Alterations in an IRE1-RNA complex in the mammalian unfolded protein response

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SUMMARY

IRE1 proteins mediate cellular responses to accumulation of malfolded proteins in the endoplasmic reticulum in the yeast and mammalian unfolded protein responses. A sensitive in vivo u.v. crosslinking assay showed that IRE1 proteins are intimately associated with RNA in mammalian cells. The IRE1-associated RNA fragments recovered by this assay were different in stressed and unstressed cells. The amount of RNA associated with IRE1 that could be revealed by end-labeling with T4 kinase was greater in IRE1-containing complexes isolated from stressed cells. Furthermore, the RNA fragments recovered from complexes found in stressed cells were shorter than those from unstressed cells, revealing a dynamic change in the IRE1-RNA complex during the UPR. Formation of the complex between IRE1 and RNA was dependent on both the kinase and endonuclease domains of IRE1, and involved pre-existing RNA species. When viewed in the context of the known importance of Ire1p-*HAC1* mRNA interactions to the yeast unfolded protein response, these findings suggest that full-length mammalian IRE1s also engage RNA molecules as downstream effectors.

Key words: Endoplasmic reticulum, Protein kinase, Ribonuclease, RNA Processing, Signal transduction

INTRODUCTION

Cells respond to the accumulation of malfolded proteins in the endoplasmic reticulum (ER) by activating signaling pathways that regulate the expression of genes whose products mitigate the effects of ER stress and attenuate protein synthesis (reviewed by Kaufman, 1999; Mori, 2000). The first ER-stress transducer to be identified was the yeast Ire1p, a transmembrane ER resident protein encoded by the *IRE1* gene (Cox et al., 1993; Mori et al., 1993). The lumenal domain of Ire1p responds to ER stress signals and its cytoplasmic C-terminal effector domain mediates downstream signaling. The C-terminus of Ire1p is a protein kinase and mutations that interfere with kinase activity block *IRE1* function (Mori et al., 1993; Tirasophon et al., 2000). In yeast, the effector function that mediates downstream gene expression is cleavage of the HAC1 mRNA by an endonuclease activity that resides in the C terminus of Ire1p (Cox and Walter, 1996; Kawahara et al., 1997; Sidrauski and Walter, 1997). In unstressed cells, the HAC1 mRNA is poorly translated. Stress-induced Ire1p-mediated cleavage liberates an intron from the HAC1 mRNA, and tRNA ligase joins the two exons to produce a translatable form of HAC1 mRNA. The encoded protein, Hac1p, is a transcription factor that binds to and activates promoters of genes involved in the yeast UPR (Chapman and Walter, 1997; Kawahara et al., 1997).

It is not known how Ire1p kinase activity controls Ire1p-mediated splicing of HAC1 mRNA – the regulated event in the yeast UPR. The bacterially expressed C-terminal effector domain of Ire1p is able to cleave the *HAC1* intron in vitro; however, it is not known if this capacity of the bacterial protein requires kinase activity (Sidrauski and Walter, 1997). Recently two mammalian homologs of *IRE1*, *IRE1*α and *IRE1*β were identified (Tirasophon et al., 1998; Wang et al., 1998). These homologs are implicated in activating target genes in the mammalian UPR and are capable of splicing the yeast *HAC1* mRNA (Tirasophon et al., 1998; Niwa et al., 1999; Tirasophon et al., 2000); however, no mammalian mRNAs that are targets for splicing by IRE1 have been identified to date. The IRE1α mRNA may be cleaved by overexpression of IRE1α, but the mechanisms involved are not known (Tirasophon et al., 2000). Both IRE1α and IRE1β possess kinase activity that is required for activating downstream signaling (Tirasophon et al., 1998), but it is not understood how this regulation is imposed.

It has recently been reported that mammalian IRE1 proteins undergo cleavage during their activation by the UPR. The cleaved C-terminal effector domain of IRE1α and IRE1β were described to migrate to the nucleus, where it is proposed they engage their yet-to-be identified substrate mRNA and effect splicing (Niwa et al., 1999). These observations suggest that mammalian IRE1 activity may be controlled at the level of access to the substrate. However, mammalian IRE1s couple ER stress to stress-activated protein kinases (Urano et al., 2000) and have been reported to activate the membrane-bound form of the transcription factor ATF6 (Li et al., 2000), activities that are thought to take place in the cytoplasm and would probably not involve interactions with an mRNA substrate. We therefore sought to explore the interaction of mammalian IRE1s with RNA as an important step towards addressing the role of mRNA splicing in downstream signaling. We now report on

the use of a sensitive in vivo method for detecting interactions between IRE1 and RNA, and show that these interactions are modulated by ER stress and are dependent on the kinase and endonuclease domains of IRE1.

MATERIALS AND METHODS

Cell culture and transfections

Rat pancreatic acinar AR42J cells and human embryonic kidney 293T cells were obtained from ATCC and were cultured in DMEM supplemented with 10% fetal calf serum.

In transfection experiments, two 10 cm dishes of 293T cells were used for each experimental point. Cells were transfected using the calcium chloride precipitation method with previously described expression plasmids (Wang et al., 1998): wild-type mIRE1β, kinase inactive mIRE1β K536A mutant, mIRE1βΔnuc mutant truncated in the endonuclease domain (amino acids 1-820) and mIRE1βΔC mutant, which lacks most of the C-terminal domain (amino acids 1-518). Cells were processed for RNA immunoprecipitation and immunoblotting 36 hours post transfection.

u.v. crosslinking and RNA immunoprecipitations

Treated or untreated cells were placed on a flat bed of crushed ice (1.5 cm thick), washed twice with 5 ml ice-cold PBS and left in 2 ml PBS per 10 cm plate. Plates on their ice bed were placed in a u.v. Stratalinker 2400 oven (Stratagene) and irradiated with 900 mJ/cm^2 (delivery of this amount of energy usually required ~5 minutes of irradiation). Immediately thereafter, cells were scraped into ice-cold PBS, recovered by a 2000 ***g*** spin for 5 minutes and solubilized for 5 minutes on ice with 150 μl of 1% Triton buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM phenylmethysulphonyl fluoride (PMSF), 4 mg ml^{-1} aprotonin and 2 mg ml^{-1} pepstatin A). Extracts were clarified by centrifugation for 10 minutes at 14,000 ***g*** 4°C. Lysates were then treated with 0.8 μg or 20 μg RNase A for 30 minutes at room temperature per AR42J cell extract or 293T cell extract respectively. SDS was added to the lysate to a final concentration of 1%, and the lysate was heated for 5 minutes at 90°C. The extracts were clarified by centrifugation for 30 minutes at 200,000 ***g*** in a TLA-100-2 rotor (Beckman).

The supernatant was diluted to 0.1% SDS in RIPA buffer without SDS (10 mM Tris HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton, 0.5% sodium deoxycholate) and pre-cleared for 1 hour at 4°C on 20 μl protein A Sepharose (Zymed). Immunoprecipitations were carried out overnight at 4°C using 4 μl of anti-IRE1α or 6 μl of anti-IRE1β polyclonal sera and 10 μl of protein A Sepharose. Immunoprecipitated material was washed three times for 5 minutes at room temperature in 1 ml RIPA buffer containing 1 M NaCl (10 mM Tris HCl pH 7.5, 1 M NaCl, 1 mM EDTA, 1% Triton, 0.5% sodium-deoxycholate, 0.1% SDS) and rinsed three times in kinase buffer (50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM DTT). Residual buffer was then carefully removed using a syringe fitted with a 30 G needle. The beads were resuspended in 30 μl of kinase reaction mix (50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM DTT, 100 μCi of [γ-^{32}P] ATP (7000Ci/mM) and 5 units of T4 polynucleotide kinase) and agitated at 1000 rpm on a Thermomixer (Eppendorf) at 37°C for 30 minutes. To minimize the autokinase activity of the immunopurified IRE1, $MnCl_2$ was omitted from the kinase reaction buffer. Beads were washed three times for 5 minutes at room temperature in 1 ml RIPA buffer containing 1 M NaCl, and resuspended in 50 μl of SDS-PAGE loading dye. Samples were loaded on 8 or 10% SDS-PAGE, and gels were exposed to autoradiography or transferred to a nitrocellulose membrane, which was sequentially exposed to autoradiography and immunoblotted for mIRE1α, as described previously (Bertolotti et al., 2000). In Fig. 3, 90% of the immunoprecipitated material solubilized in SDS-PAGE loading dye was loaded on a SDS-PAGE followed by autoradiography and 10% was used for immunoblot with anti-IRE1β antiserum (Bertolotti et al., 2000).

Recovery of labeled RNA species

To recover the immunoprecipitated RNA molecule covalently linked to IRE1α, the radiolabeled region containing to the IRE1α-RNA complex was excised from the gel, cut into small pieces with a razor blade, incubated in 350 μl of proteinase K reaction mix (20 mM Tris HCl pH 7.4, 100 mM NaCl, 0.1% SDS, 10 mM EDTA, 2 μg glycogen and 100 μg of proteinase K) twice sequentially at 37°C for 10 hours. The two eluates were combined, phenol/chloroform extracted, ethanol precipitated and resolved on a 10% acrylamide-8M Urea gel. Where indicated, the recovered labeled polynucleotide was treated with 1U RNase A before loading on the acrylamide-8M Urea gel.

RESULTS

To examine the possibility of an interaction between mammalian IRE1 and RNA, we attempted to crosslink IRE1 to any closely associated RNA species by u.v. irradiation of living cells. AR42J cells were treated with the reducing agent DTT, to promote ER stress and activate endogenous IRE1α. These cells were chosen because they express high level of IRE1α and other ER stress transducers (Bertolotti et al., 2000). The living cells were exposed to u.v. light, lysed and complexes containing IRE1α were immunoprecipitated from the lysate. To reveal complexes containing RNA covalently attached to IRE1α, we digested (partial digest) the lysate with RNase A before immunoprecipitation. This solubilizes the protein-RNA complex and leaves a free 5′ hydroxyl group on the RNA remnant. After immunoprecipitation, the 5′ OH group on the RNA part of the protein-RNA complex was radiolabeled with 32P-γATP and polynucleotide kinase, and revealed by autoradiography. A radiolabeled band identical in size to the IRE1α protein (identified by immunoblot) was observed (Fig. 1A).

The intensity of the radiolabeled IRE1α band increased with ER stress, and its presence depended on the u.v. crosslinking step and on the use of an antiserum to IRE1α in the immunoprecipitation. Specificity for IRE1 is revealed by the absence of the band in control immunoprecipitation reactions carried out with pre-immune serum or antiserum directed against the IRE1-related ER membrane protein PERK. The band was also dependent on the addition of polynucleotide kinase to the labeling reaction. The latter is a particularly important control to rule out the possibility that label is incorporated into IRE1α by its autokinase activity. The labeled band was found only in samples treated with limited quantities of RNase A, indicating that recovery of a species capable of being labeled is dependent on its solubilization by (partial) enzymatic digestion of the RNA.

To confirm the chemical nature of the IRE1α-associated species that is labeled by T4 kinase, we treated the gel slices containing the labeled band with proteinase K, to digest the protein component and recovered the labeled species. On a denaturing acrylamide-urea gel, the major labeled species co-migrated with a polynucleotide ~25 base in length. RNase A digestion of the labeled species confirmed that it had an RNA

component (Fig. 1B). Given that proteinase K rarely digests proteins to completion, the species recovered from the gel may represent a peptide-RNA fragment, and consequently the size of the RNA component cannot be estimated with accuracy in this gel.

The radiolabeled crosslinked species was induced most strongly by DTT treatment, which is also the most potent activator of IRE1α in AR42J cells (Bertolotti et al., 2000; data not shown); however, other inducers of ER stress also increased complex formation (Fig. 2A). ER stress dependence of complex formation was also confirmed by treating cells with increasing concentrations of DTT (Fig. 2C). Additional evidence for the dynamic nature of IRE1α-RNA complex and for its modification by ER stress was provided by the observation that the size of the labeled species eluted from the complex was different in stressed and unstressed cells (Fig. 2B,D). The labeled species liberated from the IRE1α immunoprecipitate from unstressed cells was larger than that from stressed cells. Moreover, the intensity of the radiographic signal revealed by labeling with T4 kinase was greater for the smaller fragments recovered from stressed cells. After purification and elution from the gel, both the longer and shorter species were degraded by treatment with RNase, indicating their RNA content (Fig. 2D).

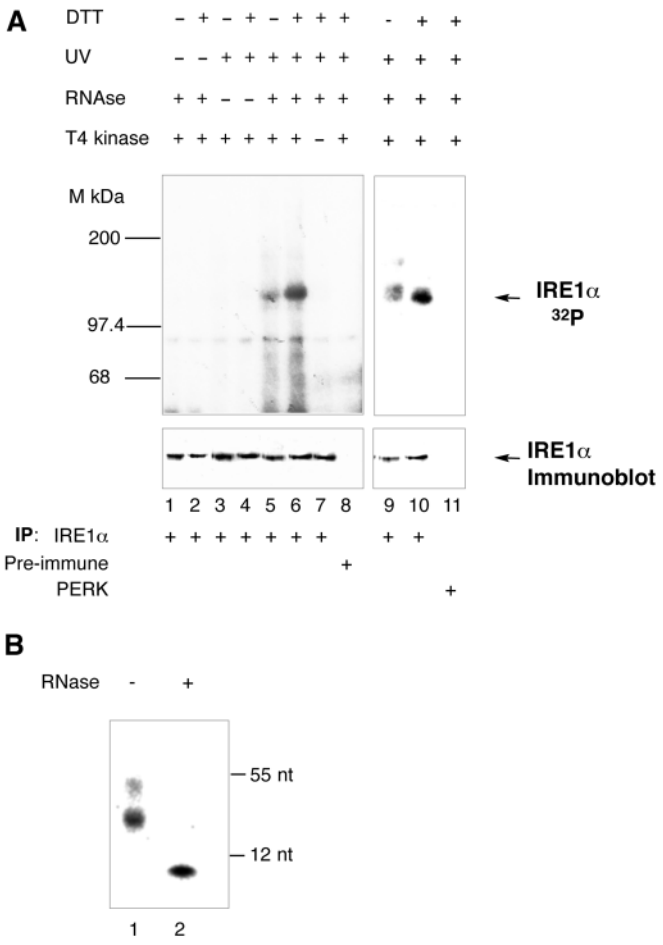

Fig. 1. Formation of stress-dependent IRE1α-RNA complex in vivo. (A) Autoradiograms of a nitrocellulose membrane containing IRE1-RNA complexes isolated from AR42J cells and ^{32}P-end-labeled using T4 kinase (top panels, IRE1α ^{32}P). The same nitrocellulose membranes were subsequently immunoblotted with antisera to IRE1α (bottom panels, IB). Where indicated, cells were treated with 10 mM DTT for 30 minutes to induce ER stress and u.v. irradiated to crosslink IRE1α to RNA. Cell extracts were treated with RNase A, to solubilize the IRE1α-RNA complexes, which were then immunoprecipitated with the indicated antisera and labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. Note that the presence of radiolabeled bands in the autoradiogram is dependant on the addition of T4 kinase, u.v. irradiation, IRE1α immunoprecipitation and treatment of the lysate with RNase A. The intensity of the band increases with addition of DTT. (B) Autoradiogram of the radiolabeled polynucleotide recovered from a gel as shown in A and resolved on a 10% acrylamide-8 M urea gel. The recovered species was digested with RNase A before electrophoresis (lane 2), revealing its RNA nature.

To examine the role of IRE1 kinase domain in formation of an in vivo complex with RNA, we expressed wild-type and mutant forms of IRE1β by transient transfection in 293T cells, and recovered the crosslinked protein-RNA complex. A labeled IRE1β band was observed only in cells expressing wild-type IRE1β. A point mutation that abolishes kinase activity (K536A) or a C-terminal truncation that retains the kinase domain but removes the nuclease domain of IRE1β both lead to loss of the protein-RNA complex (Fig. 3A). Immunoblotting controlled for the expression and immunoprecipitation efficiency of wild-type and mutant IRE1β proteins (Fig. 3B). The formation of a protein-RNA complex incorporating the overexpressed wild-type IRE1β was independent of ER stress in the transfected cells (Fig. 3C). This is consistent with the observation that the overexpression wild-type IRE1β is constitutively active, as reflected by its reduced mobility on SDS-PAGE gels (Fig. 3B,D; Bertolotti et al., 2000).

Analysis of HAC1 splicing in a yeast strain with a conditional mutation in RNA polymerase II suggested that only newly synthesized *HAC1* mRNA serves as a substrate for IRE1 (Sidrauski et al., 1996). To determine if the RNA species that interacts with mammalian IRE1α is newly synthesized or if IRE1α can associate with pre-existing RNA in a stress-dependent manner, we treated AR42J cells with Actinomycin D to block transcription and then exposed them to DTT. We have previously determined that induction of ER stress by DTT does not require new protein synthesis (Harding et al., 2000). Complexes between IRE1α and RNA were not influenced by Actinomycin D treatment (Fig. 4A). Furthermore, the characteristic shift in size of the RNA species recovered from the IRE1α-containing complexes also occurred independently of gene transcription (Fig. 4B). We conclude that the ER-stress mediated alteration in the IRE1α-RNA complex involves a pre-existing mRNA.

DISCUSSION

Two changes were observed in the u.v. crosslinked complex between IRE1 and RNA during the UPR: in stressed cells, more radiolabeled RNA was revealed by the in vitro labeling of the immunoaffinity purified complex and, the labeled species recovered from the complex with IRE1 decreased in size. These changes were observed with three different agents that cause ER stress and that activate IRE1. Formation of the IRE1-RNA complex was dependent on the kinase domain of

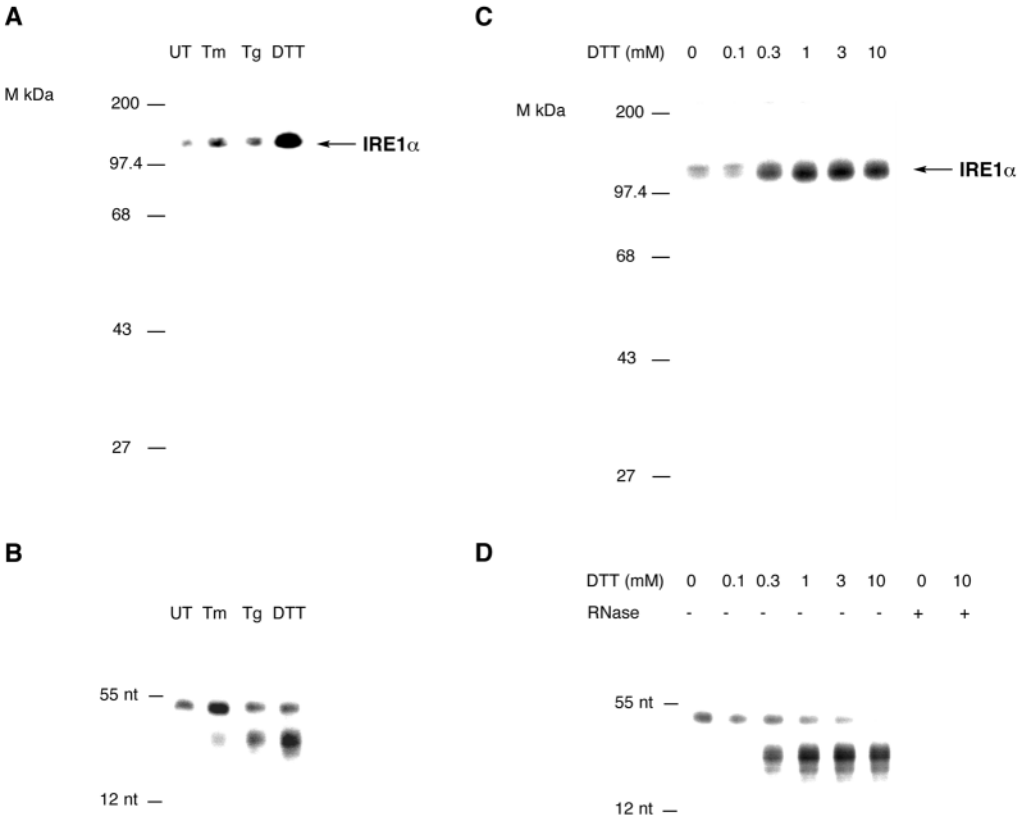

Fig. 2. ER stress modifies the IRE1α-RNA complex. (A) Autoradiogram of immunopurified, radiolabeled IRE1α-RNA complexes isolated from AR42J cells that were untreated (UT) or treated with tunicamycin (Tm, 2.5 μg/ml, 4 hours), thapsigargin (1 μM, 30 minutes) or DTT (10 mM, 30 minutes). (B) Autoradiogram of the radiolabeled polynucleotide recovered from the gel shown in A and resolved on a 10% acrylamide-8M urea gel. (C) Autoradiogram of immunopurified, radiolabeled IRE1α-RNA complexes isolated from AR42J cells that were untreated or exposed for 30 minutes to the indicated concentration of DTT in the culture media. (D) Autoradiogram of the radiolabeled polynucleotide recovered from the gel shown in C and resolved on a 10% acrylamide-8 M urea gel.

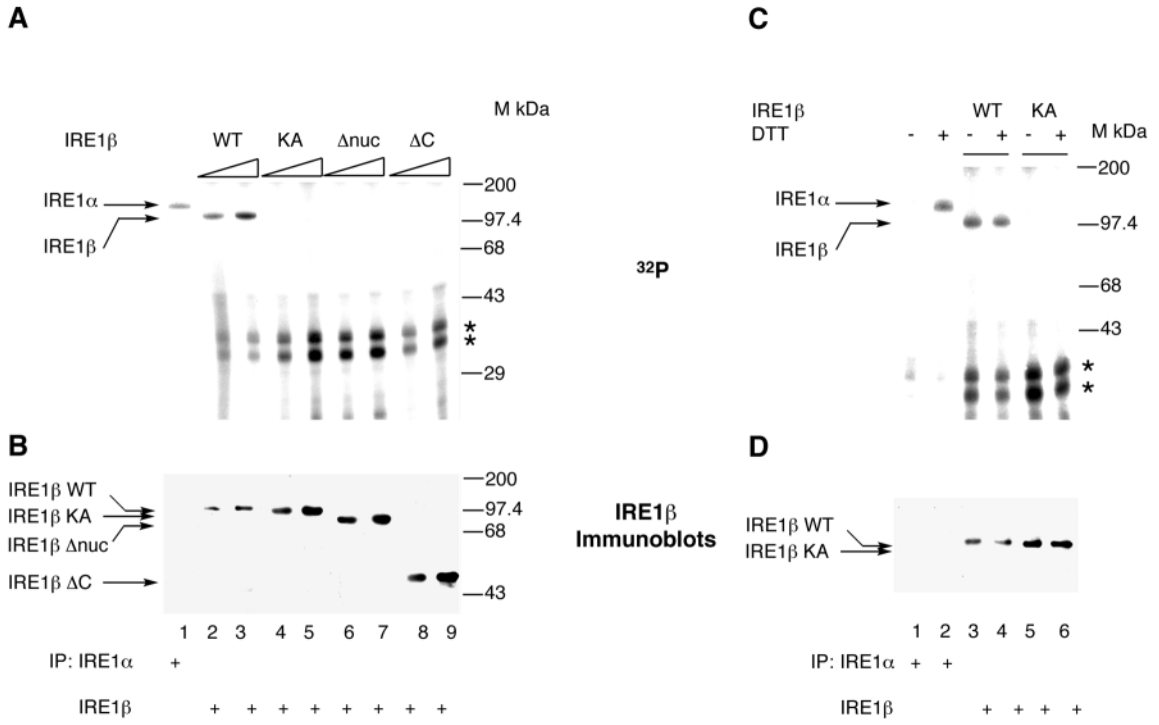

Fig. 3. Formation of the IRE1-RNA complex requires the kinase and endonuclease domains of IRE1. (A) Autoradiogram of immunopurified, radiolabeled IRE1-RNA complexes isolated from 293T cells. Lane 1 contains complexes of RNA and the endogenous IRE1α immunopurified with antiserum to IRE1α and lanes 2-9 are of complexes containing IRE1β and mutant derivatives of the protein expressed by transfection of the indicated expression plasmid and immunopurified with antiserum to IRE1β. WT, wild-type IRE1β; KA, K536A point mutant; Δnuc, mutant lacking the endonuclease domain; ΔC, mutant lacking most of the C-terminal cytoplasmic domain. The asterisks mark a nonspecific radiolabeled species present in the IRE1β immunoprecipitates. (B) Anti-IRE1β immunoblot of 1/10 of the immunoprecipitate used in A. (C) Autoradiogram of immunopurified, radiolabeled IRE1-RNA complexes isolated from 293T cells as in A. Where indicated, the cells were exposed to 10 mM DTT for 30 minutes to induce ER stress. (D) Anti-IRE1β immunoblot of lysates isolated from the cells shown in C.

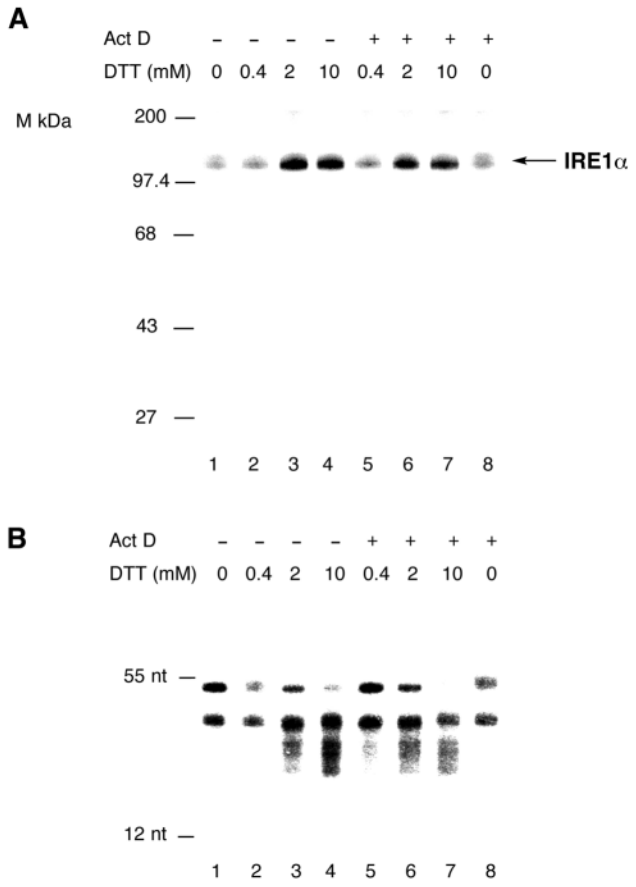

Fig. 4. Modifications of the IRE1α-RNA complex occur independently of gene transcription. (A) Autoradiogram of immunopurified, radiolabeled IRE1α-RNA complexes isolated from AR42J cells that were exposed for 30 minutes to the indicated concentration of DTT in the presence or absence of actinomycin D (5 μg/ml). (B) Autoradiogram of the radiolabeled polynucleotide recovered from the gel shown in A and resolved on a 10% acrylamide-8 M urea gel.

IRE1. Therefore, we regard it as highly likely that the changes in the complex reflect the activity of IRE1, as opposed to being a less specific consequence of the pharmacological manipulations or the stress they cause.

Because the RNA species that associate(s) with IRE1 have not yet been identified, we can only speculate about the nature of the ER stress-induced alteration in the complex. This alteration may be a consequence of IRE1-mediated splicing of the bound RNA. The splicing event may diminish the length of the protected RNA fragment directly, or alter the conformation of the RNA affecting its accessibility to RNase A used in the assay. However, to the extent that the size of the RNA fragment is determined by its susceptibility to RNase A digestion, it is equally possible that activation-mediated conformational changes in IRE1 and/or the associated RNA that precede splicing alter the cleavage pattern of the RNA and account for the abrupt decrease in size of the peptide-RNA fragment.

The increase in radiolabeling of the RNA species recovered in complex with IRE1α from stressed cells might reflect activation-mediated increase in association of IRE1 with RNA. Alternatively, ER-stress-dependent changes in the bound RNA due to cleavage by IRE1 or altered digestion by RNase A may affect accessibility of the 5′ OH group to T4 kinase used in the labeling reaction. This latter possibility is consistent with a model whereby IRE1 is pre-bound to its RNA target, facilitating rapid response to stress. It is noteworthy, in this regard, that the alterations in the IRE1-RNA complex are observed to occur in actinomycin D-treated stressed cells, indicating that the RNA species engaged by IRE1 is pre-existing in stressed cells.

Regardless of the precise identity of the RNA species crosslinked to IRE1, this assay correlated closely with IRE1 activation in the UPR. It is noteworthy in this regard that the changes in the complex occur in the absence of any evidence for IRE1 processing. At the very least, these observations indicate that substantial alterations can occur to IRE1 and its contingent molecules before the processing event that reportedly causes the effector domain of IRE1 to translocate to the nucleus (Niwa et al., 1999). If the RNA revealed by this assay represents the substrate for splicing by IRE1 and encodes effectors of the mammalian UPR, our observations would seriously question the role of nuclear translocation of the truncated effector domain of IRE1 as the proposed mechanism for effecting interactions with substrate mRNA (Niwa et al., 1999). It is also possible that activation of IRE1 by ER stress may mediate a general increase it is affinity towards RNA. However, this model too would call into question the role of IRE1 processing in mediating its effector functions, because we detect complexes only between full-length IRE1 and RNA.

The RNA detected by our assay could also represent a pre-existing structural component of the machinery that cleaves effector-encoding mRNAs in a stable complex with IRE1. Given the role of RNA in the regulation and catalysis of other RNA processing reactions in the cell, this possibility should be seriously considered. Accordingly, it is important to point out that the genetic screens for mutations that disrupt stress signaling in yeast have not been saturating and might not have uncovered an essential RNA component.

Several groups have reported recently on the use of microarray technology to identify polynucleotide species recovered by purification after crosslinking to their protein ligands (Ren et al., 2000; Takizawa et al., 2000). To adapt the cross-linking procedure described here to that application, we would need to overcome two main obstacles. Isolation of the IRE1-RNA complex and its purification from other RNAs currently requires extensive digestion of the RNA before immunoprecipitation. This severely limits the size of the RNA remnant associated with the complex and reduces its utility as a template for producing a hybridization probe in any subsequent application. Irreversible crosslinking, as effected by u.v. treatment, produces a covalent modification of the RNA; this modification may inhibit the reverse-transcriptase step that forms the basis of any procedure that seeks to display or amplify cDNA to the RNA. These difficulties not withstanding, the next step is clearly to identify or clone the RNA species complexed with IRE1 in vivo.

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