[1] Purification and Activity Assays of the Catalytic Domains of the Kinase/Endoribonuclease Ire1p from *Saccharomyces cerevisiae*

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Introduction

Ire1p is a single-spanning transmembrane protein of the endoplasmic reticulum (ER) of all eukaryotic cells. It is a bifunctional enzyme that exhibits both kinase- and site-specific endoribonuclease activities. Work with the yeast *Saccharomyces cerevisiae* showed that Ire1p provides a key regulatory switch during an intracellular signaling pathway that originates in the lumen of the ER.[1,2] In brief, when unfolded proteins accumulate in the ER, a signal is sent to induce a transcriptional program, termed the unfolded protein response or UPR, that causes an increase in the protein-folding capacity of the ER. An accumulation of unfolded proteins is initially sensed by the ER–lumenal domain of Ire1p by an unknown mechanism.[3–5] Ire1p molecules are then thought to laterally oligomerize in the plane of the membrane, which leads to *trans*-autophosphorylation of their kinase domains and concomitant activation of an endoribonuclease activity.[6] Activated Ire1p cleaves the mRNA encoding Hac1p, a UPR-specific transcription factor, at two positions, thereby excising an intron from the RNA.[7] A second enzyme, tRNA ligase, joins the two exons liberated by Ire1p cleavage to produce spliced *HAC1* mRNA that is now efficiently translated to produce Hac1p, which in turn drives the transcriptional programs that comprise the UPR.[8] The presence of the intron in the unspliced *HAC1* mRNA blocks its translation.[9] Removal of the intron by the spliceosome-independent Ire1p/tRNA ligase-mediated splicing reaction is necessary to induce the UPR.

Ire1 in *S. cerevisiae* is a 1115-amino acid protein. It is initially synthesized with an N-terminal signal sequence followed by an ER–lumenal "unfolded protein-sensing" domain [amino acids (aa) 1–526], a single-transmembrane α helix

[1] J. S. Cox, C. E. Shamu, and P. Walter, *Cell* **73,** 1197 (1993).
[2] K. Mori, W. Ma, M. J. Gething, and J. Sambrook, *Cell* **74,** 743 (1993).
[3] C. E. Shamu, J. S. Cox, and P. Walter, *Trends Cell Biol.* **4,** 56 (1994).
[4] R. Chapman, C. Sidrauski, and P. Walter, *Annu. Rev. Cell Dev. Biol.* **14,** 459 (1998).
[5] C. Sidrauski, R. Chapman, and P. Walter, *Trends Cell Biol.* **8,** 245 (1998).
[6] C. E. Shamu and P. Walter, *EMBO J.* **15,** 3028 (1996).
[7] C. Sidrauski and P. Walter, *Cell* **90,** 1 (1997).
[8] C. Sidrauski, J. S. Cox, and P. Walter, *Cell* **87,** 405 (1996).
[9] R. E. Chapman and P. Walter, *Curr. Biol.* **7,** 850 (1997).

0076-6879/01 $35.00

(aa 527–556), a linker domain (aa 557–678), a kinase domain (aa 679–973), and a C-terminal domain (aa 974–1115) that is presumed to harbor the endonuclease activity. Homologs of Ire1p have been identified in *Schizosaccharomyces pombe, Caenorhabditis elegans, Drosophila melanogaster, Arabidopsis thaliana,* and mammalian cells. By sequence analysis, the enzymes are most conserved in the kinase and presumed nuclease domains; and site-specific endoribonuclease activity has been demonstrated for two mammalian isoforms[10] and shown to be indistinguishable from that of *S. cerevisiae* Ire1p. In *HAC1* mRNA, Ire1p recognizes conserved RNA stem–loop structures, which it cleaves after invariant G residues found in the third position of a seven-nucleotide loop. RNA oligonucleotides that form such stem–loop structures are cleaved by Ire1p and hence provide convenient "minisubstrates" to characterize the reaction. In this way, it was determined that Ire1p cleaves RNA to leave a 2′,3′-phosphate on the 5′ fragment and a free 5′-hydroxyl group on the 3′ fragment.[11]

We here describe the overexpression in two different systems, purification, and activity assays for fusion proteins containing the cytosolic domains of Ire1p from *S. cerevisiae*. All expressed proteins truncate Ire1p just after the transmembrane region and hence contain the linker, kinase, and presumed nuclease domains. Because C-terminal tags reduce Ire1p activity *in vivo* (C. Shamu and P. Walter, unpublished data, 1995), all fusion proteins were constructed containing N-terminal glutathione *S*-transferase (GST) or hexahistidine (His_6) tags to facilitate purification. Detailed methods for analysis of Ire1p endonuclease cleavage products can be found elsewhere.[11]

Solutions

Buffer A: 20 m*M* HEPES (pH 7.5), 150 m*M* KCl, 1 m*M* dithiothreitol (DTT), 5 m*M* $MgCl_2$, 10% (v/v) glycerol
Buffer B: 20 m*M* HEPES (pH 7.5), 1 m*M* DTT
Buffer C: 20 m*M* HEPES (pH 7.5), 1 m*M* DTT, 500 m*M* KCl
Buffer D: 50 m*M* HEPES (pH 7.5), 150 m*M* KCl, 1 m*M* EDTA, 1 m*M* DTT
Buffer E: 20 m*M* HEPES (pH 7.5), 10 m*M* magnesium acetate, 50 m*M* potassium acetate, 1 m*M* DTT
Buffer F: 0.3 *M* sodium acetate (pH 5.2), 10 m*M* magnesium acetate
Urea buffer (2×): Combine 28 ml of 5 *M* NaCl, 4 ml of 1 *M* Tris (pH 7.5), 8 ml of 0.5 *M* EDTA, 40 ml of 10% (w/v) sodium dodecyl sulfate (SDS); add water to 200 ml

[10] M. Niwa, C. Sidrauski, R. J. Kaufman, and P. Walter, *Cell* **99,** 691 (1999).
[11] T. N. Gonzalez, C. Sidrauski, S. Dörfler, and P. Walter, *EMBO J.* **18,** 3119 (1999).

Buffer G: Combine 21 g of urea and 25 ml of 2× urea buffer; add water up to 50 ml; heat (~50°) to dissolve
Buffer H: 50 m*M* Tris (pH 8.5), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 m*M* 2-mercaptoethanol, 1% (v/v) Triton X-100
Buffer I: 50 m*M* sodium phosphate (pH 8), 300 m*M* NaCl, 10% (v/v) glycerol

Cell Growth and Protein Expression in *Escherichia coli*

For expression of GST-Ire1(l+k+t) (linker plus kinase plus tail, amino acids 556–1115) in *Escherichia coli,* the vector pCF210[7] is constructed by subcloning the Ire1p fragment into pGEX-6P-2 (Amersham Pharmacia, Piscataway, NJ). This construct contains a PreScission protease cleavage site that allows removal of the GST tag linked to the amino-terminal end of Ire1(l+k+t).

We use BL21(DE3)pLysS cells (Stratagene, La Jolla, CA), which consistently give better expression yields than DH5α or BL21(DE3) cells. Irep1 expression vectors can be unstable in *E. coli* cells; we therefore always transform cells freshly with the expression vector. Five colonies are used to start a 50-ml preculture in Luria–Bertani (LB) medium, containing carbenicillin (100 μg/ml) (GIBCO-BRL Gaithersburg, MD). The preculture is grown for 14 hr to late log phase and used to inoculate larger batches at a dilution of 1 : 200 (5 to 1000 ml) in LB medium containing carbenicillin at 100 μg/ml. Typically, cells are grown in six 1-liter batches of LB medium in shaking flasks (225 rpm) at 37° to an OD_{600} of 0.6 to 0.8. Cells are then induced with 0.7 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) (Denville Scientific, Metuchen, NJ) and shifted to 30°. About 4 hr after induction, cells are harvested by centrifugation with a GSA rotor (Sorvall, Newtown, CT) at 16,000*g* at 4° for 15 min, yielding about 20 g of *E. coli* cell paste (wet weight). The cell pellet is resuspended in buffer A (150 ml), supplemented with protease inhibitors (one tablet/50 ml, Protease Inhibitor Complete; Boehringer Mannheim, Indianapolis, IN) and 1% (v/v) Triton X-100 (Calbiochem, La Jolla, CA). The suspension is quick-frozen in liquid nitrogen and stored at −80°.

Purification of GST-Ire1(l+k+t)

The cell suspension is thawed and kept on ice. Fresh DTT (to a final concentration of 1 m*M*) and additional protease inhibitor mix (three inhibitor cocktail tablets) are added, and the cells are lysed in an ice-cold Microfluidizer (Microfluidics, Newton, MA) for three cycles. The BL21(DE3)pLysS cells used do not require the addition of lysozyme. The crude extract is centrifuged in an SS-34 rotor at 31,000*g* at 4° for 30 min to remove the cell debris. To the resulting supernatant is added a 2-ml aliquot of a slurry of 50% glutathione–Sepharose 4B (Amersham Pharmacia; equilibrated in 20 m*M* HEPES, pH 7.0) and the mixture is incubated with gentle

overhead mixing on a rotating wheel for 1 hr in three 50-ml conical tubes at 4°. The Sepharose beads are collected by centrifugation at 1000*g* for 5 min at 4°; the supernatant is discarded. The resin is washed four times with 40 ml of ice-cold buffer A, and recollected by centrifugation at 1000*g* as described above, washed a second time (four times with 10 ml of buffer A) and recollected, and resuspended in a small amount of buffer A and transferred to a 15-ml conical tube. The resin is then washed with 10 ml of buffer B (low salt), followed by a final wash with 10 ml of buffer C (high salt). All washes are done for 15 min at 4° by gently mixing.

We have found that Ire1p(l+k+t) prepared in this way, that is, without any further washes, copurifies with an almost equimolar amount of the *E. coli* heat shock protein 70 (HSP70)-like chaperone DnaK. To remove DnaK, we add another wash in 10 ml of buffer A containing 5 m*M* ATP.

Next, the resin is equilibrated with buffer D. Excess buffer is removed to produce a 50% slurry (2 ml). A 60-μl aliquot of PreScission protease (120 U; Amersham Pharmacia) is added to sever Ire1p(l+k+t) from the GST tag. The mixture is incubated at 4° for 12 hr on a rotating wheel. The completeness of the cleavage is monitored by removing a sample and subjecting it to SDS–polyacrylamide gel electrophoresis.

The resin is collected by centrifugation and washed with 500 μl of buffer A. Both supernatants are combined. To remove residual traces of uncleaved protein and PreScission protease (which also has a GST tag), 300 μl of a 50% glutathione–Sepharose 4B slurry in buffer A is added to the supernatant and gently mixed at 4° for 30 min. The resin is removed by centrifugation, and glycerol is added to a final concentration of 20% (v/v). The protein is aliquoted, quick-frozen in liquid nitrogen, and stored at −80°. The final concentration of Ire1p(l+k+t) is estimated by comparing Coomassie blue-stained bands of Ire1p (l+k+t) with bovine serum albumin (BSA) standards of known amounts after SDS–polyacrylamide gel electrophoresis (PAGE). The yield from a 6-liter culture is about 1–2 mg.

Baculovirus Expression and Purification of Yeast Ire1p(l+k+t)

Expression of Ire1p(l+k+t) in *Spodoptera frugiperda* (Sf9) insect cells, using a baculovirus vector, is more cumbersome than expression in *E. coli,* but has the advantage that Ire1p(l+k+t) does not copurify with chaperones. Similar observations have also been made with other kinases, indicating that eukaryotic cells may better promote the correct folding of kinase domains (D. Morgan, personal communication, 2000).

Baculovirus expression vector pPW463 contains the same portion of *S. cerevisiae* Ire1 as pCF210 described above. It is made by subcloning the Ire1p(l+k+t)-encoding DNA fragment after *Eco*RI–*Hin*dIII cleavage into pFastBacHTb (GIBCO-BRL). Ire1p(l+k+t) is preceded by a His_6 tag.

The expression of His_6-Ire1p(l+k+t) from the pPW463 plasmid is performed as described in the *Bac-to-Bac Baculovirus Expression Systems Manual* (GIBCO-BRL). In brief, Sf9 cells are transfected with pPW463 DNA according to the protocol. Serum-free SF-900 II SFM medium (GIBCO-BRL) is used to grow the cells at 28°, while stirring at 100 rpm. The virus is harvested after 72 hr, the titer is determined, and the virus is amplified two more times at a low multiplicity of infection (MOI of 0.01–0.1).

For the final protein expression culture, infection is done with a high MOI of 5–10 in 250 ml of SF-900 II SFM medium. The cell density is about 2×10^7 cells, and cells are harvested after 48 hr by centrifugation at room temperature, 1200*g* for 10 min. To the harvested cells, 50 μl of buffer H is added per milliliter of culture. Cells are resuspended and quick-frozen in liquid nitrogen.

For purification of His_6-Ire1p(l+k+t), the cell suspension is thawed. Cells are lysed by sonication (two 20-sec pulses, small tip—avoid heating of sample), and the resulting extract is centrifuged at 100,000*g* for 1 hr at 4°. The supernatant is diluted into 60 ml of buffer I and passed over a Hi-Trap chelating column (Amersham Pharmacia) loaded with Co^{2+}. Co^{2+}-loaded resin results in lower background binding of cellular proteins than Ni^{2+}-loaded resin. Thus, prior to use, Hi-Trap chelating resin is prepared by washing it first with 50 m*M* EDTA to strip bound cations, after which it is loaded with a solution of 0.2 *M* $CoCl_2$. The resin is then washed with water and equilibrated with buffer I.

The extract (60 ml) is chromatographed on a 1-ml column at 0.5 ml/min, washed extensively with buffer I (10 ml), followed by buffer I at pH 6 (10 ml), buffer I–1 *M* NaCl (10 ml), buffer I (10 ml), and finally buffer I containing 20 m*M* imidazole. His_6-Ire1p(l+k+t) is eluted with buffer I containing 200 m*M* imidazole. Eluted protein is analyzed and roughly quantified by Coomassie blue staining after SDS–PAGE. An 800-ml culture yields approximately 0.4 mg of His_6-Ire1p(l+k+t).

The α and β isoforms of human His_6-Ire1p(l+k+t) are expressed and purified in the same way.[10]

Kinase Assay

The kinase activity of Ire1p(l+k+t) is determined by autophosphorylation. Kinase reactions (30 μl) contain 0.1 m*M* ATP, 5 μCi of [γ-^{32}P]ATP, buffer E, and 0.5 μg of Ire1p(l+k+t). A cocktail containing buffer E, ATP, and [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia) is dispensed into 1.5-ml microcentrifuge tubes and the tubes are placed in a water bath at 30°. Reactions are initiated by adding the enzyme and incubated at 30° for 30 min. Aprotinin (3 μg) is added as carrier, and reactions are stopped with 120 μl of 20% (w/v) trichloroacetic acid (TCA) for 30 min on ice. The protein is displayed on a 7% (w/v) SDS–polyacrylamide gel and visualized by autoradiography and Coomassie blue staining.

Ire1p(1+k+t) preparations purified from both *E. coli* and Sf9 cells are already phosphorylated. This is apparent as a marked mobility shift on SDS–polyacrylamide gels when the enzymes are treated with Ptc2p (a phosphatase shown to interact with Ire1p *in vivo*[12]). Thus the kinase activity measured in our assays reflects phosphate exchange and/or phosphorylation at sites that are still available. Probably as a consequence of its already activated phosphorylation state, Ire1p(1+k+t) preparations are constitutively active and do not require the addition of hydrolyzable ATP to exhibit nuclease activity (although the addition of some form of adenosyl nucleotide, such as ADP, is required). In contrast, preparations of mutant forms of Ire1(1+k+t) designed to inactivate the kinase activity are unphosphorylated and inactive for nuclease function (M. Niwa and P. Walter, unpublished data, 2000).

In Vitro Transcription of *HAC1* 508 RNA

HAC1 508 RNA is a 508-nucleotide-long fragment of unspliced *HAC1* RNA that contains both splice junctions (181 nucleotides of the 5′ exon, 252 nucleotides of the intron, and 75 nucleotides of the 3′ exon). The template for *in vitro* transcription is prepared by linearizing 5 μg of pCF187 by digestion with *Sac*I for 2 hr at 37°. The reaction is then treated for 5 min at 37° with 10 U of T4 DNA polymerase (New England BioLabs, Beverly, MA) to produce blunt ends (to give a more homogeneous transcript), phenol extracted, and ethanol precipitated. The transcription reaction (20 μl) contains T7 RNA polymerase buffer, 1 m*M* ATP, 1 m*M* CTP, 1 m*M* GTP, 0.1 m*M* UTP (nucleotides from Boehringer Mannheim), 25 μCi of [α-^{32}P]UTP (3000 Ci/mmol; Amersham Pharmacia), 40 U of RNasin (Promega, Madison, WI), and 1 μg of linearized pCF187, and is initiated with 20 U of T7 RNA polymerase. The reaction is incubated at 37° for 2 hr. At that time, 180 μl of water and sodium acetate, pH 5.2, to a final concentration of 0.3 *M*, is added. The reaction is phenol–chloroform extracted and ethanol precipitated, using 40 μg of glycogen (Boehringer Mannheim) as carrier. The transcript is dissolved in 25 μl of gel loading buffer followed by heating to 95° for 3 min and purified on a 5% (w/v) polyacrylamide gel containing 7 *M* urea. Bands are visualized by exposing the gel to X-ray film for 5 min. The film is aligned with the gel, and the most dominant band is excised. The RNA is eluted from the gel slice by adding 400 μl of buffer F and 400 μl of phenol–chloroform followed by shaking overnight at 4°. The RNA in the extract is ethanol precipitated and dissolved in 15 μl of water. A 20-μl reaction yields 4×10^6 cpm of purified transcript, which is enough for 200 standard cleavage reactions.

[12] A. A. Welihinda, W. Tirasophon, S. R. Green, and R. J. Kaufman, *Mol. Cell. Biol.* **18,** 1967 (1998).

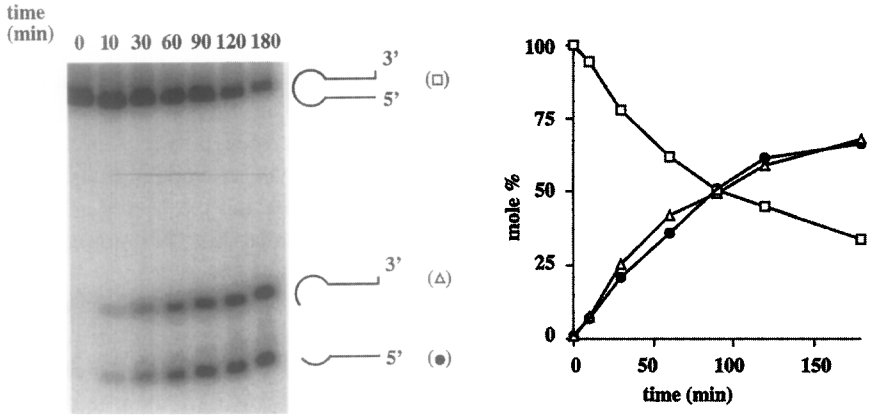

FIG. 1. Time course for hactng-10 minisubstrate cleavage by Ire1p(l+k+t). The cleavage reaction (20 μl) was performed as described in text and contained 400 n*M* Ire1(l+k+t) (expressed in *E. coli*, and with the GST tag removed) and 255 p*M* hactng-10 substrate. The symbols represent: hactng-10 minisubstrate □, 3′ cleavage product △, 5′ cleavage product ●.

In Vitro Transcription of Stem–Loop hactng-10 Minisubstrate

For preparation of the *HAC1* stem–loop minisubstrates,[11] a single-stranded DNA oligonucleotide (hactng-10; 42 nucleotides long, comprising the 3′ splice site stem–loop) is synthesized to contain a T7 promoter sequence followed by the sequences specifying the desired RNA molecule. A short oligonucleotide (T7 promoter oligonucleotide, 15 p*M*) is annealed to template (hactng-10 oligonucleotide, 0.25 p*M*) to create a double-stranded T7 RNA polymerase promoter.[13] Both oligonucleotides are gel purified prior to use. The mixture is heated to 95° for 3 min and immediately cooled on ice. The transcription reaction is performed as described above. The resulting stem–loop RNA is purified on 15% (w/v) denaturing polyacrylamide gels.

hactng-10 oligonucleotide: 5′-TGAGGTCAAA CCTGACTGCG CTTCG-GACAG TACAAGCTTG ACCTATAGTG AGTCGTATTA-3′
T7 promoter oligonucleotide: 5′-TAATACGACTCACTATAG-3′

RNA Cleavage Assay

Cleavage reactions (20 μl) contain buffer E, 2 m*M* ADP, and 215 p*M* (20000 cpm) *HAC1* 508 RNA or 255 p*M* (2000 cpm) *HAC1* stem–loop hactng-10

[13] J. F. Milligan, D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck, *Nucleic Acids Res.* **15,** 8783 (1987).

minisubstrate. Reactions are initiated by adding 400 n*M* (0.5 μg/20 μl) Ire1p (l+k+t), incubated at 30° for varying lengths of time, and stopped with 200 μl of buffer G. RNA is extracted with 200 μl of phenol–chloroform, ethanol precipitated with glycogen as carrier, dissolved in 6 μl of RNA gel loading buffer, heated to 95° for 3 min, and loaded onto a denaturing urea–polyacrylamide gel to separate the cleavage products. Gels containing polyacrylamide at 5 and 15% (w/v) are used for reactions containing *HAC1* 508 RNA and the hactng-10 minisubstrate, respectively. The reaction products are visualized by autoradiography and quantitated by phosphorimaging.

On a mole-by-mole basis, the Ire1p(l+k+t)-catalyzed cleavage of the *HAC1* 508 RNA is about three times more efficient than cleavage of the stem–loop minisubstrate. Under standard reaction conditions, Ire1p(l+k+t) (400 n*M*) cleaves 50% of *HAC1* 508 RNA (215 p*M*) in 30 min and 50% of stem–loop hactng-10 minisubstrate (255 p*M*) in 90 min (see Fig. 1).

Acknowledgments

This work was supported by a UCSF Biomedical Science Research Career Enhancement Fellowship from the National Institute of General Medical Science to T.N.G.; by NIH training grant NIH-NCI T32 CA09270, Molecular Biology of Eukaryotic Cells and Viruses, to M.N.; and by grants from the National Institutes of Health to P.W. P.W. is an Investigator of the Howard Hughes Medical Institute.

[2] Monitoring Activation of Ribonuclease L by 2′,5′-Oligoadenylates Using Purified Recombinant Enzyme and Intact Malignant Glioma Cells

By LORRAINE RUSCH, BEIHUA DONG, and ROBERT H. SILVERMAN

Introduction

RNase L is a regulated endoribonuclease present in a wide range of mammalian cell types, where it mediates antiviral, antiproliferative, and apoptotic effects of the interferons (IFNs) (reviewed in Stark *et al.*[1]). The most unusual property of RNase L is its requirement for allosteric activators consisting of 5′-phosphorylated, 2′,5′-linked oligoadenylates of the general formula: $p_x(A2'p)_nA$, where $x = 1$ to 3 and $n \geq 2$.[2] RNase L has a relatively broad specificity for cleaving single-stranded

[1] G. R. Stark, I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber, *Annu. Rev. Biochem.* **67,** 227 (1998).

[2] I. M. Kerr and R. E. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 256 (1978).

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