The abundant retinal protein of the *Chlamydomonas* eye is not the photoreceptor for phototaxis and photophobic responses

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Accepted 16 July 2001

Journal of Cell Science 114, 3857-3863 (2001) © The Company of Biologists Ltd

SUMMARY

The chlamyopsin gene (*cop*) encodes the most abundant eyespot protein in the unicellular green alga *Chlamydomonas reinhardtii*. This opsin-related protein (COP) binds retinal and was thought to be the photoreceptor controlling photomovement responses via a set of photoreceptor currents. Unfortunately, opsin-deficient mutants are not available and targeted disruption of non-selectable nuclear genes is not yet possible in any green alga. Here we show that intron-containing gene fragments directly linked to their intron-less antisense counterpart provide efficient post-transcriptional gene silencing (PTGS) in *C. reinhardtii*, thus allowing an efficient reduction of a specific gene product in a green alga. In opsin-deprived transformants, flash-induced photoreceptor currents (PC) are left unchanged. Moreover, photophobic responses as studied by motion analysis and phototaxis tested in a light-scattering assay were indistinguishable from the responses of untransformed wild-type cells. We conclude that phototaxis and photophobic responses in *C. reinhardtii* are triggered by an as yet unidentified rhodopsin species.

Key words: RNAi, Chlamyopsin, Antisense

INTRODUCTION

Green algae contain several different sensory photoreceptors controlling development of the whole organism, development of the photosynthetic apparatus, induction of gametogenesis, photomovement responses and circadian rhythms. Photomovement responses, comprising phototaxis and photophobic responses in *Chlamydomonas reinhardtii*, are controlled by rhodopsin-type photoreceptors that operate in conjunction with a directional antenna (eyespot) (Foster and Smyth, 1980; Foster et al., 1984; Uhl and Hegemann, 1990; Sineshchekov and Govorunova, 1994). Additionally, rhodopsins are responsible for non-directional processes such as retinal biosynthesis (Foster et al., 1988) and, probably in *Volvox carteri*, the final differentiation of somatic cells and gonidia (Kirk and Kirk, 1985).

Short light flashes applied to an algal eye initiate a set of photoreceptor currents, which in turn trigger biochemical and electrical processes within the flagella (Sineshchekov and Govorunova, 1999). The resulting Ca^{2+} influx into the flagella causes a transient alteration of the flagellar beating. Small vesicles still containing the whole eyespot apparatus have been teared out from *Volvox* cells. Photocurrents were recorded immediately from these vesicles, whereas the residual cell was unresponsive to light. This was the most convincing evidence that the photoreceptor and the whole signal transduction system are located within the eyespot region (Braun and Hegemann, 1999). On the basis of a Poisson-statistical analysis of flash-induced behavioral responses, it has been suggested that individual directional changes occurring at low flash intensities and photophobic responses triggered by high intensity flashes are two independent processes that may be mediated by different rhodopsin species (Hegemann and Marwan, 1988). Reconstitution experiments with retinal analogs that cannot isomerize around certain double bonds supported this finding (Zacks et al., 1993).

Supplementation of white, retinal-deficient cells with ^{3}H-retinal, or exchanging the endogenous retinal in purified eyespot membranes with ^{3}H-retinal identified only one retinal binding protein, which has been purified and sequenced (Beckmann and Hegemann, 1991; Deininger et al., 1995). Owing to its sequence homology with invertebrate opsins it was named chlamyopsin (COP). Moreover, the exon-intron organization of the *cop* gene is related to that of animal opsin genes (Deininger et al., 2000). We propose that algal opsins are part of a photoreceptor complex that includes the pore protein constituting the high light-saturating conductance responsible for phobic responses (Holland et al., 1996). This would explain the extremely fast onset of the photoreceptor current after light flash (Sineshchekov et al., 1990; Holland et al., 1996). Antisera against COP impaired the light-regulated GTPase activity of eye-specific G-proteins in the chlorophycaea *S. similis* (Calenberg et al., 1998). This finding supported data showing that the COP from *C. reinhardtii* is functioning as a photoreceptor and suggested that a G-protein is involved in the transduction process. But, the identified opsin of *C. reinhardtii* as well as its relative *V. carteri* (VOP) (Ebnet et al., 1999) are highly charged and the overall sequences are hardly compatible

with a seven-transmembrane-helix receptor, which precluded their full acceptance as a sensory photoreceptor. Although the identified algal opsins are the major proteins of the eye (Deininger et al., 1995; Fuhrmann et al., 1999), evidence arose that they may have functions other than serving as photoreceptors for behavioural responses. For example, volvoxopsin is abundant in eyes of somatic cells and is expressed in eyeless gonidia and in young embryos long before the differentiation between gonidia and somatic cells is completed (Ebnet et al., 1999).

A genetic analysis of the opsin function has been unsuccessful thus far because opsin mutants are not available and targeted gene disruption has yet to be established for any green alga. Molecular machinery for the induction of a homologous recombination process is present in *C. reinhardtii* and *V. carteri*, allowing efficient recombination between extrachromosomal elements (Sodeine and Kindle, 1993; Gumpel et al., 1994; Hallmann et al., 1997). However, the ratio between homologous and illegitimate recombination of genomic elements is only between 10^{-3} and 10^{-4} (Sizova et al., 2001). Apparently, integration of DNA occurs predominantly via non-homologous recombination at random loci of the *Chlamydomonas* genome (Debuchy et al., 1989).

To overcome the lack of methods to disrupt genes in green algae, we first tried an antisense-RNA approach in *V. carteri* (Ebnet et al., 1999). Briefly, isolated gonidia were transformed with a volvoxopsin gene (*vop*) containing exons 6-8 in reverse orientation. Biolistic transformation led to many insertions of this transgene into the genome. It was found that more than 10 gene copies were required to reduce the VOP content by a factor of 10. The reduction of the sense RNA was proportional to the *antisense/sense* ratio (*as/s*). The transformants with reduced opsin level showed a reduction of their phototactic sensitivity, whereas the phototactic rate at high light intensities was normal, exactly as expected for a reduction of the phototaxis photoreceptor. The swimming of these transformants looked normal under the microscope, but the rotation of the spheroid, which is also critical for the phototaxis, has not been tested. The transformants also showed less synchronized growth and development, which could not be explained. A more detailed analysis has been hampered by two factors. First, the *Volvox* transformants were not accessible to measurements of light-induced ionic currents. Second, the multi-copy transformants were unstable and the phenotype was lost or at least impaired within a few months (P.H., unpublished). Thus, complementary experiments in *C. reinhardtii* were deemed necessary.

Here we present the development of an efficient gene silencing method for green algae, which uses the combination of a genomic gene fragment in tandem with its cDNA counterpart in reverse orientation. The resulting transformants had reduced opsin levels, which allowed a detailed biophysical analysis of light behavioural responses.

MATERIALS AND METHODS

Strains, culture conditions and transformation

In this study we used *Chlamydomonas reinhardtii* strains *cw15 arg*$^-$A for biochemical studies and electrophysiology, whereas strain CC2454 was used for RNA assays and motion analysis. Cells were grown in standard TAP-medium (Harris, 1989) supplemented with L-arginine (50 mg/l) when necessary (*cw15 arg*$^-$A).

Plasmid-construction

A summary of DNA constructs for the expression of simple nonsense or antisense RNA in *C. reinhardtii* is shown in Fig. 1. In plasmid pMF15 one additional nucleotide was introduced into exon 1 of a 5.5 kb *Bam*HI-*Kpn*I *cop*-gene fragment. Plasmid pMF38 was made from pSP109 (Stevens et al., 1995) by exchanging *ble* against a 0.7 kb *cop*-cDNA fragment (Deininger et al., 1995). In pMF43 the 0.7 kb *cop* cDNA was inserted into the *Xba*I site of pSP124 (Lumbreras et al., 1998). Plasmid pMF47 was made by placing a 0.5 kb genomic *cop*-PCR-product between the *Msc*I and *Sac*I sites of pCOPV2.5 in inverse orientation (Fuhrmann et al., 1999). The plasmid pSR25 was designed to produce a single s/as transcript in vivo (Fig. 2). It was generated by insertion of a 0.4kb *Sac*I fragment of pKS-cop (Deininger et al., 1995) into the unique *Sac*I site of pCOPV2.5.

In vitro transcription and RNA-hybridization

Plasmid pOGF2 contains a 1.2 kb genomic PCR product, spanning the *cop*-region from exon 2 to exon 6, within the *Sma*I site of pBluescriptII KS$^-$ (Stratagene). To generate transcripts of defined length in vitro the plasmid was first linearized by cleavage with *Eco*RV or *Bam*HI, respectively. Sense and antisense strands were transcribed using either T3 or T7 RNA-polymerase (MBI Fermentas) according to the manufacturer's reaction conditions. Both transcripts were mixed, denatured at 65°C for 30 minutes and then hybridized by cooling the mixture to 25°C over a period of 3 hours. For co-transformation with plasmids pArg7.8 (Debuchy et al., 1989) or pSI103 (Sizova et al., 2001) a total of 20 μg of the in vitro RNA-transcript/μg DNA was used.

Transformation

Transformation was carried out according to the glass bead method (Kindle, 1990) with pArg7.8 (Debuchy et al., 1989) or pSI103 (Sizova et al., 2001) as selectable markers. Arginine prototroph transformants were selected on TAP-plates. *AphVIII* transformants were selected on TAP-plates supplemented with 10-20 mg/l paromomycin. The plasmids containing the antisense constructs contain no selectable marker, but integrate into the host genome in about 30-50% of recovered transformants.

Analysis of the opsin-content in wildtype and transformants

Single colonies of transformants were grown in 10 ml of selective medium to a density of 1-5×10^6/ml. Cells were harvested by centrifugation for 5 minutes at 2000 ***g*** and lysed by sonication in 10 mM MOPS, 1 mM EDTA, pH 6.8. Insoluble cell debris was removed by centrifugation for 5 minutes at 2000 ***g***. Total protein concentration of supernatants (whole cell extract) was determined by BCA-reaction (Pierce). Supernatant containing 120 μg of protein was diluted to 20 μl with 10 mM MOPS, 1 mM EDTA, pH 6.8 and mixed with 20 μl of 2× sample buffer pH 8.8, boiled for 5 minutes, and centrifuged at 14,000 ***g*** for 1 minute. 15 μl of the supernatant was applied on a 10% Tricin-SDS/polyacrylamide gel. Protein was transferred to nitrocellulose (Amersham), probed with α-COP antibodies (Deininger et al., 1995), and an alkaline phosphatase-labeled secondary antibody (Roche). Equal loading of each lane was confirmed by independent Coomassie stained gels.

RNA extraction and hybridization

RNA was extracted from 5×10^8 cells as previously described (Fuhrmann et al., 1999). Enrichment of poly-$(A)^+$ RNA was performed on oligo(dT)-cellulose columns with standard procedures. RNAs were separated on denaturing formaldehyde-agarose gels and transferred to Hybond N^+-nylon membranes (Amersham). DIG-labeled PCR-products of cDNA-fragments were used as gene probes

and detected with anti-DIG-AP-conjugates and *CDP-Star* as substrate (Roche).

Physiological analysis of transformants

Light triggered ionic currents were measured with a cell suction technique described in detail previously (Holland et al., 1996). Motion analysis of single cells was performed as described (Hegemann and Bruck, 1989). Cells were grown in 50 ml TAP to a final density of about 10^6 cells/ml and differentiated into gametes by overnight incubation in nitrogen-free medium (NMM). Three hours before the experiment was started, the culture was diluted 1:25 in NMM and kept in dim light (<1 W m^{-2} white light).

Photo-induced light scattering changes in cell suspensions were recorded as described (Uhl and Hegemann, 1990). The monitoring light (840 nm peak excitation), inefficient for either phototaxis or photosynthesis, was produced by a LED (6701SG, Hitachi). The actinic light was applied in the perpendicular direction to the monitoring light, its wavelength range was selected by a narrow-band interference filter (Schott KG, Wiesbaden, Germany; maximum transmission, 502 nm; 12 nm half band width) in combination with a heat protection filter. Light scattering changes were detected by an infrared-sensitive photodiode (860 nm, Hitachi) placed at a scattering angle of 16°, amplified by a custom-made preamplifier in series with a CyberAmp 320 signal conditioner (Axon Instruments, Union City, CA), and digitized by a TTL-1 Labmaster DMA board using pClamp 5.5 software (Axon Instruments) at 50 Hz. Cells were resuspended in NMM at a density of 1.7×10^6 cells ml^{-1} and kept overnight on a rotary shaker under cool fluorescent lamps (at about 2 W m^{-2}). A fresh cell sample was used each time after changing the stimulus intensity to avoid light adaptation of the photosensory system during the experiment.

RESULTS

Hairpin RNAs prove to be effective in lowering gene expression in *Chlamydomonas*

Because numerous experiments in *C. reinhardtii* designed for targeted disruption of the opsin (*cop*) gene had failed, several potential approaches to blockage of gene expression were tried (Fig. 1). First, we constructed an expression cassette that, after transformation into *C. reinhardtii*, should give rise to a nonsense RNA that triggers gene silencing in analogy to what similar constructs did in other organisms (Daar and Maquat, 1988; Quinn et al., 1993; Barnes, 1998). A single nucleotide insertion, leading to a frame-shift and two consecutive premature stop-codons, was introduced into exon1 of the *cop* gene. The *C. reinhardtii* strain *cw15 arg⁻A*, a cell wall-less strain that facilitates transformation with glass beads, was co-transformed with the resulting plasmid pMF15 and with a second plasmid containing the selectable gene *arg7*. Only 1.5% of the arginine prototroph transformants showed a reduction of the COP concentration, which did not exceed 50%.

Second, as the simplest approach for the production of asRNA, the complete *cop* cDNA (Deininger et al., 1995) was placed under control of the strong promoter and the 3′-end of the *rbcS2* gene (plasmid pMF38) but no changes in *cop* expression were detected in any of the transfomants. Thus, we concluded that, despite the strong promoter used, the amount of antisense RNA in these cells was insufficient to lower *cop*-gene expression. Since the phleomycin binding protein has to be expressed in large amounts to provide resistance to zeocine, the *ble* gene with two introns (pSP124) (Lumbreras et al., 1998) was inserted between the *rbcS2* promoter and the antisense *cop* cDNA (pMF43, Fig. 1) and used as the only selection marker. But still the COP reduction did not exceed 30%. Next, the strategy similar to the one used in *V. carteri* (Ebnet et al., 1999) also failed. An inverted fragment spanning exon 5 and 6, and including intron 5 linked to the forward directed exons 1 and 2 (pMF47) led to an opsin reduction of between 30 and 40%.

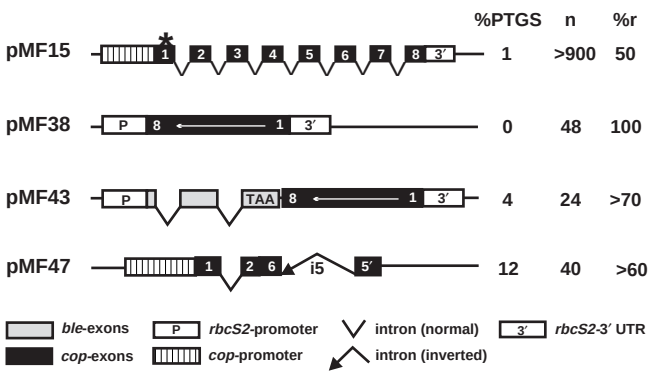

Fig. 1. Constructs for the potential triggering of *cop*-silencing in *Chlamydomonas*. The efficiency of different silencing constructs was determined by measuring the decreased opsin levels in transformants obtained with each plasmid. Construct pMF15 was designed to induce cosuppression by providing a *cop* gene containing a nonsense mutation early in the gene (*). The constructs pMF38-47 provided a single 'antisense RNA' to target *cop*-mRNA under the control of a strong promotor. Arrows show the antisense portions of the *cop*-gene. Numbers within black segments of the diagram correspond to the position of exons in the *cop*-gene.%PTGS is calculated as the number of clones showing a reduction in chlamyopsin of more than 20% divided by the number of tested clones (n).%r is the amount of residual Cop protein in the mutant with the lowest observed Cop-level in each experiment.

Interference of double-stranded RNA (dsRNA) with expression of specific genes was described first to occur in *Caenorhabditis elegans* (Fire et al., 1998). Comparable observations were made later in a large number of organisms, including *Drosophila*, protozoans such as *Trypanosoma*, and plants (Gura, 2000). Although the mechanism is still not understood in detail, RNA interference (RNAi) provides an extremely powerful tool to study gene function in many organisms. To test the potential of double stranded RNA (dsRNA) on the reduction of gene expression in *C. reinhardtii*, sense and antisense RNA of the *cop* coding region were generated in vitro and used in co-transformation experiments (Fig. 2a). 14 days after transformation the transformants were tested for their COP content. None of the 48 clones showed a reduction of more than 10%. Thus, either dsRNA was not functional in *C. reinhardtii* or the RNA was rapidly degraded before any RNA-inactivation mechanism became active. To achieve a dsRNA level that fluctuates in parallel with the endogenous *cop* RNA, a large fragment of the *cop* cDNA covering exon 1 to 5 was linked in inverse orientation to the corresponding genomic fragment under control of a 1.5 kb fragment of the endogenous *cop* promotor (pSR25 in Fig. 2a). The presence of the introns 1 to 4 prevented recombination of potential hairpin structures during cloning in *E. coli*. In analogy to the advantages of introns for expression of genes encoding

dominant selectable markers (see above), it was anticipated that the presence of introns in pSR25 might assist in maintaining an expression level of the transgene similar to that of the authentic *cop* gene. We surmised that after excision of the introns, a self-complementary hairpin RNA (hpRNA) might be formed immediately. The resulting hpRNA would be a perfect inverted repeat without a linker between the sense and antisense region, as it was reported as the most efficient trigger for PTGS in plants (Smith et al., 2000).

About half of the *C. reinhardtii* transformants obtained by co-transformation with pSR25 and pArg 7.8 showed a significant reduction in their opsin content (Fig. 2b). Transformant T7 and T12 expressed less than 5 and 2% COP, respectively, in comparison with wild-type (wt) cells. The reduction of COP is most clearly seen from the protein pattern of eyespot preparations in Fig. 2c. Expression of hpRNA seems to be the most efficient method for a specific reduction of a selected protein in *C. reinhardtii*.

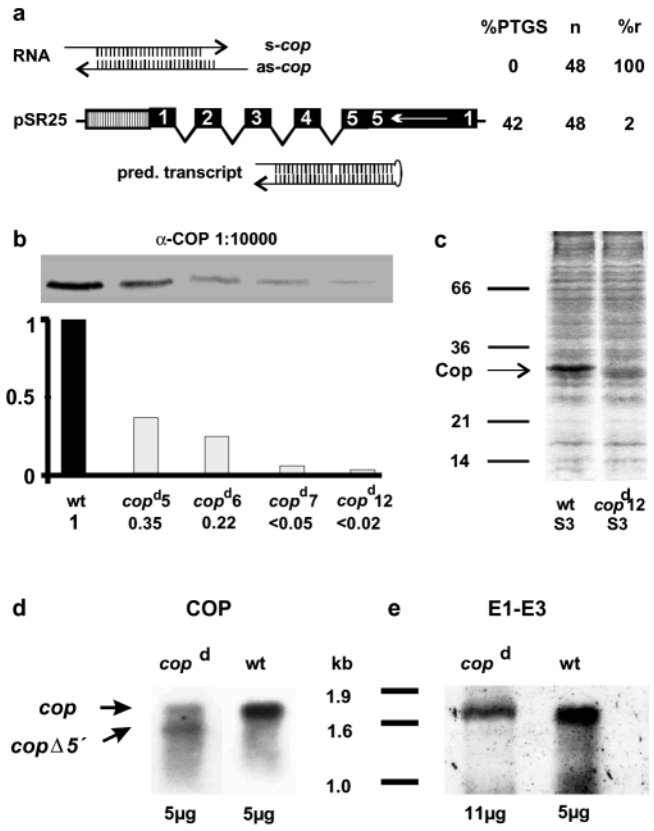

Fig. 2. Efficient silencing of *cop* gene expression mediated by 'RNAi'-like constructs. (a) DNA-constructs tested for their RNAi mediating activity. (b) Relative amounts of opsin in different pSR25 transformants compared to the wild-type as determined by densitometric measurements of the Cop-band intensity on western blots using immunodetection. (c) Comparison of purified eyespot membranes (S3-fraction) of recipient cw15 arg$^-$A cells and the transformant cop^{d}12. (d,e) Comparison of *cop*-transcripts by RNA-blot analysis. (d) Equal amounts of mRNA from wt (CC2454) cells and pSR25 transformant 35 (cop^d) were fractionated on an agarose gel, blotted and probed with full length DIG-labeled *cop*-cDNA. (e) 5 µg wt mRNA and 11 µg of transformant mRNA were hybridized with DIG-labeled *cop* fragment comprising exon 1-3.

RNA analysis

To determine the influence of hpRNA on *cop* RNA levels in the recovered transformants, RNA-blot analysis was performed in the hpRNA transformants cop^{d}12 (cop^d=Cop deficient) with a full length *cop*-cDNA as a probe. As shown in Fig. 2d, in wild-type cells a single *cop* transcript of 1.7 kb is detectable. In cop^{d}12 the amount of this transcript was reduced to only threefold, which is less pronounced than we expected from the reduction of the protein (50-fold). In addition, a smaller transcript of a defined size around 1.3 kb was detected. This was surprising, because usually truncated RNAs are rapidly degraded by a specific set of endogenous nucleases under most conditions. To further examine the nature of the shorter RNA and to test whether the truncation is related to the RNAi-construct pSR25, a different probe covering only exon 1-3 of *cop* was used. In this second experiment more transformant mRNA than wt mRNA was applied in order to enable better visibility of the truncated fragment. The smaller probe only identified the full-length *cop* transcript. This finding points to the conclusion that the 1.3 kb RNA represents an RNA truncated at its 5′-end in a region covered by the hpRNA. The attempt to analyze the 5′-end of the transcript by 5′-RACE PCR was unsuccessful even after treatment with tobacco acid pyrophosphatase to remove a potential CAP structure.

Photocurrents and behavioral responses of COP-reduced transformants

Microscopic observations and measuring the average swimming speed of the cop^d-transformants with a motion analysis system (Holland et al., 1996) revealed a significant reduction of motility in many of them, but no correlation

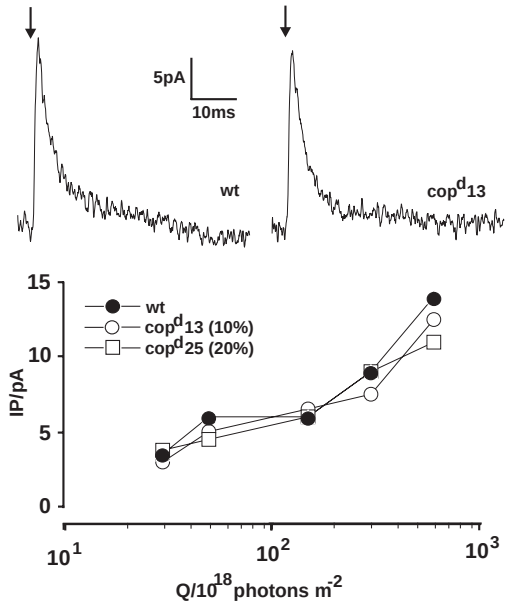

Fig. 3. Characterization of flash-induced ion currents of individual cop^d mutant cells. Photocurrents of an untransformed cw15arg$^-$A cell and a cop^{d}13 transformant. Photocurrents (I_{P1}) were plotted versus the photon exposure of the flash (Q). No intensity dependent differences in the amplitude of the photoreceptor current I_{P1} were observed in three cell lines with different Cop content.

between the reduction of motility and the amount of the *cop*-gene product assayed by the western blot analysis was found.

Since fast swimming cells with COP levels below 10% of that of wild-type were not found, electrophysiological studies were carried out with two cw15 transformants that expressed 10 to 20% of the wild-type COP level and showed normal motility. The photoreceptor currents recorded in these transformants were identical to those of the wild-type cw15 cells, with respect to kinetics and light sensitivity (Fig. 3). Recording photocurrents from the cw15 strain by means of the suction pipette technique yields a lower success ratio, and the average amplitude of the recorded currents is smaller than that of the cw2 strain used in all earlier studies (Holland et al., 1996). This is probably due to somewhat rigid remains of the cell wall present in the cw15 strain, which interfere with formation of a high-resistance seal with the pipette.

The photoreceptor current, PC, in *C. reinhardtii* comprises a low light saturating component, a, and a high light saturating component, b (Sineshchekov et al., 1990; Ehlenbeck et al., 2001). PCb dominates the photoreceptor current at high flash intensities and is the trigger for the flagellar current and subsequent photophobic responses in wt cells, whereas PCa is clearly visible at low light levels and is proposed to trigger individual directional changes or phototaxis in continuous light (Holland et al., 1997). Phobic responses were analyzed by using the motion analysis system (Hegemann and Bruck, 1989). Cells were placed under the microscope and their swimming speed was monitored with a video camera under infrared light. Upon stimulation with bright flashes the cells switch from forward swimming to slow backward swimming, which lasts for a few hundred milliseconds. This phobic response is seen as a pronounced speed reduction when the average speed of >100 cells is calculated (Fig. 4). The photophobic response of COP-reduced wt cells (5% COP) appeared identical to that of the wild-type control cells. The sensitivity of the cells in regard to this phobic response remained unchanged after reduction of the COP expression. Phototactic orientation was measured by a light scattering assay, as described (Uhl and Hegemann, 1990), because this method is characterized by both high sensitivity and statistical confidence of the results. The orientation of the cells with respect to the direction of the actinic light stimulus induces a change in the scattering pattern of the indifferent monitoring light, which can be recorded by a properly placed sensor (Fig. 5). The amplitude of this change is proportional to the degree of phototactic orientation of the cells under continuous illumination (Schaller et al., 1997). Similar time courses of the light scattering changes in *cop* antisense transformants and the wild-type showed that the cell motility was not affected by the transformation in the CC2454 strain. The light sensitivity of the photoorientation responses measured in the *cop* antisense transformants with normal and reduced levels of the expressed opsin were indistinguishable within the limits of the experimental error. This result shows that the *cop* gene product probably does not serve as the phototaxis receptor in *Chlamydomonas*.

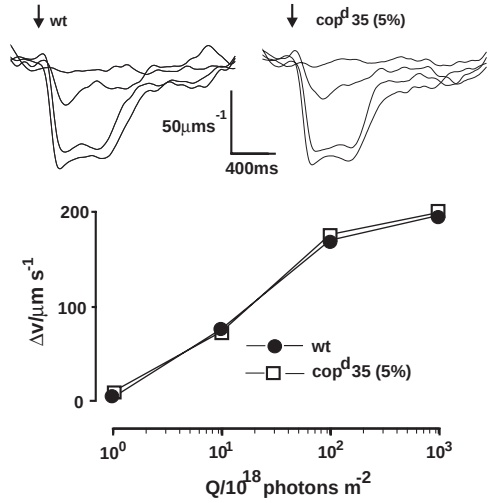

Fig. 4. Characterization of flash-induced photophobic responses of individual cop^d mutant cells. Average actual speed changes of individual cells after application of light flashes with different intensities (100, 10, 1 and 0.1%). The observed maximal speed reduction (Δv) was plotted versus the photon exposure (Q). No intensity dependent differences in the stop response are observed between wt (CC2454) and cop^{d}35 (opsin content <5%).

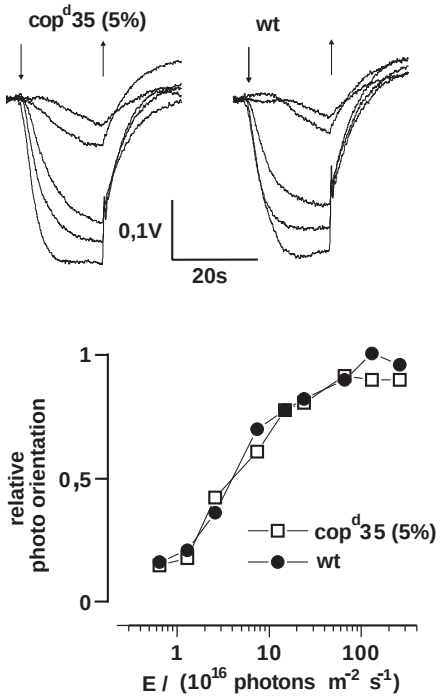

Fig. 5. Phototactic orientation measured with a light scattering assay on cell populations of opsin antisense transformants with normal and reduced levels of the expressed opsin. Light scattering changes induced in cell suspensions by a 20 second pulse of continuous 502 nm light (CC2454 cop^{d}35 with 5% opsin and cop^{d}14 with a wt-opsin level. Traces were recorded at fluence rates (from below): 261, 14.8, 7.4, 2.6 and 1.3×10^{16} photons m^{-2} s^{-1}; each trace is a mean value of two measurements in separate cell samples. Photoorientation was calculated as the absolute value of the difference between the output voltage measured after 20 seconds of illumination and 20 seconds after the light was switched off. Data points are mean values±s.d. of two measurements in separate cell samples.

DISCUSSION

PTGS in *C. reinhardtii*

Because the targeted disruption of a gene function by homologous recombination is very inefficient in green algae, we searched for alternative methods for inactivation of gene expression in order to reach a better understanding of the opsin function in green algae. As demonstrated above for *C. reinhardtii*, the use of hairpin RNA techniques for gene silencing is an effective alternative to homologous recombination. This finding is consistent with those in higher plants where the requirements for graded gene silencing by PTGS are also more favorable than for targeted gene disruption (Voucheret et al., 1998). In various models of initiation and maintenance of PTGS, dsRNA is proposed to be an important intermediate in the process. This claim is supported by the finding that in several different systems, mutants defective in RNA-dependent RNA-polymerase (RdRP) cannot mount a PTGS response after transformation with appropriate PTGS-inducing molecules (Cogoni and Macino, 1998). In the case of *Arabidopsis*, it was demonstrated that in RdRP-deficient mutants the PTGS response could be initiated by dsRNA produced by a viral polymerase that is active during normal virus replication (Gura, 2000). Another player in the PTGS reaction pathway is a double strand-specific RNAse that generates small (21-23nt) sense and antisense RNAs recently identified in plants and in *Drosophila* (Hamilton and Baulcombe, 1999; Hammond, 2000). Finally an RNA-helicase involved in PTGS was identified by insertional mutagenesis in *C. reinhardtii* (Wu-Scharf et al., 2000). Based on these findings and the discovery of the truncated RNA in *C. reinhardtii*, a hypothetical reaction sequence very similar to that proposed previously (Bass, 2000) may be envisioned for hpRNA-induced PTGS in *C. reinhardtii*: (1) a dsRNA-specific RNA-nuclease recognizes the hpRNA; (2) the nuclease domain cleaves hpRNA, generating small s/as RNA molecules that remain bound to the enzyme complex; (3) an RNA-helicase domain mediates the interaction between complementary sequences of the small associated RNAs and the target mRNAs, which leads to an exchange of the mRNA for its short analog and the release of the non-paired small sense RNA from the complex; (4) the nuclease cleaves the bound target mRNA (single strand cut) to regenerate the functional nuclease s/as RNA complex and a specifically truncated target RNA.

The photoreceptor for photomovement responses?

In RNAi transformants with reduced COP content, stimulus-response curves should unequivocally decide whether the opsin-related protein, COP, is the photoreceptor for the estimated function or not. This is because a reduction of the photoreceptor content must reduce the absolute light sensitivity of the system and shift the stimulus-response curve to higher light intensities. This is true under the assumption that most of the photoreceptor molecules in a wild-type cell are functional. However, if the majority of the 10,000 to 30,000 opsin molecules per cell were non-functional, neither the low threshold for photocurrents and phototaxis could be explained nor the large dynamic activity range extending over >4 decades of light intensity (Foster et al., 1984) (S. Ehlenbeck et al., unpublished). The unchanged phototaxis and photophobic responses and the identical stimulus-response curves in wild-type and COP-deficient transformants shown in this study should leave no doubt that the COP protein is neither the photoreceptor for photophobic responses nor for phototaxis. This claim should be regarded as proven at least for the tested time range of 1 minute and range of light stimulus intensities. This is in sharp contrast to earlier conclusions that were based on a set of biochemical and electrophysiological evidence summarized in the introduction above (Deininger et al., 1995; Holland et al., 1996; Calenberg et al., 1998).

Stimulus-response curves for phototaxis that shift to higher light intensities are difficult to interpret because phototaxis is intimately linked to a precise flagellar beating. The slightest motility change reduces the phototactic rate and possibly the phototactic threshold. This difficulty may have led to a misinterpretation of the phototaxis experiments in VOP-deficient transformats of *Volvox carteri*, where the stimulus-response curve was shifted tenfold to higher light intensities than those of wild-type spheroids (Ebnet et al., 1999).

The function of COP, and its relative VOP, as photoreceptors remained suspicious from the beginning of their identification. First, in contrast to their sequence homology to invertebrate rhodopsins, both sequences are highly charged and 7 seven transmembrane helices, typical for any rhodopsin found so far, could not be identified in any hydropathy plot (Deininger et al., 1995). Second, volvoxopsin was enriched in eyespots of somatic cells but eyeless and electrically unresponsive gonidia contain more VOP than the eye-containing somatic cells. Third, COP expressed in *E. coli*, *S. pombe* and *P. pastoris* did not form any chromophore that is red-shifted relative to free retinal (W. Deininger and P.H., unpublished). Fourth, the all-trans chromophore and its isomerization to 13-cis after light excitation, as studied in vivo on retinal supplemented white cells, favoured an archaean-type rhodopsin (Lawson et al., 1991; Hegemann et al., 1991; Takahashi et al., 1991), whereas COP and VOP are related to the animal opsin group. The conclusion that COP is not the photoreceptor for behavioural responses implies two further important consequences. First, the rhodopsin that triggers phobic responses and phototaxis still awaits identification, and second, the function of the major opsin-related retinal protein of the algal eye remains obscure.

We thank Don Weeks and Wolfgang Oertel for helpful comments and critical reading of the manuscript. The work was supported by the *Deutsche Forschungsgemeinschaft* and the *Fond der Chemischen Industrie*.

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