RESEARCH ARTICLE

Channelrhodopsin-1 Phosphorylation Changes with Phototactic Behavior and Responds to Physiological Stimuli in Chlamydomonas

Michaela Böhm\textsuperscript{a, 1}, David Bonessa\textsuperscript{a, 1}, Elisabeth Fantisch\textsuperscript{a}, Hanna Erhard\textsuperscript{a}, Julia Frauenholza, Zarah Kowalzyka, Nadin Marcinkowskia, Suneel Kateriyab, Peter Hegemann\textsuperscript{c}, and Georg Kreimer\textsuperscript{a, 2}

\textsuperscript{a} Department of Biology, Friedrich-Alexander University, 91058 Erlangen, Germany
\textsuperscript{b} School of Biotechnology, Jawaharlal Nehru University, 110067 New Delhi, India
\textsuperscript{c} Institute for Experimental Biophysics, Humboldt University, 10115 Berlin, Germany

\textsuperscript{1} These authors contributed equally to this work.
\textsuperscript{2} Corresponding Author: georg.kreimer@fau.de

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One sentence summary: Rapid changes in the phosphorylation status of the light receptor ChR1 help the motile green alga \textit{Chlamydomonas reinhardtii} adapt its phototactic sensitivity to varying environmental conditions.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Georg Kreimer (georg.kreimer@fau.de).

ABSTRACT

The unicellular alga Chlamydomonas (\textit{Chlamydomonas reinhardtii}) exhibits oriented movement responses (phototaxis) to light over more than three log units of intensity. Phototaxis thus depends on the cell’s ability to adjust the sensitivity of its photoreceptors to ambient light conditions. In Chlamydomonas, the photoreceptors for phototaxis are the channelrhodopsins ChR1 and ChR2; these light-gated cation channels are located in the plasma membrane. Although ChRs are widely used in optogenetic studies, little is known about ChR signaling in algae. We characterized the \textit{in vivo} phosphorylation of ChR1. Its reversible phosphorylation occurred within seconds as a graded response to changes in the light intensity and ionic composition of the medium and depended on an elevated cytosolic Ca\textsuperscript{2+} concentration. Changes in the phototactic sign were accompanied by alterations in the phosphorylation status of ChR1. Furthermore, compared with the wild type, a permanently negative phototactic mutant required higher light intensities to evoke ChR1 phosphorylation. C-terminal truncation of ChR1 disturbed...
its reversible phosphorylation, whereas it was normal in ChR2-knockout and eyespot-assembly mutants. The identification of phosphosites in regions important for ChR1 function points to their potential regulatory role(s). We propose that multiple ChR1 phosphorylation, regulated via a Ca^{2+}-based feedback loop, is an important component in the adaptation of phototactic sensitivity in Chlamydomonas.

INTRODUCTION

Motile green algae have diverse photoreceptors and downstream signaling pathways that allow them to monitor changes in the quality and intensity of ambient light and alter their physiological activities and developmental processes accordingly. To optimize its position in the water column, the green alga Chlamydomonas has well-defined movement responses toward or away from the light source, known as positive and negative phototaxis, respectively. One important prerequisite for highly precise phototactic behavior is the presence of a directional light-sensitive organelle, the so-called eyespot. The functional green algal eyespot contains specialized components in the chloroplast, cytosol, and plasma membrane (Kreimer, 2009). Whereas the chloroplast-localized components mainly increase the sensitivity and directionality of photoreception, the plasma membrane component harbors two type-1 rhodopsins, named Channelrhodopsin-1 (ChR1) and ChR2. Both function directly as blue-green light-gated cation channels that depolarize the plasma membrane in the eyespot region on a millisecond time scale after light excitation (Nagel et al., 2002, 2003; Deisseroth and Hegemann, 2017). Signal transduction that initiates the unoriented photoshock response at high light intensities occurs electrically via ChR-mediated membrane depolarization and secondary voltage-sensitive flagellar Ca^{2+}-channels. By contrast, the signaling cascade that initiates flagellar motility responses leading to a phototactic behavior is still unknown.

Both ChR1 and ChR2 function as receptors for phototaxis. ChR1 is more abundant than ChR2 in most vegetative Chlamydomonas strains, although the ratio of the two proteins varies substantially among strains (Sineshchekov et al. 2002, Govorunova et al., 2004; Berthold et al., 2008; Sineshchekov and Spudich, 2005; Sineshchekov et al., 2009; Greiner et al., 2017). Mass spectrometry (MS) analysis of the eyespot revealed that both ChRs are phosphorylated. Only a single phosphorylation site
was detected for ChR2, whereas ChR1 possess at least three sites in its C-terminal region. Other MS approaches revealed the presence of protein kinases and two PP2C protein phosphatases in the eyespot (Schmidt et al., 2006; Wagner et al., 2008; Wang et al., 2014). Multiple phosphorylation of rhodopsins in vertebrates and invertebrates plays a broad and central role in various visual functions, ranging from the regulation of single photon responses to adaptation (e.g. Arshavsky, 2002; Lee et al., 2004; Fu and Yau, 2007). Detailed information about the physiological relevance of ChR1 phosphorylation in Chlamydomonas is missing. The stimuli that induce changes in ChR1 phosphorylation and the dynamics of those changes remain unclear.

We therefore conducted an in vivo analysis of ChR1 phosphorylation in Chlamydomonas. Signal initiation within the eyespot and transduction toward the flagella occur rapidly, as they must correlate with the cell’s rotational speed of ~1–2 Hz. Furthermore, protein phosphorylation events are usually highly dynamic. Sensitive and rapid methods such as various gel-based methods and MS are essential for phosphorylation analysis (reviewed in: Newmann et al., 2014; Kinoshita et al., 2015; Silva-Sanchez et al., 2015). Phos-tag-SDS-PAGE allows phosphorylated and non-phosphorylated forms of a protein to be separated on a gel (Kinoshita et al., 2006, 2015). This method in combination with appropriate antisera allows qualitative and, at least, semi-quantitative time-course profiling of rapid phosphorylation events in soluble and membrane proteins, even in crude extracts (e.g. Aoki et al., 2011; Medzihradszky et al., 2013; Hosokawa et al., 2015; Longoni et al., 2015; Kinoshita et al., 2016). We therefore performed a detailed in vivo analysis of ChR1 phosphorylation using both Phos-tag and MS approaches.

Our first goal was to gain insights into the dynamics of ChR1 phosphorylation and the stimuli that initiate changes in ChR1 phosphorylation. We were also interested in the physiological function(s) of ChR1 phosphorylation. Chlamydomonas exhibits phototactic orientation over an enormous range of green light intensities (~10^{15} to >10^{21} photons m^{-2} s^{-1}), resulting in a positive or negative phototactic response. The phototactic behavior as well as the rapid, light-induced photoreceptor currents must therefore be under complex adaptational control. Accordingly, the currents undergo a fast decline during illumination (Morel-Laurens, 1987; Hegemann and Bruck, 1989; Govorunova et al., 1997;
Sineshchekov and Govorunova, 2001; Hegemann and Berthold, 2009). We thus asked whether adaptational phenomena and changes in phototactic behavior are linked to ChR1 phosphorylation. We were also interested in learning which phosphatase(s) and kinases are involved and how photoreceptor and eyespot-assembly mutations affect this response.

Our results identified novel ChR1 phosphorylation sites (phosphosites) and demonstrated that ChR1 phosphorylation in vivo is a fast, reversible process that occurs in a graded manner in response to changes in light intensity and the extracellular H⁺ and K⁺ concentrations. Furthermore, we found that ChR1 phosphorylation is prone to rapid adaptation and depends on changes in the intracellular free Ca²⁺ concentration. In conjunction with our findings that changes in phototactic behavior are accompanied by changes in the phosphorylation status of ChR1, and that higher light intensities are needed to induce ChR1 phosphorylation in a permanent-negative phototactic mutant, our data strongly suggest that multiple ChR1 phosphorylation is a mid-term regulatory element for adaptation of the phototactic sensitivity of Chlamydomonas to variations in light conditions.

RESULTS

ChR1 is Phosphorylated at Multiple Sites

Theoretical predictions of the number of phosphosites in ChR1 range from eight (score: 0.9) to 49 (score: 0.5; NetPhos 3.1; Blom et al., 1999); however, our previous MS experiments with isolated eyespots identified only three sites: Ser₃₅₈, Thr₃₇₃, and Ser₃₇₆ (Wagner et al., 2008). Therefore, it is possible that some unstable sites were lost during eyespot isolation and subsequent phosphopeptide enrichment. In the present study, we used a shorter protocol to detect the phosphosites (detailed in the Methods). Table 1 and Figure 1 summarize the phosphosites of ChR1 that we identified in four independent eyespot isolations. All phosphopeptides along with their charge and Xcorr values are listed in Supplemental Data Set 1. All of the sites are located towards the cytosol and are restricted to Ser or Thr residues. Four of the phosphosites were located within the five sequence contexts predicted with scores >0.97 by NetPhos 3.1 (Figure 1;
Supplemental Table 1). In addition to the three known phosphosites within the C-terminus close to the seventh transmembrane domain (TMD), we identified novel sites further toward its end (AS positions 658 – 678). We detected more novel sites in the intracellular vestibule between TMDs one and two, which are part of the cation-conducting pathway (Kato et al., 2012), and in the first cytoplasmic loop, which overlies the vestibule (Figure 1).

While the residues in the cytoplasmic loop and the C-terminus are probably easily accessible to kinase(s) and phosphatase(s), the situation for Ser\textsubscript{102}, as part of the inner gate of ChR1, is different. We cannot rule out the possibility that ChR1 underwent structural changes during the isolation of the eyespots, leading to unusual phosphorylation events. Such effects appeared to be minimal, however, as we otherwise only detected freely accessible cytoplasmic phosphosites. Sequence analysis of 29 ChRs from different Chlorophyta revealed that one of the phosphosites, Thr\textsubscript{117}, is highly conserved, with only one of the 29 ChRs showing a Ser residue instead of Thr at that site. The other two phosphosites in that region, Ser\textsubscript{102} and Ser\textsubscript{116}, are less conserved (Kato et al., 2012; Supplemental Figure 1A). The phosphosites located near the C-terminus of ChR1 are also well conserved (Supplemental Figure 1B and 1C).

Phos-Tag-SDS-PAGE Separates Different Phosphorylated ChR1 Forms

First, we determined whether we could separate and detect different ChR1 forms by Phos-tag-SDS-PAGE followed by immunoblot analysis. The specificity of the ChR1 serum that we used was demonstrated previously (Supplemental Figures 1 in Berthold et al., 2008 and Trippens et al., 2012). In order to differentiate between phosphorylated and non-phosphorylated forms of ChR1, we treated crude cell extracts with either Lambda Protein Phosphatase (\textlambda-PPase), \textlambda-PPase inhibited by the addition of protein phosphatase inhibitor cocktails (PIC), or \textlambda-PPase buffer. We separated the proteins by standard SDS-PAGE and by Phos-tag-SDS-PAGE in parallel and then probed the blots with anti-ChR1 serum (Figure 2A). Separation by SDS-PAGE produced only one major band in each of the three samples. Separation by Phos-tag-SDS-PAGE produced a single major band in the sample treated with active \textlambda-PPase, but it produced at least
three bands in the other two samples, indicating that different phosphorylated forms were present. The band located at $R_f$ 0.58 corresponded to non-phosphorylated ChR1, whereas the bands at $R_f$ 0.52 and $R_f$ 0.25 represented phosphorylated forms of ChR1.

In isolated green algal eyespot fractions, different kinases are present, and rapid, Ca$^{2+}$-dependent protein phosphorylation occurs in the presence of ATP (Linden and Kreimer, 1995; Schmidt et al., 2006). To further substantiate that the additional protein bands were phosphorylated forms of ChR1, we incubated isolated eyespots in kinase buffer with and without ATP and separated their proteins by Phos-tag-SDS-PAGE (Figure 2B). The samples without ATP produced three major bands with $R_f$ values similar to those observed in the crude extracts; however, the non-phosphorylated form was present only in minor amounts, while the phosphorylated forms dominated with approximately equal intensities. In addition, there were two other bands that moved only a short distance in the resolving gel. The addition of ATP induced a massive increase of the signal $\geq R_f$ 0.16, representing highly phosphorylated ChR1, concomitant with a decrease of the signals at $R_f$ ~ 0.58 and $R_f$ ~ 0.50. These results confirmed that ChR1 phosphorylation could be visualized using our approach.

Next, we asked whether different strains would produce the same pattern. We analyzed cells of six different Chlamydomonas strains at the end of the dark phase (Figure 2C). Standard SDS-PAGE resolved a single band of similar intensity across all the strains, whereas Phos-tag-SDS-PAGE resolved different phosphorylated ChR1 forms in addition to the non-phosphorylated form ($R_f$ ~ 0.60). The positions of the phosphorylated forms on the gels were very similar in all the strains, with $R_f$ ~ 0.57, $R_f$ ~ 0.37, and $R_f \geq 0.35$ corresponding to low-phosphorylated, highly phosphorylated, and hyper-phosphorylated forms, respectively. There were, however, strain-dependent differences in the relative amounts of the different forms (Figure 2C and 2D). Strain CC-4536 had the greatest relative amount of the non-phosphorylated form (54% ± 6%), while strain cw15 had the smallest relative amount of the non-phosphorylated form (19% ± 3%). To characterize the in vivo phosphorylation of ChR1 in more detail, we chose the frequently used strain wt137c and the cell wall-deficient strain cw15 for most experiments.
The Phosphorylation Status of ChR1 Changes in a Light Intensity- and Time-Dependent Manner

First, we asked whether light affects ChR1 phosphorylation. We took cells that were at the end of the dark phase, exposed them to white light of different intensities for 1 min, and then poured them directly into methanol:chloroform (2:1[v/v]). Immunoblot analysis of the precipitated proteins revealed a gradual increase in ChR1 phosphorylation with increasing light intensity (Figure 3A and 3B). The intensity of the non-phosphorylated ChR1 band at \( R_f \sim 0.50 \) decreased continuously with increasing light intensity. In parallel, the band at \( R_f \sim 0.44 \) started to split into faint bands slightly above it (\( R_f \sim 0.37 \)), indicating increasing ChR1 phosphorylation. In addition, starting at a light intensity \( \sim 200 \, \mu\text{mol m}^{-2}\text{s}^{-1} \), bands with \( R_f \) values < 0.15 appeared, indicating the presence of hyper-phosphorylated ChR1 forms. The relative intensity of the hyper-phosphorylated bands gradually increased with increasing light intensity up to the highest light intensity analyzed. By contrast, identical samples separated by standard SDS-PAGE resolved only a single ChR1 band with uniform intensity across light intensities, indicating that the illumination had no effect on the total ChR1 content of the cells (Figure 3C). Quantification of the relative contributions of the different ChR1 fractions from three independent replicates revealed that the first clear changes in the phosphorylation status occurred within 1 min of the start of illumination at white-light intensities of \( 200 \, \mu\text{mol m}^{-2}\text{s}^{-1} \) and above (Figure 3B). In cells taken out of the dark phase, the contributions of the non-phosphorylated and low-phosphorylated forms and of the high-phosphorylated and hyper-phosphorylated forms were more or less equal (56% to 44%). With increasing light intensity, the contribution of the higher-phosphorylated forms gradually increased. At \( 800 \, \mu\text{mol m}^{-2}\text{s}^{-1} \), the highest analyzed light intensity, the higher-phosphorylated forms accounted for 73% of the total ChR1, while the non-phosphorylated and low-phosphorylated forms accounted for only 27%.

Changes in the relative abundances of the phosphorylated ChR1 forms were also evident at lower light intensities (e.g. \( 60 \, \mu\text{mol m}^{-2}\text{s}^{-1} \)), although they occurred more slowly (Figure 3D). Despite clear differences between the cw15 and wt137 cells in the relative starting amounts of the phosphorylated ChR1 forms, the positions of the different forms on the gel and the time course of the changes in the relative amounts of
the different forms were similar (Figure 3D). In both strains, the relative amount of the
heavily phosphorylated ChR1 forms (≥ Rf 0.20) increased during the first 30 min after
the onset of illumination. Within the next 90 min, the relative amounts of the non-
phosphorylated and low-phosphorylated forms started to increase again. After 4 h, the
phosphorylation level was similar to that directly prior to the onset of illumination.
Separation by standard SDS-PAGE revealed that at a low light intensity (60 μmol m\(^{-2}\)
s\(^{-1}\)), there were no changes in the total ChR1 levels during the observation period
(Figure 3E). In summary, light-induced changes in ChR1 phosphorylation were evoked
not only by sudden and intense light stimulation but also by continuous stimulation with
low-intensity light on longer time scales (see also Figure 12C).

Next, we analyzed how rapidly the changes in ChR1 phosphorylation occur at a
given light intensity and whether they are reversed when the cells return to darkness.
We performed time-course experiments at high and intermediate light intensities (Figure
4). At 800 μmol m\(^{-2}\) s\(^{-1}\), the first changes in ChR1 phosphorylation were visible between
5 s and 15 s after the onset of the illumination. During that time, there was a clear
increase in the intensities of the highly phosphorylated and hyper-phosphorylated ChR1
bands at Rf ≥ 0.2. Within the next 45 s, the non-phosphorylated and low-phosphorylated
bands at Rf 0.51 and 0.45, respectively, disappeared, and the intensities of the higher-
phosphorylated bands further increased. There were no additional changes during the
next minute of illumination, indicating that the phosphorylation had reached a plateau
(Figure 4A and 4C). At 150 μmol m\(^{-2}\) s\(^{-1}\), the changes in ChR1 phosphorylation were
less pronounced, and the time course was considerably slower: even after 2 min, the
non-phosphorylated and low-phosphorylated bands were still visible, and there was no
significant increase in the hyper-phosphorylated forms (Figure 4D and 4F). These light-
induced changes in the phosphorylation status of ChR1 were reversed upon return to
darkness. The time required for the reversal depended on the magnitude of the induced
changes and was considerably less after intermediate light intensity than after high light
intensity (Figure 4C and 4F). After illumination at 150 μmol m\(^{-2}\) s\(^{-1}\), it took only 5 min of
darkness for the ChR1 forms to approach the levels observed prior to illumination, and
complete return to pre-illumination levels occurred within 15 min (Figure 4F). By
contrast, after illumination at 800 μmol m\(^{-2}\) s\(^{-1}\), the ChR1 forms returned to pre-
illumination levels more slowly, although they still almost reached pre-illumination levels within 15 min (Figure 4C). Control immunoblot analysis by standard SDS-PAGE revealed no variation in the amount of the single ChR1 band among the different samples over the observation period (Figure 4B and 4E).

In natural environments, the light intensity varies, and Chlamydomonas cells respond with well-adjusted phototactic responses. We asked whether variations in the light intensity during continuous illumination affect the phosphorylation status of ChR1. We illuminated cw15 cells for 1 h at 60 µmol m\(^{-2}\) s\(^{-1}\) and then shifted them to a light intensity of 5 µmol m\(^{-2}\) s\(^{-1}\) or 250 µmol m\(^{-2}\) s\(^{-1}\). We kept control cells at a constant 60 µmol m\(^{-2}\) s\(^{-1}\). We sampled the cells 15 min and 30 min after the shift in intensity and analyzed them by Phos-tag-SDS-PAGE and standard SDS-PAGE (Figure 5). The phosphorylation status of ChR1 in control cells remained at 60 µmol m\(^{-2}\) s\(^{-1}\), did not change, and was dominated by the highly phosphorylated and hyper-phosphorylated forms (Figure 5A and 5C). By contrast, the shifts to higher or lower light intensity induced changes in the relative amounts of the different forms. A shift to the higher intensity further reduced the amounts of the non-phosphorylated and low-phosphorylated ChR1 forms (Figure 5D and 5F), whereas a shift to the lower light intensity increased the amounts of these forms (Figure 5G and 5I). The total amount of ChR1 was stable at all intensities during the observation period (Figure 5B, 5E, and 5H).

In summary, light-induced changes in ChR1 phosphorylation (i) were fast, light-intensity-dependent, and reversible and (ii) also occurred under continuous illumination in response to variation in the light intensity. The basic requirements for effective light-dependent regulation of ChR1 phosphorylation were thus fulfilled.

**ChR1 Phosphorylation is Prone to Rapid Adaptation**

Desensitization of the cell is essential for allowing precise phototactic orientation over a broad range of light intensities. On the one hand, desensitization in Chlamydomonas is linked to the excitability of the plasma membrane (Govorunova et al., 1997). On the other hand, rapid and reversible photoreceptor phosphorylation might be an additional element of the desensitization process, as they are in other visual systems.
(Arshavsky, 2002; Lee et al., 2004). We asked whether pre-illumination affects light shock-induced ChR1 hyper-phosphorylation. We illuminated dark-adapted cells for different periods of time with low-intensity (25 µmol m⁻² s⁻¹; background) white light and measured ChR1 phosphorylation immediately (control) or after a light shock of 60 s with high-intensity (800 µmol m⁻² s⁻¹) light (Figure 6A). In the control cells, the background illumination triggered no hyper-phosphorylation response; however, after approximately 90 s, the relative proportion of the non-phosphorylated and low-phosphorylated ChR1 forms started to decrease while that of the highly phosphorylated form increased. The highly phosphorylated form dominated after 5 min of exposure to background light (Figure 6A and 6B), confirming that even low-intensity light over a longer time scale leads to changes in the relative abundances of the different ChR1 forms (see also Figure 3D). Furthermore, 25 µmol m⁻² s⁻¹ white light was sufficient to induce adaptation. The hyper-phosphorylation response to light shock gradually decreased with increasing length of pre-exposure to low-intensity light. After 5 min of low-intensity illumination, the light shock evoked no additional phosphorylation (Figure 6A). When we increased the intensity of the background illumination to 150 µmol m⁻² s⁻¹, the time required for the highly phosphorylated form to reach a stable plateau was shortened to ~ 2 min, and the duration of background illumination required for the adaptation to the light shock was shortened also to ~ 2 min (Figure 6C). These results indicate that the ChR1 hyper-phosphorylation response underlies quick and efficient adaptation.

To test whether photosynthetic electron transport or ChR activity itself is involved in adaptation, we used 660 nm (red) and 515 nm (green) light for pre-illumination. At an intensity of 150 µmol m⁻² s⁻¹, the red light alone had no effect on the phosphorylation status of ChR1 during the first 3 min; however, there was a slight increase in the amount of the higher-phosphorylated forms at 5 min (Supplemental Figure 2A). Notably, the red light did not result in significant adaptation to the white-light shock within the observation period. By contrast, the 515 nm light alone evoked a hyper-phosphorylation response and induced adaptation to the white-light shock within 1 min, suggesting that ChR activation might be involved in the adaptation process (Supplemental Figure 2B).
ChR1 activation induces rapid depolarization of the plasma membrane. The desensitization depends on the magnitude of the depolarization rather than on ChR1 bleaching (Govorunova et al., 1997; Berthold et al., 2008). We asked whether the ionic composition of the medium affects ChR1 phosphorylation. We manipulated the medium by varying either the external pH or the external K⁺ concentration. Both treatments affect the membrane potential of Chlamydomonas. Increasing the external pH from acidic to basic values leads to hyperpolarization of the plasma membrane, whereas increasing the K⁺ concentration leads to depolarization of the plasma membrane (Malhotra and Glass, 1995). Both treatments influenced the phosphorylation status of ChR1 (Figure 7). In the dark, an increase in the external pH from 5 (≈ -90 mV) to 9 (≈ -135 mV) or a decrease in the external K⁺ concentration induced an increase in the relative abundance of the non-phosphorylated and low-phosphorylated ChR1 forms (Figure 7A, 7B, and 7C). This finding indicates that membrane hyperpolarization reduced the amount of ChR1 phosphorylation independently of how the hyperpolarization was achieved. At pHₑ 5, which means that the plasma membrane is more depolarized, the non-phosphorylated and low-phosphorylated forms accounted for only ~5% and 10% of the total ChR1, respectively, while the highly phosphorylated form dominated. The relative abundances of these three ChR1 forms changed with increasing pHₑ until they became stable at pHₑ values ≥ 7. At all of the analyzed pHₑ values, light shock induced the total disappearance of the non-phosphorylated and low-phosphorylated forms. Notably, the relative abundance of the hyper-phosphorylated form progressively increased at pHₑ values > 5 (Figure 7A).

In line with these observations, increasing the external K⁺ concentration from 0 mM (≈ -140 mV) to approximately 60 mM (≈ -100 mV) also caused a successive increase in the relative proportion of the higher-phosphorylated ChR1 forms (Figure 7C). At external K⁺ concentrations of ~ 60 mM and above, additional hyper-phosphorylated forms (≥ R₁ 0.21) that were not seen with pHₑ changes appeared while the cells were still in the dark. The intensity of these bands became saturated above ~ 80 mM K⁺. On the other hand, the light shock-induced hyper-phosphorylation response gradually decreased until there was no difference between the illuminated cells and the cells that were kept in the dark (Figure 7C). In conclusion, plasma membrane
depolarization increased ChR1 phosphorylation independently of how the depolarization was achieved.

Next, we asked how rapidly ChR1 hyper-phosphorylation in the dark occurs in response to changes in the external $K^+$ concentration. We exposed cells adapted to a medium without any added $K^+$ to a sudden potassium shock and sampled the cells at the time points indicated in Figure 7D. First, small changes occurred at $30\,s$, and then a clear hyper-phosphorylation response occurred between $60\,s$ and $90\,s$ after the shock. These changes were probably not due to the activation of non-specific kinases, because the phosphorylation status of phototropin was unaffected (Figure 7D). Phototropin is a photoreceptor that also localizes to the eyespot and undergoes autophosphorylation upon activation (Schmidt et al., 2006; Christie et al., 2015). Although it is tempting to attribute changes in the $H^+$ and $K^+$ electrochemical gradients to variations in the plasma membrane potential (Malhotra and Glass, 1995), possible direct or indirect effects of both ions on proteins must also be considered. As in plants, pH and Ca$^{2+}$ signals often act in concert in Chlamydomonas. For example, intracellular acidification leads to a temporary increase in Ca$^{2+}$ influx, stimulates the phosphorylation of kinases, and affects the cytoskeleton (Pan et al., 2004; Wheeler et al., 2008; Liu et al., 2017; Behera et al., 2018).

**ChR1 Phosphorylation Depends on an Increase in Intracellular Ca$^{2+}$**

Photoreceptor currents and phototaxis strictly depend on extracellular Ca$^{2+}$ in Chlamydomonas. Although ectopically expressed ChRs are mainly $H^+$ channels, they mainly conduct $H^+$, $Na^+$, and Ca$^{2+}$ in algae (Holland et al., 1996; Nonnengäßer et al., 1996; Ehlenbeck et al., 2002; Sineshchekov et al., 2009). The reported pH optimum (pH 7.5–8.0) for photoreceptor currents in transformants enriched in either ChR1 or ChR2 point to a different situation in native cells. In algae, beside protons, Ca$^{2+}$ contributes to the photoreceptor currents, and an elevated cytosolic Ca$^{2+}$ concentration in the eyespot region is thought to play an essential role in initiating the signaling cascade(s) toward the flagella (reviewed in Hegemann and Berthold, 2009; Sineshchekov et al., 2009). We thus analyzed the role of Ca$^{2+}$ in ChR1 phosphorylation. For this, we examined the effect of extracellular Ca$^{2+}$ in several different ways: (i) light stimulation of the cells in media with defined Ca$^{2+}$ concentrations, (ii) the addition of excess EGTA 3 min before
illumination, and (iii) the reduction of Ca\(^{2+}\) influx by an increase in the extracellular Ba\(^{2+}\) concentration. Ba\(^{2+}\) carries the photocurrents in Chlamydomonas, but it does not act as a substitute for Ca\(^{2+}\) in intracellular functions (Hille, 1992; Nonnengässer et al., 1996). Reduction of the extracellular Ca\(^{2+}\) concentration down to 50 µM had no effect on light shock-induced ChR1 hyper-phosphorylation (Figure 8A); however, reduction to 20 µM and below abolished the response, although the low-phosphorylated and non-phosphorylated forms still exhibited a clear decline in levels after light shock. The addition of excess EGTA to the medium just prior to illumination inhibited the hyper-phosphorylation response, whereas its addition after illumination had no effect (Figure 8B). Gradual increases in the extracellular ratio of Ba\(^{2+}\) to Ca\(^{2+}\) from 0 to 100 slowed down the light-shock response without blocking it (Supplemental Figure 3).

To further highlight the importance of Ca\(^{2+}\) in the initiation of ChR1 hyper-phosphorylation, we increased the intracellular Ca\(^{2+}\) concentration artificially via two independent mechanisms: extracellular Ca\(^{2+}\) shock and pH shock with acetate. Both treatments are known to cause rapid increases in the cytosolic Ca\(^{2+}\) concentration, although pH shock induces higher increases than extracellular Ca\(^{2+}\) shock (Braun and Hegemann, 1999; Wheeler et al., 2008). pH shock, which leads to complete deflagellation of the cells, induced stronger ChR1 hyper-phosphorylation than Ca\(^{2+}\) shock, even while the cells were still in the dark (Figure 8C and 8D). In response to Ca\(^{2+}\) shock, only a small fraction of the ChR1 shifted toward higher R\(_f\) values compared to the control. These changes were fast, however, and most of them occurred in less than 30 s. By contrast, pH shock induced changes in ChR1 phosphorylation similar to those induced by light shock.

Under low-intensity light, ChR activation is thought to induce the opening of secondary Ca\(^{2+}\) channels, resulting in larger Ca\(^{2+}\) currents in the eyespot region (Kateriya et al., 2004; Sineshchekov et al., 2009). A putative candidate for such a secondary channel was identified recently (Awasthi et al., 2016). We analyzed two independent mutant strains in which the gene for the putative Ca\(^{2+}\) channel was disrupted (Greiner et al., 2017). If the channel is involved in the phototactic signaling cascade, the increase in intracellular Ca\(^{2+}\) concentration in the mutants should be strongly attenuated, which in turn should affect ChR1 phosphorylation. We observed no
significant difference between the mutants and the parental strain CC-3403 (Supplemental Figure 4), indicating that the channel is not part of the signaling cascade.

**Known Effectors of the Phototactic Sign Affect ChR1 Phosphorylation**

Various factors are known to affect the sign of the phototactic orientation of Chlamydomonas. For example, Takahashi and Watanabe (1993) showed that inhibition of linear photosynthetic electron transport by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) reverts the phototactic sign. As a possible mechanism, they suggested altered Ca\(^{2+}\) fluxes across the chloroplast envelope, which are also sensitive to DCMU (Kreimer et al., 1985; Nomura and Shiina, 2014). Furthermore, overexpression of the chloroplast-localized Ca\(^{2+}\)-binding protein CrCAS affects the regulation of the phototactic sign at low light intensities, and photosynthesis appears to quantitatively control the final phototactic behavior (Arrieta et al., 2017; Trippens et al., 2017). We tested the effect of DCMU on ChR1 phosphorylation during the initial illumination period and on the response to an additional high-intensity light shock (Figure 9A). Compared with the control, DCMU treatment without light shock led to the fast appearance of more highly phosphorylated ChR1 forms. This pattern resembled the pattern in untreated, dark-adapted cells after light shock. In parallel, the non-phosphorylated and low-phosphorylated forms (R~1\( \sim 0.62\) and ~0.57) disappeared rapidly. Furthermore, at 1 min after the onset of illumination, a light shock failed to stimulate further ChR1 hyper-phosphorylation in DCMU-treated cells. Even after 5 min, a considerable proportion of ChR1 was hyper-phosphorylated in the DCMU-treated cells (Figure 9A). These results might be explained by a DCMU-induced prolonged increase in cytosolic Ca\(^{2+}\) concentration, which in turn affects the phosphorylation status of ChR1. To test that assumption, we analyzed the effect of DCMU on the light intensity-dependent appearance of hyper-phosphorylated ChR1 forms, which are only seen at elevated Ca\(^{2+}\) concentrations. DCMU caused the hyper-phosphorylated forms to appear at lower light intensities as the illumination time increased (Figure 9B). The hyper-phosphorylated forms appeared in DCMU-treated cells with 200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) illumination, whereas \(\geq 400 \mu\)mol m\(^{-2}\) s\(^{-1}\) illumination was required to make them appear in control cells. After 3 min of illumination, a clear response was even detected with ~100 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) illumination.
The phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine (IBMX), which increases cellular cAMP levels and can directly induce Ca\(^{2+}\) release from intracellular stores, affects the phototactic sign in Chlamydomonas (Usachev and Verkhratsky, 1995; Boonyareth et al., 2009). IBMX provoked ChR1 hyper-phosphorylation without any illumination, reaching saturation at concentrations \(\geq 0.5\, \text{mM}\) (Figure 10A). The solvent alone had no significant effect compared to the control. Time-course analyses at a fixed IBMX concentration of 0.5 mM revealed that the first changes in ChR1 phosphorylation occurred between 30 s and 60 s after the cells were exposed to IBMX (Figure 10B). To assess whether these effects are more likely linked to increases in cAMP levels or to the release of Ca\(^{2+}\) from intracellular stores, we used the membrane-permeant cAMP analog 8-bromoadenosine 3',5'-cyclic monophosphate. Concentrations of the cAMP analog up to 10 mM failed to induce hyper-phosphorylation, suggesting that IBMX likely affected ChR1 phosphorylation via its impact on cytosolic Ca\(^{2+}\) concentration.

The cellular redox balance affects the sign of phototaxis in Chlamydomonas (Wakabayashi et al., 2011). Although the redox balance directly modulates flagellar activity (Wakabayashi and King, 2006), additional targets suggested by Wakabayashi et al. (2011) include the ChRs. To modulate the redox state of the cells, we applied the membrane-permeable compound TEMPOL, which quenches reactive oxygen species (ROS) and reduces oxidative stress, and H\(_2\)O\(_2\), which causes oxidative stress. Increasing concentrations of TEMPOL abolished the response to light shock and did not affect the relative distributions of the phosphorylated and non-phosphorylated ChR1 forms in the dark (Figure 10C). To exclude the possibility that TEMPOL inhibits ChR1 light activation and thus the necessary increase in Ca\(^{2+}\) to induce ChR1 phosphorylation, we analyzed the effect of TEMPOL on K\(^{+}\) shock-induced ChR1 hyper-phosphorylation in the dark. TEMPOL also effectively suppressed that response (Figure 10D). Given that TEMPOL is an effective spin label, it might affect the 10 Cys residues of ChR1. Therefore, we used the same samples to test whether phototropin was activated and autophosphorylated. Light activation of phototropin, which is also present in the eyespot, depends on two functional Cys residues (Schmidt et al. 2006; Christie et al., 2015). TEMPOL had no effect on the phosphorylation status of phototropin in the light or in the dark (Figure 10E). Generalized, non-specific effects therefore seem unlikely. By
contrast, oxidative stress induced ChR1 hyper-phosphorylation. Concentrations of H$_2$O$_2$
between 0.1 mM and 1.6 mM induced the appearance of higher-phosphorylated ChR1
forms in the dark in a concentration-dependent manner (Figure 10 F).

In summary, effectors known to alter the sign of phototaxis changed the
phosphorylation status of one of the primary photoreceptors of the oriented movement
response.

A Pharmacological Approach Suggests the Possible Involvement of PP2C
Phosphatases in ChR1 Dephosphorylation

The light-induced changes in ChR1 phosphorylation were reversible (Figure 4). We asked which protein phosphatase(s) were involved in the reversal and whether
blocking ChR1 dephosphorylation affects phototaxis. Proteomic studies of isolated
Chlamydomonas eyespots identified two putative signaling-related Ser-Thr
phosphatases belonging to the PP2C group (Schmidt et al. 2006). Additional studies
identified one PP2C-like protein and one member of the PP1 group (Eitzinger et al.
2015). We used inhibitors to learn which class of protein phosphatases might be
involved in ChR1 dephosphorylation. To minimize possible unspecific effects, we
restricted the total incubation time with the inhibitors to a maximum of 45 min. The PP2C
inhibitor Sanguinarine blocked ChR1 dephosphorylation in a concentration-dependent
manner (Figure 11A), with clear inhibitory effects at concentrations ≥ 2.5 µM. The
lowest effective concentration (2.5 µM) did not alter the phosphorylation status of ChR1
prior to or directly after light stimulation compared to the DMSO control; however, it
slowed the return to the pre-stimulus status. Increasing Sanguinarine concentrations
resulted in increasing amounts of hyper-phosphorylated ChR1 in the dark and a further
postponement of the return to the pre-stimulus status. Because Sanguinarine can induce
apoptosis (Aburai et al. 2010), we checked the viability of the cells by microscopy.
Concentrations ≤ 5 µM had no visible effects on cell viability within the incubation times
that we used, although higher concentrations induced flagella retraction. Inhibitors of
PP1 and PP2A phosphatases had no effect on ChR1 dephosphorylation (Supplemental
Figure 5).

Manipulation of the Phosphorylation Status of ChR1 Affects Phototaxis
To link ChR1 phosphorylation to phototactic behavior, we analyzed the movement responses of the cells after the treatments described above to increase ChR1 phosphorylation. The blocking of ChR1 dephosphorylation with Sanguinarine led to suppression of the negative phototactic orientation without affecting the swimming speed of the cells (Figure 11B and 11C). The induction of ChR1 phosphorylation with either IBMX or K+ evoked similar effects on the phototactic orientation. IBMX concentrations ≥ 0.1 mM blocked negative phototaxis (Supplemental Figure 6A; Boonyareth et al., 2009). At these concentrations, IBMX also induced ChR1 hyper-phosphorylation in the dark (Figure 10A and 10B). Similarly, increases in the external K+ concentrations up to 60 mM gradually suppressed the rate and magnitude of the negative phototactic orientation. We did not use higher concentrations of K+, because they induced the loss of flagella (Supplemental Figure 6B and 6C). It is possible, however, that the effect of K+ on phototaxis was due to a reduction of the general excitability of the cell rather than to an increase in ChR1 phosphorylation.

To further link changes in ChR1 phosphorylation to changes in phototactic behavior, we used two strains known to be permanently negative phototactic at all light intensities. The mutation(s) in strain CC-4536 are yet unknown. Ide et al. (2016) recently showed that a mutation in AGG1 (encoding a 36 kDa protein of unknown function) is involved in the negative phototactic phenotype in strain CC-124. AGG1 protein is localized to the cell body, whereas the two other members of its protein family localize to the membrane (AGG2) and matrix (AGG3) of the flagella. Mutations in AGG proteins cause negative phototaxis by reversing the beating dominance of the flagella after photo-stimulation compared to wild-type cells. AGG mutations do not affect the Ca2+-dependent flagella dominance, which suggests that the AGG pathway(s) probably functions upstream of this regulatory level Iomini et al., 2006; Wakabayashi et al., 2011; Ide et al., 2016). We compared the phosphorylation status of ChR1 in the two mutant strains after moderate-intensity illumination (60 µmol m⁻² s⁻¹). Strains wt137c and cw15 were used as references (Figure 12A and 12B). There were no clear differences in the phosphorylation patterns between strain CC-124 and strain wt137c, which is AGG1 positive. By contrast, the non-phosphorylated and low-phosphorylated ChR1 forms dominated in strain CC-4536 compared with the other three strains. This result indicates
that ChR1 phosphorylation is less sensitive to illumination in strain CC-4536 than it is in the other strains. We then compared the ChR1 phosphorylation response of strain CC-4536 with that of strain cw15 after a 10 min illumination with various light intensities. Figure 12C shows that higher light intensities were needed to induce changes in ChR1 phosphorylation in strain CC-4536. The total ChR1 levels were similar among the strains (Supplemental Figure 7).

Next, we induced changes in the phototactic behavior of all four strains under constant low-intensity illumination (1 μmol m⁻² s⁻¹) by applying an increasing level of oxidative stress. We treated the cells with increasing H₂O₂ concentrations just prior to the onset of illumination and then allowed the cells to orient for 10 min (Figure 13). Untreated cells of strains wt137c and cw15 were only slightly negatively phototactic, whereas both mutant strains exhibited strong negative phototaxis. The increasing oxidative stress affected this behavior. At H₂O₂ concentrations ≥ 0.8 mM, cells of all four strains displayed increasingly un-oriented or even positive phototactic behavior (Figure 13A and 13C) along with increasing proportions of highly phosphorylated and hyper-phosphorylated ChR1 forms (Figure 13B and 13D).

In summary, changes in the sign of phototactic behavior evoked by different treatments at a fixed light intensity were accompanied by changes in the phosphorylation status of ChR1. Predominant non-phosphorylated or low-phosphorylated ChR1 fractions were associated with negative phototactic behavior. This result is in line with the inhibition of ChR1 hyper-phosphorylation caused by the ROS-quencher TEMPOL (Figure 10C and 10D), which is known to induce negative phototaxis in wt137c cells (Wakabayashi et al., 2011).

**ChR1 Phosphorylation in Eyespot-Assembly and ChR Mutants**

A structurally well-preserved eyespot is important for the sign of phototaxis (Ueki et al., 2016). We asked whether mutants affected in eyespot assembly have altered ChR1 phosphorylation. We first looked at three mutants with altered EYE2, EYE3, and MIN1 proteins, respectively, which localize to different parts of the eyespot and are important for eyespot assembly (Boyd et al., 2011; Figure 14A). MIN1 mediates the close attachment of the plasma membrane and the chloroplast envelope in the eyespot.
region, and cells with defective MIN1 possess small, disorganized eyespots. EYE2 localizes to the chloroplast envelope and is important for assembly of the eyespot globule layer underneath the ChR patch in the plasma membrane. The stroma-localized kinase EYE3 is important for globule biogenesis (Boyd et al., 2011). First, we checked the ChR1 levels in the mutants (Figure 14B). In accordance with Mittelmeier et al. (2008), only the MIN1 mutant had a slightly reduced ChR1 level compared with the corresponding parental strains. Figure 14C shows a typical Phos-tag-SDS-PAGE analysis of the mutants. After a 1 min light shock, all three mutants exhibited a massive increase in the hyper-phosphorylated ChR1 forms comparable to that in the parental strains. Thus, disturbances in the correct placement of the components of the eyespot in the chloroplast and the plasma membrane did not significantly affect ChR1 phosphorylation.

Next, we asked whether deletion of ChR2 or truncation of the C-terminus of ChR1 affects the phosphorylation status of ChR1. First, we examined the ChR1 expression levels in two mutants, one with ChR2 deletion (ΔChR2) and one with C-terminal truncation (ChR1Δct), and in the parental strain (Figure 14D). The ChR1 expression level in the ΔChR2 mutant was unchanged compared with that in the parental strain, whereas it was severely reduced in the ChR1Δct mutant. The ChR1Δct mutant has a stop codon in exon 14, leading to a ChR1 with a predicted molecular mass of 68.25 kDa (Greiner et al., 2017). Even with a higher protein load and longer developing times, the ChR1Δct mutant produced only a weak ChR1 signal at 67 kDa. Because our polyclonal serum was raised against the C-terminal part of ChR1, we could not differentiate between a reduced ChR1 level and reduced antiserum binding to the residual C-terminal region. Also, the two mutants exhibited different light-shock responses (Figure 14E). Whereas the ΔChR2 mutant exhibited clear, light-induced ChR1 hyper-phosphorylation, the ChR1Δct mutant did not exhibit any light-induced changes in ChR1 phosphorylation. In the dark, only higher-phosphorylated forms of the truncated ChR1 were present. To exclude the possibility that the missing ChR1-phosphorylation response was due to a change in the conductance of the truncated ChR1, which might result in a reduced local Ca$^{2+}$ increase, we artificially increased the cytosolic level of Ca$^{2+}$ in the dark by acetate shock, which is capable of inducing ChR1 hyper-phosphorylation in the dark (see Figure
The acetate shock induced a transient increase in levels of the ChR1 forms at $R_f \geq 0.21$ in the parental strain, but it induced only a very weak response in the ChR1Δct mutant (Figure 14E).

**DISCUSSION**

Detailed knowledge about the structure and properties of algal ChRs in ectopic expression systems has accumulated in recent years due to their central role in optogenetic studies (Deisseroth and Hegemann, 2017). By contrast, relatively little is known about the properties and regulation of ChRs in algae, except for the well-studied photoreceptor currents (Sineshchekov and Govorunova, 2001; Hegemann and Berthold, 2009; Sineshchekov et al., 2009). More knowledge of the characteristics of ChRs is crucial for a better understanding of phototaxis and its integration and impact on the diverse light-dependent physiological responses of motile algae. One key feature of many photoreceptors across the taxonomic kingdoms is multiple (auto)phosphorylation, which modulates the signaling activity of photoreceptors in different ways. Well-known examples include animal rhodopsins and plant red-light and blue-light receptors (Arshavsky, 2002; Maeda et al., 2003; Fu and Yau, 2007; Medzihradszky et al., 2013; Christie et al., 2015). ChR1 and ChR2, the major eyespot-localized photoreceptors for light-induced movement responses in Chlamydomonas, are phosphoproteins (Wagner et al., 2008; Wang et al., 2014). However, neither the dynamics or the physiological relevance of ChR1 and ChR2 phosphorylation have been studied in detail.

Our results revealed a dynamic, rapid response of the phosphorylation status of ChR1 to changes in a variety of key physiological stimuli, including light intensity, intracellular $Ca^{2+}$ concentration, and redox balance. Changes in phototactic behavior were associated with the phosphorylation status of ChR1. Desensitization of light shock-induced ChR1 hyper-phosphorylation was rapid and dependent on the intensity and quality of the light. Based on these findings, we propose that multiple ChR1 phosphorylation has a regulatory function in the adaptation of the phototactic sensitivity of Chlamydomonas. The phosphosite positions within ChR1 support our proposal. We detected phosphorylated Ser and Thr residues in the vestibule of the cation conduction
phosphorylated ChR1 correlates with an adapted, less sensitive photoreceptive state,

Irrespective of the exact mechanism(s), targeting of the primary photoreceptors of
the movement response is an effective way to integrate different physiological signals.
and that an intermediate or lower level of phosphorylated ChR1 correlates with a more sensitive photoreceptive state. Our results support this hypothesis in several ways: (i) the relative proportions of non-phosphorylated and low-phosphorylated ChR1 forms in the dark increased under conditions in which the membrane potential of the cell became increasingly hyperpolarized (Figure 7); (ii) the proportions of the non-phosphorylated and low-phosphorylated forms gradually decreased with increasing light intensity and illumination time (Figures 3 and 12C); (iii) increased desensitization to light shock was accompanied by increased proportions of higher-phosphorylated ChR1 forms (Figures 4 and 6); (iv) induction of ChR1 phosphorylation suppressed negative phototaxis (Figures 11 and 13; Supplemental Figure 6); (v) ROS-induced reversion of negative phototactic orientation to low-intensity light was accompanied by increased ChR1 phosphorylation (Figure 13); and (vi) the ROS-quencher TEMPO, which induces negative phototaxis in wt137c cells, inhibited the ChR1 hyper-phosphorylation response (Figure 10; Wakabayashi et al., 2011). In the context of integrating the cells’ phototactic sensitivity, it is noteworthy that changes in ChR1 phosphorylation strictly depend on Ca^{2+}, but not on light. ChR-induced current desensitization in Chlamydomonas is linked to depolarization of the plasma membrane (Govorunova et al., 1997), which under most physiological circumstances causes Ca^{2+} influx. Hence, a Ca^{2+}-based feedback loop for ChR1 phosphorylation would allow regulation of ChR1 sensitivity not only within the initial and steady-state light phases but also prior to illumination. Thus, in the primordial visual system of a unicellular green alga, Ca^{2+}-induced changes in the phosphorylation status of one of the major photoreceptors appear to be a central factor in the regulation of light sensitivity, as is the case for visual phototransduction in animals (Arshavsky, 2002; Senin et al., 2013).

Inhibition of photosynthetic electron transport and manipulation of the cellular redox balance affected the sign of phototaxis. These observations are suggestive of a relationship between photosynthesis and phototaxis, which is thought to be indirect via the modulation of cytosolic Ca^{2+} and redox homeostasis (e.g. Takahashi and Watanabe, 1993; Wakabayashi et al., 2011; Arrieta et al., 2017; Trippens et al., 2017). Cyclic nucleotide levels might also play a role in conjunction with Ca^{2+} in controlling the phototactic sign and the photoacclimation of Chlamydomonas to high-intensity light.
Our data show that changes in the phosphorylation status of ChR1 are a unifying theme in a variety of treatments that induce a switch in phototactic behavior, with Ca$^{2+}$ serving as a potential common secondary signal. Ca$^{2+}$ is known to be involved in the regulation of the sign, strength, and absolute adaptation level of the phototactic response. Ca$^{2+}$ might also modulate the signal transduction chain(s) from the eyespot to the flagella, where it has well-studied, essential functions in phototactic steering (e.g. Morel-Laurens, 1987; Dolle et al., 1987; Hegemann and Bruck, 1989; reviewed by Witman, 1993; Hegemann and Berthold, 2009; Kreimer, 2009). The details of Ca$^{2+}$ action within the eyespot are still largely unknown. Our data suggest that reversible ChR1 phosphorylation is one important target. In principle, both kinase(s) and phosphatase(s) might be regulated by Ca$^{2+}$. Nothing is yet known about the kinase(s), except that phototropin and casein kinase 1 are not involved (Supplemental Figure 8). Ca$^{2+}$-dependent protein kinases are present in Chlamydomonas and its extended eyespot proteome (Liang and Pan, 2013; Eitzinger et al., 2015), and Ca$^{2+}$-dependent phosphorylation/dephosphorylation has been reported in isolated eyespots (Linden and Kreimer, 1995). Among the involved phosphatase(s), our proteomic and pharmacological evidence favors the PP2C phosphatases (Figure 11; Supplemental Figure 5; Schmidt et al., 2006), which can be regulated by Ca$^{2+}$/calmodulin in plants (Takezawa, 2003). Unequivocal identification of the involved kinase(s) and phosphatases is of paramount importance for future studies. Still, other regulatory possibilities, especially ones related to the cytoplasmic redox balance, must be considered. Although H$_2$O$_2$ is known to activate plant Ca$^{2+}$ channels (Mori and Schroeder, 2004), and the ROS-quencher TEMPOL can lead to a reduced intracellular Ca$^{2+}$ level or membrane hyperpolarization in animal systems (Kuneš et al., 2004; Xu et al., 2006), direct effects on the kinase(s) and/or phosphatase(s) cannot be excluded. Greater knowledge of the effects of such treatments on Ca$^{2+}$ levels under steady-state illumination in Chlamydomonas and on other posttranslational modifications of the involved proteins will be helpful.

A central finding of our study is the potential role of ChR1 phosphorylation in the adaptation of the phototactic sensitivity of Chlamydomonas. How does ChR1 phosphorylation fit into the current framework of phototactic sensitivity adaptation?
Chlamydomonas possesses several different adaptive mechanisms for its visual system, covering time scales ranging from milliseconds to days. The fastest known mechanism is the gradual inactivation of ChR photocurrents in continuous light, which involves photo-conversion between the all-trans and 13-cis retinal isomers and changes in the contribution of the two ChR cycling states (syn and anti). These states differ in their conductance and ion selectivity. The syn-cycle open state is less permeable to cations and contributes to the photocurrent decline under prolonged illumination (Hegemann et al., 2005; Bruun et al., 2015). On longer time scales, light affects the amount of ChR1 in the cell by regulating both the mRNA and protein level. Already 1 h after the night/day transition, the ChR1 mRNA level is reduced by ~96% (Zones et al., 2015). Furthermore, the starting ChR1 content at the onset of the day depends on the light intensity experienced by the cells during the previous day, and the eyespot size depends on the intensity and duration of the illumination (Trippens et al., 2012). Light-induced ChR1 phosphorylation and its desensitization fall in an intermediate time scale, ranging from a few seconds to minutes, depending on the stimulus intensity. This finding agrees well with the time scale of ~32 s for the dynamics of the adaptation of phototaxis and photosynthesis to a series of light on-off cycles in Chlamydomonas (Arrieta et al., 2017). Because ChR1 phosphorylation also responds to plasma membrane hyperpolarization and depolarization in the dark, it is well suited for adapting the sensitivity of the photoreceptor to the prevailing cellular requirements. ChR1 phosphorylation thus fits nicely in a cross-timescale regulatory/adaptive framework, connecting photoreception and motility responses to photosynthesis, photoprotection, and related physiological processes. To unravel the underlying molecular mechanisms, detailed correlative measurements of the phototactic behavior, the in vivo ChR1 phosphorylation status, the photocurrents, and the cytosolic Ca^{2+} concentrations under steady-state illumination will be helpful. These analyses should also be expanded to ChR2. Such approaches will be indispensable to the efforts to further decipher the in vivo regulation of the ChRs and the molecular mechanisms used by Chlamydomonas to integrate light, its cellular requirements, and the diverse strategies for adaptation into coherent movement responses.
METHODS

Strains and Culture Conditions

*Chlamydomonas reinhardtii* strain 83.81 (cw15) was obtained from the Culture Collection of Algae at Goettingen University SAG (https://www.uni-goettingen.de/de/sammlung+von+algenkulturen+(sag)/184982.html); strains CC-125 (wt137c), CC-124, and CC-3403 were obtained from the Chlamydomonas Stock Center (http://www.chlamycollection.org); strains 1330.1, EYE3, MIN1, and EYE2 were obtained from Carol Dieckmann (University of Arizona, Tucson, USA); and strains cw806 (CC-4536), 302cw, ΔPhot<sup>G5</sup>, 125-ΔPhot-C4-1, 125-ΔPhot-C4-2, ΔChR2, ChR1Δct, VGCC#1, and VGCC#2 were obtained from Peter Hegemann (Humboldt University Berlin, Germany). The strains were grown in a 14 h/10 h light/dark cycle at 15°C ± 1° (white light, 60 μmol photons m<sup>-2</sup> s<sup>-1</sup>) in standard TAP or modified Sager-Granick (CMM) liquid medium (Gorman and Levine, 1965; Harris, 2008). The non-motile strains 302cw and ΔPhot<sup>G5</sup> were grown with continuous shaking. Stock cultures were maintained in liquid medium or on TAP agar plates (1.5%) under identical conditions.

Inhibitors

Calyculin A (Santa Cruz Biotechnology), Cantharidin, CKI-7, DCMU, IBMX, Sanguinarine, and TEMPO<sub>L</sub> (all from Sigma-Aldrich) were dissolved in DMSO; 8-Bromoadenosine 3',5'-cyclic monophosphate was obtained from Sigma.

Whole-Cell Experiments

All experiments were performed with batch cultures. TAP (50 ml) was inoculated with 3 × 10<sup>6</sup> cells. Cells were used for experiments at the end of the dark phase after 5 days of growth. Biological replicates refer to independent cultures inoculated into different flasks from a starter flask. Replicate experiments were performed either in parallel or in different weeks. All necessary manipulations until protein precipitation were carried out under dim red safety light with minimal exposure of the cells to the safety light. As indicated, cells were pelleted (5 min, 1973 g; 15°C) and transferred to minimal medium (NMM; 10 mM HEPES, 81 μM MgSO<sub>4</sub>, and 100 μM CaCl<sub>2</sub>, pH 8.0), fresh TAP, or modified Sager-Granick medium (Harris, 2008) with the variations/additions indicated in
the figure legends. The volume for resuspension corresponded to 60% of the original culture volume. After a 1 h adaptation step in the dark, for each treatment, 4 ml of culture was transferred into 40 mm crystallizing dishes. The addition of and pre-incubation with inhibitors or solvents were performed directly in the dishes. The maximal solvent concentration used did not exceed 0.1% (v/v). Illumination was applied for the indicated times and intensities using LED panels above the dishes. White light was created using an equal mixture of white (30000 mcd NSPW500GS-K1) and warm white (31.000 mcd NSPL500DS) LEDs from Nichia. Emissions of other LED areas peaked at 515 nm (green) and 660 nm (red). The standard white-light shock had a fluence of 800 µmol m⁻² s⁻¹ and was administered for 1 min. To kill the cells and precipitate the proteins as quickly as possible, the cultures were directly mixed with 16 ml ice-cold MetOH:Chl to give a final ratio of methanol:chloroform:water of 8:4:3 (v/v; Schmidt et al., 2006). The precipitated proteins were dried and suspended in SDS-PAGE buffer or Phos-tag sample buffer (2× SDS-PAGE sample buffer without EDTA).

For acid-induced deflagellation, cells in TAP were rapidly mixed 1:1 with 40 mM sodium acetate (pH 4.5) supplemented with 1 mM CaCl₂, pelleted for 1 min (1600g; 15°C), and suspended in fresh TAP (pH 8.0). All manipulations were performed under dim red safety light. Light shock and protein precipitation were performed as described above. Flagella regeneration occurred in shaking cultures at room temperature in the dark. The grade of flagella shedding was evaluated by phase-contrast microscopy (Eclipse E800, Nikon) of cells fixed with Lugol’s iodine prior to or directly after deflagellation.

λ-Phosphatase Treatments

Cultures (cw15, 2 × 10 ml) were pelleted (5000 × g; 5 min, 4°C) and frozen in liquid N₂. One pellet was resuspended in 1 ml TN buffer (20 mM Tris HCl pH 7.5, 80 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail without EDTA [Roche]), and another was resuspended in TN buffer supplemented with PIC 2 and 3 (Sigma-Aldrich). The inhibitor cocktails were used according to the instructions of the supplier. The cells were homogenized by sonication (four cycles, 15 s each, interrupted by a 15 s cooling phase; 25% output intensity) in the presence of an equal volume of glass beads.
(0.25–0.5 mm). The supernatants were aspirated, and the glass beads were washed once with 1 ml of the appropriate buffer. After low-speed centrifugation (9 \( \times \) g, 5 min, 4°C), the combined supernatants were supplemented with \( \lambda \)-phosphatase buffer (NEB) and MnCl\(_2\) according to the instructions of the supplier. Finally, the samples were incubated in the dark for 2 h at 30°C in the presence or absence of \( \lambda \)-phosphatase (1000 U ml\(^{-1}\)) and PIC. The reaction was stopped by adding ice-cold MetOH:Chl to yield a final methanol:chloroform:water ratio of 8:4:3 by volume. The precipitated proteins were dried and suspended in Phos-tag sample buffer.

**SDS-PAGE, Phos-tag-PAGE, and Immunoblot Analysis**

Protein quantification and standard SDS-PAGE (high-Tris system) were conducted as previously described (Schmidt et al., 2006; Trippens et al. 2012). Phosphoproteins were separated in 6% Mn\(^{2+}\)-Phos-tag-SDS-PAGE gels (25 \( \mu \)M Phos-tag-acrylamide, 50 \( \mu \)M MnCl\(_2\)) with a current of 14 mA per gel, as described by Kinoshita et al. (2006), not more than 2 days after being resuspended in Phos-tag sample buffer (SDS sample buffer without EDTA). All Phos-tag-SDS-PAGE gels were used within the first day after casting. Loading was based on an equal protein load (usually 8 \( \mu \)g). Prior to protein transfer to PVDF membranes by tank-blotting, the Phos-Tag gels were soaked for 10 min in excess transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol) supplemented with 1 mM EDTA, followed by an additional washing step in transfer buffer. Immunoblotting and subsequent analyses were performed according to standard techniques. The blots were probed with primary rabbit antibodies against either the C-terminus of ChR1 (S. Kateriya; 1:5000) or the LOV1 domain of phototropin from Chlamydomonas (Zorin et al., 2009; 1:2000) and detected with AP-conjugated goat anti-rabbit (1:5000; Sigma-Aldrich). Coomassie brilliant blue staining of the lower part of the blots was used as a loading control. All images used in the figures were processed using Photoshop (Adobe Systems).

**Eyespot Isolation and In Vitro Phosphorylation of Eyespot Proteins**

Strain cw15 was used for eyespot isolations. The procedures for growth and isolation were as previously described (Wagner et al., 2008), except that the homogenization buffer was supplemented with PIC 2 and 3 (Sigma; dilution 1:50). Freshly isolated...
eyespots were mixed with 10× kinase buffer to yield a final concentration of 50 mM Tris HCl pH 7.5, 5 mM MgCl₂, and 2 mM DTT. The assays were started by the addition of ATP (final concentration 2 mM) and were conducted for 30 min at 25°C under dim light. Reactions were stopped by the addition of ice-cold MetOH:Chl to yield a final methanol:chloroform:water ratio of 8:4:3 by volume. Proteins were further precipitated at -20°C, washed several times with MetOH:Chl (2:1[v/v]), dried, and suspended in Phos-tag sample buffer.

**Mass Spectrometry Analyses**

To identify ChR1 phosphopeptides, eyespot proteins were precipitated directly after isolation by MetOH:Chl as described above, except that the pellets were suspended in standard SDS-PAGE sample buffer. Proteins were separated the next day in modified 10% high-Tris gels using a 30%/0.8% (w/v) acrylamide/piperazine diacrylamide stock solution and stained with Bio-Safe Coomassie G-250 (Bio-Rad). The region of the gels around the apparent molecular mass range of ChR1 was cut out, sliced into small pieces, and washed repeatedly for 10 min with 10 mM NH₄HCO₃ and then for 10 min with 5 mM NH₄HCO₃/50% (v/v) acetonitrile until no Coomassie stain was visible. The gel slices were vacuum dried and stored at -80°C until the next day. In-gel digestion with trypsin (Thermo Fisher Scientific) in 50 mM NH₄HCO₃ was carried out at 37°C overnight. The supernatant was collected, and the slices were washed once with 50% acetonitrile/0.5% trifluoroacetic acid (TFA) and once with 100% acetonitrile/0.5% TFA. Each washing step was carried out for 60 min at room temperature. The combined supernatants were vacuum dried. The peptides were suspended in 1% acetonitrile/0.1% TFA, loaded on a nanoflow Ultimate 3000 nano-HPLC (Thermo Fisher Scientific), and separated on a column (Thermo Fisher Scientific; C18 with 3 µm particle size, 15 cm × 75 µm) at a flow rate of 300 nl/min by increasing the acetonitrile concentration over 80 min. For the first stage of mass analysis (MS¹), all samples were analyzed on an Orbitrap Fusion Tribrid (Thermo Fisher Scientific) with the following settings: 2000 V spray voltage, 200–2000 (m/z) scan range, maximum injection time 50 ms, and AGC target 400,000. For the second stage of mass analysis (MS²), the most intense ions were selected for higher energy collision dissociation (HCD) with a collision energy of 27% or for electron-transfer dissociation (ETD) with a collision energy of 30%, a
maximum injection time of 250 ms, and an AGC target of 50,000. Data were analyzed using Proteome Discoverer software (Version 1.4, Thermo Fisher Scientific). The ChR1 sequence was searched with a signal-to-noise threshold of 1.5, a precursor mass tolerance of 10 ppm, and a fragment mass tolerance of 0.6 Da for tryptic peptides, allowing one missed cleavage. The parameters were set to detect dynamic modifications of 79.966 Da on Ser and Thr and of 15.995 Da on Met. For the node PhosphoRS 3.0, a fragment mass tolerance of 0.5 Da was set, and automatic neutral loss peaks for HCD and ETD were considered. To increase the confidence of the final phosphopeptide assignment, the following criteria were additionally applied: (1) only peptides with ranking 1 or 2 were accepted; (2) the maximum ΔCn value was set to 0.05; (3) scores for the cross-correlation factor Xcorr were set to 1.5 if the charge of the peptide (z) was 1, 2 if z was 2, 2.5 if z was 3, or 3 if z was ≥ 4, and partially tryptic phosphopeptides were only accepted when their Xcorr was > 3, irrespective of their charge (Washburn et al., 2001); and (4) at least four peptide spectrum matches for the phosphopeptide must be present in each run. In total, four independent eyespot isolations, each with two technical duplicates for preparation and MS analysis, respectively, were performed.

Phototaxis and Cell Motility Assays

Phototactic behavior was analyzed using vegetative cells during the first 4–5 h after the beginning of the cells’ subjective day. All manipulations were carried out at 15°C under dim red safety light, and care was taken to minimize exposure of the cells to the light. Movement responses were analyzed by a classical Petri dish (3.5 cm) assay or in a 24-well microtiter plate, as described by Trippens et al. (2017). Cells at the end of the night phase were pelleted (5 min, 1973g; 15°C), suspended in NMM, and allowed to adapt in darkness for 1 h. The cultures were then supplemented with the indicated concentrations of Sanguinarine, IBMX, KCl, or the appropriate solvent as a control and incubated for an additional 15 min in the dark prior to the start of the assays. The maximal solvent concentration did not exceed 0.1% (v/v). Illumination was applied with unilateral white light (100 µmol m⁻² s⁻¹). Treated and control cells were analyzed in parallel, and photographs (COOLPIX 990, Nikon) were taken at the indicated time points. Quantification of the response was only performed for the Petri dish assays as
described previously (Trippens et al., 2017). Cell motility was analyzed using the
computer-based cell-tracking system WinTrack (Häder et al., 2005).

Accession Numbers

Sequence data for this article can be found in the GenBank library under the following
accession numbers: ChR1 (15811379), CrChR2 (158280944).

Supplemental Data

Supplemental Figure 1: Parts of a Clustal Omega Alignments of Channelrhodopsin
Protein Sequences from Different Members of the Chlorophyta.

Supplemental Figure 2: Effect of Pre-Illumination with Intermediate Intensities of Red
and Green Light on the Phosphorylation Status of ChR1 and Adaptation in Response to
a White-Light Shock.

Supplemental Figure 3: Effect of Increasing Ba$^{2+}$ Concentrations at a Fixed Ca$^{2+}$
Concentration on High-Intensity Light-Induced ChR1 Hyper-Phosphorylation.

Supplemental Figure 4: ChR1 Phosphorylation Following Light Shock is Not Affected in
Cells Defective in a Gene of a Putative Voltage-Gated Ca$^{2+}$ Channel (VGCC).

Supplemental Figure 5: The Protein Phosphatase Inhibitors Cantharidin (A) and
Calyculin A (B) Do Not Inhibit ChR1 Dephosphorylation.

Supplemental Figure 6: Increasing IBMX and External KCl Concentrations do
Suppress Negative Phototactic Behavior of wt137c Cells.

Supplemental Figure 7: ChR1 Levels of Strains with Negative Phototaxis at All
Photon Irradiances (CC-124, CC4536) and Strains with Wild-Type Phototactic
Behavior (wt137c, cw15) as Revealed by SDS-PAGE.

Supplemental Figure 8: Casein Kinase 1 and Phototropin Do Not Phosphorylate ChR1
in vivo.

Supplemental Table 1: Predicted ChR1 Phosphorylation Sites by NetPhos 3.1 with
Scores >0.97.
Supplemental Data Set 1: List of All ChR1 Phosphopeptides Identified in the Four Independent Eyespot Isolations.

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AUTHOR CONTRIBUTIONS

G.K., M.B., D.B., and E.F. conceived and designed the research. M.B., D.B., E.F., H.E., J.F., Z.K., and N.M. performed the experiments. The ChR1 mutants and ΔPhot strains were generated in the lab of P.H.; ChR1 and anti-LOV1 antisera were from the labs of S.K. and P.H., respectively. M.B., D.B., E.F., H.E., J.F., Z.K., N.M., and G.K. analyzed the data. G.K. wrote the article, with contributions and edits from M.B., D.B., P.H., and S.K. All authors read, revised, and approved the article.
Table 1. ChR1 Phosphorylation Sites Identified by Mass Spectrometry

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Sp, phosphorylated Ser; Tp, phosphorylated Thr; M, oxidized Met. If a peptide is marked with an arrow, the peptide above it shows overlapping sequences.
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**Figure 1.** Amino Acid Sequence of Channelrhodopsin-1 from Chlamydomonas.

The positions of phosphorylated amino acids identified by mass spectrometry in four independent eyespot isolations are highlighted in red. The positions of the transmembrane domains (orange) are from a ChR1/ChR2 chimera (Kato et al. 2012). The identified peptides are summarized in Table 1. Supplemental Data Set 1 lists all identified phosphopeptides.
Figure 2. Phos-tag-PAGE Separates Phosphorylated ChR1 Forms in Whole-Cell Extracts of Different Strains and Demonstrates ChR1-Phosphorylation Activity in Isolated Eyespots.

(A) Total protein extracts from cw15 cells were treated with λ-phosphatase (λ-PPase) in the absence or presence of protein phosphatase inhibitor cocktails (PIC). As an additional control, cells were treated with only the λ-PPase buffer. Proteins were separated by standard SDS-PAGE or Phos-tag-SDS-PAGE and analyzed by immunoblotting using anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of each blot is shown as a loading control.

(B) Isolated eyespots were incubated in kinase buffer with or without ATP. After separation of the indicated amounts of protein by Phos-tag-SDS-PAGE and blotting, the samples were analyzed by probing with anti-ChR1 or Coomassie staining.

(C) In vivo ChR1 phosphorylation of dark-adapted cells of different Chlamydomonas strains grown in TAP. Cells at the end of the night phase were directly transferred into MetOH:Chl (2:1), and the precipitated proteins were separated by Phos-tag-SDS-PAGE or standard SDS-PAGE prior to immunoblot analysis with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower parts of the blots demonstrates equal loading. Typical blots from four replicates are shown. Color-coded bars at the right side of the ChR1 blot after separation by Phos-tag-PAGE indicate the areas used to determine the relative proportions shown in (D).

(D) Quantification of the major different ChR1 forms separated by Phos-tag-SDS-PAGE in (C): non = non-phosphorylated; low = low-phosphorylated; high = highly phosphorylated; hyper = hyper-phosphorylated. The sum of those forms in each lane was set to 100%. Means and standard deviations are shown (n = 4 replicates and 7 immunoblot analyses per strain).
Figure 3. The Phosphorylation Status of ChR1 Changes in a Light Intensity-Dependent Manner.

(A) In vivo ChR1 phosphorylation of dark-adapted cw15 cells in TAP in response to a 1 min white-light pulse with increasing intensity. Cells were directly injected into MetOH:Chl (2:1), and the precipitated proteins were separated by Phos-tag-SDS-PAGE prior to immunoblot analysis with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot demonstrates equal loading. A typical result from three replicates is shown. Color-coded bars at the right side of the ChR1 blot indicate the areas used to determine the relative proportions shown in (B).

(B) Quantification of the major ChR1 forms marked in (A): non = non-phosphorylated; low = low-phosphorylated; high = highly phosphorylated; hyper = hyper-phosphorylated. The sum of those forms in each lane was set to 100%. Means and standard deviations are shown (n = 3 replicates and independent immunoblot analyses).

(C) Immunoblot analysis of whole-cell extracts used in (A) separated by SDS-PAGE. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot demonstrates equal loading.

(D) Long-term changes in ChR1 phosphorylation in cw15 and 137c cells under constant illumination at low fluence (60 µmol m⁻² s⁻¹). The time course of ChR1 phosphorylation changes was determined by immunoblot analysis with anti-ChR1 after separation of whole-cell proteins with Phos-tag-SDS-PAGE. Cells were grown in TAP. Coomassie brilliant blue (Coomassie) staining of the lower parts of the blots is shown as a loading control.

(E) SDS-PAGE and immunoblot analysis of aliquots of the samples shown in (D).
Figure 4: Light-Induced ChR1 Phosphorylation is Rapid, and the Dephosphorylation Time Course in the Dark Depends on the Intensity of the Light Pulse.

(A) Time course of in vivo ChR1 phosphorylation during irradiation with high-intensity white light (800 μmol m⁻² s⁻¹) and subsequent dephosphorylation after transfer into darkness. Cw15 cells were directly injected into MetOH:Chl (2:1), and the precipitated proteins were separated by Phos-tag-SDS-PAGE prior to immunoblot analysis with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot demonstrates equal loading. A typical result from three replicates is shown. Color-coded bars indicate the areas used to determine the relative proportions of the different ChR1 forms shown in (C).

(B) Standard SDS-PAGE of whole-cell proteins treated as described in (A) followed by immunoblot analysis. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot demonstrates equal loading. Further processing was performed as described in (A).

(C) Quantification of the major ChR1 forms marked in (A): non = non-phosphorylated; low = low-phosphorylated; high = highly phosphorylated; hyper = hyper-phosphorylated. The sum of those forms in each lane was set to 100%. Means and standard deviations are shown for three replicates and independent immunoblot analyses.
(D) Time course of ChR1 phosphorylation and dephosphorylation in cells treated with an intermediate light intensity (150 μmol m⁻² s⁻¹). Further treatments were as described in (A). A typical blot from three independent experiments is shown.

(E) SDS-PAGE of whole-cell proteins treated as described in (D), followed by immunoblot analysis. Equal protein loading is demonstrated by Coomassie brilliant blue (Coomassie) staining of the lower part of the blot.

(F) Quantification of the changes in the major different ChR1 forms marked in (D) from three replicates (mean and standard deviation).
Figure 5: Shifts in the Intensity of Continuous Light Induce Changes in the Phosphorylation Status of ChR1.

(A) Dark-adapted cw15 cells were continuously illuminated at an intensity of 60 µmol m$^{-2}$ s$^{-1}$. Samples were directly injected into MetOH:Chl (2:1) at the indicated time points. The precipitated proteins were separated by Phos-tag-SDS-PAGE prior to immunoblot analysis with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot was used as a loading control. A typical result from three independently grown cultures is shown. Color-coded bars indicate the areas used to determine the relative proportions shown in (C).

(B) Standard SDS-PAGE of whole-cell proteins treated as described in (A), followed by immunoblot analysis. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot was used as a loading control.

(C) Quantification of the major ChR1 forms marked by colored bars in (A): non = non-phosphorylated; low = low-phosphorylated; high = highly phosphorylated; hyper = hyper-phosphorylated. The sum of those forms in each lane was set to 100%. Means and standard deviations are shown for three replicates and four immunoblot analyses.
(D) Dark-adapted cells were illuminated for 1 h at 60 µmol m⁻² s⁻¹ and then shifted to 250 µmol m⁻² s⁻¹. Samples were withdrawn 15 min and 30 min after the shift. Further details are described in (A).

(E) Standard SDS-PAGE of the samples separated in (D) by Phos-tag-SDS-PAGE followed by immunoblot analysis. Within the analyzed time window, no significant changes in the total ChR1 level were detected. Equal protein loading is demonstrated by Coomassie brilliant blue (Coomassie) staining of the lower part of the blot.

(F) Quantification of the changes in the major different ChR1 forms marked in (D). Means and standard deviations for three replicates and four immunoblot analyses are shown. Color code is explained in (C).

(G) Cells were shifted to 5 µmol m⁻² s⁻¹ illumination after the initial 1 h exposure to 60 µmol m⁻² s⁻¹ illumination. Further details are described in (A).

(H) Standard SDS-PAGE of samples from the experiment shown in (G) followed by immunoblot analysis. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot was used as a loading control.

(I) Quantification of the major ChR1 forms marked by colored bars in (G). Color code is explained in (C). Plotted are the means and standard deviations from three replicates and four immunoblot analyses.
Figure 6: Adaptation of High-Intensity Light Shock-Induced ChR1 Hyper-Phosphorylation Depends on the Light Intensity During Pre-Illumination.

(A) The in vivo ChR1 hyper-phosphorylation response of cw15 cells to a light shock is affected by low-intensity white-light background illumination. Dark-adapted cw15 cells in TAP were exposed for the indicated times to low-intensity light (25 µmol m⁻² s⁻¹) and then poured directly or after a 1 min light shock (800 µmol m⁻² s⁻¹) into MetOH:Chl (2:1). The precipitated proteins were separated by Phos-tag-SDS-PAGE prior to immunoblot analysis with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot shows equal protein loading. A typical blot from three replicates is shown. Colored symbols indicate the bands used for the quantifications shown in (B) and (C).

(B) Time course of the relative changes of the different phosphorylated ChR1 forms in the samples without light shock during the first 5 min after the onset of illumination. Depicted are the changes in the relative amounts of the non-phosphorylated (R₁ ~ 0.52), the low-phosphorylated (R₁ ~ 0.46), and highly phosphorylated (R₁ ~ 0.19) ChR1 bands. The sum of those forms was set to 100%. Means and standard deviations are shown for three replicates. The arrowhead indicates the time point at which the light shock evoked no clear changes compared to the control.

(C) Same as (A) and (B), but with a background light intensity of 150 µmol m⁻² s⁻¹.
Figure 7: The Phosphorylation Status of ChR1 is Affected by the Ionic Composition of the Medium.

(A) Manipulation of external pH affects the abundance of the phosphorylated ChR1 forms in the dark and the light shock-induced hyper-phosphorylation response. Cw15 cells from the end of the night phase were harvested under red safety light, suspended in TAP media with the indicated pH values, and allowed to adapt for 1 h in the dark. Samples were then precipitated either directly or after a white-light shock (1 min, 800 µmol m⁻² s⁻¹) by addition of MetOH:Chl (2:1). The proteins were separated by Phos-tag-SDS-PAGE, blotted, and analyzed with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot is shown as a loading control.

(B) Quantification of the major phosphorylated ChR1 forms in samples without light treatment at different pH values of the medium. Depicted are the changes in the relative amounts of the non-phosphorylated (R_f ~ 0.56), low-phosphorylated (R_f ~ 0.51), and highly phosphorylated (R_f ~ 0.24) ChR1 bands. The sum of those forms at each pH value was set to 100%. Means and standard deviations are shown for three replicates.

(C) Increasing external KCl concentrations induce ChR1 hyper-phosphorylation in the dark. At the end of the night phase, cw15 cells were harvested under red safety light, suspended in NMM (pH 8) with the indicated added KCl concentrations, and allowed to adapt for 1 h in the dark. Further details are described in (A). A representative blot from three replicates is shown.

(D) Time course of potassium shock-induced ChR1 hyper-phosphorylation in the dark. Cells were harvested at the end of the dark phase under red safety light, suspended in NMM (pH 8), and allowed to adapt for 1 h in the dark prior to the addition of KCl dissolved in NMM to yield a final KCl concentration of 200 mM. Samples were withdrawn at the indicated times. As a control, a sample without added KCl was withdrawn and additionally exposed to a white-light shock. Further experimental details are described in (A). As a control, the samples were also probed with an antiserum directed against the LOV1 domain of the Chlamydomonas phototropin (anti-LOV).
Figure 8: In vivo ChR1 Phosphorylation Depends on Ca\(^{2+}\).

(A) Light shock-induced ChR1 hyper-phosphorylation is reduced at low extracellular Ca\(^{2+}\) concentrations. Cells (cw15) were harvested at the end of the dark phase under red safety light, suspended in NMM (pH 8) with the indicated Ca\(^{2+}\) concentrations, and allowed to adapt for 1 h in the dark. Cultures were then precipitated either directly or after a white-light shock (1 min, 800 µmol m\(^{-2}\) s\(^{-1}\)) by the addition of MetOH:Chl (2:1). The proteins were separated by Phos-tag-SDS-PAGE, blotted, and analyzed with anti-ChR1. The lower part of the blot was stained with Coomassie brilliant blue (Coomassie) and served as a loading control. A representative blot from three replicates yielding essentially identical results is shown.

(B) Chelation of external free Ca\(^{2+}\) by EGTA reduces the hyper-phosphorylation response only when the EGTA is added before the light shock. Cultures (cw15) were pelleted at the end of the night phase and suspended in NMM (pH 8). After 1 h of adaptation in the dark, samples were treated as indicated without any additions or with the addition of EGTA (2.5 mM), either directly after or 3 min prior to the indicated illumination times (white light, 800 µmol m\(^{-2}\) s\(^{-1}\)). Further sample processing was performed as described in (A).

(C) Ca\(^{2+}\) shock evokes ChR1 hyper-phosphorylation in the dark. Dark-adapted cw15 cells in NMM (pH 8) were exposed to Ca\(^{2+}\) shocks (20 or 30 mM) in the dark. Samples taken at the indicated times were analyzed as described in (A). Arrowheads point to regions with increasing hyper-phosphorylated ChR1 forms. A representative blot from three replicates is shown.

(D) A pH shock with acetate induces flagellar shedding and ChR1 hyper-phosphorylation in the dark. Samples were taken 1 min (dark) or 2 min (1 min dark, 1 min white-light shock, 800 µmol m\(^{-2}\) s\(^{-1}\)) after deflagellation. Further sample processing was performed as described in (A). A representative blot from three replicates is shown. The presence of flagella was analyzed by phase-contrast microscopy in parallel fixed samples (n = 100 cells).
**Figure 9:** Inhibition of Linear Photosynthetic Electron Transport by DCMU Induces the Rapid Appearance and Longer Persistence of Hyper-phosphorylated ChR1 Forms.

**A** Time course of the changes in the phosphorylation status of ChR1 at an intermediate light intensity (150 µmol m\(^{-2}\) s\(^{-1}\)). Cultures (cw15) in TAP at the end of the dark phase were pre-incubated with either 100 µM DCMU or the same amount of ethanol for 30 min in the dark prior to the start of illumination with white light. Samples were taken at the indicated time points with or without an additional 1 min light shock (800 µmol m\(^{-2}\) s\(^{-1}\)), separated by Phos-tag-SDS-PAGE, and analyzed by immunoblotting with anti-ChR1. A representative blot from three independent replicates is shown.

**B** DCMU shifts the induction of ChR1 hyper-phosphorylation toward lower fluences. Cells were illuminated with the indicated fluences of white light for 1 or 3 min prior to MetOH:Chl (2:1) precipitation. Further experimental details are described in (A).
Figure 10: *In Vivo* ChR1 Phosphorylation is Affected by IBMX and by Manipulation of the Cellular Redox Balance.

(A, B) IBMX induces rapid ChR1 hyper-phosphorylation in the dark. Dark-adapted *cw15* cells in NMM were incubated for 5 min with the indicated IBMX concentrations or the solvent DMSO (A) or with a fixed IBMX concentration of 0.5 mM for the indicated times (B) prior to protein precipitation, separation by Phos-tag-SDS-PAGE and subjected to immunoblot analysis with anti-ChR1. As an additional control, cells with no IBMX addition (Cont) and after a light shock (1 min, 800 µmol m$^{-2}$ s$^{-1}$) are shown in (A) and (B), respectively. Coomassie brilliant blue (Coomassie) staining of the lower parts of the blots is shown as loading control. Representative blots from three independent replicates are shown.

(C, D) TEMPOL inhibits both light shock-induced (C) and depolarization-induced (D) ChR1 hyper-phosphorylation in the dark. Dark-adapted cells of strain *cw15* in TAP were incubated for 30 min with the indicated TEMPOL concentrations. Where indicated, a 1 min white-light shock (800 µmol m$^{-2}$ s$^{-1}$) or a 4 min KCl shock was administered prior to immune analysis with anti-ChR1.

(E) Autophosphorylation of the blue-light photoreceptor phototropin is not affected by TEMPOL or KCl shock. Protein samples from the experiment shown in (D) were analyzed by immune analysis with a serum against the LOV1 domain of phototropin from Chlamydomonas (anti-LOV).
H$_2$O$_2$ induces ChR1 hyper-phosphorylation in the dark in a concentration-dependent manner. Immunoblot analysis with anti-ChR1 of whole-cell extracts after separation by Phos-tag-SDS-PAGE. Dark-adapted cw15 cells in TAP were incubated for 30 min with the indicated H$_2$O$_2$ concentrations. A white-light shock (800 µmol m$^{-2}$ s$^{-1}$) was then administered for 1 min. A typical blot from three replicates is shown.
Figure 11: The PP2C Inhibitor Sanguinarine Blocks ChR1 Dephosphorylation and Negative Phototactic Behavior.

(A) At the end of the dark phase, wt137c cells were transferred to NMM (pH 8) under red safety light. Following a pre-incubation for 30 min in the dark with the indicated Sanguinarine concentrations or DMSO, the cells were illuminated with strong white light (800 µmol m^{-2} s^{-1}, 1 min) and then returned to darkness. Samples were taken at the indicated time points, separated by Phos-tag-SDS-PAGE, and analyzed by immunoblotting with anti-ChR1. Equal protein loading was confirmed by Coomassie brilliant blue (Coomassie) staining of the lower parts of the blots. The experiment was repeated two times, yielding identical results.

(B, C) Analysis of the phototactic behavior (B) and swimming speed (C) of dark-adapted wt137c cells in NMM (pH 8.0) after 15 min pre-incubation with 5 µM Sanguinarine or DMSO. The arrow in (B) indicates the position of the light source (100 µmol m^{-2} s^{-1}, white light). The mean swimming speed with standard deviation was calculated from tracks of 1158 (DMSO) and 1179 (Sanguinarine) cells.
Figure 12: ChR1 Phosphorylation in Strains with Negative Phototaxis at All Photon Irradiances.

(A) TAP-grown cells of the phototactic mutant strains (CC-124, CC-4536) and wild-type strains (wt137c, cw15) were transferred into MetOH:Chl either directly at the end of the dark phase or after 1 h of illumination (60 µmol m⁻² s⁻¹). The precipitated proteins were analyzed by Phos-tag-SDS-PAGE and immunoblotting with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower part of the blot was used as a loading control. Colored bars mark the major ChR1 forms: non = non-phosphorylated; low = low-phosphorylated; high = highly phosphorylated; hyper = hyper-phosphorylated. The corresponding immunoblots of samples separated by SDS-PAGE are shown in Supplemental Figure 7 A.

(B) Quantification of the immunoblots shown in (A). The sum of the different ChR1 forms in each lane was set to 100%. Means and standard deviations from three independent experiments are shown.

(C) Photon irradiance-dependent changes in the ChR1 phosphorylation status of strains cw15 (wild-type-like phototaxis) and CC-4536 (always negative phototaxis). Cells at the end of the dark phase were transferred to fresh CMM and allowed to adapt for 1 h in darkness. The cultures were then precipitated by the addition of MetOH:Chl (2:1) either directly or after 10 min illumination with white light of the indicated intensities. The proteins were separated by Phos-tag-SDS-PAGE, blotted, and analyzed with anti-ChR1. A representative blot from three replicates is shown. The corresponding immunoblot of the samples separated by SDS-PAGE is shown in Supplemental Figure 7 B.
Figure 13: H₂O₂-Induced Changes in Swimming Direction at a Steady Low-Photon Irradiance are Accompanied by an Increase in Highly Phosphorylated and Hyper-Phosphorylated ChR1 Forms.

At the end of the dark phase, cells of phototactic mutant strains (CC-124, CC-4536) and strains with normal phototactic behavior (wt137c, cw15) were transferred to fresh CMM medium under red safety light. After 1 h of dark adaptation, 3 ml of the culture (3 × 10⁶ cell ml⁻¹) was supplemented with the indicated concentration of H₂O₂ and illuminated with unidirectional white light (arrows; 1 µmol m⁻² s⁻¹) for 10 min prior to analysis. A representative example from three independent replicates is shown.

(A, C) Orientation of the indicated cultures at the end of the illumination period.

(B, D) Precipitated proteins from the samples shown in (A, B) were separated by Phos-tag-SDS-PAGE and analyzed by immunoblotting with anti-ChR1. Additionally, samples precipitated directly after the adaptation phase in the dark were separated. Coomassie brilliant blue (Coomassie) staining of a lower part of the blot was used as a loading control.
Figure 14: ChR1 Phosphorylation in Eyespot Assembly and ChR Mutants.

(A) Schematic drawing of the eyespot from Chlamydomonas demonstrating the localization of the eyespot-related proteins analyzed in (B–E). PM: plasma membrane; OM and IM: outer and inner chloroplast envelope membrane, respectively; ST: stroma thylakoid; GT: grana thylakoid.

(B) ChR1 levels in the different eyespot-assembly mutants compared with those in the parental strains. Whole-cell extracts of the indicated strains were separated by SDS-PAGE, blotted, and analyzed with anti-ChR1. Data represent the mean ± standard deviation of the quantified blots from five replicates. *significant difference (P = 0.0296) compared with wt137c, as revealed by paired t test (two-tailed).

(C) Immunoblot analysis (anti-ChR1) of whole-cell extracts from the indicated dark-adapted strains after separation by Phos-tag-SDS-PAGE reveals no significant difference in the response to a white-light shock (800 μmol m⁻² s⁻¹, 1 min) between the eyespot-assembly mutants and their corresponding parental strains. Representative blots from at least three replicates are shown.
(D) ChR1 levels in the ∆ChR2 and ChR1.Δct mutants compared to those in the parental strains. Whole-cell extracts of the indicated strains were separated by SDS-PAGE, blotted, and analyzed with anti-ChR1. Coomassie brilliant blue (Coomassie) staining of the lower parts of the blots was used as a loading control. For the strain ChR1.Δct, a fourfold-higher protein load was used.

(E) Immunoblot analysis (anti-ChR1) of whole-cell extracts separated by Phos-tag-SDS-PAGE from the indicated dark-adapted strains prior to and after a white-light shock (800 µmol m$^{-2}$ s$^{-1}$, 1min). In addition, for strains CC-3403 and ChR1.Δct, samples taken 1 min and 2 min after deflagellation by acetate shock in the dark were analyzed. For strain ChR1.Δc, a fourfold-higher protein load was used. Representative blots from three replicates are shown.
Channelrhodopsin-1 phosphorylation changes with the phototactic behavior and responds to physiological stimuli in Chlamydomonas

Michaela Böhm, David Boness, Elisabeth Fantisch, Hanna Erhard, Julia Frauenholz, Zarah Kowalzyk, Nadin Marcinkowski, Suneel Kateriya, Peter Hegemann and Georg Kreimer

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