The turnover of damaged proteins is critical to cell survival during stressful conditions such as heat shock or oxidative stress. The accumulation of misfolded proteins in the endoplasmic reticulum (ER) is toxic to cells. Therefore, these proteins must be efficiently exported from the ER and degraded by the proteasome or the vacuole. Previously it was shown that proteins must be efficiently exported from the ER and degraded during stressful conditions such as heat shock or oxidative stress. The accumulation of misfolded proteins in the endoplasmic reticulum (ER), a specialized organelle for protein synthesis, has negative effects on protein structure, some of which can be reversed by molecular chaperones, such as the heat shock protein family. Upon the loss of eEF1Bγ, a non-guanine nucleotide exchange factor, strains lacking eEF1Bγ show severe defects in protein turnover during conditions of oxidative stress. Furthermore, these strains accumulate a greater amount of oxidized proteins, which correlates with changes in heat shock chaperones. These strains show severe defects in vacuole morphology and defects related to the maturation of carboxypeptidase Y that is not dependent on the catalytic subunit of the eEF1B complex as a guanine nucleotide exchange factor. Finally, eEF1Bγ co-immunoprecipitates with an essential component of ER-Golgi transport vesicles. Taken together, these results support a broader protein metabolism role for eEF1Bγ.

Cellular stresses such as heat shock and oxidative stress can have negative effects on protein structure, some of which can be reversed by molecular chaperones, such as the heat shock family of proteins (HSPs) (1). In more severe cases, the damage is irreversible, and the proteins must be degraded by the proteasome or vacuole (2–4). When this damage occurs to proteins in the endoplasmic reticulum (ER), a specialized turnover pathway is required called ER-associated degradation. In addition to vacuole and proteasome function, this turnover pathway also requires the ER-to-Golgi transport system for proper function. Misfolded proteins must be transported to the vacuole for protease degradation or exported to the cytoplasm for proteasomal degradation (5).

The eukaryotic elongation factor 1Bγ (eEF1Bγ) is part of the elongation factor 1 complex, which also contains the eukaryotic elongation factor 1A (eEF1A) and eukaryotic elongation factor 1Bα (eEF1Bα) subunits. The canonical role of this complex is to recruit aminoacylated tRNA to the ribosome during translation elongation (6). Although eEF1A and eEF1Bα are essential for this process and viability in the yeast Saccharomyces cerevisiae (7), deletion of eEF1Bγ does not result in any obvious defects in protein synthesis or growth in yeast (8) despite the fact that in mammalian cells, eEF1Bγ has been linked to translation and the keratin cytoskeleton (9). Yeast strains lacking eEF1Bγ do, however, exhibit significant resistance to oxidative stress in the form of CdSO4 and H2O2 (10). Additionally, eEF1Bγ has been identified both as a calcium-dependent membrane-binding protein and as a high copy suppressor of a mutation in the DR5 gene, whose product has been shown to play a role in vesicle budding from the Golgi (11–13). These findings strongly suggest a role for eEF1Bγ in the membrane systems of the cell, although the exact mechanism by which eEF1Bγ affects this process and the link to gene expression is unknown.

In this study, protein metabolism is examined in strains lacking eEF1Bγ. Although a mutant strain lacking both genes encoding this protein does not exhibit observable changes in total protein synthesis, there are dramatic changes in protein expression patterns. These changes correlate with altered vacuolar protease regulation and a greater accumulation of oxidized proteins in cells lacking eEF1Bγ. Furthermore, changes in the pattern of specific heat shock chaperones appear in a strain lacking eEF1Bγ similar to a wild type strain exposed to oxidative stress. These include longer expression of some proteins in an eEF1Bγ-deficient strain following stress treatment, whereas others appear to be a result of altered mobility. Strains lacking eEF1Bγ also displayed morphological defects in vacuoles during stationary phase and defects in maturation of carboxypeptidase Y (CPY), a protein that requires ER-Golgi transport for maturation. Interestingly, the CPY maturation was normal upon the loss of eEF1Bα, the catalytic subunit of the guanine nucleotide exchange factor complex, indicating that this effect is not due to the canonical catalytic function of this complex. Lastly, eEF1Bγ was found to co-immunoprecipitate with an essential component of Golgi-to-ER transport vesicles. These results support the model that eEF1Bγ may play a broader role in protein metabolism than initially thought.
**Non-translation Role of eEF1βγ**

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Yeast strains used in this study are described in Table 1. Candidate genes were HA-tagged at the C terminus using pFA6-3HA-HIS3 with F2 R1 primers and candidate gene-specific sequences as described in Ref. 14. Yeast were grown on yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone, 2% dextrose) and transformed using Flo transformation II kit (Open Biosystems, Huntsville, AL). Strains were grown in synthetic complete-methionine medium to an OD₅₉₅ of 0.4 and treated with 150 μM CdSO₄ for up to 4 h. At indicated time points, cells were harvested and lysed by vortexing with glass beads in lysis buffer (100 mM Tris, 1.5 mM NaCl, 0.05% Tween 20). TAP-tagged protein blots were probed with 1:1,000 dilution of polyclonal anti-CPY antibody (Open Biosystems) in TBST. CPY blots were probed with 1:1,000 dilution of polyclonal anti-CPY antibody (Millipore) in TBST with 5% dry milk and 1% BSA. Western blot analysis was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

**SDS-PAGE and Western Blot Analysis**—Strains were grown in YPD to an A₅₉₅ of 0.4 and treated with 150 μM CdSO₄ for up to 4 h. At indicated time points, cells were harvested and lysed by vortexing with glass beads in lysis buffer (100 mM Tris, pH 8.0, 20% glycerol, and 1 mM PMSF). The protein concentration was determined using the Bradford protein assay (Bio-Rad), and 5 μg of protein was resolved by SDS-PAGE using 10% gels. Protein gels were stained using GelCode Blue stain (Thermo Fisher). HA-tagged protein blots were probed with 1:500 dilution of monoclonal anti-HA antibody in TBST (100 μM Tris, 1.5 mM NaCl, 0.05% Tween 20). TAP-tagged protein blots were probed with 1:10,000 dilution of polyclonal anti-TAP antibody (Open Biosystems) in TBST. CPY blots were probed with 1:1,000 dilution of polyclonal anti-CPY antibody (Millipore in TBST with 5% dry milk and 1% BSA). Western blots were quantitated with the ImageJ software (National Institutes of Health). For HA-tagged blots, protein levels at each time point were normalized to the levels of the phosphoglycerate kinase control, and each time point was expressed as a ration to the zero time point. For OxyBlot, all samples were quantitated and normalized to untreated wild type.

**[35S]Methionine Incorporation**—Strains were grown in Synthetic complete-methionine medium to an A₅₉₅ of 0.4. At time 0, a final concentration of 50 μM cold methionine and 1 μCi/ml of [35S]methionine was added to the cultures. At the indicated time points, samples were lysed in 50% trichloroacetic acid and filtered through 25-mm glass microfiber filters. Radioactivity was measured using an LKB 1209 Rack-beta liquid scintillation counter and normalized to cell number as described previously (17).

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**TABLE 1**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| BY4741 | Mata ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 | Open Biosystems |
| TKY603 | Mata ura3Δ2 trp1Δ101 lys2-801 leu2Δ1 met2::His3-713 tef5::TRP1 pTEF5 LEU2 | 25 |
| TKY604 | Mata ura3Δ2 trp1Δ101 lys2-801 leu2Δ1 met2::His3-713 tef5::TRP1 pTEF2 URA3 | 25 |
| TKY656 | Mata leu2Δ1, trp1Δ101, ura3Δ2, lys2-801, his3Δ201, lys2-801 | 10 |
| TKY659 | Mata leu2Δ1, trp1Δ101, ura3Δ2, lys2-801, his3Δ201, lys2-801 tef3::LEU2 tef6::TRP1 | 10 |
| TKY677 | Mata ura3Δ2 leu2Δ1 his3Δ200 trp1Δ101 lys2-801 | 10 |
| TKY680 | Mata ura3Δ2 leu2Δ1 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 | 10 |
| TKY781 | Mata ura3Δ2 trp1Δ101 lys2-801 leu2Δ1 met2-1 his3Δ201 tef5::TRP1 TEF2 SBD5 (TEF1-4 E122K) | 25 |
| TKY782 | Mata ura3Δ2 trp1Δ101 lys2-801 leu2Δ1 met2-1 his3Δ201 tef5::TRP1 TEF1 SBD6 (TEF1-7 D156N) | 25 |
| TKY133 | Mata ura3Δ2 leu2Δ0 his3Δ200 lys2-802 | This study |
| TKY1355 | BY4741 Mata ura3Δ2 leu2Δ0 his3Δ1 met15Δ0 pep4::KanMX | Open Biosystems |
| TKY1350 | BY4741 Mata ura3Δ20 leu2Δ0 his3Δ1 met15Δ0 prb1::KanMX | Open Biosystems |
| TKY1385 | Mata ura3Δ2 leu2Δ0 his3Δ200 lys2-801 SSA1·HA | This study |
| TKY1357 | Mata ura3Δ2 leu2Δ0 his3Δ200 lys2-801 SSA2·HA | This study |
| TKY1415 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 SSA1·HA | This study |
| TKY1417 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 SSA2·HA | This study |
| TKY1418 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 SSA4·HA | This study |
| TKY1460 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 HIS3·HA | This study |
| TKY1461 | Mata ura3Δ2 leu2Δ0 his3Δ200 lys2-800 HIS2·HA | This study |
| TKY1462 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 HIS2·HA | This study |
| TKY1463 | Mata ura3Δ2 leu2Δ0 his3Δ200 trp1Δ101 lys2-801 tef3::LEU2 tef6::TRP1 HIS2·HA | This study |
| TKY1507 | BY4741 Mata ura3Δ20 leu2Δ0 his3Δ1 met15Δ0 prb1::KanMX | Open Biosystems |
| TKY1509 | BY4741 Mata ura3Δ20 leu2Δ0 his3Δ1 met15Δ0 prb1::KanMX | Open Biosystems |
Polyribosome Analysis—Yeast strains were grown in YEPD to an A\textsubscript{595} of 0.4 and then treated with 340 \mu M cycloheximide and lysed using glass beads. Samples were separated by centrifugation for 4 h at 130,000 \times g on 7–47% sucrose gradients and analyzed with an ISCO model UA-5 monitor at A\textsubscript{254} as described previously (18).

OxyBlot Assay—Yeast strains were grown in YEPD to an A\textsubscript{595} of 0.4 and treated with H\textsubscript{2}O\textsubscript{2} for 4 h. Ten \mu g of lysate was derived with 2,4-dinitrophenylhydrazine (DNPH) using the OxyBlot kit (Millipore). SDS-PAGE and Western blot analysis were performed using standard methods and probed using an anti-DNPH antibody as described.

Electron Microscopy—Yeast strains grown to stationary phase (A\textsubscript{600} = 4) in YEPD were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer and analyzed by the RWJMS Core Imaging Lab. Cells were rinsed briefly in 0.1 M buffer (cacodylate) and post-fixed in 1% osmium tetroxide for 1 h followed by dehydration in a graded series of acetone (10 min each) and infiltrated in Epon resin overnight. Two changes of fresh resin were done for 30 min each and placed at 70 °C overnight. Thin sections (90 nm) were cut with a DiATOME diamond knife and placed on a 200-mesh copper grid. The grids were stained with saturated uranyl acetate for 10 min followed by lead citrate for 2.5 min. Microscopic analysis of grids was performed with a JEOL 1200 Electron microscope at 80 kV, and images were obtained with an AMT XR41 CCD camera.

Fluorescent Labeling of Vacuoles—Yeast strains grown to stationary phase (A\textsubscript{600} = 4) in YEPD were labeled using FM 4-64 (Invitrogen) as described (19). Briefly, 1 ml of cells was resuspended in 50 \mu l of YEPD and incubated with 100 \mu M FM 4-64 for 20 min at 30 °C. Cells were washed with 1 ml of YEPD and then resuspended in YEPD and incubated at 30 °C for 1.5 h. Cells were pelleted and resuspended in 50 \mu l of YEPD, and 5 \mu l of cells was placed on a microscope slide with a 1% agarose layer as described in Ref. 20. Cells were viewed using an Olympus IX70 inverted fluorescence microscope.

Co-immunoprecipitation—Co-immunoprecipitations were performed essentially as described previously (10). Briefly, yeast strains with TAP-tagged Sec27p were grown to log phase (A\textsubscript{600} = 0.8) and lysed in immunoprecipitation buffer (50 mM NaCl, 50 mM Tris, pH 7.5, 0.02% NaN\textsubscript{3}, 1 mM PMSF, 0.1% Triton X-100, and 1 \times protease inhibitor cocktail (Sigma)). Forty \mu g of lysate was incubated with polyclonal anti-eEF1B\gamma antibody at 4 °C for 1 h with constant rocking. Protein A-Sepharose beads were added, and incubation continued at 4 °C for 1 h. The pellet was collected by centrifugation and washed three times in immunoprecipitation buffer and then boiled in loading buffer before SDS-PAGE analysis and Western blotting with the anti-TAP antibody (Open Biosystems, 1:5,000 dilution).

RESULTS

Strains Lacking eEF1B\gamma Exhibit Altered Expression of Heat Shock Chaperones—To observe the effects of the loss of the eEF1B\gamma subunit of eEF1 on the yeast proteome, two-dimensional gel electrophoresis (2DGE) was performed on wild type and strains lacking both the TEF3 and the TEF4 genes encoding eEF1B\gamma with and without treatment with 150 \mu M CdSO\textsubscript{4} over a mass range of 10–220 kDa and a pI range of 4–7 (Fig. 1). Comparison of expression patterns revealed several spots that were present in treated but not untreated wild type samples (Fig. 1, spots A, B, and D–I). Very similar spots also appeared in treated samples from strains lacking eEF1B\gamma, and surprisingly, in untreated samples from strains lacking eEF1B\gamma (Fig. 1, spots A–F, H, and I). These spots were excised...
from the wild type treated and Δtef3 Δtef4 untreated gels and analyzed by MALDI-TOF mass spectrometry to identify the protein or proteins represented by each spot. Candidate proteins were compared with gel spots, and candidates with molecular weights or pI values that were inconsistent with the gel spot were removed from the analysis in addition to any candidate protein having a log(e) value greater than 20, indicating a low likelihood of a match. The remaining high probability candidate proteins for each gel spot are listed in order of probability value in Table 2. Eight of these ten high probability candidates were found to be molecular chaperones, primarily members of the heat shock chaperone family, Ssa1p, Ssa2p, Sse1p, Hsc82p, Hsp82, Sba1p, Hsp60p, and Kar2p. The remaining two candidates are Cdc48p, involved in transport of proteins from ER to cytosol for degradation, and Def1p, involved in the degradation of RNA polymerase 2.

**Table 2**

| Name    | Mass | pI | Description                                                                 | log(e) |
|---------|------|----|-----------------------------------------------------------------------------|--------|
| Spot A  | 94   | 5–6| HSP70 family; component of Hsp90 chaperone complex; binds unfolded proteins  |
| SSE1    | 77   | 4  |                                                                             |
| KAR2    | 74   | 4  | Protein folding and import into the ER; regulation of unfolded protein response|
| SSE1    | 77   | 4  |                                                                             |
| CDC48   | 91.9 | 4.7| Transport of ubiquitinated proteins from the ER to the cytosol for degradation|
| Spot C  | 87   | 5–6| HSP70 family; involved in protein folding and vacuolar import of proteins    |
| SSA2    | 69.4 | 4.7|                                                                             |
| SSA1    | 69.7 | 4.82|                                                                             |
| KAR2    | 74   | 4  |                                                                             |
| HSC82   | 80.8 | 4.6| Hsp90 chaperone required for pheromone signaling, negative regulation of Hsf1p |
| HSP82   | 81.4 | 4.7|                                                                             |
| Spot D  | 30   | 4–5| Mitochondrial chaperonin required for folding of precursor polypeptides       |
| HSP60   | 60   | 5.06|                                                                             |
| Spot E  | 90   | 5–6|                                                                             |
| SSA2    | 69.4 | 4.7|                                                                             |
| SSA1    | 69.7 | 4.82|                                                                             |
| DEF1    | 84   | 4.76|                                                                             |
| Spot F  | 87   | 4–5|                                                                             |
| SSA1    | 69.7 | 4.82|                                                                             |
| Spot G  | 90   | 5–6|                                                                             |
| HSC82   | 80.8 | 4.6|                                                                             |
| HSP82   | 81.4 | 4.7|                                                                             |
| CDC48   | 91.9 | 4.7|                                                                             |
| Spot H  | 30   | 4–5|                                                                             |
| SBA1    | 24   | 4.3| Co-chaperone that binds to and regulates Hsp90 family chaperones             |

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**Figure 2. Hsc82p and Ssa2p show altered expression in Δtef3 Δtef4 strain following oxidative stress.** Strains containing HA-tagged copies of indicated candidate genes in pairs of isogenic wild type and Δtef3 Δtef4 strains (TKY1385 and TKY1386, SSA1; TKY1415 and TKY1416, SSA2; TKY1417 and TKY1418, SSA4; TKY1460 and TKY1462, HSC82; TKY1461 and TKY1463, HSP82) were treated with 150 μM CdSO4 for the times indicated. Expression was monitored by Western blotting of samples using anti-HA antibodies with untagged strains shown as controls. Quantitation below each band indicates the ratio of protein at each time point to the level at time 0, normalized to phosphoglycerate kinase expression.
eEF1Bγ (0.74 of the level at time 0; Fig. 2). Because so many members of the Hsp70 family were candidates, the stress-inducible Hsp70 family member, Ssa4p, was also examined; however, there was no observable difference in the expression of Ssa4p in wild type and strains lacking eEF1Bγ (Fig. 2). Although Hsc82p and Ssa2p showed an increase in the duration of their expression in the tef3 tef4 strain when compared with wild type, all of the candidates did not show the same constitutive activation observed in the 2DGE. This could indicate that the changes in migration of spots in 2DGE were caused by modifications that alter the isoelectric point of candidate genes and thus result in a shifting of spots in the 2DGE, which cannot be detected by standard Western blot analysis.

**Global Protein Stability in Response to Stress Is Altered in Strains Lacking eEF1Bγ**—To look more globally at protein stability, wild type (TKY680) and eEF1Bγ-deficient strains (TKY680) were treated with 25 or 100 μM CdSO₄ for 4 h and visualized by GelCode Blue staining of SDS-PAGE gels. At time 0, cycloheximide-treated samples were treated with 178 μM cycloheximide in addition to CdSO₄. log phase strains as in A were treated with indicated concentrations of H₂O₂ for 4 h, and lysates were analyzed using the OxyBlot kit (anti-DNP, anti-dinitrophenyl antibody treated). Non-DNPH modified lysate (NODNP) is shown as a negative control. C, log phase wild type and vacuolar protease-deficient strains, TKY1335 Δpep4 and TKY1350 Δprb1, were treated with 150 μM CdSO₄ for up to 4 h, and total protein was visualized as in A.

**FIGURE 3.** Strains lacking eEF1Bγ are deficient in vacuolar-dependent turnover of oxidized proteins. A, wild type (TKY1333) and eEF1Bγ-deficient strains (TKY680) were treated with 25 or 100 μM CdSO₄ for 4 h and visualized by GelCode Blue staining of SDS-PAGE gels. At time 0, cycloheximide-treated samples were treated with 178 μM cycloheximide in addition to CdSO₄. B, log phase strains as in A were treated with indicated concentrations of H₂O₂ for 4 h, and lysates were analyzed using the OxyBlot kit (anti-DNP, anti-dinitrophenyl antibody treated). Non-DNPH modified lysate (NODNP) is shown as a negative control. C, log phase wild type and vacuolar protease-deficient strains, TKY1335 Δpep4 and TKY1350 Δprb1, were treated with 150 μM CdSO₄ for up to 4 h, and total protein was visualized as in A.
teinase turnover pathway.

Amino acid oxidation is dependent on the vacuolar proteasome. Examination of lysates from cells treated with 150 μM CdSO4 for 1 h. Both Δtep3 Δtep4 cells treated with 1 mM H2O2 accumulated large amounts of oxidized proteins, whereas treatment with a lower concentration of H2O2 (0.5 mM) caused significantly more protein oxidation in Δtep3 Δtep4 strains (2.18 times untreated wild type) than in wild type (1.34 times untreated wild type).

Protein Turnover during Oxidative Stress Requires Vacuolar Proteinas A and B—To determine whether the altered levels of proteins were due to altered protein turnover, total protein expression under stress conditions in strains containing null alleles of PEP4 (protease A) and PRB1 (protease B) was examined by SDS-PAGE analysis of lysates from cells treated with 150 μM CdSO4 for up to 4 h (Fig. 3C). Both Δtep4 and Δprb1 strains displayed phenotypes similar to Δtep3 Δtep4 cells, showing minimal changes in total protein expression in response to stress, suggesting that the disappearance of high molecular mass protein bands in wild type strains under conditions of oxidative stress is dependent on the vacuolar proteinase turnover pathway.

Altered Protein Expression in Strains Lacking eEF1Bγ Is Not Due to Changes in Global Protein Synthesis—To determine whether these alterations in total protein expression in strains lacking eEF1Bγ were due to a change in total protein synthesis under stress conditions, incorporation of [35S]methionine was measured in exponentially growing cells under normal and stress conditions (150 μM CdSO4 for 1 h). Both wild type and eEF1Bγ-null strains showed very similar levels of [35S]methionine incorporation in the absence of CdSO4 exposure, whereas both showed a 30% decrease in total incorporation after 60 min of CdSO4 treatment (Fig. 4A). To confirm that the deletion of the genes encoding eEF1Bγ did not affect any specific stage of translation under normal or stress conditions, polyribosomal analysis of strains lacking eEF1Bγ was performed under normal and stress conditions (150 μM CdSO4 for 1 h). Both wild type and eEF1Bγ-deficient strains showed very similar polyribosomal profiles under normal conditions as well as during stress conditions. Both strains showed an increase in the 80 S peak, indicating that the known initiation block in response to oxidative stress is not disrupted by the deletion of the gene encoding eEF1Bγ (23). These results suggest that the loss of eEF1Bγ does not affect global protein synthesis under normal conditions or oxidative stress conditions when compared with wild type yeast. Thus, the changes in the global protein expression pattern are likely due to changes in protein turnover.

Strains Lacking eEF1Bγ Display Altered Vacuole Morphology—Because strains lacking eEF1Bγ display decreased protein turnover, an alteration in global protein levels in response to stress that is similar to protease-deficient strains, vacuoles were visualized in wild type and Δtep3 Δtep4 strains. In wild type cells grown to stationary phase, the formation of a single large central vacuole was observed in most cells (Fig. 5, A and B) as published previously (24). In strains lacking eEF1Bγ and grown to stationary phase, however, the vacuole was absent in most cells (Fig. 5C), and the few cells where vacuoles were visible displayed abnormal morphology such as fragmentation (Fig. 5D). These findings were confirmed by utilizing FM 4-64, a vital stain for the labeling of vacuoles (19), to observe vacuole morphology in a wild type strain and a strain lacking eEF1Bγ. In the wild type strain, most cells contained one or more large vacuoles (Fig. 5, E and F). In the strain lacking eEF1Bγ, many cells did not display a vacuole, instead exhibiting a more diffuse staining pattern characteristic of a class C vacuole mutant (Fig. 5, G and H).
The cells that did exhibit a detectable vacuole in the strain lacking eEF1Bγ exhibited significantly smaller vacuoles than wild type, similar to the vacuole fragmentation observed in class B vacuole mutant (Fig. 5, G and H).

Strains Lacking eEF1Bγ Exhibit Defects in Vacular Protein Maturation—Because strains lacking eEF1Bγ exhibit severe defects in vacuole formation, the integrity of other membrane systems that function upstream of the vacuole, specifically the ER and Golgi apparatus essential for the full maturation of many vacuolar proteins, was assessed. Strains lacking eEF1Bγ were tested for sensitivity to tunicamycin, which prevents ER glycosylation of glycoproteins, causing a buildup of unfolded proteins in the ER that require efficient ER-Golgi transport to be properly degraded (5). Wild type and eEF1Bγ-deficient strains were treated with 0–3 nM tunicamycin for 6 h. The Δtef3 Δtef4 strain was significantly more sensitive to tunicamycin than wild type strain, suggesting a defect in either ER-Golgi transport or the turnover of these unfolded proteins (Fig. 6A).

The integrity of the ER and Golgi apparatus in strains lacking eEF1Bγ was assessed by examining the maturation of CPY because this protein requires transport through both the ER and the Golgi apparatus to be fully glycosylated. Western blot analysis revealed a decrease in the size of CPY in a Δtef3 Δtef4 strain equivalent to that seen in a strain lacking the α-1,3-mannosyltransferase encoded by the MNN1 gene, a Golgi native protein required for protein glycosylation (Fig. 6B). Further, the treatment of lysates from wild type and eEF1Bγ-deficient cells with endoglycosidase H to remove the entire carbohydrate moiety from CPY resulted in the same size band in both wild type and eEF1Bγ-null strains, confirming that the size difference of CPY in untreated lysates is due to a difference in glycosylation in wild type and eEF1Bγ-deficient strains. The strain lacking CPY (Δprc1) had no band at the size of interest, confirming this band to be CPY (Fig. 6B). Because eEF1Bγ forms a complex with the catalytic subunit of the guanine nucleotide exchange factor for translation elongation factor eEF1α, CPY maturation was analyzed in a strain lacking the essential TEF5 gene encoding eEF1α using two different methods. First, CPY maturation was analyzed in strains overexpressing eEF1A, which bypasses the necessity for eEF1α (Fig. 6B, Δtef5). Additionally CPY maturation was analyzed in strains containing suppressor of Bα deficiency (SBD) mutations. These strains contain the eEF1A mutants E122K (SBD5) or D156N (SBD6) that allow cells to survive in the absence of the normally essential eEF1α subunit (25). Strains lacking eEF1α exhibited normal CPY maturation, indicating that the Golgi glycosylation of CPY requires the eEF1Bγ subunit but not the eEF1α subunit of the eEF1B complex (Fig. 6B).

eEF1Bγ Interacts with Sec27p, an Essential COPI Vesicle Component—Because the size of CPY in strains lacking eEF1Bγ was the same as in Δmnn1 strains, in which CPY receives its ER glycosylation but not its Golgi glycosylation, it is possible that eEF1Bγ is required either for Mnn1p activity or for CPY to be transported from the ER to the Golgi. It has previously been found by genome-wide screens that TEF4-encoded eEF1Bγ immunoprecipitates with TAP-tagged Sec27p, a COPI-coated vesicle subunit required for ER-Golgi transport (26, 27). To confirm this finding, we performed co-
Non-translation Role of eEF1Bγ

immunoprecipitation experiments, pulling down eEF1Bγ using anti-eEF1Bγ antibody and analyzing TAP-tagged Sec27p. Under these conditions, Sec27p was enriched in the pellet in TAP-tagged Sec27p but not untagged lysates (Fig. 6C). This interaction with an essential component of COPI vesicles further supports the model that eEF1Bγ is required for the transport of at least a subset of proteins.

DISCUSSION

Although eEF1Bγ associates with the essential translation elongation factor eEF1Bα, the loss of eEF1Bγ appears to have very little effect on global translation in yeast. Although eEF1Bα is the catalytic subunit of the guanine nucleotide exchange factor complex, the role of eEF1Bγ has remained unclear. Significant evidence exists, however, implicating a non-translation-related role for eEF1Bγ. First, eEF1Bγ was found to be a high copy suppressor of cold-sensitive mutations of DRS2, a gene that has been shown to play a role in vesicle budding from the Golgi (12, 13). Further, eEF1Bγ was identified in a screen for calcium-dependent membrane-binding proteins (11). Genome-wide genetic screens have revealed that the loss of the TEF4 gene encoding one form of eEF1Bγ is synthetically lethal with the loss of either YPT6, the Rab family GTPase of the secretory pathway, or RIC1, which forms heterodimer with Rgp1p that acts as a GTP exchange factor for Ypt6 (28). Because both of these proteins are involved in vesicle transport, together with the data presented, they support a role for eEF1Bγ in a membrane-associated function in the cell.

In this study, extracts of strains lacking eEF1Bγ showed protein expression patterns that differed from wild type extracts under normal and stress conditions. The 2DGE revealed many stress-inducible changes in protein pattern from wild type cells after 1 h of CdSO₄ treatment. Further, comparison of treated wild type cell extracts with untreated eEF1Bγ-deficient extracts showed very few significant changes between the two samples, suggesting a constitutively active stress response in the absence of eEF1Bγ.

The candidate proteins were tagged with an HA epitope to determine whether the protein levels are altered by Western blot analysis. The majority of these changes were identified as proteins related to the stress response. Some of the identified proteins, however, did not show altered levels or stability when analyzed by one-dimensional SDS-PAGE and Western analysis, indicating that some of these changes are more likely modifications of the proteins. Two of the proteins analyzed by 2DGE, Hsc82p and Ssa2p, displayed altered expression in response to stress in strains lacking eEF1Bγ. Similar levels of Hsc82p were present in wild type and eEF1Bγ-deficient strains at time 0, but after 1 h of treatment, Hsc82p level had decreased in wild type cells but not in eEF1Bγ-deficient strains. The protein was undetectable in both strains after a 2-h treatment. Ssa2p displayed a similar trend at the 2-h time point, where the protein level had been reduced in wild type but not in cells lacking eEF1Bγ. This apparent change in protein levels suggests alterations in protein turnover in eEF1Bγ-deficient strains that may result in prolonged presence of specific stress response proteins in these mutants.

The SDS-PAGE analysis of total protein extracts further supported this hypothesis because many proteins disappeared in wild type extracts over time in response to CdSO₄ treatment but not in extracts from cells lacking eEF1Bγ. This suggests a defect in protein turnover in response to stress in the absence of eEF1Bγ. The finding that the addition of cycloheximide to these samples prevented the disappearance of protein bands in response to stress indicated that new protein synthesis is required, which has previously been shown for certain forms of protein turnover (29).

Changes in protein amounts are not due to altered protein synthesis in strains lacking eEF1Bγ relative to wild type cells. Both total protein synthesis as measured by [³⁵S]methionine incorporation and polyribosomal profile analysis showed identical stress-induced changes in wild type cells and eEF1Bγ-deficient cells, which exhibited an increase in the 80 S monoribosome peak and a reduction in total protein synthesis.

If a defect exists in the turnover of oxidized proteins, it would be expected that during oxidative stress conditions, a greater amount of oxidized proteins would accumulate in response to an oxidative stressor. This is the case for the eEF1Bγ-deficient strain, which accumulated a greater amount of protein carbonylation when compared with the wild type.
strain exposed to the same sublethal level of oxidative stress. Thus, the stress is affecting the cellular components as determined by this method; however, the lack of turnover pathways leads to their accumulation.

It is has been previously shown that both vacuolar protein turnover and proteasomal protein turnover play a role in oxidative stress, often by degrading proteins damaged by oxidation (1, 2). Because strains lacking eEF1Bγ appeared to show altered protein turnover in response to oxidative stress, defects in vacuolar protease function were assessed. Lyssates from strains lacking the vacuolar proteinase A or B showed protein levels in response to stress that were similar to strains lacking eEF1Bγ when visualized by SDS-PAGE. In vacuolar protease mutants, as in eEF1Bγ-deficient cells, treatment with CdSO₄ did not result in the expected reduction of protein levels. This suggests that strains lacking eEF1Bγ have a defect in the vacuolar functions that are required for turnover in response to oxidative damage. This was further supported by electron microscopy and FM 4-64 labeling of vacuoles. There is a lack of a visible vacuole in most Δtef3 Δtef4 cells at stationary phase in rich medium, a condition that leads to the formation of a single large vacuole in wild type strains. The Δtef3 Δtef4 strain exhibited a mixed population of cells with many cells showing characteristics of class C vps mutants, lack of a clearly defined vacuole in the EM staining and a diffuse staining pattern with FM 4-64. The remaining Δtef3 Δtef4 cells, which did exhibit visible vacuoles, exhibited vacuolar fragmentation characteristic of a class B vps mutant.

Because many vacuolar proteins must pass through the ER and Golgi apparatus for proper maturation and defects in the transport between the membrane systems have been linked to vacuolar defects, the integrity of the transport through these systems was evaluated in a strain lacking eEF1Bγ. The Δtef3 Δtef4 strain showed sensitivity to tunicamycin, suggesting a defect in the ER-associated degradation pathway, a process that requires proteasomal and vacuolar turnover as well as ER-Golgi transport (5, 30, 31). Strains lacking eEF1Bγ also had a defect in the Golgi-dependent glycosylation of CPY, further supporting the link between eEF1Bγ and protein trafficking. The maturation of CPY did not require the catalytic eEF1Ba subunit of the eEF1B complex, suggesting that the trafficking function of eEF1Bγ is not related to the canonical guanine nucleotide exchange factor function of this complex in protein synthesis.

It was also found that eEF1Bγ immuno precipitates with Sec27p, an essential subunit of the COP1 complex required for retrograde vesicular transport between the Golgi apparatus and ER, which when defective can alter CPY maturation (27). This provided more evidence that eEF1Bγ plays a role in transport of proteins between the Golgi apparatus and ER. These findings also fit well with the previous identification of eEF1Bγ as a high copy suppressor of cold-sensitive mutations of DR52, a gene shown to play a role in vesicle budding from the Golgi (12, 13), as well as its identification as a calcium-dependent membrane-binding protein (11).

These data support the model that eEF1Bγ plays a non-canonical role in protein metabolism. One explanation that links these findings is that eEF1Bγ binds to COP1 vesicles for retrograde transport between the Golgi apparatus and the ER. In the absence of eEF1Bγ, the block in retrograde transport causes an uneven distribution of SNAREs and perhaps some HSPs, which affects the anterograde transport of either vacuolar proteins such as CPY or the Golgi enzymes, which glycosylate CPY. The defects in maturation of these vacuolar proteins result in the lack of a visible vacuole and a decrease in vacuolar activity in response to oxidative stress-induced protein damage, both of which were observed in the Δtef3 Δtef4 strain. This buildup of damaged proteins then leads to an increase or altered modifications of molecular chaperones also seen in the Δtef3 Δtef4 strain. These functions of the eEF1Bγ subunit are independent of its interaction with the catalytic eEF1Ba subunit as strains lacking eEF1Ba displayed normal CPY maturation. Taken together, these results strongly suggest a novel and unique role for the eEF1Bγ subunit of a guanine nucleotide exchange factor complex.

Acknowledgments—We thank the members of the Kinzy laboratory, Drs. Paul Copeland and Barth Grant for helpful comments, and Dr. Estela Jacinto for plasmids.

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