Photoactivated Adenylyl Cyclase Controls Phototaxis in the Flagellate Euglena gracilis

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Euglena gracilis, a unicellular freshwater protist exhibits different photomovement responses, such as phototaxis (oriented movement toward or away from the light source) and photophobic (abrupt turn in response to a rapid increase [step-up] or decrease [step-down] in the lightfluence rate) responses. Photoactivated adenylyl cyclase (PAC) has been isolated from whole-cell preparations and identified by RNA interference (RNAi) to be the photoreceptor for step-up photophobic responses but not for step-down photophobic responses (M. Iseki, S. Matsunaga, A. Murakami, K. Ohno, K. Shiga, C. Yoshida, M. Sugai, T. Takahashi, T. Horii, M. Watanabe [2002] Nature 415: 1047–1051). The present study shows that knockdown of PAC by RNAi also effectively suppresses both positive and negative phototaxis, indicating for the first time that PAC or a PAC homolog is also the photoreceptor for photoorientation of wild-type E. gracilis. Recovery from RNAi occurred earlier for step-up photophobic responses than for positive and negative phototaxis. In addition, we investigated several phototaxis mutant strains of E. gracilis with different cytological features regarding the stigma and paraxonemal body (PAB; believed to be the location for the phototaxis photoreceptor) as well as Astasia longa, a close relative of E. gracilis. All of the E. gracilis mutant strains had PAC mRNAs, whereas in A. longa, a different but similar mRNA was found and designated AlPAC. Consistently, all of these strains showed no phototaxis but performed step-up photophobic responses, which were suppressed by RNAi of the PAC mRNA. The fact that some of these strains possess a cytologically altered or no PAB demonstrates that at least in these strains, the PAC photoreceptor responsible for the step-up photophobic responses is not located in the PAB.

The protist Euglena gracilis, a unicellular freshwater flagellate, is capable of both autotrophy and heterotrophy. The cell is characterized (Fig. 1A) by one flagellum emerging from the reservoir (invagination of the anterior plasma membrane) and a second nonemerging flagellum. The paraxonemal body (PAB) is a photosensing organelle (Ghetti et al., 1985) located inside the reservoir close to the connecting point of the two flagella. The stigma, formerly known as the eyespot, is positioned inside the cytoplasm and adjacent to the PAB. It contains carotenoids and is not involved in photoorientation (Lebert and Hader, 1997) by shading the PAB as the cell rotates around its longitudinal axis during forward swimming. A detailed description of E. gracilis can be found in Buetow (1968).

E. gracilis uses light and gravity for orientation to move to and stay at optimal growth conditions in the water column. Light-induced responses (Lebert and Hader, 2000) can be categorized into photokinesis, a light-dependent swimming velocity; phototaxis, an oriented movement toward (positive phototaxis) or away (negative phototaxis) from the light source (Hader et al., 1981); and photophobic responses (Mikolajczyk, 1984; Walne et al., 1984; Doughty, 1993). The latter ones occur when the cells experience a sudden change in light intensity and are characterized by a period of tumbling and subsequent swimming seemingly in a random direction. Photophobic responses caused by an abrupt increase in light fluence rate are called step-up photophobic responses. Those caused by an abrupt decrease are referred to as step-down photophobic responses. Recently, the receptor of step-up photophobic responses in E. gracilis has been isolated and identified (Iseki et al., 2002) to be a flavoprotein photoactivated adenylyl cyclase (PAC). PAC represents a novel blue-light receptor consisting of two α-subunits (PACα) and two β-subunits (PACβ). Each subunit contains two flavin-binding domains and two adenylyl cyclase catalytic domains (Iseki et al., 2002). Excitation of the receptor protein in vitro by UV-17 blue light (peaks at 370 and 450 nm) results in the formation of cAMP, which is thought to alter the flagellar beat pattern and trigger step-up photophobic responses. The overall similarity between PACα and PACβ is 72% at the nucleotide
Reverse genetics (RNA interference [RNAi]; see below) revealed that PAC is not the photoreceptor of step-down photophobic responses (Iseki et al., 2002), which is consistent with the different action spectrum of this response (Matsunaga et al., 1998). The photoreceptor of phototaxis was up to now also unknown, but action spectroscopy suggested the involvement of flavins and pterins (Brodhun and Hader, 1990; Hader and Lebert, 1998). Other researchers proposed that the phototaxis receptor is not a flavoprotein but a rhodopsin-like protein (Walne et al., 1998; Barsanti et al., 2000).

The aim of the present study was to investigate the function of PAC in step-up photophobic responses in colorless *E. gracilis* mutants and in *A. longa*, a non-photosynthetic close relative of *E. gracilis* which lacks a PAB. Furthermore, we aimed at clarifying the role of PAC in both positive and negative phototaxis in wild-type *E. gracilis*.

**RESULTS**

Presence and Localization of PAC or PAC Homolog in *E. gracilis* Mutants and *A. longa*

The presence of an intact PAB has been detected only in the cells of wild-type *E. gracilis* (normal PAB; Fig. 1A) and the mutant strain FB (smaller PAB; Fig. 1B) by autofluorescence and light microscopy, whereas no PAB was detected in the strains 1F and 9F, as well as in *A. longa* (Fig. 1C; Lebert and Hader, 1997). Irrespective of the presence of an intact PAB, PAC mRNAs were detected by reverse transcriptase-PCR in all of the *E. gracilis* strains (Fig. 2, A and B). After several attempts to detect PACα and PACβ in *A. longa* by reverse transcriptase-PCR, we found two similar but distinct mRNAs. The deduced amino acid sequences were very similar to PACα and PACβ. Because consensus amino acids in the flavin-binding domains and those in the adenylyl cyclase catalytic domains are conserved well, these proteins are expected to constitute an adenylyl cyclase that can be activated by light. The proteins, which seem to be orthologs of PACα and PACβ, were named AlPACα and AlPACβ, respectively (accession nos. AB085169 and AB085170; Table I).

Upon examination of the role of PAC in photomovement responses in these strains by RNAi, step-up photophobic responses were suppressed in all of the strains tested regardless of their anatomical and molecular differences. RNAi for one of the PAC subunits was sufficient to suppress the responses. Recovery of step-up photophobic responses occurred first in *A. longa* (3 weeks) followed by the other strains (5–6 weeks).

**Involvement of PAC in Phototaxis**

Among all strains investigated, only wild-type *E. gracilis* exhibits normal phototaxis; we therefore examined the possible function of PAC in phototaxis in this strain quantitatively using an in-house-developed computerized motion analysis system (Hader and Lebert, 2000). Gene silencing of PAC by RNAi resulted in complete suppression of negative and positive phototaxis (Fig. 3). This suppression persisted for more than 2 months (Figs. 4 and 5). As in the case of step-up photophobic responses, no...
differences were observed between silencing PACα or PACβ alone or both subunits. Recovery of phototaxis, however, differed significantly from that of step-up responses: Whereas the suppression of step-up photophobic responses persisted for up to 6 weeks, suppression of phototaxis persisted for more than 3 months (Figs. 4 and 5). Both positive and negative phototaxis reappeared almost simultaneously. Other parameters observed by motion analysis, including velocity and form factor (indicator of cell shape), remained normal after RNAi (data not shown), indicating that the silencing process did not affect such cell functions.

### DISCUSSION

Light is an important parameter for growth of *E. gracilis*. Although *E. gracilis* can live heterotrophically, optimal growth occurs in light. Positive phototaxis, supported by negative gravitaxis (Lebert et al., 1999), leads the cells to the light source to facilitate photosynthesis, whereas negative phototaxis protects the cells from damage induced by excessive solar radiation at the surface. So far, the photoreceptor had been characterized only for step-up photophobic responses (Iseki et al., 2002). Here, we demonstrate that PAC is responsible not only for the step-up photophobic responses in the wild type but also in all *E. gracilis* mutant strains investigated, even in those with a smaller PAB (mutant strain FB) or without a visible PAB (mutant strains 1F and 9F). Therefore the presence of a PAB is not a necessary prerequisite for step-up photophobic responses. Furthermore, *A. longa*, a colorless close relative of *E. gracilis*, lacks a PAB altogether but shows step-up photophobic responses that were also inhibited by...
RNAi. This result strongly confirms that the PAC is not necessary for step-up photophobic responses. At least in these cases, PAC or AlPAC must be located somewhere else in the cell, e.g. in the flagellum. The chloroplasts are not the location because all strains investigated except for wild-type *E. gracilis* do not possess chloroplasts.

RNAi is a powerful tool that has been applied to a wide range of organisms from unicellular protists (Ngo et al., 1998) to mammals (Zhang et al., 2003). Studies conducted so far indicate that specificity of RNAi depends mainly on the organism. Nonspecific degradation of homolog genes has been described for lower eukaryotes (Alder et al., 2003) and plants (Hamilton et al., 2002). In contrast, RNAi is highly specific in mammals (Elbashir et al., 2001; Chi et al., 2003) when assayed with small interfering RNAs of 21 bp in length. Nonspecific degradation of mRNAs can also be triggered in mammals if longer double-stranded RNA (dsRNA) is used leading to general inhibition of translation (Bass, 2001). The interference of *E. gracilis* PAC dsRNA with *A. longa* AlPAC mRNA indicates a nonspecific inhibition also found in other lower eukaryotes.

In addition to step-up photophobic responses, gene silencing of PAC inhibited phototaxis, another light-dependent response of *E. gracilis*. Inhibition occurred for positive as well as for negative phototaxis and was characterized by random swimming. Interestingly, recovery occurred simultaneously for positive and negative phototaxis but significantly later than for step-up photophobic responses in the same cells. Either a lower threshold level of requirement of PAC in the latter or sequential recovery of the PAC compartmentation for the two different photomovement responses would be a possible explanation.

These data suggest, but do not give definite proof, that the PAC receptor responsible for step-up photophobic responses also serves as photoreceptor for phototaxis. Alternatively, a related PAC homolog might be responsible for light perception for phototaxis in *E. gracilis*. If the latter option is the case, the PAC homolog might be degraded nonspecifically as shown for AlPAC. Finally, PAC could influence downstream events in phototaxis.

**CONCLUSIONS**

PAC is the photoreceptor of step-up photophobic responses in *E. gracilis* wild type and the mutant strains 1F, 9F, and FB as well as in *A. longa*, a close relative of *E. gracilis*. Because in some strains, the PAC has an altered structure or is even absent, the PAC photoreceptor for step-up photophobic responses must have a different location in the cell. A PAC photoreceptor also controls positive and negative phototaxis of *E. gracilis* (wild type). Knockdown cells could not orient in low- and high-light conditions, and swimming was at random.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**

*Euglena gracilis* Z wild type, mutant strains 1F (1224-5/1F), 9F (1224-5/9F), and *Astasia longa* wild type were obtained from Sammlung für Algenkulturen (Göttingen, Germany). Mutant strain FB was a kind gift of Prof. R. Hertel (University Freiburg, Freiburg, Germany) originally isolated by Dr. L. Barnanti (Consiglio Nazionale delle Ricerche, Pisa, Italy). All strains were grown in a rich medium described previously (Starr, 1964) at 22°C under low-light conditions (25 W m⁻²) in static Erlenmeyer flasks.

**Identification of AlPAC**

Total RNAs were purified from 7-d-old culture of *A. longa* using RNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized using ReverTra Ace (TOYOBO, Osaka) and the Oligo dt 3 ts Adaptor Primer (TaKaRa, Otsu). PCR amplification of the cDNA was done with LA-TaqDNA polymerase (TaKaRa) and sets of primers originally designed for PACα (*EgBR2S1*, 5’-GGGTATGGCATCCCTG-3’; *EgBR2A10*, 5’-TCAACCTTACATACCAAG-3’) and PACβ (*EgBR2S7*, 5’-ACATCTCGAAGCCACTC-3’; *EgBR2A7*, 5’-CCGGTTAGGAAATCGAC-3’). According to the sequence of the PCR products, specific primers for AlPACα (*EgBR51*, 5’-GAACACTTCTCTATG-3’) and AlPACβ (*EgBR44*, 5’-CATCAGTCGACACTTCTCT-3’) were synthesized. 5’ ends of the cDNAs encoding AlPACα and AlPACβ were amplified by PCR using the specific reverse primers and the primer (EgLS1, 5’-TCTTACTAGTGTCTATTTTTTCC-3’) that was designed according to the consensus sequence of the leader sequence of *E. gracilis* mRNAs (Tessier et al., 1991). 3’ ends of the cDNAs were extended by conventional 3’-RACE technique using the specific forward primers and the 3 sites Adaptor Primer (TaKaRa, Japan). Sequences of the PCR products were determined by direct sequencing using DYEEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) and ABI PRISM 310 (Applied Biosystems, Foster City, CA).

**RNAi**

Knockdown of PAC by RNAi was accomplished by synthesizing dsRNA fragments corresponding to the N-terminal site of PACα and PACβ. These fragments of 616 bases for PACα and 618 bases for PACβ contained the first flavin-binding domain in both subunits. Each strain was treated three times with RNAi for PACα and PACβ alone and both subunits simultaneously. dsRNA was generated as described (Seki et al., 2002; supplemental material at http://www.nature.com). The procedure entails amplification of a fragment corresponding to the 5′ end of each gene from pGEM (Promega, Madison, WI) containing either PACα or PACβ DNA with Pfu proofreading polymerase (Invitrogen, Carlsbad, CA). A T7 promoter was ligated to the cDNA fragments of 616 bases for PACα alone and both subunits simultaneously. Sequences of the PCR products were determined by direct sequencing using DYEEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) and ABI PRISM 310 (Applied Biosystems, Foster City, CA).

**Step-Up Photophobic Responses and Phototaxis**

Motion analysis was conducted with a computer-based cell tracking system using Wintrack 2000 (Häder and Lebert, 2000). Before measurement, cells were adapted to darkness for 1 h, and all further manipulations were performed under dim red light. For observation, cells were transferred into glass chambers consisting of two microscope slides sealed at the edges with silicone. The light source was a slide projector and desired intensities were achieved using neutral glass filters. The fluorescence rate was 800 W m⁻² for step-up photophobic responses and negative phototaxis and 10 W m⁻² for...
positive phototaxis. Cells were tracked for 2 min, and all measurements were done in duplicate. Precision of orientation was described by the r value, a statistical parameter ranging from 0 (random orientation) to 1 (perfect orientation; Batschelet, 1981). Cell shape was calculated from the form factor starting from 1 (round cells) and increasing as the cells become more elongated. The elongated cells of *E. gracilis* tend to become more rounded in various conditions like the presence of toxins or excessive irradiances. The form factor is therefore used as an indicator for cell fitness. At the time of measurement, cultures were 1 week old.

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**LITERATURE CITED**


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