Inhibition of Protein Synthesis by TOR Inactivation Revealed a Conserved Regulatory Mechanism of the BiP Chaperone in *Chlamydomonas*[1][W]

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The target of rapamycin (TOR) kinase integrates nutritional and stress signals to coordinately control cell growth in all eukaryotes. TOR associates with highly conserved proteins to constitute two distinct signaling complexes termed TORC1 and TORC2. Inactivation of TORC1 by rapamycin negatively regulates protein synthesis in most eukaryotes. Here, we report that down-regulation of TOR signaling by rapamycin in the model green alga *Chlamydomonas reinhardtii* resulted in pronounced phosphorylation of the endoplasmic reticulum chaperone BiP. Our results indicated that *Chlamydomonas* TOR regulates BiP phosphorylation through the control of protein synthesis, since rapamycin and cycloheximide have similar effects on BiP modification and protein synthesis inhibition. Modification of BiP by phosphorylation was suppressed under conditions that require the chaperone activity of BiP, such as heat shock stress or tunicamycin treatment, which inhibits *N*-linked glycosylation of nascent proteins in the endoplasmic reticulum. A phosphopeptide localized in the substrate-binding domain of BiP was identified in *Chlamydomonas* cells treated with rapamycin. This peptide contains a highly conserved threonine residue that might regulate BiP function, as demonstrated by yeast functional assays. Thus, our study has revealed a regulatory mechanism of BiP in *Chlamydomonas* by phosphorylation/dephosphorylation events and assigns a role to the TOR pathway in the control of BiP modification.

The abundance and quality of nutrients are key factors that determine cell growth (increase in cell mass) in all living organisms. In eukaryotes, the availability of nutrients as well as other environmental cues is transmitted to the cell growth machinery through a number of important signaling proteins, including the target of rapamycin (TOR) kinase (for review, see Crespo and Hall, 2002; De Virgilio and Loewith, 2006; Wullschleger et al., 2006; Ma and Blenis, 2009; Sengupta et al., 2010). TOR is a Ser/Thr kinase that associates with other proteins to form two distinct multiprotein complexes, termed TORC1 and TORC2, which modulate two major signaling branches in eukaryotic cells (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Wedaman et al., 2003; Jacinto et al., 2004). TOR and other core components of these two complexes are evolutionarily conserved from lower to higher eukaryotes, indicating that these proteins must play central roles in nutrient signaling (Wullschleger et al., 2006).

The TOR pathway stimulates cell growth by promoting anabolic processes, including translation and ribosome biogenesis, and inhibiting catabolic processes such as autophagy (Crespo and Hall, 2002). Control of protein synthesis is one of the best-characterized processes downstream of the TOR pathway. In yeast and mammals, TORC1 controls protein synthesis at different levels by positively regulating translation initiation and ribosome biogenesis (De Virgilio and Loewith, 2006; Ma and Blenis, 2009). The functions of a number of translation factors, including eIF4E, eIF4G, and eIF2, are regulated by TORC1 signaling (Beretta et al., 1996; Redpath et al., 1996; Raught et al., 2000), whereas key proteins in promoting translation, such as the mammalian S6K or yeast SCH9 protein kinase A/protein kinase G/protein kinase C family kinases, are direct substrates of TORC1 (Pearson et al., 1995; Urban et al., 2007). Under favorable growth conditions, yeast TORC1 phosphorylates several Ser and Thr residues in the C terminus of SCH9, and phosphorylated SCH9 participates in the activation of all three RNA polymerases (Urban et al., 2007). In mammalian cells, phosphorylated S6K promotes phosphorylation of the ribosomal protein S6, which in turn results in the up-regulation of translation initiation (Chung et al., 1992). In addition to activating protein synthesis, TORC1 inhibits autophagy when nutrients

[1] This study was supported by the Spanish Ministry of Science and Innovation and Fondo Social Europeo (grant nos. BFU2009–07368 to J.L.C. and BFU2010–15708 to F.J.F.).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: José L. Crespo (crespo@ibvf.csic.es).

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.179861

are available (Noda and Ohsumi, 1998). Autophagy is a membrane-trafficking process by which starved cells degrade and recycle cytosolic proteins and organelles. During this catabolic process, cytosolic components are nonselectively enclosed within a double-membrane structure (autophagosome) and delivered to the vacuole/lysosome for degradation (Xie and Klionsky, 2007; Nakatogawa et al., 2009). TORC1 has been identified as an essential component in transmitting nutrient starvation signals to the autophagic machinery in yeast and higher eukaryotes. TORC1 negatively controls autophagy via inhibition of the evolutionarily conserved protein kinase ATG1 that mediates an early activation step in the autophagic process (for review, see Díaz-Troya et al., 2008b).

The TOR kinase has been described in plants and algae, indicating that this signaling pathway is also conserved in photosynthetic organisms (Menand et al., 2002; Crespo et al., 2005). In both systems, TOR has been involved in cell growth control, since disruption of the AtTOR gene in Arabidopsis (*Arabidopsis thaliana*) is lethal (Menand et al., 2002) and rapamycin-mediated inactivation of CrTOR in *Chlamydomonas reinhardtii* results in strong inhibition of cell growth (Crespo et al., 2005). Components of TORC1 signaling are conserved in Arabidopsis and *Chlamydomonas*. AtRaptor1 and AtRaptor2 have been described as homologs of KOG1/raptor in Arabidopsis (Anderson et al., 2005; Deprost et al., 2005), and direct interaction of AtRaptor1 with AtTOR has been demonstrated, indicating that TORC1 might be conserved in plants (Mahfouz et al., 2006). Besides KOG1/raptor and TOR proteins, two putative LST8 homologs can be identified in the Arabidopsis genome (Moreau et al., 2010; Dobrenel et al., 2011). In *Chlamydomonas*, it has been demonstrated that CrTOR localizes in high-molecular-mass complexes that include a highly conserved LST8 homolog (Díaz-Troya et al., 2008a). These findings indicate that TORC1 might be functionally and structurally conserved in photosynthetic eukaryotes. However, no obvious homologs exist for TORC2-specific partners in plants or algae (Moreau et al., 2010; Pérez-Pérez and Crespo, 2010b; Dobrenel et al., 2011).

Dissection of the TOR pathway in plants has been hampered by the natural resistance of these organisms to rapamycin, likely due to the inability of plant FKBP12 to bind to this drug (Xu et al., 1998). Nevertheless, the generation of inducible RNA interference lines that allow conditional silencing of the AtTOR gene (Deprost et al., 2007) or rapamycin-sensitive plants (Sormani et al., 2007; Leiber et al., 2010) has importantly contributed to our understanding of how TOR controls plant cell growth. The finding that silencing of AtTOR expression arrests plant growth and reduces mRNA translation (Deprost et al., 2007) strongly suggests that control of protein synthesis by TOR is conserved in plants. Unlike plants, *Chlamydomonas* is sensitive to rapamycin, and inactivation of TOR signaling by this drug leads to cell growth inhibition (Crespo et al., 2005) and autophagy activation (Pérez-Pérez and Crespo, 2010a; Pérez-Pérez et al., 2010), demonstrating that TOR controls this degradative process in photosynthetic organisms. Recently, it has also been shown that TOR inhibits autophagy in plants (Liu and Bassham, 2010). As in other systems, the identification of components of the TOR pathway in plants and algae will be essential to understanding how TOR controls cell growth in these organisms.

In this study, we show that the endoplasmic reticulum (ER) chaperone BiP becomes phosphorylated in *Chlamydomonas* cells treated with rapamycin. BiP phosphorylation also occurred in response to the inhibition of protein synthesis, whereas ER stress induced by tunicamycin or heat shock resulted in the complete dephosphorylation of the protein. Our findings indicate that a rapamycin-sensitive TOR pathway controls the phosphorylation state of BiP in *Chlamydomonas* and reveals a conserved regulatory mechanism of this protein by phosphorylation/dephosphorylation events.

RESULTS

Rapamycin Treatment Induces Thr Phosphorylation of a 70-kD Protein

In order to identify proteins regulated by TOR in *Chlamydomonas* and whose phosphorylation state is under the control of this signaling pathway, we performed western-blot analysis of total extracts from *Chlamydomonas* cells treated with rapamycin using anti-phospho-Ser (anti-P-Ser) and anti-phospho-Thr (anti-P-Thr) antibodies. Whereas no significant difference was observed in extracts from untreated cells compared with rapamycin-treated cells with the anti-P-Ser antibody, levels of Thr phosphorylation of a 70-kD protein were increased, as detected with an anti-P-Thr antibody (Fig. 1A). In order to resolve the 70-kD molecular mass range, we conducted two-dimensional (2D) gel electrophoresis analysis of *Chlamydomonas* cells treated with rapamycin. Total extracts obtained from rapamycin-treated or untreated cells were subjected to isoelectric focusing followed by SDS-PAGE and examined by Coomassie blue staining and western blotting with the anti-P-Thr antibody (Fig. 1B). The comparative analysis of the different 2D gels obtained allowed us to identify a few spots in the 70-kD region that became phosphorylated (Fig. 1, B and C). Three spots whose mobility was altered by rapamycin treatment were detected in untreated cells by Coomassie blue staining (Fig. 1C). Accordingly, the signal obtained in this region with the anti-P-Thr antibody was more intense in rapamycin-treated cells, indicating the presence of phosphorylated proteins in at least some of these spots (Fig. 1B). Proteins present in the selected spots from control and treated samples were identified by mass spectrometry (MS). Most of the spots included a mixture of three proteins that belong to the same category, namely HSP70B and the BiP1/BiP2 chaperones (Supplemental Table S1). HSP70B and the two

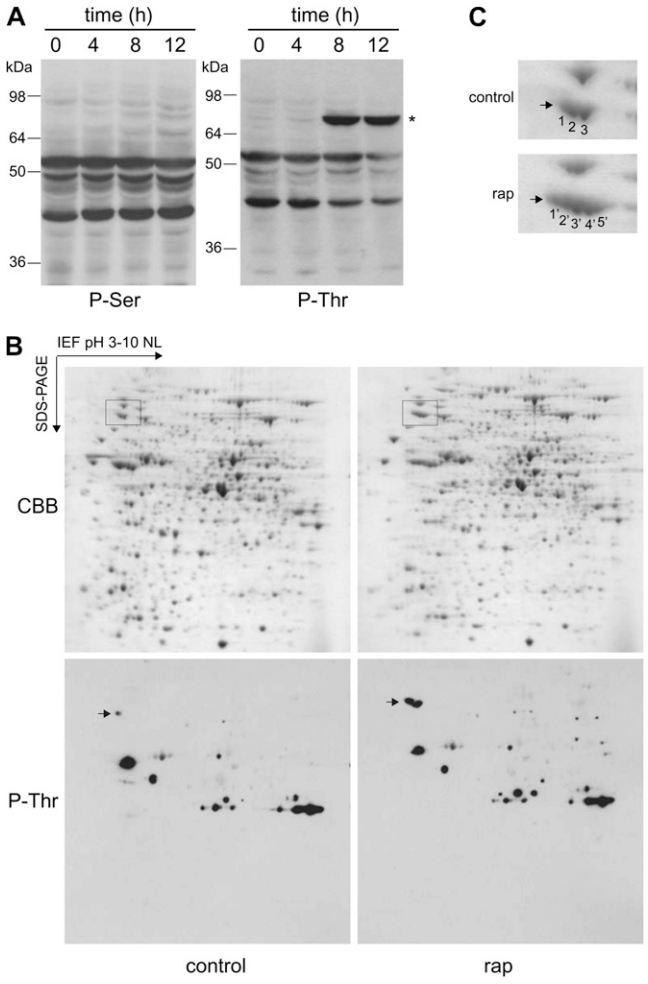

Figure 1. Rapamycin treatment induces alterations in the Thr phosphorylation pattern in *Chlamydomonas* soluble extract. A, Western-blot analysis of *Chlamydomonas* soluble extracts with phospho-Ser (P-Ser) and phospho-Thr (P-Thr) antibodies. Log-phase wild-type cells were treated with 500 nM rapamycin or drug vehicle, and samples were taken at the indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western blot. The asterisk denotes the 70-kD band detected with the P-Thr antibody. B, Soluble extracts of *Chlamydomonas* cells treated (rap) or not (control) with 500 nM rapamycin for 8 h were resolved by 2D electrophoresis as described in "Materials and Methods." Gels were stained with Coomassie Brilliant Blue (CBB) or processed for immunodetection with the phospho-Thr (P-Thr) antibody. C, Enlarged images of box sections on 2D gels shown in B. The arrow indicates spots whose mobility differs in untreated and rapamycin-treated cells. Numbers refer to spots where proteins have been identified by MS (for details, see Supplemental Table S1).

isoforms of the BiP chaperone are members of the HSP70 superfamily (Schroda, 2004) and have a molecular mass of about 72 kD and similar isoelectric points, which explains their comigration in the same spots. However, these proteins are localized in different compartments of the cell, since HSP70B resides in the chloroplast while BiP1/2 proteins are predicted to be ER proteins (Schroda, 2004). BiP1 and BiP2 share 90% identity at the amino acid level, and both proteins were present from spots 1′ to 5′ from rapamycin-treated cells (Fig. 1C; Supplemental Table S1). Given the high identity of BiP1 and BiP2, these proteins could not be differentially identified by available antibodies; therefore, we will refer to them as BiP chaperones. In addition to the spots containing BiP and HSP70B proteins, other spots with reduced intensity were differentially detected in control and rapamycin-treated samples with the anti-P-Thr antibody (Fig. 1B). Some new spots were found in response to rapamycin treatment, indicating that other proteins became phosphorylated upon TOR inactivation. However, the identities of these proteins could not be verified.

Identification of BiP Chaperones as the Phosphorylated Proteins upon TOR Inhibition

The former experiments indicated that HSP70B and/or BiP might be modified by Thr phosphorylation in response to rapamycin-mediated inhibition of TOR signaling. To investigate whether this posttranslational modification is due to an increase of newly synthesized protein or an induction of protein phosphorylation, HSP70B and BiP levels were analyzed by western blot. No significant accumulation of HSP70B or BiP chaperones was found after 8 h of rapamycin addition to *Chlamydomonas* cultures, indicating that the signal detected by the anti-P-Thr antibody is due to an increase in protein phosphorylation (Fig. 2A). Furthermore, phosphorylation of HSP70B and/or BiP was not a rapid event, since it occurred after 4 to 6 h of rapamycin addition (Fig. 2A). To demonstrate that this phosphorylation is specific to the inhibition of TOR signaling by rapamycin, we performed a similar experiment in the rapamycin-resistant mutant *rap2*, which lacks the rapamycin primary target FKBP12 (Crespo et al., 2005). As expected, no phosphorylation induction was found in this mutant in response to rapamycin (Fig. 2A).

Next, we investigated if the signal detected with the anti-P-Thr antibody is caused by the modification of HSP70B and BiP chaperones or only by one of these proteins. To this aim, we performed 2D gel electrophoresis followed by western-blot analysis using antibodies specific to HSP70B or BiP. Samples were separated in the first dimension by isoelectric focusing on pH 4 to 7 strips in order to obtain a better resolution of HSP70B and BiP spots. The signal of the anti-P-Thr antibody was strongly induced in certain spots of rapamycin-treated cells (Fig. 2B), indicating the modification of the protein(s) localized in these spots. The mobility of the HSP70B chaperone was not altered in response to rapamycin, which strongly suggests that this protein is not responsible for the increased signal obtained with the anti-P-Thr antibody (Fig. 2B). We cannot exclude, however, the possibility that HSP70B is phosphorylated, although this unlikely phosphorylation should have no obvious effect on the migration of the protein. In contrast to HSP70B, the mobility of BiP was clearly influenced by rapamycin, since new

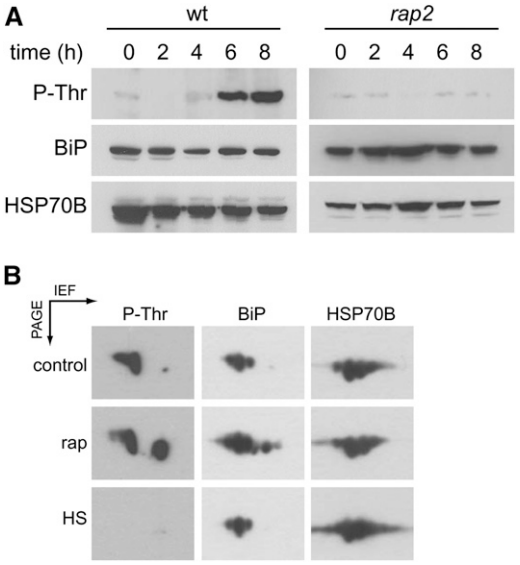

Figure 2. Rapamycin induces Thr phosphorylation of the BiP chaperone in its peptide-binding domain. A, Phosphorylation time course of the 70-kD protein following rapamycin treatment. Rapamycin (500 nM) or drug vehicle was added to wild-type (wt) or rapamycin-insensitive (*rap2*) *Chlamydomonas* cells, and samples were collected at the indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western-blotting, probing with phospho-Thr (P-Thr), BiP, and HSP70B antibodies. B, 2D western-blot analysis of BiP and HSP70B. Wild-type *Chlamydomonas* cells were treated with 500 nM rapamycin for 12 h (rap), heat shocked at 40°C for 1 h (HS), or remained untreated (control). Soluble extracts were resolved by 2D electrophoresis as described in "Materials and Methods" using pH 4 to 7 strips, and gels were processed for immunodetection with phospho-Thr (P-Thr), BiP, and HSP70B antibodies. IEF, Isoelectric focusing.

spots were detected in the region where the phospho-Thr signal was induced (Fig. 2B). Therefore, our results indicated that treatment of *Chlamydomonas* cells with rapamycin results in the phosphorylation of BiP chaperones.

Inhibition of Protein Synthesis by Cycloheximide or Rapamycin Induces BiP Phosphorylation

It is well established that inactivation of TOR by rapamycin results in, among other effects, the inhibition of protein synthesis in most eukaryotes. Therefore, we investigated a possible link of protein synthesis inhibition and BiP phosphorylation by treating *Chlamydomonas* cells with cycloheximide. Western-blot analysis using the anti-P-Thr antibody revealed that cycloheximide induced BiP phosphorylation even more efficiently than rapamycin, since phosphorylation was detected within 1 h of treatment (Fig. 3A). Addition of cycloheximide and rapamycin simultaneously did not result in a more pronounced phosphorylation of BiP, suggesting that both drugs act on the same pathway.

These results indicate that BiP phosphorylation may be induced as a result of protein synthesis inhibition mediated by rapamycin or cycloheximide. To test this hypothesis, we investigated if rapamycin has a negative effect on translation, as described in other eukaryotes. *Chlamydomonas* cultures were treated with rapamycin, and protein synthesis was examined in vivo by labeling cells with [^{14}C]Arg. Rapamycin-mediated inhibition of TOR resulted in a 30% to 40% reduction in protein synthesis, demonstrating that indeed TOR promotes translation in *Chlamydomonas* (Fig. 3B). Similar experiments were conducted with cycloheximide instead of rapamycin, and about 90% of protein synthesis was inhibited (Fig. 3B). The specificity of rapamycin on translation inhibition was confirmed by the null effect of the drug in the *rap2* mutant (Fig. 3B). A time-course experiment revealed that cycloheximide and rapamycin have different kinetics in inhibiting protein synthesis, since cycloheximide quickly and efficiently reduced the incorporation of [^{14}C]Arg into proteins while rapamycin caused a progressive and less intense effect (Fig. 3C), in agreement with the faster phosphorylation of BiP detected with cycloheximide compared with rapamycin treatments (Fig. 3A). Thus, our results strongly suggest that BiP phosphorylation occurred primarily in response to a decrease in the rate of protein synthesis.

Dephosphorylation of BiP Correlates with Chaperone Activation

In general, chaperone activity of BiP is up-regulated under stress conditions that increase the level of misfolded proteins in the ER (Otero et al., 2010). Stress-induced activation of BiP has been correlated with in vivo dephosphorylation of the protein in mammalian cells (Hendershot et al., 1988; Freiden et al., 1992; Satoh et al., 1993). To investigate whether Thr phosphorylation of BiP detected in *Chlamydomonas* is related to a regulatory mechanism of this protein, *Chlamydomonas* cells were subjected to heat stress and the phosphorylation state of BiP was examined. The basal phosphorylation level of BiP was strongly reduced within 15 min of heat shock, being undetectable after 60 min (Fig. 4A). 2D gel electrophoresis analysis of heat-shocked cells further confirmed that this stress caused the complete dephosphorylation of all spots initially detected in untreated cells with the anti-P-Thr antibody (Fig. 2B). Dephosphorylation of BiP by heat shock was also determined in cells that have been previously treated with rapamycin to increase the phosphorylation level of BiP prior to heat stress. Heat shock also resulted in undetectable levels of BiP phosphorylation despite the elevated modification of the chaperone after rapamycin treatment (Fig. 4B). These results indicated that BiP is dephosphorylated upon heat shock, likely due to increased levels of unfolded polypeptides in the ER.

To strengthen this model, a specific stress in the ER was generated by treating cells with tunicamycin, which inhibits *N*-linked glycosylation of nascent proteins at this compartment, and the phosphorylation

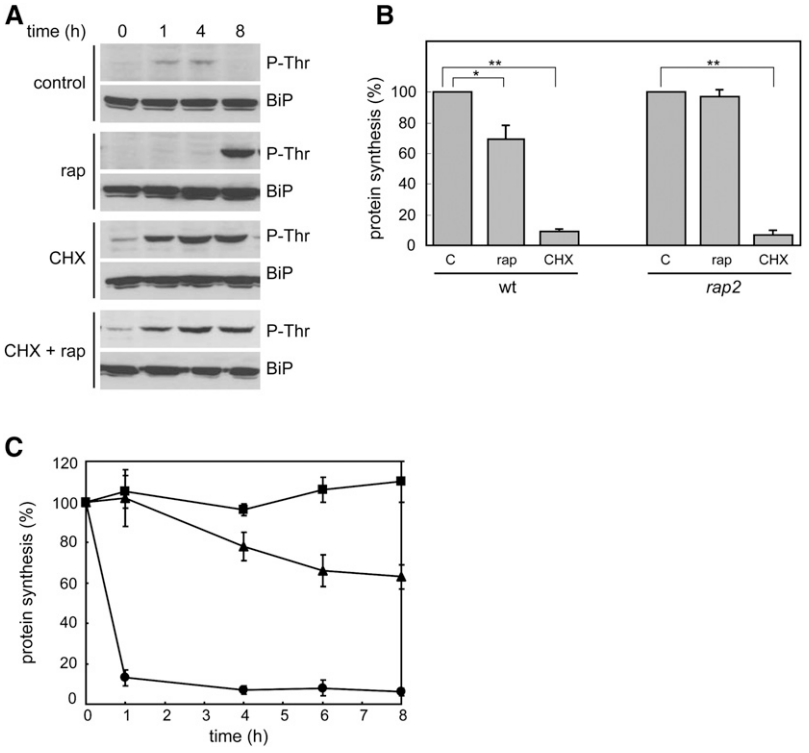

Figure 3. Inhibition of protein synthesis by rapamycin or cycloheximide induces BiP phosphorylation. A, Time course of BiP phosphorylation following rapamycin and/or cycloheximide treatment. Rapamycin (500 nM [rap]), 10 μg mL^{-1} cycloheximide (CHX), or both drugs simultaneously (CHX + rap) were added to wild-type *Chlamydomonas* cells, and samples were taken at the indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western blotting, probing with P-Thr or BiP antibodies. B, Incorporation of [^{14}C]Arg in *Chlamydomonas* cells. Wild-type (wt) and rapamycin-insensitive (*rap2*) *Chlamydomonas* cells were treated with 500 nM rapamycin for 6 h, 10 μg mL^{-1} cycloheximide for 30 min, or drug vehicle (C) and processed for the analysis of [^{14}C]Arg incorporation as described in "Materials and Methods." Values are represented in relation to the [^{14}C]Arg incorporation detected in control, untreated cells and correspond to six independent determinations (* $P < 0.05$, ** $P < 0.01$). C, Effect of cycloheximide and rapamycin on protein synthesis. *Chlamydomonas* cultures were treated with 500 nM rapamycin (triangles), 10 μg mL^{-1} cycloheximide (circles), or drug vehicle (squares), and samples were taken at different times for protein synthesis determination. Data correspond to results from three independent experiments made in duplicate.

state of BiP was examined by western blot. Compared with untreated cells, tunicamycin abolished the basal level of BiP phosphorylation (Fig. 4C, compare lanes 6 and 7 with lanes 2 and 3). In addition to the effect of tunicamycin on basal BiP phosphorylation, we investigated whether tunicamycin may induce the dephosphorylation of BiP under conditions where BiP is strongly phosphorylated. As described above, *Chlamydomonas* cells were first treated with rapamycin to induce BiP phosphorylation and then with tunicamycin without removing rapamycin from the medium. The phosphorylation state of BiP was elevated at the time of tunicamycin addition (Fig. 4C, compare lane 8 with lane 2). Tunicamycin reduced BiP phosphorylation to undetectable levels in rapamycin-treated cells (Fig. 4C, compare lane 9 with lane 5). Based on these results, tunicamycin treatment must activate BiP in the ER. Taken together, these results indicated that phosphorylation of BiP is strongly reduced under conditions that induce stress in the ER and consequently require the activity of this chaperone at this cellular compartment.

The Highly Conserved Thr-520 Plays a Role in BiP Function

To further characterize the modification of BiP, we tried to identify rapamycin-sensitive phosphorylation sites by MS. Total extracts from untreated or rapamycin-treated cells were resolved by 2D gel electrophoresis, and BiP proteins were excised from the gel, digested with the endoproteinase GluC, and analyzed by MS for phosphopeptide identification (see "Materials and Methods"). A phosphopeptide containing a single phosphate was identified in BiP samples from rapamycin-treated cells. This phosphopeptide was not detected in BiP samples prepared from untreated cells. The phosphopeptide is localized in a highly conserved region of the substrate-binding domain of BiP and extended from Glu-504 to Lys-525 of BiP1 (Fig. 5A). An alignment of BiP proteins from different organisms revealed that a Thr residue (Thr-520 in *Chlamydomonas* BiP1) from this phosphopeptide is widely conserved within eukaryotes (Fig. 5B). Interestingly, a modeling study of BiP1 indicated that Thr-520 might be in an exposed region of the chaperone accessible to other proteins (Fig. 5C), while the NetPhosK server (Blom et al., 2004) predicted Thr-520 as the residue with the highest probability of phosphorylation in BiP1. Our results, therefore, indicated that inhibition of TOR signaling by rapamycin leads to phosphorylation of BiP in a conserved region of the peptide-binding domain and strongly suggested that Thr-520 might play an important role in this modification.

To explore this hypothesis, we performed a functional study of *Chlamydomonas* BiP1 in yeast where conditional BiP mutants have been previously reported (Vogel et al., 1990). Although *KAR2* (yeast BiP) is an essential gene (Normington et al., 1989; Rose et al., 1989), temperature-sensitive mutants have been generated. The *kar2-159* mutation was isolated in a genetic screen of temperature-sensitive alleles of *KAR2* and allows growth at 23°C but not at 35°C to 37°C (Vogel et al., 1990). Wild-type and *kar2-159* mutant cells were

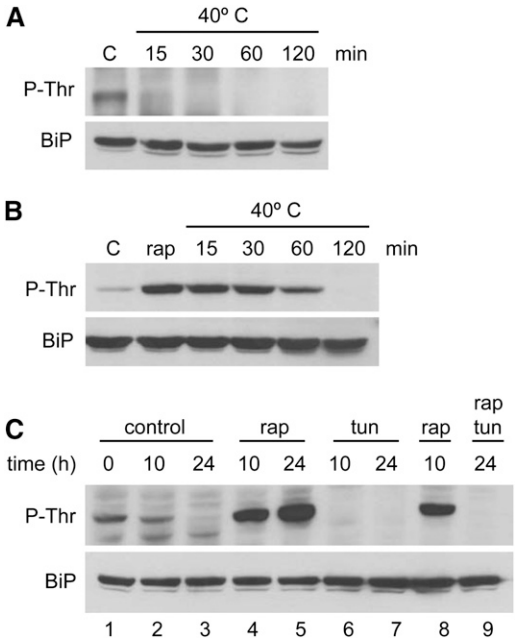

Figure 4. BiP dephosphorylation by ER stress-inducing agents. A and B, BiP dephosphorylation by heat stress. *Chlamydomonas* cells pretreated (B) or not (A) with 500 nM rapamycin (rap) for 12 h were subjected to heat stress at 40°C, and samples were taken at the indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western blotting, probing with P-Thr and BiP antibodies. C, BiP dephosphorylation by tunicamycin treatment. *Chlamydomonas* cells were treated with 500 nM rapamycin, 5 μg mL^{-1} tunicamycin (tun), or first treated with rapamycin (lane 8) and then with tunicamycin (lane 9). Samples were taken at the indicated times, and soluble extracts were processed and analyzed by western blotting.

transformed with a plasmid expressing the *Chlamydomonas* BiP1 cDNA from the strong, constitutive GPD promoter (for details, see "Materials and Methods"). Yeast complementation assays indicated that while *kar2-159* cells transformed with an empty vector were not able to grow at the restrictive temperature of 37°C, expression of *Chlamydomonas* BiP1 partially corrected the growth defect of *kar2-159* cells at 30°C but not at 37°C (Fig. 6A). Moreover, BiP1 was not able to functionally replace yeast KAR2 in a heterozygous *Δkar2* mutant strain (Supplemental Fig. S1). Western-blot analysis of these strains confirmed that BiP1 is expressed in *kar2-159* cells, although to a lower level than in *Chlamydomonas* cells (Fig. 6B). Compared with *Chlamydomonas* extracts, most of the BiP1 protein expressed in yeast was detected as a band of higher molecular mass (Fig. 6B). It is well established that KAR2 is itself translocated into the ER in yeast via a mechanism that depends on KAR2 function, and mature and precursor forms of the protein can be detected under certain circumstances (Rose et al., 1989; Vogel et al., 1990). Therefore, the lower mobility band of BiP1 might very likely correspond to the precursor form of the protein that cannot be properly translocated into the ER.

With the availability of a functional assay for *Chlamydomonas* BiP1, we next investigated whether the conserved Thr-520 of this protein is involved in BiP function. Thr-520 was replaced by Ala, Glu, or Arg, and the point mutants (T520A, T520E, and T520R, respectively) were expressed in yeast cells. Similar to wild-type BiP1, T520A and T520R proteins were able to promote the growth of *kar2-159* cells at 30°C (Fig. 6A). However, the T520E mutation, which mimics a constitutive phosphorylation of the residue, could not fully complement BiP function at 30°C, indicating that the T520E mutation decreased BiP function (Fig. 6A). None of the BiP1 mutants were able to promote growth at 37°C (Fig. 6A). Interestingly, all mutations in Thr-520 resulted in a pronounced increase in the abundance of the precursor and mature forms of this protein in yeast cells compared with wild-type BiP1 (Fig. 6B), which indicates that this residue is important for the stability and processing of BiP1 in yeast cells.

Cellular Relocalization of BiP upon ER Stress Induced by Tunicamycin

BiP has been localized in *Chlamydomonas* to discrete punctae all over the cell that are more abundant around the flagellar basal bodies (Díaz-Troya et al., 2008a; Fig. 7), a membrane-enriched region known as the peribasal body region where intraflagellar transport particle polypeptides concentrate (Cole et al., 1998). We wanted to investigate whether phosphorylation/dephosphorylation events of BiP might result in alterations in the cellular localization pattern of this protein. Compared with control cells, no difference was observed in cells where BiP phosphorylation was induced by rapamycin (Fig. 7). However, tunicamycin-mediated dephosphorylation of BiP resulted in a relocalization of this protein in the cell, being accumulated in a central region of the cell near the nucleus instead of the distal peribasal body region (Fig. 7). The cellular distribution of BiP in cells subjected to heat shock did not significantly differ from untreated cells (data not shown). These results demonstrate that dephosphorylation and activation of BiP by a specific stress in the ER correlates with a mobilization and concentration of this protein to this cellular compartment and may reflect the requirement of dephosphorylated BiP to overcome this stress.

DISCUSSION

We have previously reported that, unlike in plants, the growth of *Chlamydomonas* cells is sensitive to rapamycin, indicating that a rapamycin-sensitive TOR pathway operates in this unicellular green alga to govern cell growth (Crespo et al., 2005; Díaz-Troya et al., 2008a). In this study, we show that inactivation of TOR signaling by rapamycin caused an inhibition of protein synthesis in *Chlamydomonas* (Fig. 3B), which demonstrates that TOR promotes protein synthesis in this

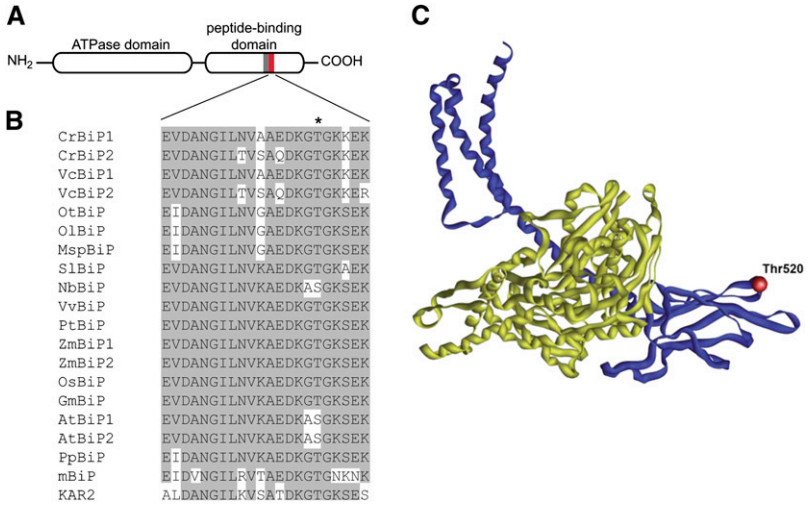

Figure 5. Identification of a phosphopeptide in *Chlamydomonas* BiP. A, Schematic representation of the BiP protein, and identification of a phosphopeptide in the peptide-binding domain. The scheme shows the ATPase and peptide-binding domains of BiP. The position of the phosphopeptide identified in *Chlamydomonas* is indicated by the red box, whereas the gray box shows the position of the phosphopeptide identified in mammalian BiP (Gaut, 1997), which spans from Thr-455 to Asp-506 of *Chlamydomonas* BiP. B, Amino acid sequence alignment of the phosphopeptide identified in *Chlamydomonas* BiP (from Glu-504 to Lys-525) with the corresponding region of BiP proteins from other organisms: Cr, *Chlamydomonas reinhardtii*; Vc, *Volvox carteri*; Ot, *Ostreococcus tauri*; Ol, *Ostreococcus lucimarinus*; Msp, *Micromonas* sp. RCC299; Sl, *Solanum lycopersicum*; Nb, *Nicotiana benthamiana*; Vv, *Vitis vinifera*; Pt, *Populus trichocarpa*; Zm, *Zea mays*; Os, *Oryza sativa*; Gm, *Glycine max*; At, *Arabidopsis thaliana*; Pt, *Physcomitrella patens*; mBiP, *Mus musculus*. C, Modeling of *Chlamydomonas* BiP1 based on the crystal structure of mammalian 70-kD heat shock protein (3c7nA). Conserved Thr-520 was mapped onto the crystal structure. Structure prediction was performed using the SWISS-MODEL bioinformatics tool, and the image was generated by iMol software. ATPase and peptide-binding domains are shown in yellow and blue, respectively.

photosynthetic organism. Control of protein synthesis by TOR has been widely characterized in yeast and mammalian cells. In these systems, inhibition of TORC1 signaling by rapamycin resulted in a decrease in protein synthesis (Barbet et al., 1996; Beretta et al., 1996; Loewith et al., 2002). In Arabidopsis, inducible RNA interference of the *AtTOR* gene or rapamycin treatment of plant lines expressing yeast FKBP12 caused a reduction in the abundance of high-M_r polysomes (Deprost et al., 2007; Sormani et al., 2007), indicating that TOR also controls protein translation in plants, likely through a conserved TORC1 signaling branch, since core components of this complex are conserved (Anderson et al., 2005; Deprost et al., 2005; Mahfouz et al., 2006).

Our analysis of rapamycin-treated cells identified the ER chaperone BiP as a phosphoprotein whose phosphorylation state is regulated by TOR through the control of protein synthesis in *Chlamydomonas*. Inhibition of TOR signaling by rapamycin strongly induced the phosphorylation of BiP on Thr residues (Figs. 1 and 2), and a phosphopeptide in a regulatory region of BiP that contains a highly conserved Thr (Thr-520) could be identified in rapamycin-treated cells (Fig. 5). Although our study identified a single phosphopeptide in this chaperone, we cannot rule out that other residues might be phosphorylated as well. Actually, our results indicate that rapamycin further induced a basal, low level of phosphorylation in BiP, suggesting the presence of additional phosphorylation sites (Figs. 1–4). Posttranslational modification of BiP, including phosphorylation and ADP-ribosylation, has been previously reported in mammalian cells (Carlsson and Lazarides, 1983; Welch et al., 1983; Hendershot et al., 1988; Freiden et al., 1992). Phosphoamino acid analysis of purified mammalian BiP demonstrated that this protein is predominantly phosphorylated on Thr residues, with only a trace of detectable phospho-Ser (Hendershot et al., 1988; Gaut, 1997). Moreover, in vivo Thr phosphorylation has been mapped to a region of the peptide-binding domain of mammalian BiP (Gaut, 1997) adjacent to the phosphopeptide identified in *Chlamydomonas* BiP (Fig. 5).

Modification of mammalian BiP by phosphorylation or ADP-ribosylation seems to be restricted to a pool of BiP that does not bind to substrate proteins (Hendershot et al., 1988; Freiden et al., 1992). Furthermore, it has been demonstrated that stresses that increase the need for active BiP in mammalian cells, such as Glc starvation or tunicamycin treatment, promote the dephosphorylation of BiP and its association with proteins (Hendershot et al., 1988; Satoh et al., 1993). These findings led to the theory that modified BiP represents an inactive form of the protein and that unmodified BiP is able to bind to proteins and promote their folding and assembly (Hendershot et al., 1988). It has also been proposed that posttranslational modification of BiP might provide a storage pool of BiP that

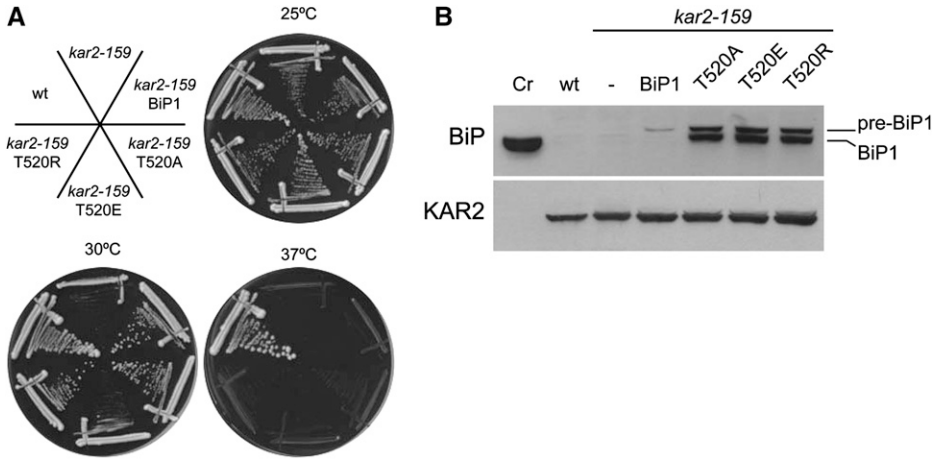

Figure 6. *Chlamydomonas* BiP1 partially complements KAR2 function in a *kar2-159* temperature-sensitive mutant. A, Wild-type MS10 (wt) and *kar2-159* strains transformed with empty plasmid or plasmids expressing *Chlamydomonas* BiP1, T520A, T520E, or T520R proteins were streaked for single colonies. Plates were incubated at the indicated temperatures for 3 to 4 d. B, Wild-type MS10 and a *kar2-159* strain expressing the indicated *Chlamydomonas* BiP1 proteins were grown at the permissive temperature of 25°C, and expression of BiP1 and endogenous KAR2 proteins in these cells was detected by western blotting. Precursor and mature forms of BiP1 are indicated. Cr, Soluble extract from *Chlamydomonas* cells grown in TAP medium. Fifty micrograms of total protein was loaded per lane.

can be recruited back to the active form in response to need (Gething, 1999). Our study here strongly suggests that regulation of BiP by phosphorylation is conserved in the photosynthetic alga *Chlamydomonas* and assigns a novel role to the TOR pathway in the control of BiP phosphorylation. Similar to rapamycin, treatment of *Chlamydomonas* cells with cycloheximide resulted in increased phosphorylation of BiP (Fig. 3). Both drugs have a negative effect on protein synthesis, although with different efficiencies, since cycloheximide is more effective than rapamycin in translation inhibition (Fig. 3). Interestingly, BiP phosphorylation occurred faster in response to cycloheximide, since phosphorylated BiP was detected within 1 h in cycloheximide-treated cells, whereas rapamycin induced BiP phosphorylation after at least 4 h (Fig. 3). Thus, our results establish a correlation between phosphorylation of BiP and protein synthesis through the TOR pathway in *Chlamydomonas*.

Supporting the model that unmodified BiP is active (Hendershot et al., 1988), we found in *Chlamydomonas* that stress conditions that require the chaperone activity of BiP, such as heat shock or inhibition of protein glycosylation in the ER by tunicamycin, triggered the fast dephosphorylation of BiP (Fig. 4). Moreover, complete BiP dephosphorylation in response to heat shock or tunicamycin occurred even in cells where BiP phosphorylation was previously induced by inhibition of TOR signaling, emphasizing the linkage between dephosphorylation and activation of BiP. Our results also demonstrated that the cellular localization of BiP is regulated by the phosphorylation state of the protein. Tunicamycin-induced dephosphorylation and activation of BiP clearly modified the cellular distribution of this chaperone in the cell, as it accumulated in a central region of the cell near the nucleus where ER and Golgi compartments likely localize (Fig. 7). Our findings in *Chlamydomonas* indicated that modification of BiP by phosphorylation/dephosphorylation events may play a regulatory role in the function of this chaperone and that this molecular mechanism of regulation proposed in mammals (Hendershot et al., 1988; Freiden et al., 1992; Satoh et al., 1993) is conserved in photosynthetic eukaryotes. In vivo phosphorylation of BiP has been described in maize (*Zea mays*; Fontes et al.,

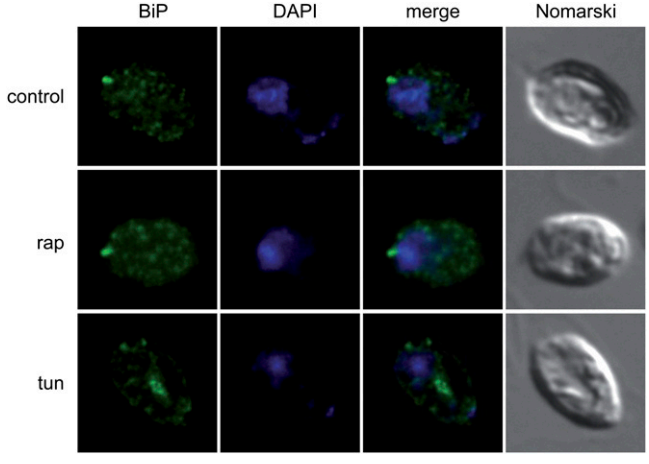

Figure 7. Relocalization of BiP in tunicamycin-treated cells. Wild-type *Chlamydomonas* cells growing exponentially in acetate medium were treated with 500 nM rapamycin (rap) or 5 μg mL^{-1} tunicamycin (tun) for 8 h. Cells were collected and processed for immunofluorescence microscopy analysis. The signal recognized by the BiP antibody is shown in green. DNA staining is shown in blue (4′,6-diamino-phenylindole [DAPI]).

1991) and spinach (*Spinacia oleracea*; Anderson et al., 1994), although to our knowledge a link between the phosphorylation state of the protein and its activity has not been shown in plants.

Yeast complementation assays performed with *Chlamydomonas* BiP1 indicated that this protein is able to replace only partially endogenous KAR2 function (Fig. 6), despite the high identity between both proteins. Functional complementation of *kar2* mutants has been achieved with mammalian and plant BiP proteins (Normington et al., 1989; Denecke et al., 1991). However, neither cytosolic nor mitochondrial yeast HSP70s are able to substitute functionally for KAR2 (Brodsky et al., 1993, 1998), likely due to the inability of these proteins to productively interact with proteins such as cochaperones or components of the ER translocation machinery. The partial complementation of *kar2-159* mutant cells by *Chlamydomonas* BiP1 might be due to an ineffective interaction of this protein to substrates or specific partners. Accordingly, most *Chlamydomonas* BiP1 was detected in yeast extracts as a precursor form, indicating that, unlike endogenous KAR2 (Rose et al., 1989; Vogel et al., 1990), this protein is not efficiently translocated into the ER lumen. Mutation of the conserved Thr-520 in BiP1 led to a reduction in protein function (Fig. 6A). These results suggest that this residue plays a role in the control of BiP1 activity in yeast cells and are in agreement with the finding that Thr phosphorylation inhibits BiP activity in mammals (Hendershot et al., 1988; Satoh et al., 1993; Gaut, 1997). Given that phosphorylation maps to the peptide-binding domain, this modification might interfere with substrate binding and/or interaction with partners such as cochaperones, as proposed for mammalian BiP (Gaut, 1997). The finding that Thr-520 mutation in *Chlamydomonas* BiP1 stimulated the maturation of this protein in yeast cells indicated that this residue is indeed important for the regulation of BiP1.

Biochemical fractionation and indirect immunofluorescence microscopy studies revealed that TOR complexes in *Chlamydomonas* might associate, at least in part, with membranes from the ER (Díaz-Troya et al., 2008a). Actually, the cellular distribution of *Chlamydomonas* TOR and BiP proteins was coincident in some respects. Both proteins localize to discrete bodies and accumulate in the peribasal body region (Díaz-Troya et al., 2008a; Fig. 7). The common distribution of TOR and ER proteins may reflect functional interactions between the TOR pathway and this cellular compartment in *Chlamydomonas*. Several lines of evidence have recently associated TOR signaling and ER stress in mammals. Loss of the tuberous sclerosis tumor suppressor complex, an upstream negative regulator of mTORC1 (Garami et al., 2003; Saucedo et al., 2003; Tee et al., 2003; Zhang et al., 2003), causes increased translation in certain mouse or human tumors due to the up-regulation of mTORC1 signaling, which in turn induces ER stress (Ozcan et al., 2008). This finding also indicates that mTORC1 genetically functions upstream of ER stress (Ozcan et al., 2008). Interestingly, it has been reported that the accumulation of misfolded proteins in mice induces mTORC1 signaling, which is directly regulated by chaperone availability (Qian et al., 2010). These studies have revealed the important role that control of protein synthesis by TORC1 plays in the regulation of ER homeostasis. Components of TORC1 signaling controlling protein synthesis, such as raptor or the S6 kinase, are conserved in plants and seem to participate in the general response of the plant to osmotic stress signals (Mahfouz et al., 2006; Deprost et al., 2007). However, it remains to be explored if, as in *Chlamydomonas*, TOR may function in ER stress through the control of protein synthesis.

In conclusion, our studies show that the ER chaperone BiP can be regulated by reversible phosphorylation in *Chlamydomonas* and connect TOR signaling to this process. A better understanding of BiP regulation by phosphorylation/dephosphorylation events will come with the identification of kinases and phosphatases involved in the posttranslational modification of this chaperone and their possible control by TOR and/or other signaling pathways.

MATERIALS AND METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii wild-type strain 6C+ was obtained from the laboratory of J.-D. Rochaix (University of Geneva), and the rapamycin-insensitive *rap2* strain has been described previously (Crespo et al., 2005). Cells were grown as described by Harris (1989) under continuous illumination at 25°C. If required, Tris-acetate-phosphate (TAP) medium was solidified with 1.2% Bacto agar (Difco). When specified, cells were treated with 500 nM rapamycin (LC Laboratories) from a 4 mM stock in 90% ethanol and 10% Tween 20, 5 μg mL^{-1} tunicamycin (Calbiochem) from a 5 mg mL^{-1} stock in dimethylformamide, or 10 μg mL^{-1} cycloheximide (Sigma) from a 3 mg mL^{-1} stock in water. *Saccharomyces cerevisiae* wild type (MS10) and *kar2-159* were obtained from the laboratory of M.D. Rose. YJL034W (*KAR2*) heterozygous mutant was obtained from the EUROSCARF mutant collection. Yeast cells were grown in rich or synthetic medium as described previously (Sherman, 1991).

Protein Preparation

Chlamydomonas cells from liquid cultures were collected by centrifugation (3,000*g*, 5 min), washed once in lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM NaF, 10 mM NaN_3, 10 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate, and 10 mM β-glycerophosphate), and resuspended in a minimal volume of the same solution supplemented with Protease Inhibitor Cocktail (Sigma). Cells were lysed by two cycles of slow freezing to −80°C followed by thawing to room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15,700*g*, 15 min) at 4°C and stored at −80°C until they were analyzed. Yeast extracts from logarithmically growing cells were prepared in lysis buffer (phosphate-buffered saline buffer, 1 mM phenylmethylsulfonyl fluoride, 0.5% Tween 20, 10 mM NaF, 10 mM NaN_3, 10 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate, and 10 mM β-glycerophosphate) by vortexing them 10 times for 1 min each time with glass beads (Sigma-Aldrich). Crude extracts were cleared by centrifugation at 15,000*g* for 15 min at 4°C. Proteins were quantitated with the Coomassie dye-binding method (Bio-Rad).

2D Gel Electrophoresis

For 2D electrophoresis, proteins from soluble cell extract samples were precipitated with the 2D Clean-Up Kit (GE Healthcare), resuspended in rehydration solution, and quantitated with RC DC Protein Assay (Bio-Rad) according to the manufacturers' instructions. Isoelectric focusing for the first dimension was carried out by rehydrating 11-cm-long pH 3-10NL or pH 4-7

Immobiline DryStrips (GE Healthcare) with 200 μL of DeStreak Rehydration Solution (GE Healthcare) supplemented with 0.5% Bio-Lyte 3/10 Ampholytes (Bio-Rad) containing 150 μg of protein. Following overnight active rehydration (50 V, 20°C), isoelectric focusing was performed for 15 kVh (for pH 3-10NL strips) or 20 kVh (for pH 4-7 Immobiline DryStrips) at 20°C using the Protean IEF Cell (Bio-Rad). After the first-dimensional separation, strips were blotted to remove mineral oil and stored at −80°C. Prior to the second-dimensional separation (SDS-PAGE), thawed strips were first equilibrated with 10 mg mL^{-1} dithiothreitol in equilibration buffer (6 M urea, 75 mM Tris-HCl [pH 8.8], 29.3% glycerol, 2% SDS, and 0.002% bromphenol blue) and then with 25 mg mL^{-1} iodoacetamide in equilibration buffer. A RE 600 Ruby chamber system (GE Healthcare) was used for the second-dimensional separation. Equilibrated strips were placed on 10% SDS-PAGE gels and sealed with 0.5% agarose. Proteins were separated by M_r using a 70-mA constant intensity. Gels were Coomassie blue stained or processed for western blotting.

Western Blotting

Proteins resolved by SDS-PAGE or 2D gel electrophoresis were transferred to a polyvinylidene difluoride membrane (for phospho-Thr detection) or a nitrocellulose membrane (for BiP, HSP70B, and FKBP12 detection) for antibody binding. Immunoblots were visualized using horseradish peroxidase-conjugated secondary antibodies (Sigma) and the ECL Plus chemiluminescent system (GE Healthcare). The *Chlamydomonas* FKBP12 polyclonal antibody has been described previously (Crespo et al., 2005). The Arabidopsis (*Arabidopsis thaliana*) BiP, *Chlamydomonas* HSP70-B, and phospho-Thr polyclonal antibodies were purchased from Santa Cruz Biotechnology, Agrisera, and Zymed Laboratories, respectively. The KAR2 antibody was obtained from Santa Cruz Biotechnology.

Fluorescence Microscopy

Wild-type *Chlamydomonas* cells were fixed and stained for immunofluorescence microscopy as described previously (Pérez-Pérez et al., 2010). The primary antibody used was rabbit polyclonal anti-BiP (1:500; Santa Cruz Biotechnology). For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500; Sigma) was used. Preparations were photographed on a DM6000B microscope (Leica) with an ORCA-ER camera (Hamamatsu) and processed with the Application Suite Advanced Fluorescence software package (Leica). Deconvolution analysis of images was performed with the same software.

Protein Identification by Matrix-Assisted Laser-Desorption Ionization Time of Flight

Coomassie Brilliant Blue-stained proteins were excised from the gel using the EXQuest Spot Cutter excision robot (Bio-Rad) and in-gel trypsin digested using a Proteineer dp Digester (Bruker Daltonics). Gel plugs were incubated in acetonitrile (50 μL) and treated with 10 mM dithiothreitol in 50 mM ammonium bicarbonate followed by 55 mM iodoacetamide in 50 mM ammonium bicarbonate. Trypsin digestion was performed at 30°C for 4 h using 13 μg mL^{-1} trypsin in 50 mM ammonium bicarbonate. Digestion was stopped and peptides extracted using 0.5% trifluoroacetic acid. Tryptic digests were analyzed using an Autoflex matrix-assisted laser-desorption ionization time of flight instrument (Bruker Daltonics), and proteins were identified as the highest ranked result by searching the National Center for Biotechnology Information nonredundant database, version from February 2008, for Viridiplantae (Green Plants) sequences (from a total of 474,525 sequences) by the Protein Mass Fingerprint technique using the MASCOT search tool (Matrix Science; http://matrixscience.com). The search parameters included carbamidomethylation of Cys, oxidation of Met, one miscleavage by trypsin, and 50 ppm mass accuracy.

Identification of Phosphorylated Peptides by MS

To identify phosphorylated peptides, the gel slice containing BiP was reduced with 10 mM Tris(2-carboxyethyl)phosphine at 37°C for 1 h and alkylated with 50 mM iodoacetamide for 15 min at room temperature. In-gel digestion was with 250 ng of trypsin or 250 ng of endoproteinase GluC at 37°C overnight. The peptides were analyzed on an LTQ Orbitrap instrument (Thermo Fisher) coupled to an Agilent 1200 nano pump. The solvents used for peptide separation were 0.1% acetic acid in water (solvent A) and 0.1% acetic acid and 80% acetonitrile in water (solvent B). Peptides were injected via a 2-μL loop onto a C18 trap column (0.15 × 10 mm; SGE Analytical Science) with the capillary pump of an Agilent 1200 system set to 5 μL min^{-1}. After 15 min, the trap column was switched into the flow path of the C18 separating column (0.1 × 150 mm; Swiss Bioanalytics). A linear gradient from 2% to 35% solvent B in solvent A in 60 min was delivered with an Agilent 1200 nano pump at a flow rate of 300 nL min^{-1}. After 60 min, the percentage of solvent B was increased to 60% in 10 min and further increased to 80% within 2 min. The eluting peptides were ionized at 1.7 kV. The mass spectrometer was operated in a data-dependent fashion. The precursor scan was done in the Orbitrap set to 60,000 resolution, while the fragment ions were mass analyzed in the LTQ instrument. A top-five method was run so that the five most intense precursors were selected for fragmentation. The MS/MS spectra (Supplemental Fig. S2) of the identified peptides (Supplemental Table S2) were then searched against the National Center for Biotechnology Information nonredundant database, version from August 2008, using TurboSequest software (Gatlin et al., 2000). The databank was searched with Bioworks version 3.3.1. SP1 by setting the precursor ion tolerance to 10 ppm while the fragment ion tolerance was set to 0.5 D. Cleavage rules were set to fully enzymatic, cleaves at both ends, allowing two missed cleavages. The modifications were set to carbamidomethyl Cys as fixed modification, while phosphorylation on Ser/Thr, and Tyr was set to variable modifications. Post filtering was set to the following parameters: ΔCN, 0.1; Xcorr versus charge state was 1.50 (1+), 2.00 (2+), 2.50 (3+); peptide probability was 0.01; protein probability was 0.01.

Cloning of *Chlamydomonas* BiP1 and Plasmid Construction

The BiP1-coding region from *Chlamydomonas* (1,971 bp) was amplified by PCR from a *Chlamydomonas* cDNA library using the following primers, BiP1 *Spe*I 5′ (5′-CCGGACTAGTATGGCGCAGTGGAAGGCTGCTGTGCTG-3′) and BiP1 *Spe*I 3′ (5′-CCGGACTAGTTTAGAGCTCGTCGTGGTCGCCCAAGTC-3′), designed to contain a *Spe*I site (underlined). The resulting product was digested with *Spe*I and inserted into p425GPD (Mumberg et al., 1995) for expression in *S. cerevisiae*. The point mutations in Thr-520 of BiP1, denoted as T520A, T520E, and T520R, were obtained by overlapping PCR using the wild-type version of BiP1 and the following primers: T520A 5′ (5′-GCCGCTGAGGACAAGGGCGCCGGCAAGAAGGAGAAGATC-3′), T520A 3′ (5′-GATCTTCTCCTTCTTGCCGGCGCCCTTGTCCTCAGCGGC-3′), T520E 5′ (5′-GCCGCTGAGGACAAGGGCGAGGGCAAGAAGGAGAAGATC-3′), T520E 3′ (5′-GATCTTCTCCTTCTTGCCCTCGCCCTTGTCCTCAGCGGC-3′), T520R 5′ (5′-GCCGCTGAGGACAAGGGCCGCGGCAAGAAGGAGAAGATC-3′), and T520R 3′ (5′-GATCTTCTCCTTCTTGCCGCGGCCCTTGTCCTCAGCGGC-3′). The final PCR products were cloned into p425GPD for expression in yeast cells.

Labeling of Cells with [^{14}C]Arg

Cell labeling was performed essentially as described by Stavis and Hirschberg (1973) with some modifications. *Chlamydomonas* cells from liquid TAP cultures at a density of 10^6 cells mL^{-1} were harvested by centrifugation (3,000*g*, 5 min), washed with TAP-N medium, and cultured in the same medium for 6 h to improve [^{14}C]Arg incorporation. Next, cells were treated with 500 nM rapamycin for 6 h, 10 μg mL^{-1} cycloheximide for 30 min, or drug vehicle. Reactions mixtures containing 10^7 cells and 1 μCi of [^{14}C]Arg in 1 mL of medium were incubated at 25°C in the light with gentle shaking. Samples of 0.1 mL were collected and added to 0.5 mL of cold 10% TCA. The precipitated material was collected on Whatman glass fiber filters, washed four times with cold 5% TCA, and dried, and radioactivity was measured by a liquid scintillation counter.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *Chlamydomonas* BiP1 cannot fully replace yeast KAR2.

Supplemental Figure S2. Annotated spectra of phosphorylated peptides.

Supplemental Table S1. Proteins identified by matrix-assisted laser-desorption ionization time of flight.

Supplemental Table S2. Phosphopeptide identified in *Chlamydomonas* BiP.

ACKNOWLEDGMENTS

We thank Mark Rose for providing MS10 and *kar2-159* yeast strains, Robbie Loewith for critical comments on the manuscript, Jesús de la Cruz and Dieter Kessler for providing the yeast heterozygous *KAR2*::*kar2* mutant, and Néstor García and Ralf Wellinger for micromanipulation of yeast cells.

Received May 11, 2011; accepted August 1, 2011; published August 8, 2011.

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