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# Two redundant systems maintain levels of resident proteins within the yeast endoplasmic reticulum

CHRISTOPHER T. BEH AND MARK D. ROSE*

Lewis Thomas Laboratory, Princeton University, Princeton, NJ 08544

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**ABSTRACT The *Saccharomyces cerevisiae* gene *ERD2* is responsible for the retrieval of lumenal resident proteins of the endoplasmic reticulum (ER) lost to the next secretory compartment. Previous studies have suggested that the retrieval of proteins by *ERD2* is not essential. Here, we find that *ERD2*-mediated retrieval is not an essential process only because, on its failure, a second inducible system acts to maintain levels of ER proteins. The second system is controlled by the ER membrane-bound kinase encoded by *IRE1*. We conclude that *IRE1* and *ERD2* together maintain normal concentrations of resident proteins within the ER.**

The contents of the endoplasmic reticulum (ER) lumen are continually in flux as proteins begin the secretory stream by translocation into the ER and then flow through transport vesicles to the next secretory compartment. Soluble proteins that are resident in the ER seemingly resist the forward flow of secretion. These proteins are retained because in the next secretory compartment a specific retrieval system sorts out ER proteins and returns them back to the ER (1, 2). In the yeast *Saccharomyces cerevisiae*, retrieval is mediated by the binding of the *ERD2* receptor to the C-terminal peptide sequence HDEL present on soluble ER resident proteins (3, 4). Since *ERD2* is an essential gene, retrieval might appear to be essential for viability. However, bypass suppressors (*SED1*–*SED6*) of the lethality caused by deletion of *ERD2* restore viability but do not restore ER retrieval (5–7). *SED5*, for instance, is a Golgi-localized t-SNARE and is not likely to affect ER function. Furthermore, cells depleted of Erd2p exhibit defects in the Golgi, not the ER (4). Taken together these results suggested that *ERD2* is essential for Golgi function, but not for ER protein retrieval. Moreover, the removal of the HDEL-retrieval tag from the essential protein Kar2p/BiP, the ER-resident Hsp70, results in no loss in cell viability (4). From these observations two alternatives are possible: either *ERD2*-mediated retrieval is dispensable for viability or another system acts in conjunction or in place of *ERD2* to maintain proper levels of ER resident proteins.

The ER-localized transmembrane, $cdc2^{+}$/*CDC28*-like kinase, Ire1p, mediates another system controlling ER homeostasis (8, 9). Mutants of *IRE1* were isolated in a genetic selection for new inositol auxotrophs (10) and in screens for mutants that fail to induce transcription of Kar2p/BiP (8, 9). *IRE1* mediates the transcriptional induction of *KAR2* in response to agents that specifically perturb ER function. These agents include tunicamycin, which blocks core glycosylation, mutants in genes important for ER function (e.g., *SEC53* and *SEC18*), and mutant alleles of *KAR2* itself (refs. 11–14; unpublished observations). In addition to Kar2p/BiP, *IRE1* induces transcription of other resident ER proteins under the same stress conditions (8, 9). *IRE1* is a nonessential gene but is required under conditions that compromise ER function. Presumably the inducible system controlled by Ire1p mediates recovery from stresses on the ER. However, the normal physiological conditions to which Ire1p responds are not known.

In this paper we resolve the paradox of why *ERD2*-mediated ER retrieval is not an essential process. We show that *IRE1* and *ERD2* share functional redundancy for maintaining normal levels of ER proteins. In addition, we show the phenotypic consequence of defective ER protein retrieval in the absence of *IRE1*.

## MATERIALS AND METHODS

**Yeast Strains, Media, and Genetic Methods.** The strains used for this study are as follows: MS10 (*MAT***a** *ura3-52 ade2-101 leu2-3,112*); MS776 [*MAT***a** *ura3-52 leu2-3,112 kar2ΔL148::LEU2* (pMR1341)]; MS3548 (*MAT***a** *ura3-52 ade2-101 leu2-3,112 ire1Δ1::URA3*); MS3855 (*MAT***a** *ura3-52 ade2-101 ire1Δ1::URA3 leu2-3,112*::pMR3002); MY3330 (*MATα ura3-52 leu2-3,112 his4-519 suc2-d9 erd2-B36*) (4); MY3331 (*MATα ura3-52 leu2-3,112 his4-519 suc2-d9 erd2-B25*) (4); MY3553 (*MAT***a** *ura3-52 erd2-B25 ire1Δ1::URA3 leu2-3,112*::pMR3002); MY3557 (*MATα ura3-52 erd2-B25 his4-519 suc2-d9 leu2-3,112*::pMR3002); MY3558 (*MATα ura3-52 his4-519 suc2-d9 leu2-3,112*::pMR3002); MY3806 [*MAT***a** *ura3-52 leu2-3,112 kar2ΔL148::LEU2 erd2-B25* (pMR1341)]; SEY2102 (*MATα ura3-52 leu2-3,112 his4-519 suc2-d9*) (15); YFGR (*MATα ura3-52 leu2-3,112 his4-519 suc2-d9 kar2ΔHDEL*) (15).

Culture media and genetic manipulations were as described elsewhere (16).

**Plasmid Construction.** The construction of the *GAL1*-regulated *ERD2* integration plasmid is as follows. An *Eco*RI fragment containing the *GAL1* promoter was cloned into the *Eco*RI site immediately upstream of *ERD2* in JS209 (4). In addition, in this vector 2-μm sequences were removed by plasmid reclosure following *Xba* I digestion. In place of the 2-μm segment, a 3.4-kb *Nhe* I–*Spe* I fragment from pMR2780 bearing the *LEU2* gene was inserted. The final construct, pMR3002, contains *URA3* and *LEU2* genes; integration is directed to *leu2-3,112* by linearization of the vector with *Cla* I. This plasmid was integrated to produce strains MS3855, MY3553, MY3557, and MY3558.

Plasmid pMR1341 is a *CEN4/ARS1* episome bearing a fusion of *GAL1* regulatory sequences to the structural gene of *KAR2*. pMR1341 also bears the *URA3* selectable marker.

**Immunoblots.** Immunoblots for the detection of secreted Kar2p/BiP were performed on dissected spores as follows. To ensure even distribution, colonies from dissection plates were dispersed in water and 5 μl of each suspension, with roughly the same number of cells, was spotted on YPD and SC(−Ura) plates. Colonies grown for 3 days were replica printed onto nitrocellulose filters, onto which any secreted Kar2p/BiP was bound. Secreted Kar2p/BiP was visualized with anti-Kar2p/BiP antiserum (1:5000) and a horseradish peroxidase-con-

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Abbreviation: ER, endoplasmic reticulum.

*To whom reprint requests should be addressed.

jugated anti-rabbit secondary antibody, followed by the ECL chemiluminescent system (Amersham).

**Microscopy.** Prior to fixation for microscopy, exponentially growing cells were cultured in 4% galactose/2% raffinose SC(−Leu) and subcultured in either 4% galactose/2% raffinose or 2% glucose SC(−Leu) for 30 hr at 30°C. Cells were fixed for 45 min in formaldehyde; nuclei were visualized with 1 μg of 4′,6-diamidino-2-phenylindole per ml; Kar2p/BiP was visualized by indirect immunofluorescence using 1:1000 anti-Kar2p/BiP polyclonal antiserum and a fluorescein isothiocyanate-conjugated anti-rabbit secondary antiserum. The procedures used for the immunofluorescent staining of fixed cells were as described elsewhere (16).

## RESULTS AND DISCUSSION

In cells unable to retrieve ER lumenal proteins the levels of these proteins in the ER remain constant despite their active secretion out of the cell and into the surrounding media (ref. 4; unpublished observations). This suggested that yeast cells can compensate for inefficient retrieval by specifically inducing the expression of ER lumenal proteins. To test directly whether this induction is mediated by the *IRE1* kinase, we examined the consequence of crossing an *ire1*Δ strain to strains bearing mutations in *ERD2*. The two mutant alleles of *ERD2* tested, *erd2-B25* and *erd2-B36*, have distinct ER protein retrieval defects but otherwise show no gross physiological defect (4). Crosses of each single mutant to wild type resulted in high spore viability. The cross of *erd2-B36* and the isogenic, wild-type parent (MS10) of the *ire1*Δ strain produced 97% viable spores (16 tetrads dissected). The cross of *erd2-B25* to the isogenic, wild-type parent (MS10) of the *ire1*Δ strain produced 98% viable spores (22 tetrads dissected). The cross of *ire1*Δ with a wild-type strain congenic to *erd2-B25/36* (SEY2102) produced 98% viable spores (48 tetrads dissected).

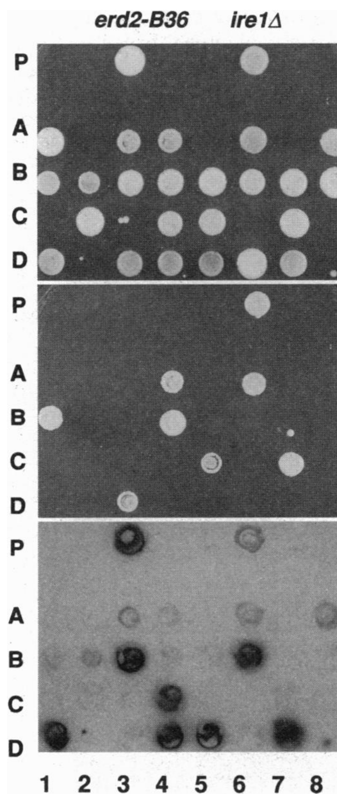


FIG. 1. *ire1*Δ is synthetically lethal with *erd2-B36*. (*Top*) Representative dissection of *ire1*Δ*ERD2/IRE1 erd2-B36* sporulated diploids on YPD rich medium. Note the high spore inviability. Each tetrad is numerically labeled; individual spores from each tetrad are lettered A–D. Parent strains (P) are shown as labeled. (*Middle*) The same cells spotted onto a SC(−Ura) plate. Cells that grew on these plates bore the *ire1*Δ::*URA3* allele. (*Bottom*) Kar2p/BiP immunoblot of cells from the YPD plate. ER proteins not recycled are secreted out of the cell and onto the membrane. Darkened spots indicate cells defective for ER recycling.

In contrast to crosses to wild-type strains, crosses between *ire1*Δ strains and either of the two *erd2* mutants resulted in low spore viability (Fig. 1). Overall viability from the cross between *erd2-B25* and *ire1*Δ strains was 71% (44 tetrads dissected); *erd2-B36* and *ire1*Δ also yielded 71% viable spores (33 tetrads dissected). Segregation of the *ire1* deletion was followed through the cross by the presence of the *URA3* gene. Mutant *erd2* spores were identified by their defect in ER recycling, specifically the failure to retain Kar2p/BiP within the cell. Of 16 *ire1*Δ*ERD2/IRE1 erd2* tetrads analyzed (9 tetratypes, 3 nonparental ditypes, 4 parental ditypes), none contained viable Kar2p/BiP secreting/Ura$^+$ spores (15 *ire1*Δ*erd2* double mutants predicted, none observed). The lethality of *ire1*Δ*erd2* double mutant could be rescued by wild-type *ERD2* on an autonomous construct (see below). Dissected *ire1*Δ*erd2* spores germinate and form microcolonies of two to eight cells before growth arrest. Presumably these spores have enough ER lumenal protein, or Ire1p or Erd2p, to survive one to three cell divisions. The inviability of a cell bearing two mutations, which otherwise cause no growth defect on their own, is referred to as synthetic lethality. Synthetic lethality is indicative of two gene products that overlap in function and/or physically interact.

The relationship between these two mutations suggests that together, Ire1p and Erd2p maintain ER homeostasis. However, the synthetic lethality of *ire1*Δ with the two *erd2* mutants may reflect a process, unrelated to any function in the ER, in which both genes otherwise participate. Given that *ERD2* controls the retrieval of essential ER proteins, one might expect that any single essential ER protein that cannot be

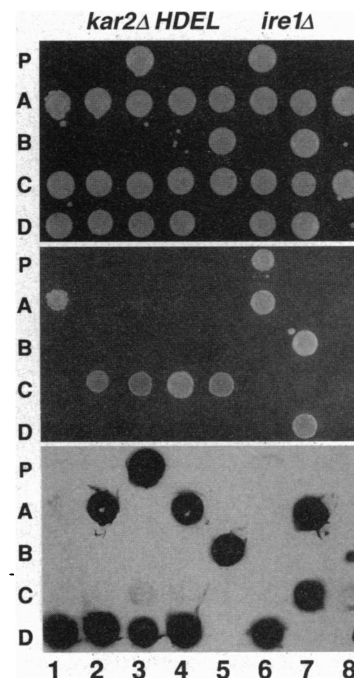


FIG. 2. Synthetic lethality of dissected *ire1*Δ*kar2*Δ*HDEL* spores. (*Top*) Representative dissection of *ire1*Δ*KAR2/IRE1 kar2*Δ*HDEL* diploid on YPD rich medium. Low spore viability was noted. (*Middle*) Same cells on SC(−Ura) plates; *ire1*Δ::*URA3* cells grow. (*Bottom*) Kar2p/BiP immunoblot of cells from the YPD plate. The *kar2*Δ*HDEL* cells are recognized by their secretion of Kar2p/BiP onto the blot and are detected as darkened spots.

retrieved might also be synthetically lethal with *ire1Δ*. We tested whether *kar2ΔHDEL* is also synthetically lethal with *ire1Δ*. The *kar2ΔHDEL* mutant bears a deletion of its C-terminal ER-retrieval signal, HDEL; this mutant is viable and intracellular levels of Kar2p/BiP are normal (ref. 4; unpublished observations). If *IRE1* compensates for the lack of Kar2p/BiP in the ER of *kar2ΔHDEL* strains, then the *ire1Δkar2ΔHDEL* double mutant should also be inviable. As shown in Fig. 2, *kar2ΔHDEL* was synthetically lethal with *ire1Δ*. Of 16 *ire1ΔKAR2/IRE1 kar2ΔHDEL* tetrads analyzed, none produced viable Kar2p/BiP secreting/Ura$^+$ spores. The cross of *kar2ΔHDEL* and *ire1Δ* strains yielded a spore viability of 71% (47 tetrads dissected). The diploid resulting from the cross between the *kar2ΔHDEL* parent strain (SEY2102) and *ire1Δ* produced spores with 98% viability overall (48 tetrads dissected). Crossing *kar2ΔHDEL* with the *ire1Δ1::URA3* parent (MS10) resulted in a spore viability of 94% (35 tetrads dissected). Synthetic lethality with *ire1Δ* is therefore a general effect of mutations defective for ER retrieval.

The lethality of the *ire1Δerd2* double mutant was rescued with an extragenic copy of the wild-type *ERD2* gene under the control of the regulated *GAL1* promoter. Using this strain, the terminal phenotype of *ire1Δerd2* cells, after depletion of Erd2p, was examined. First, cells were cultured in galactose, allowing expression of *ERD2*. Then glucose was added to repress wild-type Erd2p expression; growth stopped by 24 hr. If this strain can neither retrieve nor replace lumenal ER proteins by new synthesis, then cells arrested in glucose should have little or no resident ER proteins. Fig. 3 shows the localization of Kar2p/BiP in cells bearing the regulated *ERD2* gene. In galactose-grown cells, Kar2p/BiP showed exclusive ER and nuclear envelope localization. After Erd2p depletion in *ire1Δerd2* cells, Kar2p/BiP immunofluorescence was more diffuse and punctate (Fig. 3*D*). Little, if any, Kar2p/BiP could be discerned in the ER/nuclear envelope. Mislocalization was not seen in cells wild type for either *IRE1* or *ERD2*, or both (Fig. 3 *A, C, E,* and *F*). When Erd2p synthesis was repressed, total levels of intracellular Kar2p/BiP did not change in the *ire1Δerd2* strain and the amount of Kar2p/BiP secreted from these cells was only nominally higher (unpublished observation). These results imply that depletion of ER proteins leads to a block in the secretory pathway before significant amounts of Kar2p/BiP are secreted out of the cell. Nevertheless, we conclude that the immediate cause of growth arrest is the loss of proteins from the ER to subsequent compartments.

How does Ire1p compensate for the failure of ER recycling? The *IRE1* pathway is not constitutively required for the expression of ER proteins but is required for an inducible response (8). Comparison by Northern analysis of *ERD2* mRNA in wild-type, *ire1Δ1::URA3, erd2B25,* and *erd2B36* strains showed no significant differences (data not shown). Therefore, in mutant *erd2* strains, Ire1p does not directly compensate for the failure in ER retrieval by inducing more mutant *erd2* protein. To test whether *erd2* and *ire1Δ* were synthetically lethal because of the trivial possibility that *erd2* affects Kar2p levels only, we determined whether the overexpression of Kar2p/BiP alone (expressed from the *GAL1* promoter) could rescue the *ire1Δerd2* synthetic lethality. A diploid transformed with *pGAL1-KAR2*, and heterozygous for *ire1Δ* and *erd2-B25*, was sporulated. Of 26 tetrads dissected on galactose, none gave rise to viable *ire1Δerd2* double mutant spores. However, the *GAL1* promoter may not be able to express Kar2p to levels high enough to compensate for the loss of Kar2p due to lack of retention in an *erd2* mutant. To test this directly we determined whether an *erd2* mutant bearing a complete deletion of *KAR2* could be suppressed by *pGAL1-KAR2* when grown on galactose. In 5 complete tetrads from a cross between MS776 and MY3331, we recovered 5 *kar2Δerd2* (*pGAL1-KAR2*) spores whose viability depended upon growth

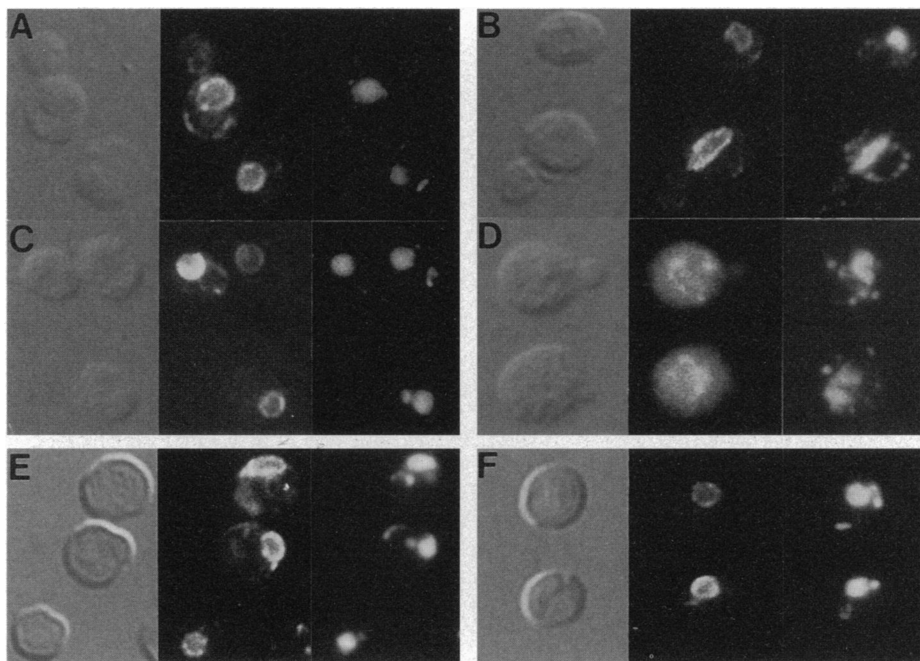


FIG. 3. Repression of Erd2p synthesis in an *ire1Δerd2* cell leads to mislocalization of Kar2p/BiP. In each series (*A–F*), the left panel shows whole cell morphology by differential interference contrast microscopy, the middle panel shows the ER/nuclear envelope as shown by Kar2p immunofluorescence, and the right panel shows nuclei by 4′,6-diamidino-2-phenylindole staining. Wild-type cells bearing the *pGAL1-ERD2* construct (MY3558) in galactose (*A*) and in glucose (*C*) showed ER/nuclear envelope localization of Kar2p/BiP. ER/nuclear Kar2p/BiP localization is also seen in wild-type cells without the *pGAL1-ERD2* construct (not shown). (*B*) The *ire1Δerd2* double mutant (bearing *pGAL1-ERD2*, MY3553) in galactose shows ER/nuclear envelope Kar2/BiP localization as in wild type. (*D*) In glucose, Kar2p/BiP localization in the *ire1Δerd2* double mutant (bearing *pGAL1-ERD2*) was no longer ER localized but diffuse and punctate; Kar2p/BiP was mislocalized. Arrested cells were enlarged and frequently had multiple small buds, which often detached with zymolyase treatment (a phenotype shared with *kar2* mutants). The parent strains, *ire1Δ pGAL1-ERD2* (MY3855) and *erd2B25 pGAL1-ERD2* (MY3557), seen in *E* and *F*, respectively, show wild-type ER/nuclear Kar2p/BiP localization when grown in glucose (or galactose, not shown).

on galactose. We conclude that expression from the *GAL1* promoter is at least comparable to that from the *KAR2* promoter under the relevant conditions of Ire1p induction. Therefore, these results suggest that Kar2p/BiP cannot be the only essential ER protein retrieved by *ERD2*. Most likely, Ire1p compensates for the loss of resident ER proteins such as Kar2p/BiP, Pdi1p, and Eug1p by the global induction of their transcription. In *erd2* mutants, *IRE1* is effectively acting as a "backup" for the loss of active ER retrieval, though this may reflect only a part of its wild-type function.

A number of different conditions that perturb ER function lead to the Ire1p-mediated transcriptional response. In a manner analogous to the cytoplasmic heat shock response, protein misfolding within the ER is assumed to be the basis for the induction by these various conditions (17). The transcriptional induction in these cases is separable from the heat shock response as it is totally independent of the *HSF1* heat shock-transcription factor (unpublished results). Two models can be proposed as to how Ire1p senses the failure of ER retrieval. The first model proposes that reduced retrieval of the ER proteins involved in protein folding might cause an accumulation of misfolded secretory proteins within the ER. In turn, the misfolded proteins would induce transcription via Ire1p. Alternatively, Ire1p might directly monitor levels of proteins either retrieved (i.e., Kar2p/BiP) or part of the retrieval machinery (i.e., Erd2p). In both models, any mutation that compromises ER retrieval should induce expression of ER resident proteins.

Although Erd2p and Ire1p control very different processes, both act to accomplish the same result: to maintain appropriate levels of resident proteins within the ER. Since separately each system is not essential for cell viability, we expect other genes in these two processes to be also nonessential (unless needed for another cellular function). Given that most known *sec* mutants do affect cell growth, the components of ER retrieval may be distinct from already-characterized *SEC* genes. Neither *sec21-1* [implicated in the retrieval of dilysine-tagged ER proteins (18)] nor *sec18* is synthetically lethal with *ire1*Δ (unpublished observation). Thus, synthetic lethality between mutants of *IRE1* signal transduction and *ERD2* retrieval may be used to select for more mutants in these systems as well as novel genes regulating ER function.

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