Running head: TOR controls BiP phosphorylation in Chlamydomonas

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Title: Inhibition of protein synthesis by TOR inactivation revealed a conserved regulatory mechanism of the BiP chaperone in Chlamydomonas

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FOOTNOTES

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ABSTRACT

The TOR kinase integrates nutritional and stress signals to coordinately control cell growth in all eukaryotes. TOR associates to 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 downregulation 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.

INTRODUCTION

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 signalling proteins including the target of rapamycin (TOR) kinase (for reviews 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 to other proteins to form two distinct multiprotein complexes, termed TORC1 and TORC2, which modulate two major signalling 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 signalling (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 function of a number of translation factors, including eIF4E, eIF4G and eIF2 are regulated by TORC1 signalling (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 AGC (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 favourable growth conditions, yeast TORC1 phosphorylates several

serine and threonine residues in the carboxy 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 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 (reviewed in (Diaz-Troya et al., 2008)).

The TOR kinase has been described in plants and algae, indicating that this signalling 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 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 signalling are conserved in Arabidopsis and Chlamydomonas. AtRaptor1 and AtRaptor2 have been described as homologues 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 homologues can be identified in the Arabidopsis genome (Moreau, 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 homologue (Diaz-Troya et al., 2008). These findings indicate that TORC1 might be functionally and structurally conserved in photosynthetic eukaryotes. However, no obvious homologues exist for TORC2-specific partners in plants or algae (Moreau, 2010; Pérez-Pérez, 2010; 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 RNAi 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) have importantly contributed to understand 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 signalling by this drug leads to cell growth inhibition (Crespo et al., 2005) and autophagy activation (Perez-Perez and Crespo, 2010; Perez-Perez 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). Like in other systems, the identification of components of the TOR pathway in plants and algae will be essential to understand 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 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 threonine phosphorylation of a 70 kDa protein

In order to identify proteins regulated by TOR in Chlamydomonas and whose phosphorylation state is under the control of this signalling pathway, we performed western blot analysis of total extracts from Chlamydomonas cells treated with rapamycin using anti-phosphoserine (anti-P-Ser) and anti-phosphothreonine (anti-P-Thr) antibodies. Whereas no significant difference was observed in extracts from untreated cells compared to rapamycin-treated cells with the anti-P-Ser antibody, levels of Thr phosphorylation of a 70-kDa protein were increased, as detected with an anti-P-Thr antibody (Figure 1A). In order to resolve the 70 kDa 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 staining and western blot with the anti-P-Thr antibody (Figure 1B). The comparative analysis of the different 2D gels obtained allowed us to identify a few spots in the 70 kDa region that become phosphorylated (Figures 1B and 1C). Three spots whose mobility was altered by rapamycin treatment were detected in untreated cells by Coomassie staining (Figure 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 (Figure 1B). Proteins present in the selected spots from control and treated samples were identified by mass spectrometry. Most of the spots included a mixture of three proteins that belong to the same category, namely HSP70B and the BiP1/BiP2 chaperones (Suppl. Table 1). HSP70B and the two isoforms of the BiP chaperone are members of the HSP70 superfamily (Schroda, 2004) and have a molecular mass of about 72 kDa and

similar isoelectric points, which explains the co-migration 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 spot 1' to 5' from rapamycin-treated cells (Figure 1C and Suppl. Table 1). Given the high identity of BiP1 and BiP2, these proteins could not be differentially identified by available antibodies, and we will therefore 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 (Figure 1B). Some new spots were found in response to rapamycin treatment, indicating that other proteins became phosphorylated upon TOR inactivation. However, the identity 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 threonine phosphorylation in response to rapamycin-mediated inhibition of TOR signaling. To investigate whether this post-translational 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 hours 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 (Figure 2A). Furthermore, phosphorylation of HSP70B and/or BiP was not a rapid event since it occurred after

four to six hours of rapamycin addition (Figure 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* that lacks the rapamycin primary target FKBP12 (Crespo et al., 2005). As expected, no phosphorylation induction was found in this mutant in response to rapamycin (Figure 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 in pH 4-7 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 (Figure 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 (Figure 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 spots were detected in the region where the phosphothreonine signal was induced (Figure 2B). Therefore, our results indicated that treatment of Chlamydomonas cells with rapamycin results in 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 among other effects in the inhibition of protein synthesis in most eukaryotes. We therefore 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 one hour of treatment (Figure 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 labelling cells with [^{14}C]arginine. Rapamycin-mediated inhibition of TOR resulted in a 30-40% reduction in protein synthesis, demonstrating that indeed TOR promotes translation in Chlamydomonas (Figure 3B). Similar experiments were conducted with cycloheximide instead of rapamycin and about 90% of protein synthesis was inhibited (Figure 3B). The specificity of rapamycin on translation inhibition was confirmed by the nule effect of the drug in the *rap2* mutant (Figure 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]arginine into proteins while rapamycin caused a progressive and less intense effect (Figure 3C),

in agreement with the faster phosphorylation of BiP detected with cycloheximide compared to rapamycin treatments (Figure 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 threonine phosphorylation of BiP detected in Chlamydomonas is related with a regulatory mechanism of this protein, Chlamydomonas cells were subjected to heat stress and the phosphorylation state of BiP was examined. Basal phosphorylation level of BiP was strongly reduced within 15 min of heat shock, being undetectable after 60 min (Figure 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 (Figure 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 (Figure 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 state of BiP was examined by western blot. Compared to untreated cells, tunicamycin abolished the basal level of BiP phosphorylation (Figure 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 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 (Figure 4C, compare lane 8 to lane 2). Tunicamycin reduced BiP phosphorylation to undetectable levels in rapamycin-treated cells (Figure 4C, compare lane 9 to 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 Thr520 plays a role in BiP function

To further characterize the modification of BiP, we tried to identify rapamycin-sensitive phosphorylation sites by mass spectrometry (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 Glu504 to Lys525 of BiP1

(Figure 5A). An alignment of BiP proteins from different organisms revealed that a threonine residue (Thr520 in Chlamydomonas BiP1) from this phosphopeptide is widely conserved within eukaryotes (Figure 5B). Interestingly, a modelling study of BiP1 indicated that Thr520 might be in an exposed region of the chaperone accessible to other proteins (Figure 5C) while the NetPhosK server (Blom et al., 2004) predicted Thr520 as the residue with the highest probability of phosphorylation in BiP1. Our results therefore indicated that inhibition of TOR signalling by rapamycin leads to phosphorylation of BiP in a conserved region of the peptide-binding domain and strongly suggested that Thr520 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 (ts) mutants have been generated. The *kar2-159* mutation was isolated in a genetic screen of ts alleles of *KAR2* and allows growth at 23°C but not at 35-37°C (Vogel et al., 1990). Wild type and *kar2-159* mutant cells were transformed with a plasmid expressing the Chlamydomonas BiP1 cDNA from the strong, constitutive GPD promoter (see Materials and Methods for details). 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 37ºC (Figure 6A). Moreover, BiP1 was not able to functionally replace yeast KAR2 in a heterozygous *Δkar2* mutant strain (Suppl. Fig. 1). Western blot analysis of these strains confirmed that BiP1 is expressed in *kar2-159* cells although to a lower level than in Chlamydomonas cells (Figure 6B). Compared to Chlamydomonas extracts, most of the BiP1 protein expressed in yeast was detected as a band of higher

molecular mass (Figure 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 Thr520 of this protein is involved in BiP function. Thr520 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 growth of *kar2-159* cells at 30ºC (Figure 6A). However, T520E mutation, which mimics a constitutive phosphorylation of the residue, could not fully complement BiP function at 30ºC, indicating that T520E mutation decreased BiP function (Figure 6A). None of the BiP1 mutants were able to promote growth at 37ºC (Figure 6A). Interestingly, all mutations in Thr520 resulted in a pronounced increase in the abundance of the precursor and mature forms of this protein in yeast cells compared to wild-type BiP1 (Figure 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 ((Diaz-Troya et al., 2008) and Figure 7), a membrane-enriched region known as peri-basal body region where intraflagellar transport (IFT) 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 to control cells, no difference was observed in cells where BiP phosphorylation was induced by rapamycin (Figure 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 peri-basal body region (Figure 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 plants, 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; Diaz-Troya et al., 2008). In this study we show that inactivation of TOR signalling by rapamycin caused inhibition of protein synthesis in Chlamydomonas (Figure 3B), which demonstrates that TOR promotes protein synthesis in this photosynthetic organism. Control of protein synthesis by TOR has been widely characterized in yeast and mammalian cells. In these systems, inhibition of TORC1 signalling by rapamycin resulted in a decrease in protein synthesis (Barbet et al., 1996; Beretta et al., 1996; Loewith et al., 2002). In Arabidopsis, inducible RNAi of the *AtTOR* gene or rapamycin treatment of plant lines expressing yeast FKBP12 caused a reduction in the abundance of high-molecular weight 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 signalling by rapamycin strongly induced phosphorylation of BiP on threonine residues (Figures 1 and 2) and a phosphopeptide in a regulatory region of BiP that contains a highly conserved threonine (Thr520) could be identified in rapamycin-treated cells (Figure 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 (Figures 1 to 4). Post-translational 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 threonine residues with only a trace of detectable phosphoserine (Hendershot et al., 1988; Gaut, 1997). Moreover, *in vivo* threonine 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 (Figure 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 which increase the need for active BiP in mammalian cells such as glucose starvation or tunicamycin treatment promote 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 been also proposed that post-translational modification of BiP might provide a storage pool of BiP that can be recruited back to the active form in response to need (Gething, 1999). The present study 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 (Figure 3). Both drugs have a negative effect on protein synthesis although with different efficiencies since cycloheximide is more effective than

rapamycin in translation inhibition (Figure 3). Interestingly, BiP phosphorylation occurred faster in response to cycloheximide since phosphorylated BiP was detected within one hour in cycloheximide-treated cells whereas rapamycin induced BiP phosphorylation after at least four hours (Figure 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 (Figure 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 signalling, emphasising 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 (Figure 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 (Fontes et al., 1991) and spinach (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 (Figure 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; Brodsky et al., 1998) likely due to the inability of these proteins to productively interact with proteins such as cochaperones or components of the ER translocation machine. 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 Thr520 in BiP1 led to a reduction in protein function (Figure 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 threonine 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 Thr520 mutation in Chlamydomonas BiP1 stimulated 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 (Diaz-Troya et al., 2008). Actually, the cellular distribution of

Chlamydomonas TOR and BiP proteins was coincident in some aspects. Both proteins localize to discrete bodies and accumulate in the peri-basal body region ((Diaz-Troya et al., 2008) and Figure 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 signalling and ER stress in mammals. Loss of the tuberous sclerosis (TSC) 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 signalling, 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 signalling, 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 on 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, like 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 signalling 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 post-translational modification of this chaperone and their possible control by TOR and/or other signalling 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 rapamycin-insensitive *rap2* strain has been previously described (Crespo et al., 2005). Cells were grown as described by Harris (1989) under continuous illumination at 25ºC. If required, Tris-acetate phosphate medium (TAP) was solidified with 1.2% Bacto agar (Difco). When specified, cells were treated with 500 nM rapamycin (LC Labs) from a 4 mM stock in 90% ethanol-10% Tween20, 5 μg/ml tunicamycin (Calbiochem) from a 5 mg/ml stock in dimethylformamide, or 10 μg/ml cycloheximide (Sigma) from a 3 mg/ml 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 previously described (Sherman, 1991).

Protein preparation

Chlamydomonas cells from liquid cultures were collected by centrifugation (3000 x *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 NaPPi, 10 mM beta-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 -80ºC followed by thawing to room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15700 x *g*, 15 min) at 4ºC, and stored at -80ºC until they were analyzed. Yeast extracts from logarithmically growing cells were prepared in lysis buffer (PBS buffer, 1 mM PMSF,

0.5% Tween20, 10 mM NaF, 10 mM NaN_3, 10 mM p-Nitrophenylphosphate, 10 mM NaPPi, 10 mM beta-glycerophosphate) by vortexing them 10 times for 1 min each time with glass beads (Sigma-Aldrich). Crude extracts were cleared by centrifugation at 15,000g for 15 min at 4_C. Proteins were quantitated with the Coomassie dye binding method (BioRad).

2D gel electrophoresis

For 2D electrophoresis, proteins from soluble cell extract samples were precipitated with 2D Clean-Up Kit (GE Healthcare), resuspended in rehydration solution, and quantitated with RC DC Protein Assay (BioRad) according to the manufacturers' instruction. Isoelectric focusing (IEF) 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 (BioRad) containing 150 µg of protein. Following overnight active rehydration (50V, 20°C), IEF was performed for 15kVh (for pH 3-10NL strips) or 20kVh (for pH 4-7 strips) at 20°C using the Protean IEF Cell (Bio-Rad). After the first dimension separation, strips were blotted to remove mineral oil and stored at -80°C. Prior to the second dimension separation (SDS-PAGE), thawed strips were first equilibrated with 10 mg/ml DTT in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29,3% glycerol, 2% SDS, and 0.002% bromophenol blue), and then with 25 mg/ml iodoacetamide in equilibration buffer. A RE 600 Ruby chamber system (GE Healthcare) was used for the second dimension separation. Equilibrated strips were placed on 10% SDS-PAGE gels and sealed with 0.5% agarose. Proteins were separated by molecular weight using a 70 mA constant intensity. Gels were Coomassie blue stained or processed for western blot.

Western blotting

Proteins resolved by SDS-PAGE or 2D-gel electrophoresis were transferred to PVDF membrane (for phosphothreonine detection) or 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 BiP, Chlamydomonas HSP70-B, and phosphothreonine 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 IF microscopy as described previously (Perez-Perez et al., 2010). The primary antibody used was rabbit polyclonal anti-BiP (1:500; Santa Cruz Biotechnology). For signal detection, a fluorescein isothiocyanate (FITC)-labelled 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 Leica Application Suite Advanced Fluorescence software package (Leica). Deconvolution analysis of images was performed with the same software.

Protein identification by MALDI-TOF

CBB-stained proteins were excised from the gel using the EXQuest Spot Cutter excision robot (BioRad) 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 DTT 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 trypsin in 50 mM ammonium bicarbonate. Digestion was stopped and peptides extracted using 0.5% trifluoroacetic acid. Tryptic digests were analysed using an Autoflex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA, USA) and proteins were identified as the highest ranked result by searching the NCBI non-redundant database, version from February 2008, for Viridiplantae (Green Plants) sequences (from a total of 474525 sequences) by the Protein Mass Fingerprint technique using the MASCOT search tool (Matrix Science; http://matrixscience.com). The search parameters included carbamidomethylation of cysteines, oxidation of methionines, 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 TCEP at 37^{o}C for 1 hour and alkylated with 50 mM iodoacetamide for 15 min at room temperature. In-gel digestion was with 250 ng trypsin or 250 ng endoproteinase GluC at 37^{o}C overnight. The peptides were analyzed on an LTQ Orbitrap instrument (Thermo Fisher, San José, CA, USA) 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 x 10 mm, SGE Analytical Science, Victoria, AU) with the capillary pump of an Agilent 1200 system set to 5

µl/min. After 15 min, the trap column was switched into the flow path of the C18 separating column (0.1 x 150 mm, Swiss Bioanalytics, Birsfelden, CH). 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. After 60 min the percentage of solvent B was increased to 60% in ten minutes 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 were then searched against the NCBI non-redundant 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 Da. Cleavage rules were set to Fully enzymatic – cleaves at both ends, allowing 2 missed cleavages. The modifications were set to carbamidomethyl cysteine 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, 0.01; protein probability 0.01.

Cloning of Chlamydomonas BiP1 and plasmid construction

The BiP1 coding region from Chlamydomonas (1971 bp) was amplified by PCR from a Chlamydomonas cDNA library using the following primers: BiP1 SpeI 5' (5'-CCGGACTAGTATGGCGCAGTGGAAGGCTGCTGTGCTG-3') and BiP1 SpeI 3' (5'-CCGGACTAGTTTAGAGCTCGTCGTGGTCGCCCAAGTC-3'), designed to

contain a *SpeI* site (underlined). The resulting product was digested with *SpeI* and inserted into p425GPD (Mumberg et al., 1995) for expression in *S. cerevisiae*. The point mutations in Thr520 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.

Labelling of cells with [^{14}C]arginine

Cell labelling was performed essentially as described in Stavis and Hirschberg (Stavis and Hirschberg, 1973) with some modifications. Chlamydomonas cells from liquid TAP cultures at a density of 10^6 cells/ml were harvested by centrifugation (3000 x *g*, 5 min), washed with TAP-N medium and cultured in the same medium for 6 h to improve [^{14}C]arginine incorporation. Next, cells were treated with 500nM rapamycin for 6h, 10 μg/ml cycloheximide for 30 min or drug vehicle. Reactions mixtures containing 10^7 cells and 1 μCi [^{14}C]arginine 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% trichloroacetic acid (TCA). The precipitated material was collected on Whatman glass fiber filters, washed four times with cold 5% TCA, dried, and radioactivity measured by a liquid scintillation counter.

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FIGURE LEGENDS

Figure 1. Rapamycin treatment induces alterations on the threonine phosphorylation pattern in Chlamydomonas soluble extract. **A,** Western blot analysis of Chlamydomonas soluble extracts with phosphoserine (P-Ser) and phosphothreonine (P-Thr) antibodies. Log-phase wild-type cells were treated with 500 nM rapamycin (rap) 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 kDa band detected with the P-Thr antibody. **B,** Soluble extracts of Chlamydomonas cells treated (rap) or not (control) with 500 nM rapamycin for 8 hours were resolved by 2D electrophoresis as described in Experimental Procedures. Gels were stained with Coomassie Brilliant Blue (CBB) or processed for immunodetection with the phosphothreonine (P-Thr) antibody. **C,** Enlarged images of box sections in 2D gels shown in panel B. Arrow indicates spots whose mobility differs in untreated and rapamycin-treated cells. Numbers refer to spots where proteins have been identified by mass spectrometry (see Suppl. Table I for details).

Figure 2. Rapamycin induces threonine phosphorylation of the BiP chaperone in its peptide-binding domain. **A,** Phosphorylation time course of the 70 kDa protein following rapamycin treatment. 500 nM rapamycin or drug vehicle were 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 phosphothreonine (P-Thr), BiP and HSP70B specific antibodies. **B,** 2D western blot analysis of BiP and HSP70B. Wild type Chlamydomonas cells were treated with 500 nM rapamycin for 12 hours (rap), heat shocked at 40ºC for 1 hour (HS) or remained untreated (control). Soluble extracts were

resolved by 2D electrophoresis as described in Experimental Procedures using pH 4-7 strips and gels were processed for immunodetection with phosphothreonine (P-Thr), BiP and HSP70B specific antibodies.

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. 500 mM rapamycin (rap), 10 μg/ml 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 blot probing with P-Thr or BiP specific antibodies. **B,** Incorporation of [^{14}C]arginine in Chlamydomonas cells. Wild-type (wt) and rapamycin-insensitive (*rap2*) Chlamydomonas cells were treated with 500nM rapamycin for 6 hours (rap), 10 μg/ml cycloheximide for 30 min (CHX), or drug vehicle (C) and processed for the analysis of [^{14}C]arginine incorporation as described in Experimental Procedures. Values are represented in relation to the [^{14}C]arginine incorporation detected in control, untreated cells and correspond to six independent determinations (*$p<0.05$, **$p<0.01$). **C,** Effect of CHX and rapamycin on protein synthesis. Chlamydomonas cultures were treated with 500 nM rapamycin (triangles) or 10 μg/ml CHX (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.

Figure 4. BiP dephosphorylation by ER stress-inducing agents. BiP dephosphorylation by heat stress. Chlamydomonas cells pretreated (**B**) or not (**A**) with 500 nM rapamycin (rap) for 12 hours were subjected to heat stress at 40ºC and samples were taken at the

indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western blot probing with P-Thr and BiP specific antibodies. **C**, BiP dephosphorylation by tunicamycin treatment. Chlamydomonas cells were treated with 500 nM rapamycin (rap), 5 μg/ml tunicamycin (tun), or first treated with rapamycin (rap, lane 8) and then with tunicamycin (rap tun, lane 9). Samples were taken at the indicated times and soluble extracts were processed and analyzed by western blot.

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 a red box whereas the grey box shows the position of the phosphopeptide identified in mammalian BiP (Gaut, 1997), which spans from Thr455 to Asp506 of Chlamydomonas BiP. **B**, Amino acid sequence alignment of the phosphopeptide identified in Chlamydomonas BiP (from Glu504 to Lys525) 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 kDa heat shock protein (3c7nA). Conserved Thr520 was mapped onto the crystal structure. Structure prediction was performed using the SWISS-MODEL bioinformatics tool and image was generated by iMol software. ATPase and peptide-binding domains are shown in yellow and blue, respectively.

Figure 6. Chlamydomonas BiP1 partially complements KAR2 function in a *kar2-159* ts mutant. **A**, 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 3-4 days. **B**, MS10 (wt) and *kar2-159* strain expressing the indicated Chlamydomonas BiP1 proteins were grown at the permissive temperature of 25ºC and expression of BiP1 and endogenous KAR2 proteins in these cells were detected by western blot. Precursor and mature forms of BiP1 are indicated. Cr, soluble extract from Chlamydomonas cells grown in TAP medium. 50 μg of total protein were loaded per lane.

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 tunicamycin (tun) for 8 h. Cells were collected and processed for IF microscopy analysis. The signal recognized by the BiP antibody is shown in green. DNA staining is shown in blue (DAPI).

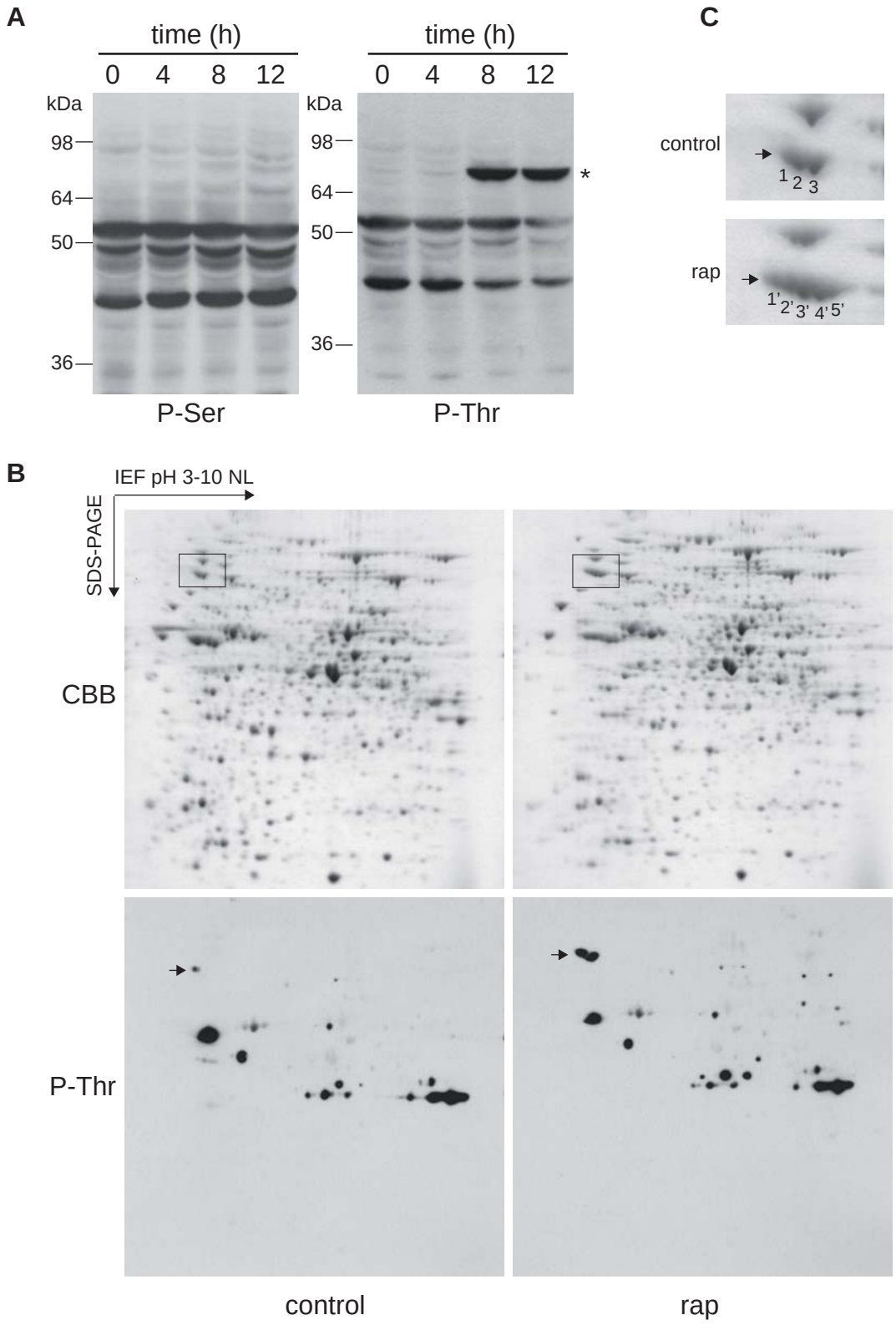


Figure 1. Rapamycin treatment induces alterations on the threonine phosphorylation pattern in Chlamydomonas soluble extract. **A**, Western blot analysis of Chlamydomonas soluble extracts with phosphoserine (P-Ser) and phosphothreonine (P-Thr) antibodies. Log-phase wild-type cells were treated with 500 nM rapamycin (rap) 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 kDa band detected with the P-Thr antibody. **B**, Soluble extracts of Chlamydomonas cells treated (rap) or not (control) with 500 nM rapamycin for 8 hours were resolved by 2D electrophoresis as described in Experimental Procedures. Gels were stained with Coomassie Brilliant Blue (CBB) or processed for immunodetection with the phospho-threonine (P-Thr) antibody. **C**, Enlarged images of box sections in 2D gels shown in panel B. Arrow indicates spots whose mobility differs in untreated and rapamycin-treated cells. Numbers refer to spots where proteins have been identified by mass spectrometry (see Suppl. Table I for details).

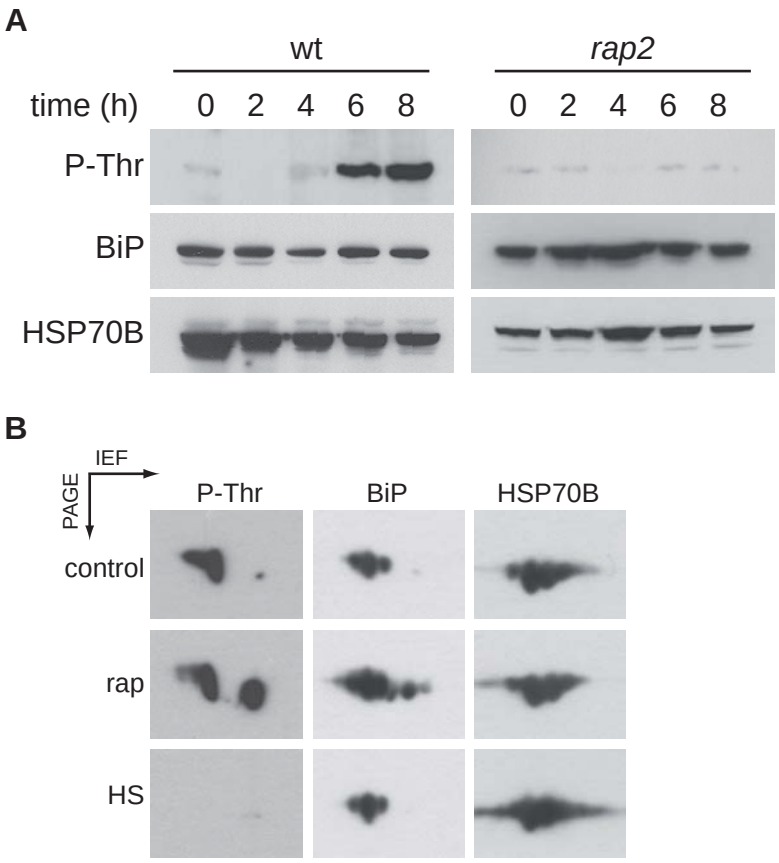


Figure 2. Rapamycin induces threonine phosphorylation of the BiP chaperone in its peptide-binding domain. **A**, Phosphorylation time course of the 70 kDa protein following rapamycin treatment. 500 nM rapamycin or drug vehicle were added to wild-type (wt) or rapamycin-insensitive (rap2) Chlamydomonas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

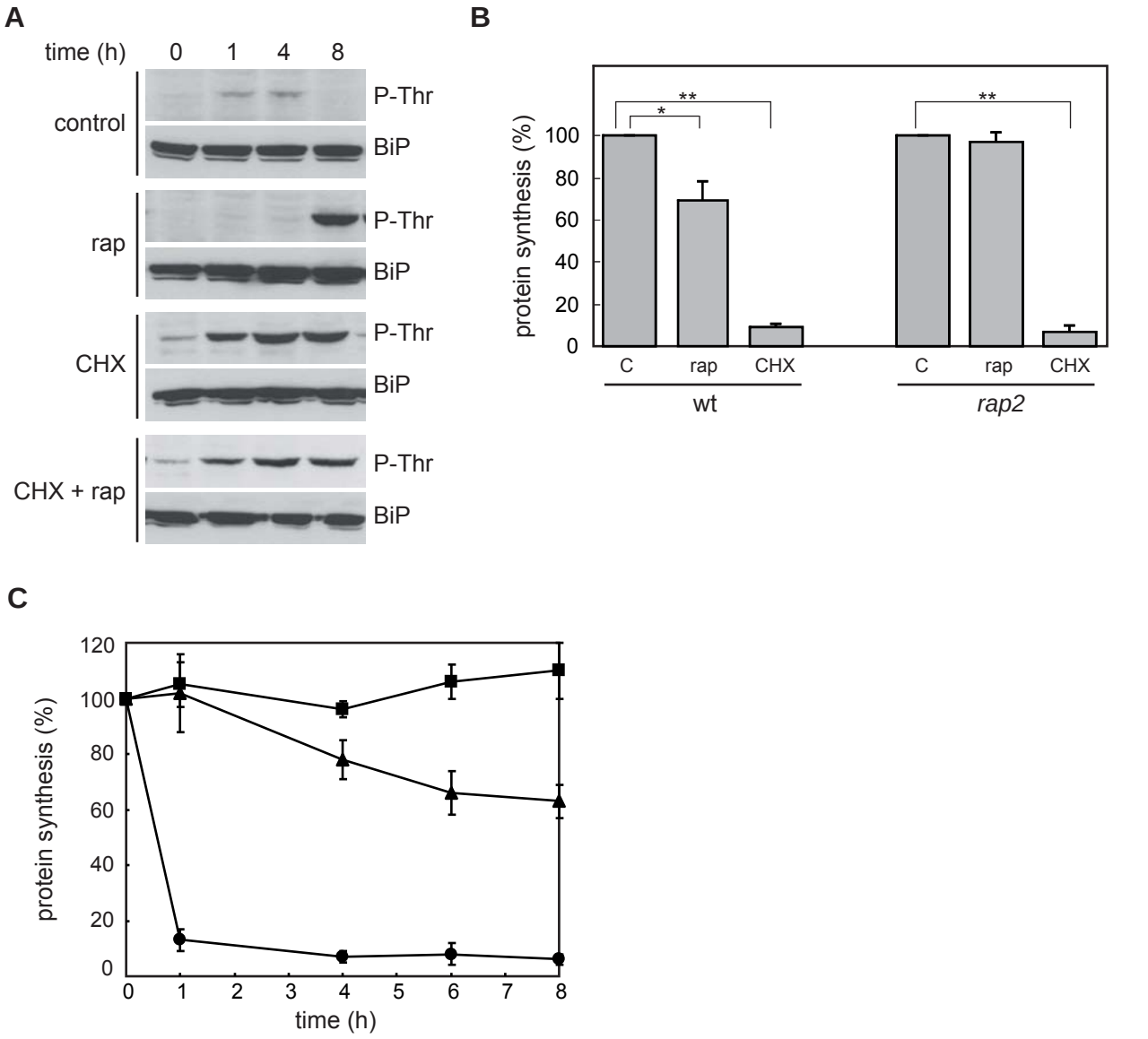


Figure 3. Inhibition of protein synthesis by rapamycin or cycloheximide induces BiP phosphorylation. **A,** Time course of BiP phosphorylation following rapamycin and/or cycloheximide treatment. 500 mM rapamycin (rap), 10 µg/ml cycloheximide (CHX) or both drugs simultaneously (CHX + rap) were added to wild-type Chlamydomonas cells and samples were taken at the indicated times. Soluble extracts were fractionated by SDS-PAGE and analyzed by western blot probing with P-Thr or BiP specific antibodies. **B**, Incorporation of [14C]arginine in Chlamydomonas cells. Wild-type (wt) and rapamycin-insensitive (rap2) Chlamydomonas cells were treated with 500nM rapamycin for 6 hours (rap), 10 µg/ml cycloheximide for 30 min (CHX), or drug vehicle (C) and processed for the analysis of [14C]arginine incorporation as described in Experimental Procedures. Values are represented in relation to the [14C]arginine incorporation detected in control, untreated cells and correspond to six independent determinations (*p<0.05, **p<0.01). **C,** Effect of CHX and rapamycin on protein synthesis. Chlamydomonas cultures were treated with 500 nM rapamycin (triangles) or 10 µg/ml CHX (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.

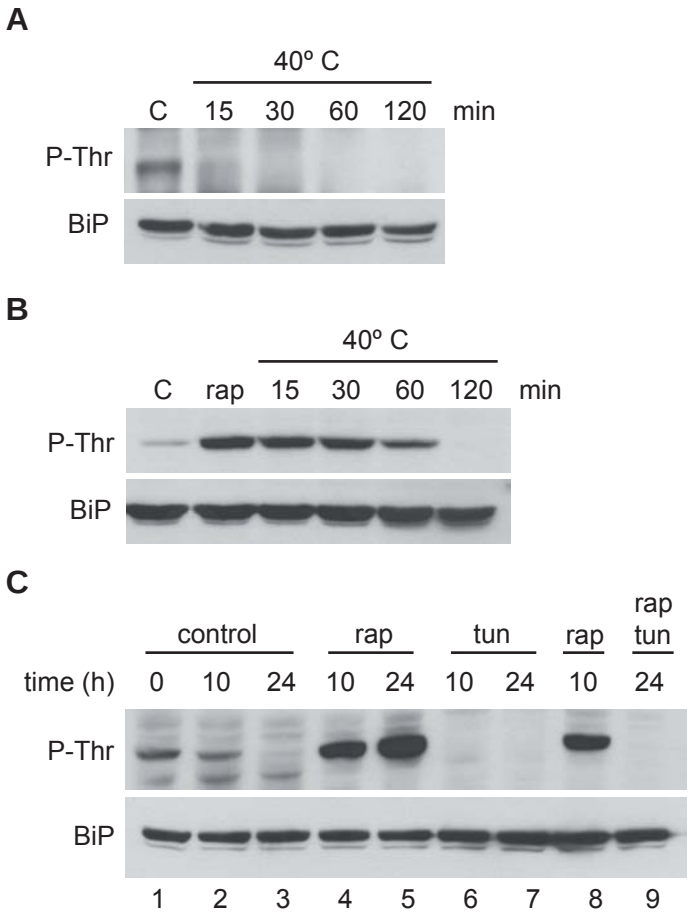


Figure 4. BiP dephosphorylation by ER stress-inducing agents. BiP dephosphorylation by heat stress. Chlamydomonas cells pretreated (**B**) or not (**A**) with 500 nM rapamycin (rap) for 12 hours were subjected to heat stress at 40ºC and samples were taken at the indicated times. Soluble extracts were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

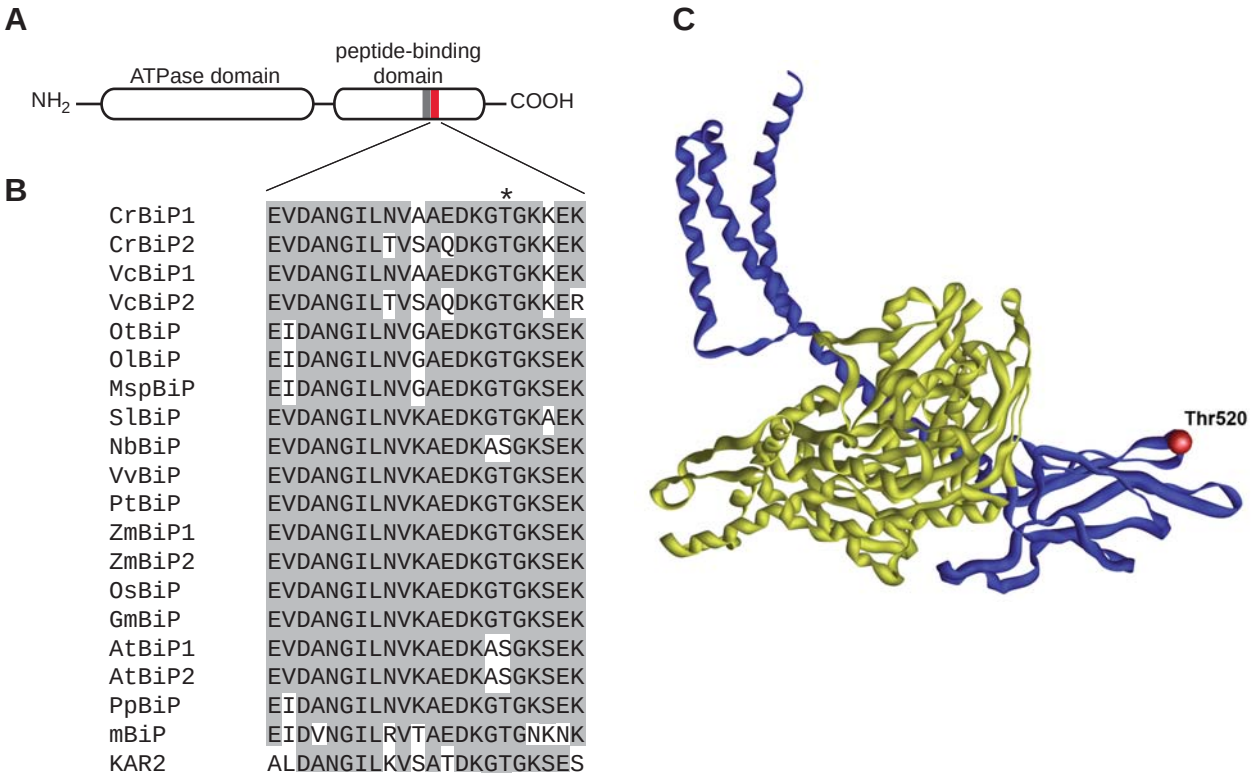
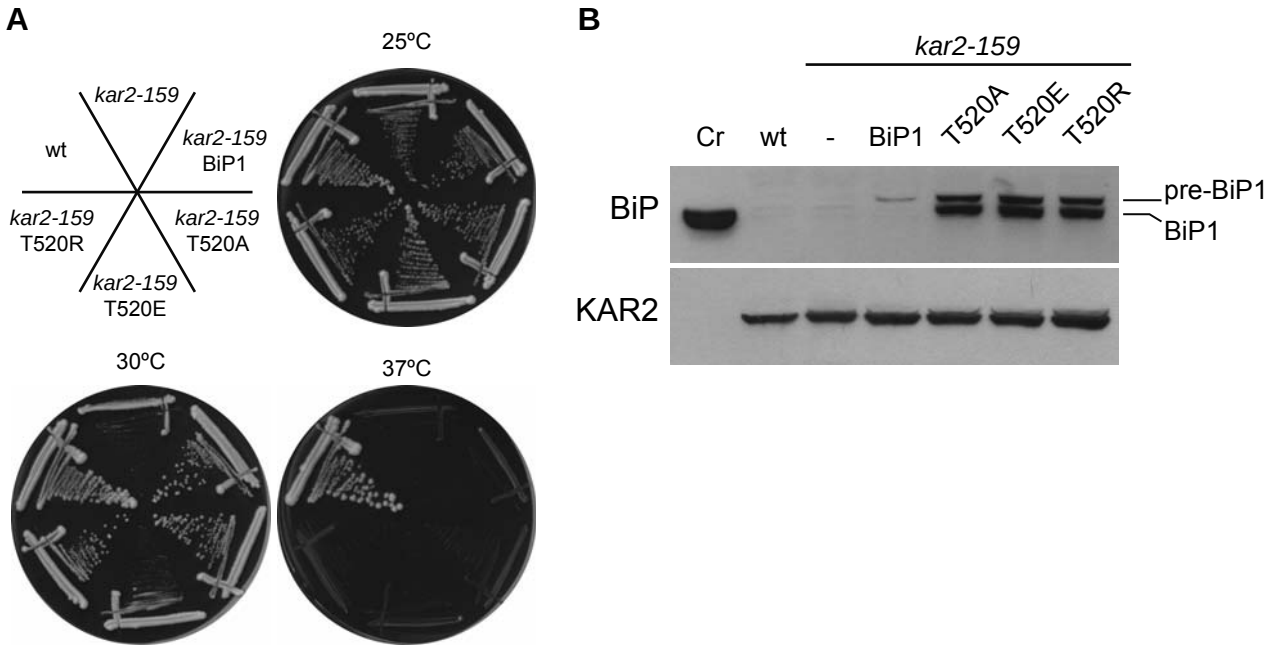


Figure 5. Identification of a phosphopeptide in Chlamydomonas BiP. **A**, Schematic representation of the BiP protein and identification of a phosphopeptide in the peptide-binding domain. The scheme shows the ATPase and peptide-binding domains of BiP. The position of the phosphopeptide identified in Chlamydomonas is indicated by a red box whereas the grey box shows the position of the phosphopeptide identified in mammalian BiP (Gaut, 1997), which spans from Thr455 to Asp506 of Chlamydomonas BiP. **B**, Amino acid sequence alignment of the phosphopeptide identified in Chlamydomonas BiP (from Glu504 to Lys525) 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 kDa heat shock protein (3c7nA). Conserved Thr520 was mapped onto the crystal structure. Structure prediction was performed using the SWISS-MODEL bioinformatics tool and image was generated by iMol software. ATPase and peptide-binding domains are shown in yellow and blue, respectively.



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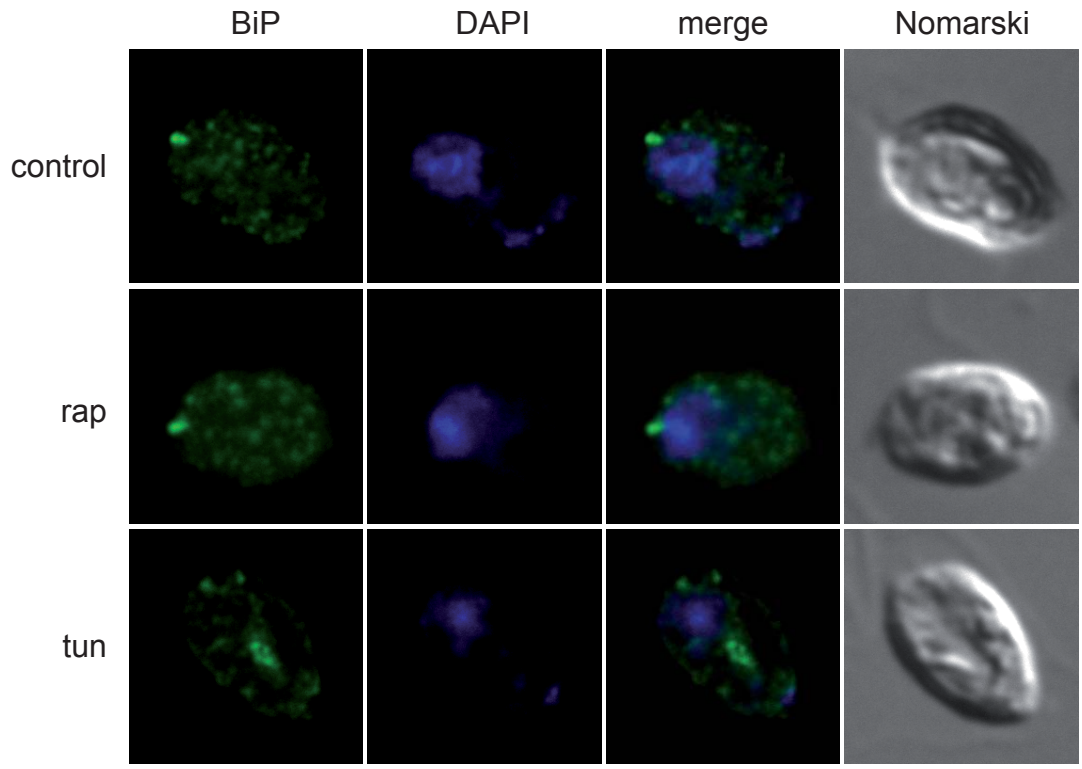

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