p, the receptor that senses the load of unfolded proteins in the ER and transduces the signal across the ER membrane. The IRE1/ERN1 gene encodes the first type 1 transmembrane receptor kinase characterized in yeast (Mori et al., 1993; Cox et al., 1993) and the first such receptor in eukaryotic cells known to signal across an internal membrane. The C-terminal portion of Ire1p carries a UPR stress-activated endoribonuclease domain that participates in splicing the mRNA precursor encoding the Hac1p transcription factor (Sidrauski and Walter, 1997). The question arose as to how the cytoplasmic domain of Ire1p could be targeted to the inner nuclear membrane, the location of the Rlg1p ligase that completes the unconventional splicing reaction (Clark and Abelson, 1987). In this study we analyzed the capacity of a highly basic sequence in the linker region of Ire1p to function as an NLS both in vivo and in vitro. We found that the 18 residue basic sequence, which includes a stretch of six residues that closely resembles a
randomly extracted basic sequences. The consensus sequence demands only five positions that should be basic residues, but the sequences identified frequently matched the Ire1p NLS (Kaps 108p, 111p, and 114p). The fourth and fifth boxes contain examples of ribosomal and nonribosomal nuclear proteins identified by

The 19-residue combined Ire1p NLS is shown with the 13-residue minimal importin β-binding sequence highlighted in bold. A consensus motif (third box, Z denotes K or R) was defined on the basis of sequence similarities (bolded residues) between the Ire1p NLS and sequences within either ribosomal and histone proteins (first box) or other proteins (second box) previously reported to interact with one or more importin β proteins. Yeast importin proteins are designated a (Kap60p), β1 (Kap95p), β2 (Kap104p), β3 (Kap121p), β4 (Kap123p), and βp (Kaps 108p, 111p, and 114p). The fourth and fifth boxes contain examples of ribosomal and nonribosomal nuclear proteins identified by interrogation of mammalian and yeast protein sequence databases as containing an exact or very close fit to the consensus sequence. The consensus sequence demands only five positions that should be basic residues, but the sequences identified frequently matched the Ire1p NLS at additional positions (in particular a pair of basic residues aligning with K653 and K654 of Ire1p), providing confidence that we have not randomly extracted basic sequences. y, yeast; h, human; m, mouse; n, Aspergillus nidulans; r, rat; and x, Xenopus. The references cited are 1. Schlenstedt et al. (1997); 2. Jäkel and Gärlich (1998); 3. Rosorius et al. (2000); 4. Claussen et al. (1999); 5. Muhlhauser et al. (2001); 6. Mosammeparast et al. (2002); 7. Chan et al. (1998); 8. Nikolaev et al. (2003); 9. Kahle et al. (2005). The two sequences lie within the portion of the conserved C-terminal sequence of NF-YA that contains the ncNLS; 10. Xiao et al. (2000); 11. Schedlitch et al. (2000). IGBP denotes insulin-like growth factor binding protein; 12. Tiganis et al. (1997). TCPTP denotes T-cell protein tyrosine phosphatase; 13. Lam et al. (1999). PTHrP denotes parathyroid hormone-related protein; 14. J. R. Aris, annotation of MRH1/YDR033W in the Stanford Saccharomyces Genome Database; 15. Ursic et al. (1995). The consensus sequence identified lies within the 231 amino acid sequence reported to contain the Sen1p NLS. 16. Bourquin et al. (1997). Human and rat CAR-SCYP are members of the cyclophilin family of peptidylprolyl isomerases. The motif identified is located outside the cyclophilin domain, in a region not shared with cyclophilin family members that function in other cellular locations.
cNLS (Mori et al., 1993), is capable of targeting GFP to the nucleus of yeast cells in a process that requires proteins involved in the Ran GTPase cycle that facilitates nuclear import. The UPR, and in particular stress-induced HAC1 mRNA splicing, is inhibited by point mutations in the Ire1p NLS that inhibit nuclear localization and also require functional RanGAP and Ran GEF proteins.

Mutagenic analysis and importin binding studies demonstrated that the Ire1p linker region contains overlapping potential NLSs: at least one cNLS (within sequences 642KKKRKR647 and/or 653KKGR656) that can be recognized efficiently by yeast importin α (Kap60p), but only poorly by murine importin α2, and a novel βNLS (665KKGR-GKKGRK676) that is recognized by several yeast β importins and by murine importin β1. In vivo in yeast, Ire1p thus has the capacity to interact with Kap60p or an array of different importin βs; hence, it can traffic to the nucleus either via the classical importin α/β heterodimer pathway or via various importin β-mediated, importin α-independent pathways. Clearly, our kinetic data suggest that binding to importin β proteins would predominate.

Jäkel and Görlich (1998) suggested that BIB domains contain an archetypal import signal, still present in many ribosomal proteins, which was originally the recognition motif for the evolutionary progenitor of present importin α Somal proteins, which was originally the recognition motif still present in many ribosomal proteins. We think that the importin family members and displays the M9 NLS. The crystal structure of importin β1 (residues 1–485) bound to the nonclassical NLS of PTTHrp (Cingolani et al., 2002) supports this hypothesis by defining a ‘prototypical’ cargo binding site that is distinct from the site that interacts with the importin α BIB domain. We think that the βNLS we have identified in Ire1p corresponds to an archetypal BIB motif: it is recognized by at least seven different importin β family members and displays significant sequence similarity to a portion of the BIB domains defined for the yeast L25 and human L23 ribosomal proteins. The ancient character of this motif seems consistent with other seemingly archaic features of the UPR signaling pathway, such as the use of a nonconventional splicing mechanism (Cox and Walter, 1996; Kawahara et al., 1997), which shares components of the tRNA maturation system, to mediate transcriptional control.

Ire1p, which contains an N-terminal hydrophobic signal sequence for targeting to the ER (Mori et al., 1993), must initially be inserted across the ER membrane with its N-terminal receptor domain located in the ER lumen, and its C-terminal linker, kinase, and endonuclease domains located in the cytoplasm. Because the outer nuclear membrane is contiguous with the rest of the ER membrane system of the cell, Ire1p could either be co- or posttranslationally inserted across the outer nuclear membrane or move to this location by diffusion from other portions of the ER. Two possible mechanisms could then be envisaged to transfer the C-terminal domains of the molecule into the nucleus. The first model, which has well-characterized precedents in the activation in mammalian cells of the SREBP receptor in response to low cholesterol (Brown and Goldstein, 1997) or of the ATF6 bZIP protein in response to ER stress (Haze et al., 1999; Yoshida et al., 2000), involves proteolytic cleavage of Ire1p at or near the cytoplasmic face of the membrane, followed by import of the released C-terminal domain into the nucleus. However, we and others have consistently failed to detect any stress-induced cleavage of Ire1p (Figure 2A; Shamu and Walter, 1996), so we support an alternative model, which involves movement of the intact Ire1p transmembrane protein around the periphery of the nuclear pore, such that the N-terminal receptor domain remains in the ER lumen, whereas the C-terminal domains are translocated into the nuclear matrix. Lateral diffusion of transmembrane proteins around the pore membrane from the ER membrane to the inner nuclear membrane of mammalian cells has been demonstrated for the lamin B receptor and emerin by using fluorescence recovery after photobleaching (for review, see Holmer and Worman, 2001). However, this diffusion process is very slow and although sequences promoting localization to the inner nuclear membrane have been mapped to the nucleocytoplasmic and transmembrane domains of these and other inner nuclear membrane proteins (Holmer and Worman, 2001; Wu et al., 2002), there is no evidence to indicate that the nuclear import apparatus actively facilitates the transport process. Instead, localization is apparently driven by trapping of the proteins in the inner nuclear membrane by their association with nucleoplasmic components such as lamins and/or heterochromatin, or by interaction between their transmembrane segments and those of other inner nuclear membrane proteins (Holmer and Worman, 2001; Wu et al., 2002). Our findings that Ire1p contains an essential importin β-binding NLS that targets membrane-anchored GFP–Ire1p to the inner nuclear membrane, together with the very recent report of importin-mediated inner nuclear membrane localization of the S. cerevisiae Heh1 and Heh2 proteins (King et al., 2006), document the interaction of integral membrane proteins with the nuclear import machinery. This interaction can apparently occur either via the importin α/importin β (karyopherin-α/karyopherin-β) complex, reported by King et al. (2006) to be the sole route for the Heh1 and Heh2 proteins, or in the case of Ire1p, via a variety of different importin family members. The distinct importin-binding specificities of the Heh and Ire1 proteins probably reflect differences in their NLSs; thus, although a 14-residue NLS (124PKKKRRKKSSKANK137) identified in Heh2p displays significant sequence identity with the N-terminal “cNLS” region of the Ire1p NLS (642KKKKRRSRRGGKKGRK657), little identity is evident with the extended “βNLS” region (italicized).

The data reported in this article, as well as our unpublished data on the localization and splicing activity of various GFP–Fv–Ire1 fusion proteins (Vodala, S., and Gething, M.-J., unpublished data) demonstrate a strong correlation between the degree to which Ire1p is localized in the nucleus and its capacity to cleave the HAC1 mRNA precursor and signal the UPR. However, Ruegsegger et al. (2001) reported that splicing of HAC1 mRNA precursors that have accumulated on stalled polyribosomes can occur in the cytoplasm. To reconcile these apparently disparate findings, we suggest that the import of Ire1p into the nucleus is not required for processing of the preexisting pool of stalled polyribosome-associated HAC1 mRNA immediately upon induction of ER stress, but, as the UPR continues, is essential for splicing of newly synthesized HAC1 mRNA precursor in the nucleus. Nuclear import of Ire1p may also be necessary to facilitate its interaction with Ada5p, which is essential for HAC1 mRNA splicing (Wellhinda et al., 2000).
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