Running title: Redox control of autophagy upon ER stress

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Research Area:

Signaling and Response
Oxidative stress contributes to autophagy induction in response to endoplasmic reticulum stress in Chlamydomonas

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Summary:

Alteration of the intracellular redox state during endoplasmic reticulum stress contributes to autophagy activation in Chlamydomonas.
Footnotes:

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ABSTRACT

The accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER) results in the activation of stress responses such as the unfolded protein response or the catabolic process of autophagy to ultimately recover cellular homeostasis. ER stress also promotes the production of reactive oxygen species (ROS), which play an important role in autophagy regulation. However, it remains unknown whether ROS are involved in ER stress-induced autophagy. In the present study, we provide evidence connecting redox imbalance caused by ER stress and autophagy activation in the model unicellular green alga Chlamydomonas reinhardtii. Treatment of Chlamydomonas cells with the ER stressors tunicamycin or dithiothreitol resulted in upregulation of the expression of genes encoding ER resident ERO1 oxidoreductase and protein disulfide isomerases. ER stress also triggered autophagy in Chlamydomonas based on the protein abundance, lipidation, cellular distribution and mRNA levels of the autophagy marker ATG8. Moreover, an increase in the oxidation of the glutathione pool and in the expression of oxidative stress-related genes were detected in tunicamycin-treated cells. Our results revealed that the antioxidant glutathione partially suppressed ER stress-induced autophagy and decreased the toxicity of tunicamycin, suggesting that oxidative stress participates in the control of autophagy in response to ER stress in Chlamydomonas. In close agreement, we also found that autophagy activation by tunicamycin was more pronounced in the Chlamydomonas sor1 mutant, which shows increased expression of oxidative stress-related genes.
INTRODUCTION

All living organisms have evolved sophisticated mechanisms to efficiently respond and adapt their growth and metabolism to different types of stress. A well-documented example of such stress-induced responses is the process of autophagy or self-degradation, which is structurally and functionally conserved in all eukaryotes. During autophagy (also known as macroautophagy), cytoplasmic components including proteins, membranes and even organelles are non-selectively enclosed within a double-membrane vesicle known as autophagosome and delivered to the vacuole/lysosome for degradation of toxic or damaged components and recycling of needed nutrients (Xie and Klionsky, 2007; Nakatogawa et al., 2009; Li and Vierstra, 2012; Liu and Bassham, 2012).

Autophagy is mediated by a set of proteins coded by ATG (autophagy-related) genes that are widely conserved from yeasts to humans. Homologs of ATG genes have been reported in plant and algal genomes, indicating that autophagy is also conserved in photosynthetic organisms (Thompson and Vierstra, 2005; Bassham et al., 2006; Diaz-Troya et al., 2008; Avin-Wittenberg et al., 2012). Some ATG proteins play a structural role in autophagy and are essential for the formation of the autophagosome. For instance, the ATG8 protein anchors to the autophagosome membrane through its covalent binding to phosphatidylethanolamine (PE), and the ATG8-PE conjugate is essential for the formation and completion of the autophagosome (Ichimura et al., 2000). ATG genes are conserved in Chlamydomonas (Diaz-Troya et al., 2008; Perez-Perez et al., 2010), and autophagy has been investigated in this model system by monitoring the abundance, lipiddation state and cellular distribution of the ATG8 protein under several growth
and stress conditions. As reported for other systems, nitrogen or carbon depletion triggers autophagy in Chlamydomonas (Perez-Perez et al., 2010). Moreover, entry of Chlamydomonas cells into stationary growth phase activates autophagy in a reversible manner since the process is quickly downregulated when cells return to exponential growth (Perez-Perez et al., 2010). Autophagy is also induced in Chlamydomonas cells treated with H₂O₂ or methyl viologen (MV), indicating that oxidative stress triggers this process in algae (Perez-Perez et al., 2010; Perez-Perez et al., 2012a). Reactive oxygen species (ROS) are potent inducers of autophagy in Chlamydomonas and plants (Liu and Bassham, 2012; Perez-Perez et al., 2012b). Indeed, a link between photo-oxidative damage, ROS accumulation and autophagy activation has been shown in Chlamydomonas cells with a decreased carotenoid content caused by either the mutation of phytoene synthase or the inhibition of phytoene desaturase by the herbicide norflurazon (Perez-Perez et al., 2012a). Moreover, ROS generated in the chloroplast of carotenoid-deficient cells or in the chloroplast of wild-type cells subjected to high light stress activate autophagy (Perez-Perez et al., 2012a).

The accumulation of un/misfolded proteins in the endoplasmic reticulum (ER) is known to trigger autophagy in yeasts (Bernales et al., 2006; Yorimitsu et al., 2006) and mammals (Ogata et al., 2006). More recently, induction of autophagy by ER stress has also been reported in land plants and algae (Perez-Perez et al., 2010; Liu et al., 2012), indicating that the signaling pathways controlling autophagy activation in response to this intracellular stress might be conserved in photosynthetic organisms. In Chlamydomonas, tunicamycin, which induces ER stress by inhibition of N-linked glycosylation, strongly increases the abundance and lipidation of ATG8 and modifies its cellular distribution (Perez-Perez et al.,
The accumulation of misfolded proteins in the ER is a potent stress signal that induces the expression of chaperones and other proteins required for the reestablishment of cell homeostasis, a signaling process known as the unfolded protein response (UPR) (Walter and Ron, 2011). ER stress is perceived in the cell by key signaling proteins such as the highly conserved IRE1 kinase, which transduces stress signals to the nucleus by promoting the splicing of bZIP-like transcription factors (Walter and Ron, 2011). Eukaryotic cells employ a quality control mechanism that recognizes aberrantly folded proteins in the ER for their degradation via the proteasome, a process that is known as ER-associated degradation (ERAD) (Walter and Ron, 2011). Prolonged ER stress also triggers autophagy to remove unfolded proteins and to counterbalance ER expansion caused by UPR (Bernales et al., 2006).

Unlike other cellular compartments, the ER provides, through ER-resident oxidoreductases, an oxidative environment that facilitates the oxidation of cysteines and thereby the formation of disulfide bonds (Tu and Weissman, 2004). In yeasts and mammals, it has been shown that the ER oxidoreductin 1 (ERO1) is a major source of ROS in the ER and in the cell (Haynes et al., 2004; Tu and Weissman, 2004). Oxidative protein folding in the ER occurs in part through the formation of disulfide bonds via protein disulfide isomerases (PDIs). To introduce disulfides into client proteins, PDI must be maintained in an oxidized state, and ERO1 is the main responsible for keeping PDI oxidized and active in the ER (Frand and Kaiser, 1999). ERO1 utilizes molecular oxygen as final electron acceptor and hence forms one molecule of H$_2$O$_2$ for every disulfide it introduces (Tu and Weissman, 2004). Several studies have established an association between ER stress and ROS generation (Malhotra and Kaufman, 2007; Rutkowski and Kaufman, 2010).
2007; Ozgur et al., 2014), and the molecular mechanisms by which ROS are produced during UPR have been thoroughly reviewed (Santos et al., 2009).

ROS have been proposed to play an important role in the mechanisms of autophagy induction in response to various stress conditions in mammals, plants and algae (Huang et al., 2011; Scherz-Shouval and Elazar, 2011; Szumiel, 2011; Li et al., 2012; Perez-Perez et al., 2012b). Although ER stress promotes ROS production and induces autophagy, it remains unknown whether ROS are involved in ER stress-induced autophagy. In the present study, we provide evidence connecting autophagy activation in ER-stressed cells with redox imbalance generated from the UPR-regulated oxidative folding machinery in Chlamydomonas, and propose that oxidative stress contributes to the induction of autophagy by ER stress.
RESULTS

Tunicamycin triggers ER stress and autophagy in Chlamydomonas

In a previous study, we showed that tunicamycin triggers autophagy in Chlamydomonas (Perez-Perez et al., 2010). To further characterize the effect of tunicamycin on autophagy, we analyzed the expression of the ATG8 gene by quantitative real-time PCR (qPCR). Tunicamycin treatment resulted in a progressive increase of ATG8 transcript levels (Fig. 1A). In close agreement, an increase in ATG8 protein abundance and the appearance of modified ATG8 forms were observed in ER-stressed cells (Fig. 1B). No effect was observed on the amount of FKBP12, an ER stress unrelated protein (Crespo et al., 2005). Transcription of ATG3, encoding an E2-like enzyme involved in ATG8 lipidation (Ichimura et al., 2000), was also analyzed in tunicamycin-treated cells to investigate the participation of other ATG genes in ER stress. qPCR analysis revealed that similar to ATG8, ATG3 expression was upregulated with tunicamycin and also with other stressors previously shown to induce autophagy such as H2O2, methyl viologen or norflurazon (Perez-Perez et al., 2010; Perez-Perez et al., 2012a) (SFig. 1). As expected, tunicamycin had no effect on the expression of FKBP12 (SFig 2).

The cellular response to ER stress in Chlamydomonas is still poorly characterized and no markers have been established to investigate this process in this model alga. In Arabidopsis, it has been reported that expression of some UPR genes such as the ER-resident chaperone Calreticulin-2 (At1g09210) increases in response to tunicamycin (Martinez and Chrispeels, 2003). We identified a member of the calreticulin family in Chlamydomonas nuclear genome (SFig. 3), which we denoted
as CAL2. To investigate whether the expression of CAL2 is subject to UPR regulation in Chlamydomonas, transcription of this gene was determined by qPCR analysis in cells treated with tunicamycin or with other stressors that should not trigger ER stress. CAL2 mRNA levels were increased with tunicamycin (Fig. 1C) but not with H2O2, methyl viologen or norflurazon (Sfig. 1A), demonstrating that this gene can be used to monitor ER stress in Chlamydomonas.

ERO1 has not been previously described in algae but based on its high evolutionary conservation we identified an ERO1 homologue in the Chlamydomonas genome (Sfig. 3). To investigate the participation of Chlamydomonas ERO1 in ER stress, ERO1 expression was analyzed by qPCR in cells treated with tunicamycin. We found a strong induction of ERO1 expression in ER-stressed cells (Fig. 1C). In yeasts, ERO1 and PDI expression are coordinately regulated and subjected to UPR regulation (Frand and Kaiser, 1998; Pollard et al., 1998). Rb60/PDI1A is the only canonical PDI that has been studied in Chlamydomonas, although other PDI-like proteins appear to be conserved in the Chlamydomonas genome (Lemaire and Miginiac-Maslow, 2004; Filonova et al., 2013). Rb60 appears to have a dual localization in the ER and the chloroplast (Levitan et al., 2005) but its role in ER stress has not been investigated. We analyzed the expression of Rb60 in response to tunicamycin treatment and found that, similar to ERO1, this gene was strongly upregulated (Fig. 1C). Searching for PDI proteins other than Rb60 in Chlamydomonas, we identified a PDI-like protein that we denoted as PDI6 containing an N-terminal J domain (Sfig. 3). This domain structure is conserved in ER proteins involved in the recognition and binding of misfolded proteins that fail to achieve their correct conformation in the ER (Schroda, 2004). We investigated the participation of PDI6 in ER stress by
analyzing the expression of this gene in tunicamycin-treated cells. *PDI6* mRNA level dramatically increased in ER-stressed cells (Fig. 1C), strongly suggesting that this gene is involved in the cellular response to this stress. Taken together, these results indicated that *CAL2, ERO1, Rb60* and *PDI6* expression can be used to monitor ER stress in Chlamydomonas.

In addition to tunicamycin, we tested the effect of thapsigargin, an inhibitor of the ER Ca\(^{2+}\)/ATPase that triggers ER stress by depletion of luminal calcium stores (Urano et al., 2000), on autophagy and ER stress markers. Like tunicamycin treatment, thapsigargin led to ER stress and autophagy activation in Chlamydomonas (SFig. 4).

**Tunicamycin triggers oxidative stress**

We explored the possible induction of oxidative stress caused by ER stress through different approaches. On the one hand, we analyzed the expression of *GPXH* and *GSTS1* genes in ER-stressed cells. Both genes can be induced by several ROS although *GPXH* is more significantly induced by singlet oxygen (Ledford et al., 2007; Fischer et al. 2007; Fischer et al., 2012). Our results revealed a high induction of *GSTS1* and a moderate increase of *GPXH* expression in response to tunicamycin treatment (Fig. 2B), suggesting that ER stress may result in the activation of oxidative stress signaling in Chlamydomonas. On the other hand, the intracellular pool of total glutathione, comprising reduced (GSH) and oxidized (GSSG) glutathione, was determined in cells treated with tunicamycin. Our results indicated that ER stress raised the intracellular concentration of GSSG (Fig. 2B),
decreasing the GSH/GSSG ratio (Fig. 2C), a hallmark of redox imbalance (Foyer and Noctor, 2011). Taken together, these results indicated that ER stress triggers oxidative stress in Chlamydomonas.

**Dithiothreitol causes ER stress and autophagy activation**

Disruption of disulfide bonds by dithiothreitol (DTT) results in accumulation of unfolded proteins in the ER and consequently triggers ER stress. Massive aggregation of misfolded proteins caused by DTT has been shown to induce autophagy in various systems including yeasts, mammals and more recently plants (Bernales et al., 2006; Yorimitsu et al., 2006; Liu et al., 2012). We investigated the effect of DTT on ER stress and autophagy in Chlamydomonas by different approaches. Autophagy was investigated in DTT-treated cells by examining ATG8 expression. Our results showed that DTT induces ATG8 expression (Fig. 3A), suggesting that DTT efficiently triggers autophagy in Chlamydomonas. Activation of autophagy by DTT was confirmed by western blot and immunofluorescence analysis of ATG8. The abundance of this protein progressively increased in response to DTT as shown by western blot (Fig. 3B). In agreement with this result, the cellular distribution of ATG8 drastically changed upon DTT treatment as observed by immunofluorescence microscopy. In untreated cells, the ATG8 signal was very weak and localized in a single spot in some cells whereas this signal was much stronger and detected as multiple spots in response to DTT or tunicamycin treatment (Fig. 3C).
ER stress was monitored in DTT-treated cells by qPCR analysis of \textit{CAL2}, \textit{ERO1}, \textit{PDI6} and \textit{Rb60} genes. DTT treatment resulted in the upregulation of these four genes (Fig. 3D). This result confirmed that \textit{CAL2}, \textit{ERO1}, \textit{PDI6} and \textit{Rb60} genes might be used as ER stress markers in Chlamydomonas. Given the tight correlation found between ER and oxidative stresses in tunicamycin treated cells (Fig. 2), we also analyzed the expression of the oxidative stress-related genes \textit{GSTS1} and \textit{GPXH}. Interestingly, reductive stress induced by DTT may indirectly lead to oxidative stress in the cell since transcription of both \textit{GSTS1} and \textit{GPXH} genes was enhanced (Fig. 3E).

\textbf{Glutathione partially suppresses ER stress-induced autophagy}

To test whether redox imbalance contributes to autophagy activation during ER stress, autophagy and ER stress markers were analyzed in cells treated with DTT, which massively reduces disulfide bonds. DTT was used alone or in combination with reduced glutathione (GSH), an abundant antioxidant in the cell that plays an important role in ROS scavenging and in the maintenance of cellular redox homeostasis (Foyer and Noctor, 2011). Our results show that \textit{ATG8} induction caused by DTT is decreased by 50\% in the presence of GSH (Fig. 4A), indicating that autophagy might be partially suppressed by GSH. Interestingly, ER stress was also decreased by exogenous GSH in DTT treated cells, as revealed by the lower expression of \textit{PDI6} (Fig. 4A) and other ER stress-induced genes (SFig. 5A). Since GSH decreased both ER stress and autophagy, it was not possible to separate these two processes and therefore downregulation of autophagy might be due to the partial suppression of ER stress or to ROS scavenging or redox signaling.
mechanisms dependent on GSH. Thus, further investigation of the role of oxidative stress in the control of ER stress-induced autophagy should rather be performed under experimental conditions where ER stress and oxidative stress can be independently tested. We found that GSH was also able to partially suppress tunicamycin-induced expression of ATG8. However, unlike DTT, GSH did not significantly decrease the tunicamycin-induced expression of ER stress markers (Fig. 4B and SFig. 5B). In agreement with decreased ATG8 transcription, the abundance of the ATG8 protein was also downregulated in cells treated with both tunicamycin and GSH compared to cells treated only with tunicamycin (Fig. 4C). Moreover, when the GSH concentration was increased from 5 to 10 mM, a stronger decrease of ATG8 protein level was observed (Fig. 4D). To analyze the specificity of the response to GSH, we also investigated whether ascorbate, an abundant metabolite in plant cells with antioxidant properties (Foyer and Noctor, 2011), is able to mitigate the increase of ATG8 protein level caused by tunicamycin, but unlike GSH, no effect was observed on ATG8 abundance (Fig. 4C). We found however that ascorbate potently and rapidly increased the expression of GSTS1, indicating that this antioxidant is acting on the expression of some genes (SFig. 6).

The effect of GSH on ER stress-induced autophagy was also examined by immunolocalization assays of ATG8 in Chlamydomonas cells. As previously shown (Perez-Perez et al., 2010), tunicamycin treatment strongly increased the ATG8 signal, which accumulated in a few intense spots (Fig. 5A). However, the presence of GSH in the culture medium significantly decreased the intensity of the ATG8 signal although some spots were still clearly visible, indicating that autophagy was reduced but not fully abolished in these cells (Fig. 5A). Quantification of the immunofluorescence signal from individual cells confirmed the inhibitory effect of
GSH on autophagy induced by ER stress (Fig. 5B). These results are in close agreement with qPCR and western blot assays of ATG8 performed in tunicamycin-treated cells in the presence of exogenous GSH (Fig. 4) and strongly suggest that oxidative stress might be involved in the induction of autophagy in ER-stressed cells.

Glutathione decreases the toxicity of tunicamycin in Chlamydomonas

Protein glycosylation in the ER is essential for cell growth and thus its inhibition by tunicamycin is toxic for the cell. Indeed, we found that tunicamycin reduced cell viability in Chlamydomonas after 24 hours of treatment (Fig. 6A). Our results indicated that the presence of exogenous GSH in the medium decreased the toxicity of tunicamycin as revealed by Evans blue staining (Fig. 6A) or serial spot dilution assays (Fig. 6B). Interestingly, cells were still fully viable at the time of autophagy activation by tunicamycin (6-8 hours; Fig. 6A), supporting a pro-survival role of this catabolic process in response to ER stress. In relation to the effect of tunicamycin on cell viability, we analyzed the expression of the DAD1 gene, a homologue of defender against apoptotic cell death 1 that is conserved in many eukaryotes including Chlamydomonas (Kelleher and Gilmore, 1994; Gallois et al., 1997; Kelleher and Gilmore, 1997; Moharikar et al., 2007). The DAD1 protein is part of the oligosaccharyltransferase complex and appears to play an important role in N-linked glycosylation in the ER (Kelleher and Gilmore, 1994), a process specifically inhibited by tunicamycin. Our results revealed that DAD1 expression was upregulated upon prolonged exposure (48 hours) to tunicamycin (Fig. 6C), when cell viability is significantly compromised (Fig. 6A). No induction of DAD1
was observed in cells treated with tunicamycin for 6 hours (SFig. 7), indicating that
DAD1 expression is associated to tunicamycin-induced loss of cell viability. In agreement with the protective role of GSH on cell growth in ER-stressed cells, the presence of this antioxidant decreased tunicamycin-mediated DAD1 expression (Fig. 6C).

**The sor1 mutant displays increased autophagy in response to ER stress**

To further investigate the redox regulation of ER stress-induced autophagy in Chlamydomonas we analyzed the activation of this degradative pathway by tunicamycin in the sor1 mutant. This strain lacks the SOR1 protein, a putative bZIP transcription factor that controls the expression of a large number of oxidative stress response and detoxification genes including GSTS1 (Fischer et al., 2012). Treatment of sor1 cells with tunicamycin revealed a more pronounced accumulation of the ATG8 protein in the sor1 mutant compared to wild-type cells (Fig. 7A), suggesting a higher induction of autophagy in this mutant in response to ER stress. Expression analysis of the ATG8 gene also showed a higher response to tunicamycin in sor1 compared to cw15 cells (Fig. 7B). As previously reported (Fischer et al., 2012), we found that sor1 mutant cells show a constitutively higher expression of GSTS1 (Fig. 7B). Interestingly, despite this elevated expression, tunicamycin further increased GSTS1 mRNA abundance in the sor1 mutant (Fig. 7B). Taken together, these results strongly suggested that autophagic activity triggered by ER stress is higher in cells with anomalous expression of oxidative stress-related genes.
DISCUSSION

In a previous report we demonstrated that ER stress induces autophagy in Chlamydomonas (Perez-Perez et al., 2010). In this study we show that ER stress mediated by tunicamycin or DTT treatment strongly increased the expression of the ATG8 gene, in agreement with the pronounced accumulation of the ATG8 protein and its modified forms detected in ER stressed cells (Figs. 1 and 3). Induction of autophagy by ER stress is evolutionarily conserved and has been reported in yeasts (Bernales et al., 2006; Yorimitsu et al., 2006), mammals (Ogata et al., 2006) and more recently in plants (Liu et al., 2012). Studies performed in yeasts strongly suggest that autophagy serves to counterbalance ER expansion that occurs as a consequence of UPR signaling (Bernales et al., 2006). Degradation of ER membranes via autophagy during ER stress is conserved in plants since this type of membrane has been detected inside autophagic bodies in Arabidopsis plants treated with tunicamycin (Liu et al., 2012).

The present study also demonstrated that the accumulation of unfolded proteins in the ER triggers oxidative stress in Chlamydomonas. Formation of disulfide bonds in the ER and associated oxidative protein folding are linked to the generation of H₂O₂ by the activity of the ERO1 oxidoreductase that catalyzes the re-oxidation and activation of PDI (Tu and Weissman, 2004). Originally identified in yeasts (Frand and Kaiser, 1998; Pollard et al., 1998), ERO1 is widely conserved in all eukaryotes including plants (Aller and Meyer, 2012). In this study, we found that ERO1 is conserved in Chlamydomonas and its expression is highly induced by the ER-stressors tunicamycin and DTT (Figs. 1 and 3). ERO1 being an H₂O₂-generating enzyme, its high expression may lead to increased levels of H₂O₂ under ER stress.
Indeed, our results indicated that Chlamydomonas cells subjected to ER stress displayed elevated expression of oxidative stress-related genes and higher levels of the intracellular pool of GSSG (Fig. 2B, 2C and 3E). Thus, different lines of evidence indicate that ER stress triggers oxidative stress in Chlamydomonas. In yeasts, ROS such as H$_2$O$_2$ are generated during ER stress and overexpression of ERO1 causes a significant increase in ROS and GSSG pool, suggesting that upregulation of the oxidative protein folding machinery by the UPR contributes to ROS accumulation (Haynes et al., 2004). In a recent report, it has also been shown that ER stress triggers ROS signaling in plants (Ozgur et al., 2014), indicating that ROS production upon ER stress is widely conserved.

In addition to ERO1, we identified other genes subjected to UPR regulation in Chlamydomonas. The expression levels of two PDI genes, Rb60 and PDI6, and the CAL2 chaperone were increased in response to ER stress (Figs. 2 and 4). Rb60 is a canonical PDI with an atypical dual localization in the ER and in the chloroplast (Levitan et al., 2005) where it regulates psbA translation by light (Danon and Mayfield, 1991). The function of Rb60 in the ER is unclear although based on its high identity to classical PDI and its localization, it likely participates in oxidative protein folding in the ER. PDI6 encodes a PDI-like protein containing an N-terminal J domain that is conserved among ER co-chaperones (Schroda, 2004) and a C-terminal arginine-rich domain involved in ER stress quality control in mammals (Mizobuchi et al., 2007). The domain architecture of PDI6 appears to be well conserved in microalgae and less represented in plants, where the J domain-containing protein ATERDJ3A from Arabidopsis thaliana seems to be the closest homologue (SFig. 3). No clear homologues of this protein have been found in yeasts or humans. Our results revealed that PDI6 mRNA levels are particularly
sensitive to ER stress and a massive increase in the expression of this gene was observed with tunicamycin or DTT (Fig. 1, 3 and 4). Expression of the calreticulin \textit{CAL2} gene was also found to be upregulated by the UPR in Chlamydomonas (Fig. 1, 3 and 4). Similar to ERO1, CAL2 is conserved in plants and other systems (SFig. 3), and microarray analysis of Arabidopsis seedlings treated with tunicamycin or DTT suggested that this gene is upregulated under ER stress conditions (Martinez and Chrispeels, 2003). Our results indicate that the abundance of \textit{ERO1}, \textit{Rb60}, \textit{PDI6} or \textit{CAL2} mRNAs can be used as markers of the UPR in Chlamydomonas and strongly suggest that the mechanisms of oxidative protein folding described in other eukaryotes are conserved in green algae.

Given that autophagy is regulated by ROS (Perez-Perez et al., 2012b) and ER stress triggers oxidative stress responses in Chlamydomonas (Fig. 2), ER stress-derived ROS may likely contribute to autophagy regulation in response to this stress (Fig. 8). In close agreement with this hypothesis, we found that the antioxidant GSH downregulates autophagy in ER-stressed cells (Figs. 4 and 5). Glutathione is the main free soluble thiol in the cell and acts as a redox buffer by maintaining the intracellular environment in a reduced state (Foyer and Noctor, 2011). Moreover, glutathione has also been shown to play a protective role from ER-generated oxidative stress. Prolonged UPR activation in a yeast strain that is genetically predisposed to sustained ER stress causes oxidative stress ultimately leading to cell death (Haynes et al., 2004). In this strain, the presence of GSH in the culture medium relieved oxidative stress and prevented cell death without diminishing UPR activation, indicating that ROS accumulate during ER stress and are toxic for the cell (Haynes et al., 2004). The effect of GSH in alleviating oxidative stress and promoting growth of ER-stressed cells might be conserved in Chlamydomonas.
since our results showed that GSH decreases the toxicity of tunicamycin (Fig. 6). The protective role of GSH was emphasized by the finding that this antioxidant decreased the expression of the DAD1 gene, which is specifically upregulated when cell viability is compromised due to prolonged ER stress (Fig. 6C). DAD1 is a putative anti-apoptotic gene widely conserved in eukaryotes including humans, plants, yeasts and algae (Nakashima et al., 1993; Kelleher and Gilmore, 1994; Gallois et al., 1997; Kelleher and Gilmore, 1997; Moharikar et al., 2007). The DAD1 protein is localized in the ER and forms part of the oligosaccharyltransferase complex, which catalyzes N-linked glycosylation (Kelleher and Gilmore, 1994). Although the precise function of this protein is not clear, DAD1 appears to be involved in preventing cell death and in regulating N-linked glycosylation. Our results are in agreement with these putative roles since expression of the Chlamydomonas DAD1 gene is induced by tunicamycin treatment, which is toxic for the cell and specifically inhibits N-linked glycosylation.

The hypothesis that ER stress-induced autophagy and redox signaling are linked was strengthened by the finding that tunicamycin triggers autophagy more potently in sor1 mutant cells (Fig. 7). The sor1 strain was isolated in a screen for mutants with increased tolerance to singlet oxygen and shows a constitutively higher expression of a large number of oxidative stress response and detoxification genes (Fischer et al., 2012). Mapping of the sor1 mutation identified a putative bZIP transcription factor denoted as SOR1 that controls among others the expression of the glutathione-S-transferase gene GSTS1 (Fischer et al., 2012), which is upregulated in ER-stressed cells (Fig. 2B). The high autophagic activity observed in sor1 mutant cells treated with tunicamycin might be related to the enhanced expression of ROS-induced genes reported in this mutant since our
results indicated that ER stress-induced autophagy is linked to redox signaling in Chlamydomonas (Figs. 2, 4 and 5). Given the role of SOR1 in the control of glutathione-based ROS scavenging by regulating the expression of genes such as GSTS1 or GPXH (Fischer et al., 2012) and the regulatory function of this antioxidant in ER stress-induced autophagy (Figs. 4 and 5), it is possible that the upregulation of autophagy observed in the sor1 mutant is related to the high expression of these genes, which may result in reduced intracellular levels of glutathione in sor1 cells.

Overall, our results indicate that ER stress triggers autophagy and oxidative stress in Chlamydomonas. Under ER stress triggered by tunicamycin, GSH was found to decrease autophagy induction while UPR markers remained unaffected. This uncoupling of UPR and autophagy in the presence of an antioxidant participating in diverse ROS scavenging mechanisms suggests that ROS production may participate in the control of ER stress-induced autophagy in Chlamydomonas (Fig. 8). However, GSH effects on ER-stress may involve other mechanisms not associated with ROS-scavenging. For example, given the protective role that glutathione plays in the ER (Haynes et al., 2004), it may also be possible that glutathione specifically regulates autophagy by restoring the GSH/GSSG ratio in the ER, which is critical for maintaining the functionality and redox balance at this cellular compartment. Glutathione or redox imbalance may also play a signaling role during ER stress, e.g. through glutathionylation of specific target proteins (Zaffagnini et al., 2012). In the present work we have established the importance of the intracellular redox state and glutathione during ER-stress induced autophagy. Further studies will be required to unravel how redox active molecules control autophagy induction and to examine the interplay between these mechanisms and other ER stress signaling pathways.
MATERIALS AND METHODS

Strains and growth conditions

*Chlamydomonas reinhardtii* cell wall-deficient cw15 4B+ and sor1 mutant strains were obtained from the Chlamydomonas Culture Collection. Chlamydomonas cells were grown under continuous illumination at 25°C in Tris-acetate phosphate (TAP) medium as described (Harris, 1989). When required, cells in exponential growth phase (10^6 cells/ml) were treated with 5 µg/ml tunicamycin (Calbiochem, 654380) from a 5 mg/ml stock in dimethylformamide, 2,5 mM DTT (Applichem, A2948), 1 mM H$_2$O$_2$ (Sigma, H1009), 1 µM methyl viologen (Sigma-Aldrich, 856177) or 20 µM norflurazon (Sigma-Aldrich, PS1044).

Gene accession numbers

Chlamydomonas genes analyzed in this study were identified at the Phytozome website (http://www.phytozome.net/cgi-bin/gbrowse/chlamy/) under the following accession numbers: *ATG3*, Cre02.g102350.t1.2; *ATGB*, Cre16.g689650.t1.2; *CAL2*, Cre01.g038400.t1.2; *ERO1*, Cre17.g723150.t1.3; *PDI6*, Cre12.g518200.t1.3; *Rb60*, Cre02.g088200.t1.2; *GPXH*, Cre10.g458450.t1.3; *GSTS1*, Cre16.g688550.t1.2; *DAD1*, Cre02.g108400.t1.2.

RNA isolation and quantification

Chlamydomonas total RNA was isolated from frozen cell pellets as previously described (Crespo et al., 2005). First-strand cDNA was produced using 2 µg total
RNA, oligo(dT) primer, and 100 units of SuperScript II RNase H- reverse transcriptase (Invitrogen, 18064-014) in a 50 µl reaction. Reverse transcriptase quantitative PCR was performed with a StepOne Real-Time PCR System (Applied Biosystems). PCR reactions, in a final volume of 20 µl, contained 10 µl of FastStart Universal SYBR Green Master (Roche, 04913850001), 1 µl of cDNA dilution, 250 nM of each primer, and distilled water up to 20 µl. Conditions used for amplification in the thermocycler were: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing and elongation at 55-58°C depending on the gene analyzed for 60 s. All reactions were performed in triplicate with 2-4 biological replicates. The CBLP gene was used as a control constitutively expressed gene (Pootakham et al., 2010). The primer pairs used for qPCR were: 5’-CTTCTCGCCCATGACCAC-3’ and 5’-CCACCAGGTTGTTCTTCAG-3’ for CBLP; 5’-CGAGTTCAAGGTGAGCAGC-3’ and 5’-CCACCCACAGACATGTGA-3’ for ATG3; 5’-GCCGCATATCAGACAAGAG-3’ and 5’-TGCGGATGACGTACAAAT-3’ for ATG8; 5’-ACCTGACTACGTCCAGAC-3’ and 5’-GCCTCTAAGAATTGGACCT-3’ for CAL2; 5’-TGCTACCTGCTCATCAACC-3’ and 5’-CTGCTGCTACTG-3’ for ERO1; 5’-GGTGCTGGTGGTGTACCT-3’ and 5’-CTCTTTTGCGCCCTACAGT-3’ for PID1; 5’-CCAAGCCTTTTAAGAGTG-3’ and 5’-GTAGGGAAGCCCTTGACCTC-3’ for Rb60; 5’-AGTTCTGGATGCTCTCCTA-3’ and 5’-ACACAGTCAAGGAGAG-3’ for DAD1; 5’-GGCTGGCACAATAACCAAT-3’ and 5’-AAGGCTGTCGCCAAAGC-3’ for GPXH (Fischer et al., 2009); 5’-CTGACCACATCACGCCACACT-3’ and 5’-ACATCGAAGCAAGGTAGC-3’ for FKBP12; 5’-CAGAGGTGAAAGGCGGATAC-3’ and 5’-GTGTTGCAATGGAGCTCCAGC-3’ for GSTS1 (Fischer et al., 2012).
Protein preparation and immunoblot analysis

Chlamydomonas cells from liquid cultures were collected by centrifugation (4,000\(g\), 5 min), washed once in 50 mM Tris-HCl (pH 7.5) buffer, and resuspended in a minimal volume of the same solution. Cells were lysed by two cycles of slow freezing to -80°C followed by thawing at room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15,000\(g\), 15 min) in a microcentrifuge at 4°C. For immunoblot analyses, total protein extracts (30 µg) were subjected to 15% SDS-PAGE, and then transferred to nitrocellulose membranes (Bio-Rad, 162-0115). Anti-CrATG8 (Perez-Perez et al., 2010) and secondary antibodies were diluted 1:2,500 and 1:10,000, respectively, in phosphate-buffered saline containing 0.1% Tween 20 (Applichem, A4974) and 5% milk powder. The ECL-Plus immunoblotting detection system (GE Healthcare, RPN2132) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Sigma-Aldrich, A6154). Anti-FKBP12 antibody was diluted 1:3,000 and used as loading control as described previously (Crespo et al., 2005). Proteins were quantified with the Coomassie Brilliant Blue dye-binding method as described by the manufacturer (Bio-Rad, 500-0006).

Glutathione determination

Chlamydomonas cells were collected by centrifugation (5000\(g\), 5 min), washed once in 50 mM sodium phosphate (pH 7.5) buffer, resuspended in 0.2 N HCl and lysed by two cycles of frozen/thawed at -80°C. Crude extracts were cleared by centrifugation at 15000\(g\) for 20 min at 4°C. 500 µl of sample were neutralized by
adding 50 μl of 50 mM NaH$_2$PO$_4$ (pH 7.5) and 0.2 N NaOH to a final pH between 5 and 6. The neutralized sample was directly used for measuring total glutathione (reduced (GSH) plus oxidized (GSSG) glutathione) by the recycling assay initially described by Tietze (Tietze, 1969) and adapted from Queval and Noctor (Queval and Noctor, 2007). The method relies on the GR (Glutathione Reductase)-dependent reduction of 5,5’-dithiobis(2-nitro-benzoic acid) (DTNB; Sigma, D8130). Oxidized glutathione was measured after treatment of neutralized sample with 10 mM 4-vinylpyridine (VPD, Sigma V320-4) for 30 min at 25°C. To remove excess VPD, the derivatized sample was centrifuged twice at 15000g for 20 min at 4°C. To measure total glutathione or GSSG, sample was added to a mix containing 120 mM NaH$_2$PO$_4$ (pH 7.5), 300 μM DTNB, 500 μM NADPH, 1 mM EDTA (pH 8), 1 U/ml GR (Sigma, G3664), and DTNB reduction was measured at 412 nm. Different GSH (Sigma, G4251) concentrations ranging from 0 to 5 μM were used as standards.

**Viability assay**

Cell viability was estimated by determining the percentage of Chlamydomonas cells that excluded Evans blue dye (Sigma, E2129), which only stains nonviable cells. 450 μl of Chlamydomonas cells were incubated with 0.1% Evans blue for 5 min, washed once with 500 μl of TAP medium and resuspended in an equal volume of TAP medium. Cells were examined in a phase contrast microscope to visualize uptake of the dye.
Fluorescence microscopy

Chlamydomonas cells were fixed and stained for immunofluorescence microscopy as previously described (Diaz-Troya et al., 2008; Perez-Perez et al., 2010). Affinity-purified polyclonal anti-ATG8 was used as primary antibody at 1:500 dilution. For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500; Sigma-Aldrich, F4890) 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). For the comparative analysis of the fluorescein isothiocyanate signal from different samples, the same acquisition time was fixed. Immunofluorescence signals in individual cells were quantified using the ImageJ software.
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FIGURE LEGENDS

Figure 1. Tunicamycin triggers autophagy and ER stress in Chlamydomonas. Log phase cells grown in TAP medium were treated with 5 µg/ml tunicamycin (tun) and samples were taken at the indicated times and processed for expression analysis of ATG8 (A), CAL2, ERO1, PDI6 and Rb60 (C) by qPCR or western blot (B). Thirty micrograms of total extracts were resolved by 15% SDS-PAGE followed by western blotting with anti-ATG8 and anti-FKBP12 antibodies. Values are means of three independent experiments.

Figure 2. Tunicamycin triggers oxidative stress. (A), Expression analysis of GSTS1 and GPXH genes by qPCR in cells treated with 5 µg/ml tunicamycin at the indicated times. (B), Intracellular pools of reduced (GSH) and oxidized (GSSG) glutathione were determined in Chlamydomonas cells treated with 5 µg/ml tunicamycin (tun) or drug vehicle (control); a 100% glutathione pool corresponds to 837 ± 58 and 744 ± 64 pmoles per million cells for control and tunicamycin-treated cells, respectively. (C), GSH/GSSG ratio in tunicamycin-treated cells compared to untreated cells. Values are means of three independent experiments. "*", Differences were significant at P<0.05 according to Student’s t-test.

Figure 3. DTT triggers autophagy and ER stress in Chlamydomonas. Log phase cells grown in TAP medium were treated with 2.5 mM DTT and samples were taken at the indicated times for qPCR (A), western blot (B) or immunofluorescence (C) analysis of ATG8. Immunofluorescence images correspond to 6 h of treatment. In addition to ATG8, expression of the UPR-regulated genes CAL2, ERO1, PDI6 and
Rb60 (D) or the oxidative stress-related genes GSTS1 and GPXH genes (E) was analyzed by qPCR. Values are means of three independent experiments.

**Figure 4.** GSH partially prevents ER stress-induced autophagy. Log phase cells grown in TAP medium were treated with 2.5 mM DTT (A) or 5 µg/ml tunicamycin (tun) (B) alone or in combination with 10 mM GSH. After 8 h of treatment, cells were processed for expression analysis of ATG8 and PDI6 genes by qPCR. Values are means of three independent experiments. **“*”** Differences were significant at $P<0.05$ according to Student’s t-test between DTT and DTT/GSH or tun and tun/GSH. (C, D), Western blot analysis of ATG8 and FKBP12 proteins in log phase cells treated with 5 µg/ml tunicamycin (tun), 5-10 mM GSH or 2 mM ascorbate (asc) or combinations of these compounds. Thirty micrograms of total extracts were resolved by 15% SDS-PAGE followed by western blotting with anti-CrATG8 and anti-CrFKBP12 antibodies.

**Figure 5.** GSH decreases tunicamycin-induced cellular accumulation of ATG8 in Chlamydomonas. (A), Immunolocalization of ATG8 in Chlamydomonas cells treated for 8 h with 5 µg/ml tunicamycin (tun) alone or in combination with 10 mM GSH. Scale bar, 5 µm. (B), Quantification of the immunofluorescence signal detected in individual cells from the experiment described in (A). For each condition, a minimum of 100 individual cells was analyzed using ImageJ software. **“***”** Differences were significant at $P<0.01$ according to Student’s t-test between tun and tun/GSH.
Figure 6. GSH reduces the toxicity of tunicamycin in Chlamydomonas. (A) Log phase cells grown in TAP medium were treated with tunicamycin (5 µg/ml; tun), GSH (10 mM) or both compounds in combination. Untreated cells were used as control. Cell viability was determined by Evans blue staining at the indicated times. Results are means of four independent experiments. (B) Cells were subjected to 10-fold serial dilutions and spotted onto TAP plates containing the indicated concentrations of tunicamycin (tun) and GSH. Plates were grown at 25°C under continuous illumination for 5 days. (C) Expression analysis of the DAD1 gene by qPCR. Log phase cells were treated with 5 µg/ml tunicamycin (tun) alone or in combination with 10 mM GSH for 48 hours and then processed for RNA isolation and analysis. Values are means of three independent experiments. "***" Differences were significant at P<0.01 according to Student’s t-test between tun and tun/GSH.

Figure 7. The sor1 mutant displays increased autophagy in response to ER stress. (A) Log phase cw15 and sor1 cells were treated with tunicamycin (5 µg/ml) and at the indicated times samples were taken and processed for western blot analysis. (B) Expression analysis of ATG8 and GSTS1 genes by qPCR. Log phase cw15 and sor1 cells were treated with tunicamycin (5 µg/ml) for 6 hours and subsequently subjected to RNA isolation and analysis. Values are means of three independent experiments.
Figure 8. Control of autophagy by ER stress and ER-derived ROS in Chlamydomonas. Tunicamycin (tun) treatment results in toxic accumulation of unfolded proteins in the ER, leading to autophagy activation as a defensive mechanism. ER stress also increases ROS production in the ER via de oxidoreductase ERO1, which contributes to the upregulation of autophagy. The antioxidant properties of GSH counterbalance ROS signaling to the autophagic machinery but do not abrogate ER stress, resulting in partial inactivation of autophagy.
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