Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response

Anne Bertolotti*, Yuhong Zhang*, Linda M. Hendershot*†, Heather P. Harding* and David Ron*‡

**Skirball Institute of Biomolecular Medicine, Departments of Medicine and Cell Biology and the Kaplan Cancer Center, New York University School of Medicine, New York, New York 10016, USA*
†Department of Tumour Cell Biology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA
‡e-mail: ron@saturn.med.nyu.edu

PERK and IRE1 are type-I transmembrane protein kinases that reside in the endoplasmic reticulum (ER) and transmit stress signals in response to perturbation of protein folding. Here we show that the lumenal domains of these two proteins are functionally interchangeable in mediating an ER stress response and that, in unstressed cells, both lumenal domains form a stable complex with the ER chaperone BiP. Perturbation of protein folding promotes reversible dissociation of BiP from the lumenal domains of PERK and IRE1. Loss of BiP correlates with the formation of high-molecular-mass complexes of activated PERK or IRE1, and overexpression of BiP attenuates their activation. These findings are consistent with a model in which BiP represses signalling through PERK and IRE1 and protein misfolding relieves this repression by effecting the release of BiP from the PERK and IRE1 lumenal domains.

Conditions that alter protein folding in the ER elicit two principal cellular responses. The first involves upregulation of genes encoding chaperones and other proteins that prevent polypeptide aggregation and participate in polypeptide folding, post-translational assembly of protein complexes and protein degradation[1]. The second consists of a transient attenuation in rates of protein synthesis[2]. Both responses minimize the accumulation and aggregation of misfolded proteins, the first by increasing the capacity of the machinery for folding and degradation and the second by reducing the burden placed on them. Two ER-resident proteins are thought to have an important function in transducing the stress signal initiated by misfolded proteins to the afore-mentioned downstream effects.

In yeast, activation of gene expression by the unfolded-protein response (UPR) is dependent on the function of a type-I transmembrane protein kinase, IRE1, which resides in the ER. The lumenal domain of IRE1 is thought to respond to an ER stress signal that induces oligomerization of the protein, transphosphorylation by its kinase domain, activation of its effector functions and subsequent down-stream signalling to the transcriptional apparatus[3,4]. However, the nature of the lumenal signal that activates IRE1 is unknown. Two mammalian homologues of IRE1 have been identified and found to have a similar function in ER stress signalling[5,6]. A second transmembrane ER stress-signal transducer has also been identified. PKR-like ER kinase (PERK, also known as PEK[7]), is conserved in all known metazoans and seems to respond to a similar set of signals to those that influence IRE1, using an apparently related ER-lumenal domain[8]. PERK is also a protein serine/threonine kinase that undergoes transphosphorylation in response to ER stress. Activated PERK phosphorylates the α-subunit of translation-initiation factor 2 (eIF2α), which results in inhibition of translation initiation[8]. Thus, these two stress-response mechanisms in the ER use proteins with similar topology and similar overall organization of domains to respond to a lumenal signal or signals, the nature of which is the focus of this study.

Results

Association of Bip with PERK and IRE1. To identify the proteins that interact with the lumenal domains of ER stress-signal transducers and that may therefore participate in signalling, we began our studies with PERK, for which an enriched source was found in the pancreas[7]. We used an antiserum against the carboxy-terminal portion of PERK to co-immunopurify associated cellular proteins in lysates of the pancreatic acinar cell line AR42J. PERK co-purified with a cellular protein of relative molecular mass 70,000 (M_r70K) that was absent from control immunoprecipitation reactions carried out using antisera against GCN2, a related eIF2α kinase, or ribophorin-1, a transmembrane protein abundantly present in the ER (Fig. 1a). The protein of M_r70K was indistinguishable in size from the ER chaperone BiP, which we immunoprecipitated using a specific anti-BiP serum (Fig. 1a). BiP is allosterically regulated by nucleotide binding, and complexes between BiP and target proteins

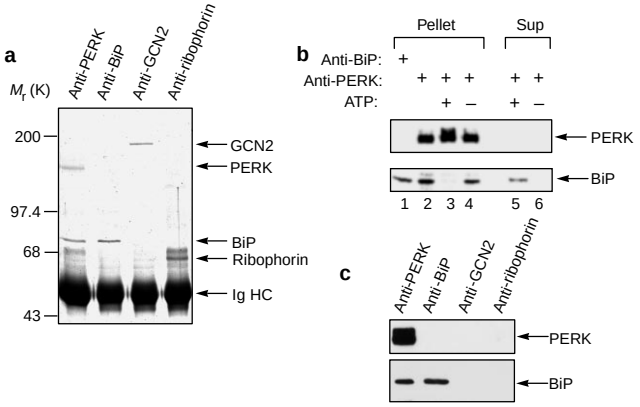

Figure 1 **PERK forms a complex with BiP in unstressed cells. a**, Stained gel of proteins immunopurified from AR42J cells, using the indicated antisera. Proteins were resolved by SDS–PAGE and stained with Coomassie blue. Ig HC, immunoglobulin heavy chain. **b**, Autoradiogram showing the proteins retained in the PERK immune complex (pellet) or released into the supernatant (sup) following incubation of the immunoprecipitates *in vitro* for 30 min at 25 °C, in the presence (+) or absence (–) of 2 mM ATP. **c**, Immunoblots, after immunoprecipitation with the indicated antisera.

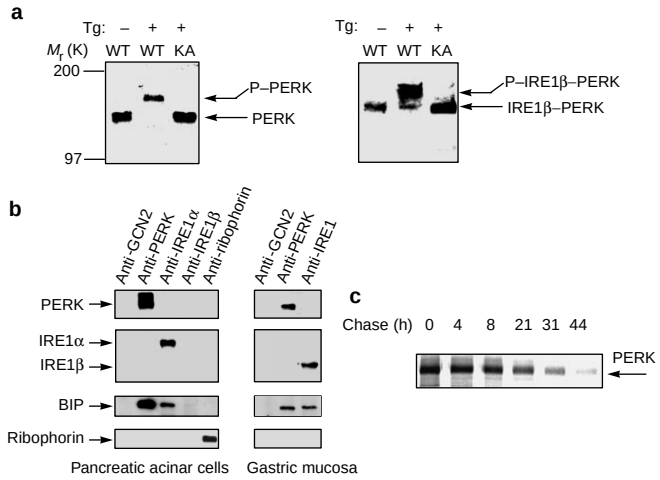

Figure 2 **Functional similarities of the lumenal domains of PERK and IRE1. a**, Immunoblots against PERK from lysates of COS-1 cells transfected with the indicated expression vectors in the absence (–) or presence (+) of 1 μM thapsigargin (Tg). WT, wild type; KA, K618A mutant lacking PERK kinase activity; P–PERK, phosphorylated PERK. The signal from endogenous PERK is not observed at the exposure shown here. **b**, Immunoblots of proteins from the pancreatic acinar cells (AR42J) or the gastric mucosa of mice. **c**, Autoradiogram showing the half-life of PERK in metabolically labelled AR42J cells. PERK was immunoprecipitated from cells labelled by a brief pulse of [^{35}S]methionine–cysteine and chased in unlabelled media for the indicated time periods.

can be disrupted by incubation *in vitro* with ATP[9,10]. We therefore incubated PERK-containing complexes immunopurified from metabolically labelled AR42J cells in a buffer containing magnesium and ATP. This resulted in rapid dissociation of the protein of M_r70K, showing that its interaction with PERK can be regulated by ATP and further indicating that it may belong to the HSP (heat-shock protein) 70 family of chaperones (Fig. 1b). We subjected the protein of M_r70K to tryptic digestion within the gel and to mass spectrometry, and identified it as rat BiP (48% of the predicted tryptic fragments of BiP were present in the digest and these accounted for >50% of the peaks in the mass spectra). This identification was confirmed by immunoblotting with antisera against rodent BiP (Fig. 1c).

PERK activation during ER stress correlates with phosphorylation of its cytoplasmic kinase domain. This retards the mobility of PERK on SDS–polyacrylamide gels and serves as a convenient marker for its activation status[8] (Fig. 2a). To examine the functional relationship between the lumenal domains of IRE1 and PERK, we tested the ability of the lumenal domain of IRE1β to replace that of PERK in mediating responsiveness to ER stress. When expressed in COS-1 cells, the chimaeric IRE1β–PERK protein, like authentic PERK, exhibited a mobility shift in response to ER stress (Fig. 2a). A similar chimaera prepared using a mutant form of PERK that lacks kinase activity (IRE1β–PERK(K618A)) did not shift in response to ER stress. This indicates that PERK and IRE1 may respond to common lumenal signals.

We examined the ability of IRE1 to associate with BiP. Mammals possess two *IRE1* genes — *IRE1*α, which is expressed at highest levels in the pancreas[5], and *IRE1*β, the expression of which is restricted to epithelial cells in the gut (our unpublished observations). Antise-

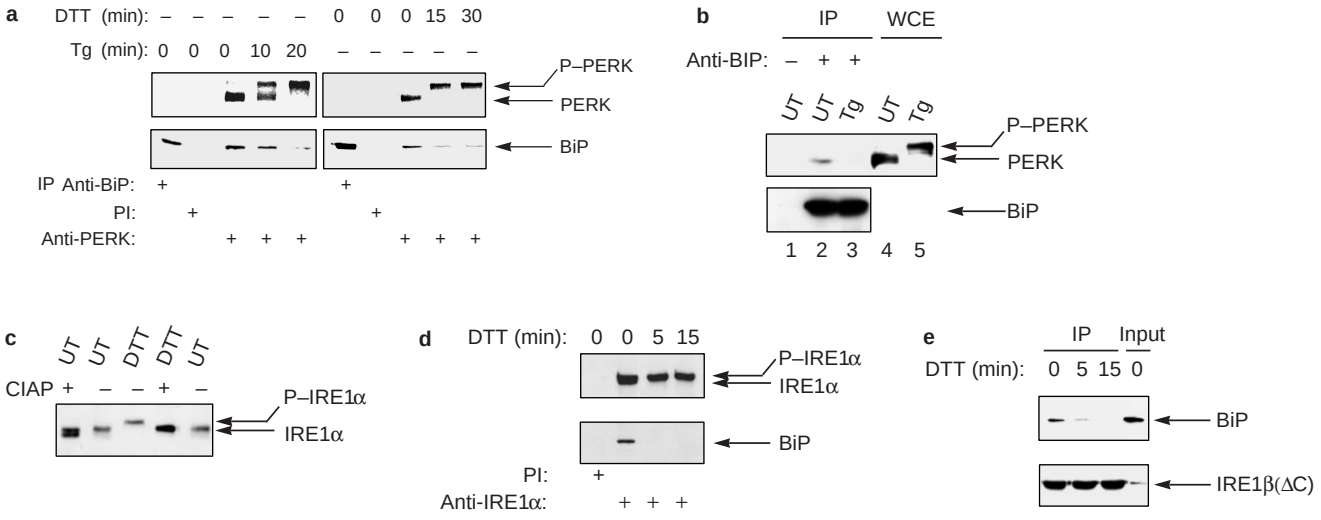

Figure 3 **ER stress leads to dissociation of the BiP–PERK and BiP–IRE1α complexes. a**, Autoradiogram of metabolically labelled proteins from AR42J cells treated with thapsigargin (Tg) or dithiothreitol (DTT) for the indicated time periods, and immunoprecipitation with the indicated antisera. Complex components were resolved by SDS-PAGE. PI, pre-immune serum; P–PERK, phosphorylated PERK. **b**, Contents of BiP-containing complexes immunopurified from untreated (UT) or thapsigargin-treated CHO cells using a rat monoclonal antibody against hamster BiP (IP, lanes 2, 3; lane 1 represents a control). Complex components were analysed by immunoblotting against PERK (upper panel) and BiP (lower panel). Lanes 4, 5 represent whole-cell extracts (WCE) and serve to mark the migration of phosphorylated and unphosphorylated PERK. **c**, Immunoblot against IRE1α from untreated (UT) and DTT-treated AR42J cells. Phosphorylated (P–IRE1α) and unphopsphorylated IRE1α were resolved by SDS–PAGE. Where indicated, immune complexes were treated *in vitro* with calf-intestine alkaline phosphatase (CIAP). **d**, Contents of IRE1α–BiP complexes immunopurified using an antiserum against the IRE1α C terminus from cells treated with DTT for the indicated time periods. Complex components were analysed by immunoblotting against IRE1α (upper panel) and BiP (lower panel). **e**, Contents of complexes containing a truncated form of IRE1β that lacks most of the C-terminal domain (IRE1β(ΔC)[6]. Complexes were immunopurified from NIH 3T3 cells stably expressing IRE 1β(ΔC) that were treated with DTT for the indicated time periods. Complex components were analysed by immunoblotting against BiP (upper panel) and IRE1β(ΔC) (lower panel). The lane labelled 'input' was loaded with 1/500 of the lysate volume used in immunoprecipitation reactions.

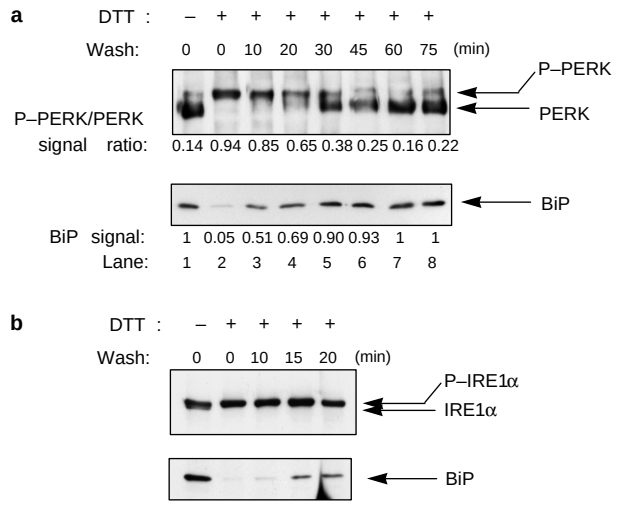

Figure 4 **BiP reassociates with PERK and IRE1α upon removal of ER stress.** **a**, Dynamics of PERK–BiP complex formation, as shown by co-immunoprecipitation using anti-PERK antiserum from lysates of untreated AR42J cells and AR42J cells treated with dithiothreitol (DTT) for 15 min and then placed in DTT-free media for the indicated time periods. The ratio of the signal from phosphorylated PERK (P–PERK) to the total PERK signal, and the intensity of the BiP signal in each lane were densitometrically quantified. Note that reformation of the PERK–BIP complex precedes PERK dephosphorylation. **b**, Dynamics of IRE1α–BiP complex formation in AR42J cells, determined as in **a** using an anti-IRE1 antiserum. Activation-dependent changes in IRE1α mobility are not evident under the electrophoresis conditions required to resolve both IRE1α and BiP.

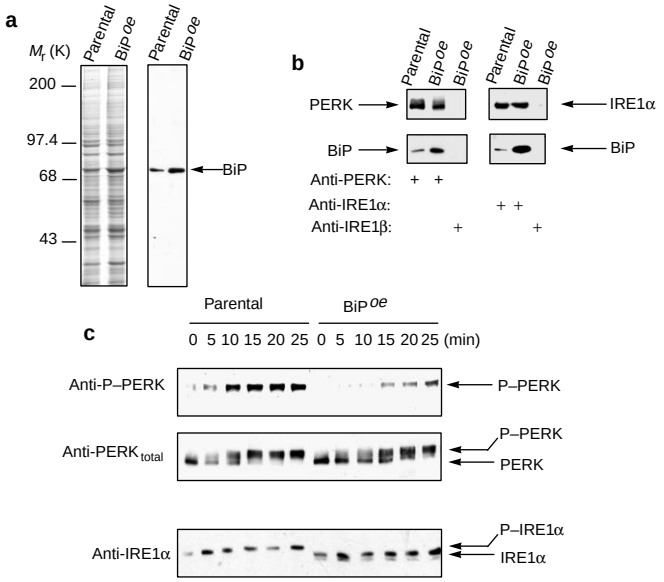

Figure 5 **BiP binding and activation of stress-signal transducers in BiP-overexpressing CHO cells.** **a**, Levels of BiP in whole-cell extracts from parental CHO cells and CHO cells overexpressing BiP (BiP^{oe}), as determined by SDS–PAGE with Coomassie-blue staining (left panel) and immunoblotting against BiP (right panel). **b**, Contents of PERK–BIP (left panels) and IRE1α–BiP (right panels) complexes immunopurified from parental and BiP-overexpressing CHO cells, as determined by immunoblotting with the indicated antisera. **c**, Immunoblots, after immunoprecipitation, of PERK and IRE1α from parental and BiP-overexpressing CHO cells treated with 1 μM thapsigargin (upper and middle panels) or 10 mM DTT (lower panel) for the indicated time periods. PERK was detected using antisera against phosphorylated PERK (P–PERK; upper panel) and against all forms of PERK (PERK$_{total}$; middle panel); IRE1α was detected using an antiserum against all forms of IRE1α. Note that, in BiP-overexpressing cells, phosphorylation of PERK and IRE1α is incomplete. A faint, faster-migrating band was revealed in BiP-overexpressing cells by IRE1α antiserum; its significance is not known.

rum against the C terminus of IRE1α co-immunoprecipitated BiP from extracts of AR42J cells, whereas antiserum against IRE1β co-immunoprecipitated BiP from extracts of mouse-stomach mucosa but not from AR42J extracts (Fig. 2b). Recovery of BiP in the immunoprecipitate therefore requires the presence of its binding partner, indicating that IRE1 and PERK may associate with BiP.

BiP associates transiently with many proteins during their synthesis and maturation[11]. However, PERK has an estimated half-life of 13 h (Fig. 2c), and it is therefore unlikely that much of the PERK present in cells at any one time represents newly synthesized protein undergoing BiP-assisted folding. Thus, the association between PERK and BiP is unlikely to represent binding of the chaperone to nascent PERK.

Reversible dissociation of BiP complexes. We studied the PERK–BiP complex during the ER stress response. We treated AR42J cells with thapsigargin or dithiothreitol (DTT), two agents that interfere with protein folding in the ER, and examined the association between PERK and BiP by immunoprecipitating PERK from lysates of metabolically labelled cells. The quantity of BiP associated with PERK decreased within a few minutes of application of ER stress (Fig. 3a). A marked reduction was also observed in the amount of BiP associated with IRE1α in stressed AR42J cells (Fig. 3d). Loss of BiP binding correlated with PERK and IRE1α activation, as reflected by a shift in their mobility to more slowly migrating forms. This mobility shift was easier to detect in PERK than in IRE1α, particularly in the 7% SDS–polyacrylamide gels used to resolve BiP (see above). In both cases, however, mobility shifts were caused by phosphorylation events, as they were readily reversed by *in vitro* treatment with alkaline phosphatase (Fig. 3c and ref. 8). DTT treatment caused a more obvious shift in IRE1α mobility and more pronounced BiP dissociation than did thapsigargin treatment (data not shown), whereas PERK was equally responsive to both agents; the reason for this is unknown.

We were unable to detect PERK (or IRE1) in immunoprecipitates using polyclonal rabbit anti-BiP serum (Fig. 1a, c). This may be a result of the low cellular concentrations of PERK and IRE1 relative to that of BiP, or of steric hindrance of the antibody-binding site of BiP by bound PERK or IRE1. We therefore used a rat monoclonal antibody to immunoprecipitate BiP from hamster CHO-K12 cells and determined the presence of BiP-associated PERK by immunoblotting. PERK was found to be associated with BiP in unstressed cells but not in stressed cells (Fig. 3b). Furthermore, BiP-associated PERK exhibited high mobility on SDS–polyacrylamide gels, indicating that it may have been inactive (unphosphorylated). This reciprocal immunoprecipitation confirms the link between dissociation of the PERK–BiP complex and PERK activation. We were unable to detect IRE1α in BiP-immunoprecipitation reactions, probably because of the lower level of expression of IRE1α compared with that of PERK in CHO-K12 cells (the monoclonal antibody does not react with rat BiP and could not be used in AR42J cells, an enriched source of IRE1α).

Signal transduction during the ER stress response is likely to be initiated on the lumenal side of the ER membrane, favouring a model in which dissociation of BiP from the lumenal domains of stress-signal transducers is an early upstream event leading to their phosphorylation and activation. To critically examine this hypothesis, we investigated the effect of ER stress on the association of endogenous BiP with a stably expressed, truncated version of IRE1β, IRE1β(ΔC)[6], which contains the lumenal and transmembrane domains but lacks most of the cytoplasmic kinase domain.

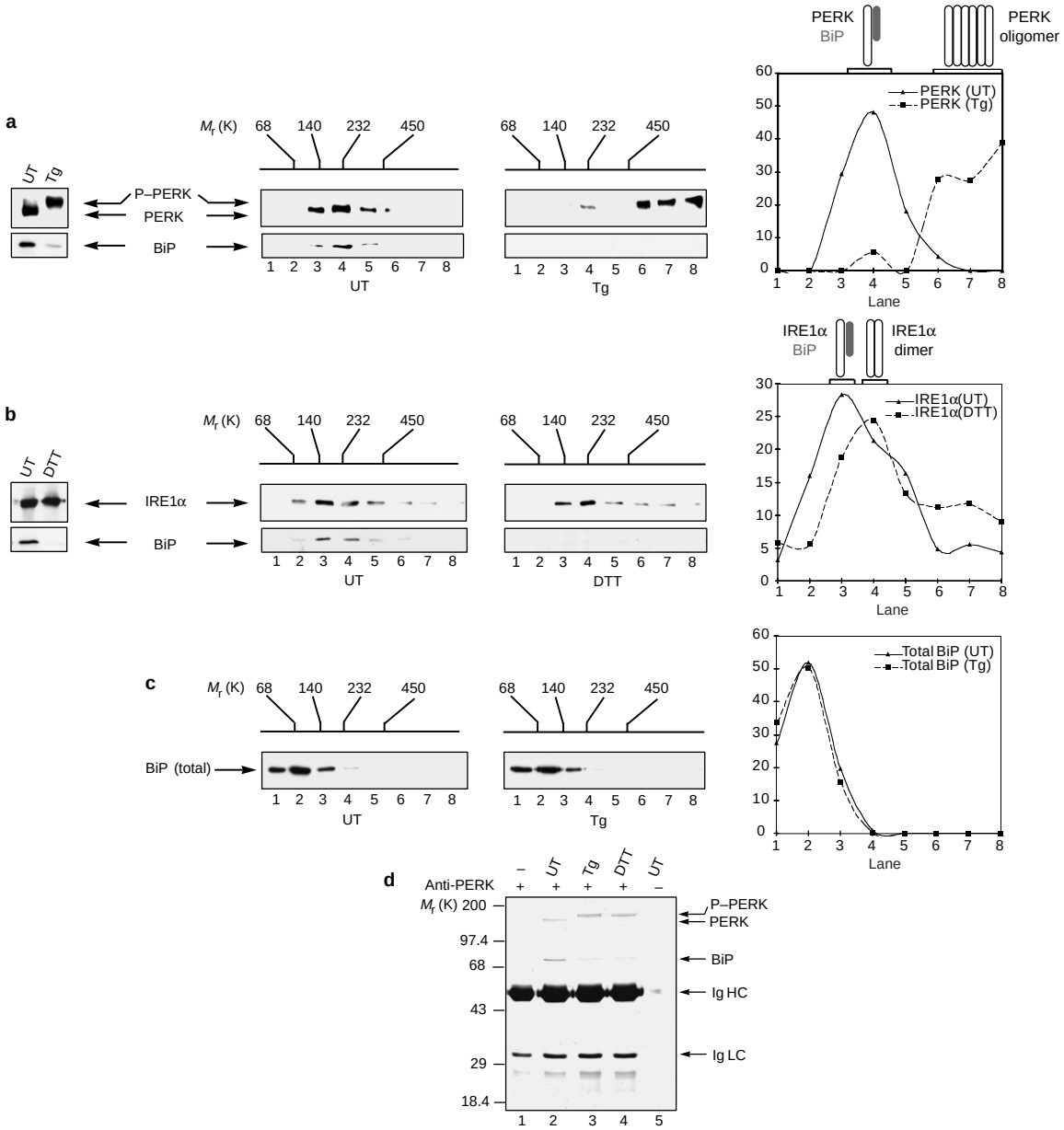

Figure 6 **ER-stress-induced formation of high-molecular mass complexes containing PERK or IRE1α but lacking BiP. a**, Left panel, contents of PERK–BiP complexes from lysates of untreated (UT) or thapsigargin-treated (Tg; 1 μM, 30 min) AR42J cells. Complexes were immunopurified using anti-PERK antiserum and their components identified by immunoblotting. P–PERK, phosphorylated PERK. Middle panels, analysis of complex contents as above after size fractionation of lysates by sedimentation on a 20–40% glycerol gradient. The positions of protein M_r markers, fractionated in parallel on an identical gradient, are indicated. Right panel, quantified levels of protein in size-fractionated blots. **b**, Left panel, contents of IRE1α–BiP complexes from lysates of untreated or dithiothreitol-treated (DTT; 10 mM, 30 min) AR42J cells. Complexes were immunopurified using anti-IRE1α antiserum and their components identified by immunoblotting. Middle panels, analysis of complex contents after size fractionation as in **a**. Right panel, quantification of data from size-fractionated blots as in **a**. **c**, Left and middle panels, immunoblots showing the total quantity of BiP in size-fractionated lysates of untreated and thapsigargin-treated AR42J cells. Lanes were loaded with 1/1,000 of the volume used in **a**. Right panel, quantification of data from blots as in **a**, **b**. **d**, Contents of PERK-containing immune complexes isolated from untreated, thapsigargin-treated or DTT-treated AR42J cells using anti-PERK antiserum (lane 5 represents a control). Complex components were resolved by SDS–PAGE and stained with Coomassie blue. The positions of unphosphorylated and phosphorylated PERK, BiP and the immunoglobulin heavy (Ig HC) and light (Ig LC) chains are indicated. Lane 1, control containing no cell lysate.

Previous experiments using mammalian cells and yeast have shown that truncated IRE1 localizes to the ER in the same way as the wild type[6,14]. Using NIH 3T3 cells stably expressing 9E10-tagged IRE1β(ΔC), we immunopurified IRE1β(ΔC)-containing complexes and determined their BiP contents by western blotting. Treatment of cells with DTT resulted in a marked decrease in the quantity of BiP associated with the recombinant protein (Fig. 3e). Because IRE 1β(ΔC) lacks most of the C-terminal portion of IRE1β, it is incapable of undergoing autotransphosphorylation, and is also unlikely to serve as a substrate for endogenous IRE1. Dissociation

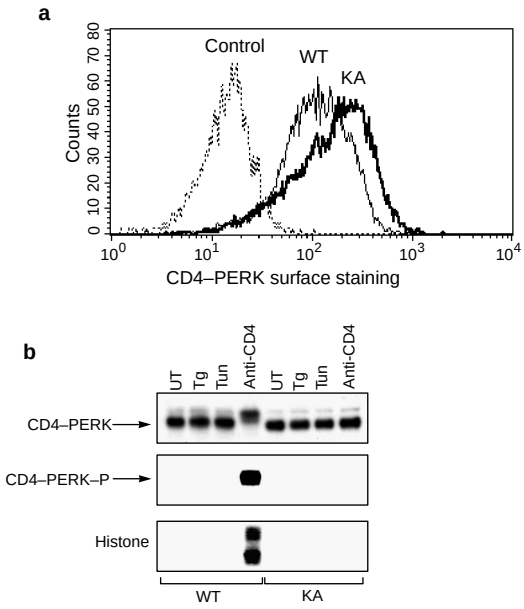

Figure 7 **Oligomerization-induced activation of PERK kinase *in vivo*. a**, FACS analysis of NIH 3T3 cells stably expressing fusions of the extracellular domain of CD4 with either wild-type (WT) or K618A-mutant (KA) PERK kinase domains. Untransfected cells were used as a control. Surface proteins were stained with a monoclonal anti-CD4 antibody. **b**, Upper panel, immunoblot against CD4–PERK immunoprecipitated from untreated, thapsigargin-treated (Tg; 1 μM, 30 min), tunicamycin-treated (Tun; 2.5 μg ml^{-1}, 4 h) or anti-CD4-antibody-treated NIH 3T3 cells. Middle and lower panels, autoradiograms showing *in vitro* PERK kinase activity after incubation with [γ-^{32}P]ATP. Middle panel, autophosphorylation (CD4–PERK–P, phosphorylated CD4–PERK); lower panel, phosphorylation of a histone substrate. Note the presence of two bands of differing mobility.

of the IRE1–BiP complex during ER stress therefore occurs independently of phosphorylation of the IRE1 effector domain. This result supports the idea that complex dissociation initiates signalling in the unfolded-protein response.

To examine further the dynamics of the BiP–PERK interaction, we made use of the fact that ER stress induced by DTT is readily reversible. Brief exposure to DTT was sufficient to cause dissociation of the PERK–BiP complex and activation of PERK (Fig. 4a, lane 2). However, the BiP–PERK complex rapidly reformed after DTT was washed out (Fig. 4a, lane 3). Reassociation of BiP with PERK preceded conversion of PERK from a phosphorylated, lower-mobility form to a dephosphorylated, higher-mobility form (Fig. 4a, lanes 3–8). PERK was therefore presumably still activated during reformation of the BiP–PERK complex, indicating that formation and dissociation of this complex may respond rapidly to changes in the lumenal environment, and may precede changes in the activation status of the PERK effector domain. The interaction of BiP with IRE1α exhibited similarly rapid complex reformation when DTT was washed out (Fig. 4b). These results are consistent with a function for BiP binding in determination of the activation status of ER stress-signal transducers.

CHO cells stably overexpressing BiP have a markedly attenuated unfolded-protein response[12,13]. In these cells, the amount of chaperone associated with PERK or IRE1α was considerably greater than in parental CHO cells expressing normal levels of endogenous BiP (Fig. 5a, b). Phosphorylation of PERK was both delayed and incomplete in BiP-overexpressing CHO cells compared with parental cells (Fig. 5c, upper and middle panels), and activation of IRE1α by ER stress, as reflected by its shift to lower mobility on SDS–polyacrylamide gels, was absent in BiP-overexpressing cells (Fig. 5c, lower panel). These results support the idea that overexpression of BiP attenuates ER stress signalling upstream of PERK and IRE1 activation.

Oligomerization of PERK and IRE1. It has been proposed that oligomerization has an important function in activation of ER stress-signal transducers[14,15]. When fractionated by glycerol-gradient sedimentation, PERK from untreated cells migrated as a single peak with an apparent M_r of ~230K, which is consistent with the predicted size of a PERK–BiP heterodimer. Treatment of cells with thapsigargin (or DTT, data not shown) resulted in rapid formation of a PERK-containing complex of high M_r (estimated at >600K). The retarded mobility of this complex on SDS–polyacrylamide gels showed that its PERK was phosphorylated (Fig. 6a). BiP was readily detected in anti-PERK immunoprecipitates from the lower-M_r complex obtained from untreated cells, but was undetectable in anti-PERK immunoprecipitates obtained from the higher-M_r complex (Fig. 6a).

We examined the composition of the PERK-containing complex of M_r>600K by protein staining of the anti-PERK immunoprecipitates obtained from ER-stressed cells (Fig. 6d). The only proteins detected in these immunoprecipitates were immunoglobulin heavy and light chains, PERK and BiP; the amount of BiP was reduced during ER stress (Fig. 6d, lanes 3, 4). We carried out these immunoprecipitation reactions under conditions that preserved the integrity of the high-M_r PERK-containing complex — the same conditions that were used to prepare the glycerol gradients. The absence of other species therefore indicates that this complex may principally consist of PERK oligomers.

In untreated cells, IRE1α was present in a complex of M_r 140K–230K, which is consistent with a subunit stoichiometry of one IRE1α and one BiP molecule. ER stress resulted in a modest, but reproducible, shift of the IRE1α peak to a heavier fraction. Mass estimates for the IRE1-containing peaks in the two conditions were consistent with loss of BiP binding and formation of IRE1α homodimers during DTT treatment (Fig. 6b), and the magnitude of the DTT-induced shift in the IRE1α peak was consistent with the small difference in mass between IRE1α–BiP heterodimers and IRE1α homodimers. ER stress did not affect the mobility of the majority of BiP in AR42J cells, indicating that the observed alterations in the compositions of the anti-PERK and anti-IRE1α immunoprecipitates may reflect changes to PERK- or IRE1α-containing complexes, rather than changes in the state of BiP (Fig. 6c).

The formation of PERK- and IRE1-containing complexes of higher M_r during the unfolded-protein response is consistent with an oligomerization step preceding transphosphorylation. PKR, an eIF2α kinase related to PERK, is also activated by oligomerization[16]. To determine whether oligomerization can directly lead to PERK activation, we fused the cytoplasmic kinase domain of PERK to the extracellular and transmembrane domains of the T-lymphocyte co-receptor CD4. CD4–PERK was stably expressed on the surfaces of NIH 3T3 cells and its kinase activity was found to be dormant (Fig. 7a, b). However, we observed a marked increase in CD4–PERK kinase activity upon incubation of cells with a monoclonal antibody that crosslinks the CD4 extracellular domain[17]. This activation was reflected in the lower mobility, on SDS–polacrylamide gels, of the CD4–PERK fusion protein, in its *in vitro* autokinase activity and in its kinase activity with respect to a substrate protein (Fig. 7b). We used a CD4–PERK chimaera that lacks kinase activity as a control. Thus, oligomerization is sufficient to activate the kinase activity of PERK in a heterologous location. Furthermore, the wild-type CD4–PERK chimaera was unresponsive to ER stress, indicating that PERK activation by ER stress may not be an intrinsic property of its cytosolic kinase domain, and may depend on its lumenal domain.

Discussion

Although IRE1 and PERK share only weak sequence similarity in their lumenal domains, our data indicate that they may use a similar mechanism to sense ER stress. We have shown that BiP binds to the

lumenal domains of both of these proteins. The interactions of BiP with IRE1 and with PERK are disrupted by conditions that perturb protein folding in the ER, and are rapidly restored upon termination of stress. Activated PERK and IRE1 are found, probably as oligomers, in high-M_r complexes that lack BiP; oligomerization can induce the kinase activity of PERK even in the absence of ER stress. These findings are consistent with a model in which BiP binds to the lumenal domains of PERK or IRE1 and constitutively inhibits their oligomerization. In this model, therefore, activation is dependent on a derepression step effected by release of BiP from stress-signal transducers.

This BiP release could be caused by the accumulation of misfolded proteins that compete with the lumenal domains of stress-signal transducers for BiP binding. However, if the *in vivo* stability of the complexes bears any relationship to their stability *in vitro* (Fig. 1b, lane 4), the low off-rates observed would not be consistent with complex dissociation simply on the basis of competition for BiP binding by mass action. Perturbations in the ER environment could lead to modification of BiP, causing its release from stress-signal transducers; BiP would thus serve as a regulatory repressive subunit of a holoreceptor complex. The BiP component of the holoreceptor could be the target for the primary lumenal event; alternatively, ER homologues of co-factors that modify the activity of the HSP70 chaperone[18] may be upstream mediators in the dissociation step. Another possibility is that the lumenal domains of IRE1 and PERK may be the primary recipients of the lumenal signal and that their modification would result in loss of BiP binding and subsequent oligomerization of stress-signal transducers. Finally, although PERK complexes isolated from stressed cells do not contain further species detectable by protein staining (Fig. 6d), it remains possible that activation of stress-signal transducers also involves ligands that were not observed in our assay.

We have not addressed the mechanism by which BiP retains bound stress-signal transducers in inactive forms. It is possible that BiP binds to a single high-affinity site, thereby masking a domain that is essential for oligomerization. Alternatively, BiP may bind to several sites in the lumenal domain, interfering with oligomerization in a manner that is biochemically less specific and stochastic. A precedent for BiP binding, with low sequence specificity, to several segments of a polypeptide was recently provided by analysis of the function of BiP in post-translational translocation of proteins into the yeast ER[19]. The estimated size of the PERK–BiP and IRE1–BiP complex from non-stressed AR42J cells is consistent with a stoichiometric ratio of 1:1 (Fig. 6a, b). However, PERK and IRE1α immunopurified from BiP-overexpressing CHO cells were associated with many BiP molecules (Fig. 5b). This may be because of the presence of several potential BiP-binding sites in the lumenal domains of stress transducers that are revealed when BiP is overexpressed. It also possible, however, that BiP binds to a single site on the transducers, but can do so as an oligomer, which would account for the unusual stoichiometry of transducer–BiP complexes in BiP-overexpressing cells.

Our findings support a model that is similar to the negative-feedback mechanism by which heat-shock proteins control their own expression. Under normal conditions in *Escherichia coli*, the σ32 subunit of RNA polymerase, which is specific to the heat-shock promoter, is engaged in an inactive complex by the HSP70-like BiP homologue DNAK[20,21]. Accumulation of misfolded proteins releases σ32 from its inactive complex with DNAK, thereby increasing transcription from heat-shock promoters[22]. A conceptually similar mechanism applies to the constitutive repression of mammalian heat-shock transcription factor HSF by HSPs. Heat shock leads to loss of HSP binding, HSF trimerization, promoter binding and activation of HSP-encoding genes[23,24]. Thus, in examples from several phyla and from different cellular compartments, overexpression of HSPs attenuates stress responses[12,13,22,25]. Our findings indicate that, in the mammalian ER, this may be because of the ability of BiP to repress the activity of ER stress-signal transducers. We thus provide a missing link in the model, originally proposed over seven years ago[26], to explain a key function of BiP in ER stress signalling. □

Methods

Antibodies.

Rabbit polyclonal antisera were directed against the C-terminal 507 residues of mouse IRE1α, the C-terminal 516 residues of mouse IRE1β and residues 588–1,024 of mouse GCN2. Antisera against total PERK were directed against a bacterially expressed protein fragment lacking kinase activity (the C-terminal 417 residues of mouse PERK). Antiserum against phosphorylated PERK was directed against a peptide fragment (the C-terminal 501 residues of mouse PERK) that is heavily autophosphorylated[8]. Specific antibodies against phosphorylated PERK were obtained by depleting the anti-phospho-PERK antiserum against a protein identical to the immunogen except for a mutation, K618A, that inactivated its kinase domain and rendered the protein unable to undergo autophosphorylation[8]. Antibodies against mouse CD4 were produced as a tissue culture supernatant of the GK1.5 rat anti-mouse-CD4 hybridoma cell line (ATCC). Polyclonal rabbit and monoclonal rat anti-BiP immunochemicals were as described[27,28]

Expression plasmids and transfections.

The IRE1β–PERK plasmid encoded the amino-terminal 499 residues of mouse IRE1β, encompassing the lumenal and transmembrane domains, and the cytoplasmic C-terminal kinase domain of mouse PERK (residues 537–1,114); its expression was driven by the viral promoter in the pBABE.puro plasmid. The CD4–PERK plasmid encoded the extracellular and transmembrane domains of mouse CD4 (residues 1–396) and the cytoplasmic domain of mouse PERK; its expression was driven by the viral promoter described above. COS-1 cells were transfected in 6-cm dishes with 3μg of the indicated expression plasmid, using the DEAE–dextran method. Protein extracts were prepared 36h later in 50μl 1% Triton buffer (20mM HEPES pH7.5, 150mM NaCl, 1% Triton X-100, 10% glycerol, 1mM EDTA, 10mM tetrasodium pyrophosphate, 100mM NaF, 17.5mM B-glycerophosphate, 1mM phenylmethysulphonyl fluoride (PMSF), 4mg ml^{-1} aprotonin and 2mg ml^{-1} pepstatin A); 10μl of each extract were analysed by immunoblotting against PERK, as described below.

NIH 3T3 cell lines stably expressing CD4–PERK were prepared by retroviral infection and selection with puromycin. Individual colonies were isolated and screened by immunoblotting for expression of CD4–PERK. Surface expression of the fusion protein was confirmed by FACS analysis of cells ($n = 5 \times 10^5$) stained with a 1/10 dilution of GK1.5 culture supernatant, followed by washing and incubation with a 1/50 dilution of phycoerythrin-labelled goat anti-rat antibody (Caltag, Burlingame, California, USA).

Induction of ER stress, immunoprecipitation and immunoblotting.

Dishes of 10-cm diameter containing confluent AR42J cells, CHO-K12 cells, BiP-overexpressing CHO cells or CD4–PERK-expressing NIH 3T3 cells were either left untreated or treated with 2.5μg ml^{-1} tunicamycin, 10mM DTT or 1μM thapsgargin or with a 1:10 dilution (*v*/*v*) of GK1.5 hybridoma culture supernatant, for 30 min or as indicated in (Figs 3–7). Plates were washed in ice-cold PBS containing 1 mM EDTA, lysed in 200μl 1% Triton buffer (see above) and clarified by centrifugation at 16,000*g* for 10 min and by preincubation for 1h with 10μl protein-A–sepharose. Soluble proteins were immunoprecipitated with 1μl of the anti-PERK or anti-IRE1 polyclonal antisera bound to 10μl protein-A–sepharose and washed three times in RIPA buffer; bound proteins were resolved by 7% SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes. Blots were incubated with rabbit anti-PERK antibody (10,000 dilution) or antisera against IRE1α (1/2,000), IRE1β (1/2,000), BiP (1/1,000), ribophorin I (1/1,000) or GCN2 (1/1,000); signals were observed using protein A tagged with horseradish peroxidase (1/5,000) and enhanced chemiluminescence. For Coomassie-blue staining of immunopurified protein complexes from AR42J cells in ten plates of 10-cm diameter, 3μl of polyclonal antibodies (Fig. 1a) or 4μg of affinity-purified anti-PERK antibody (Fig. 5d) were used. Immunoprecipitation–kinase assays were carried out at 30 °C for 30 min, in a 20-μl reaction volume containing washed immunoprecipitated proteins attached to protein-A–sepharose beads in kinase buffer (20mM HEPES pH7.5, 50mM KCl, 2mM magnesium acetate, 2mM $MnCl_2$, 1.5mM DTT and 2.5mM ATP) supplemented with 5μCi [γ-^{32}P]ATP (4,500Ci mM^{-1}; ICN). Where indicated (Fig. 7b), 5μg of calf-thymus histone mix (Sigma) were added to the reaction.

Metabolic labelling.

AR42J cells at 70% confluence in 6-cm dishes were starved for 30 min in methionine/cysteine-free media, pulse-labelled with 500μCi [^{35}S]Met–Cys express-labelling mix (ICN) for 30min, and then cold-chased in complete media for the indicated time periods (Fig. 2c) or for 20h (Figs 1a, 3a). This long chase was required to completely reverse the induction of the unfolded-protein response that took place when cells were deprived of methionine. Extracts and immunoprecipitations were carried out as described above; labelled protein complexes were resolved by SDS–PAGE and observed using autoradiography.

Glycerol-gradient sedimentation.

Confluent AR42J cells in 10-cm dishes were lysed in 300μl 1% Triton buffer (see above). Extracts or high-M_r markers (Pharmacia) were centrifuged through 20–40% glycerol gradients in polyallomer tubes of 11 × 60mm as described[29]. Each 4-ml gradient was divided into eight equal fractions of 500μl. Aliquots of each fraction (0.5μl) were subjected to SDS–PAGE; the BiP content was measured by immunoblotting. The contents of PERK- and IRE1α-containing complexes were analysed, after adjustment of the glycerol concentration in each fraction to 20%, by sequential immunoprecipitation and immunoblotting using anti-PERK or anti-IRE1α antisera as described above.

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Correspondence and requests for materials should be addressed to D.R.