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Characterization of Blue Light Signal Transduction Chains That Control Development and Maintenance of Sexual Competence in *Chlamydomonas reinhardtii*[1]

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Blue light induces the differentiation of *Chlamydomonas reinhardtii* pregametes to gametes. The light-induced conversion of pregametes to gametes is protein synthesis dependent and proceeds only after a lag phase. Upon incubation in the dark, gametes lost their mating ability, resulting in dark-inactivated gametes. Reillumination rapidly restored mating competence and this was shown to be independent of protein synthesis. Apparently, differentiation and maintenance of gametic competence are both regulated by light. Whether one or two light-activated signal pathways are involved was investigated using pharmacological compounds that affect signal transduction. Compounds that affected pregamete-to-gamete conversion affected the expression of a gamete-specific gene in a similar fashion. Other drugs affected only dark-inactivated gametes, suggesting that reactivating gametes requires a separate signaling pathway. Combined treatments provided evidence for the consecutive action of a phosphatase and a protein kinase C-like kinase in the light-induced reactivation process.

Blue light responses have been observed in many higher and lower plants. They are evolutionary among the most ancient and are detected in lower eukaryotes, such as the green alga *Chlamydomonas reinhardtii*. Analysis of molecules involved in perceiving blue light has made progress recently with the cloning and characterization of a blue light photoreceptor from *Arabidopsis thaliana* (Ahmad and Cashmore, 1996). However, analysis of mutants defective in this photoreceptor showed that only a subset of blue light responses was affected. This is taken as an indication for the presence of additional molecules involved in blue light perception, one candidate for which is a plasma membrane protein that is phosphorylated upon irradiation (Short and Briggs, 1994; Liscum and Briggs, 1995). Little is known about immediate downstream signaling events, although a membrane-bound GTP-binding activity in pea seedlings has been shown to be specifically activated by blue light (Warpeha et al., 1991; Kaufman, 1993). Downstream components of blue light signal pathways have been identified genetically by the isolation of mutants defective in signal transmission (Kaufman, 1993; Quail, 1994; McNellis and Deng, 1995) but the molecular nature of these components remains to be determined.

The unicellular green alga *C. reinhardtii* is well suited for the investigation of blue light-signaling cascades. One system studied is the blue light induction of genes encoding enzymes for early steps of chlorophyll and heme biosynthesis (Matters and Beale, 1995), where an involvement of Ca^{2+} and calmodulin in signal transduction has been demonstrated (Im et al., 1996). Blue light is also required for sexual differentiation of this alga (Weissig and Beck, 1991). Gametogenesis is initiated by nitrogen deprivation, resulting in mating-incompetent pregametes when cells are kept in the dark. Blue light induces the differentiation of pregametes into mating-competent gametes (Treier et al., 1989; Weissig and Beck, 1991; Beck and Acker, 1992; Quarmby, 1994; Beck and Haring, 1996). In higher plants, the analysis of blue light-signaling pathways is confronted with an extra challenge caused by the involvement of phytochrome in various blue light-mediated responses (Quail, 1994). This has led to the proposal of some kind of coaction between the photoreceptors (Mohr, 1994). Since neither phytochrome itself (Bonenberger et al., 1994) nor phytochrome-mediated responses has been detected in *C. reinhardtii*, the analysis of blue light-signaling chains should be more straightforward.

In *C. reinhardtii* gametogenesis controlling a blue light-signaling pathway with components that promote signal flux and others that attenuate signal flux is suggested by studies with agonists/antagonists of signal transduction. The data obtained with *C. reinhardtii* support the concept of a basal flux passing through a signal chain in the absence of extrinsic stimulation, as postulated for signal pathways in higher plants (Bowler and Chua, 1994). A PKC-like activity that appears to operate against signal flux was defined by inhibitor/activator studies. Inhibition of its activity caused constitutive activation of the signal pathway; its activation resulted in inhibition of signal transduction. A second pro-

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Abbreviations: db-cAMP, dibutyryl-cAMP; *GLE*, gamete lytic enzyme gene; PKC, protein kinase C; SC-10, *N*-heptyl-5-chloro-1-naphthalenesulfonamide; TAP, Tris-acetate-phosphate.

tein kinase was shown to promote signal flux downstream from the PKC-like enzyme (Pan et al., 1996).

Here we report on a blue light-signaling pathway that maintains the mating competent state of gametes. Darkness inactivates gametes, a process that can be reverted by light. Using inhibitors of protein synthesis and pharmacological agents known to interfere with signal transduction, we provide evidence for a blue light-signaling pathway that reactivates dark-inactivated gametes and that is different from that controlling the conversion of pregametes to gametes.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii wild-type strain CC-124 (*mt*−) was used as the tester strain. CF14, a sibling from a cross between CC-1010 (*mt*+) and 137c (*mt*−), served as the mating partner. Another wild-type strain tested that exhibits strict light dependence for gametogenesis was C-9 (*mt*−) (obtained from T. Saito, Kobe University, Japan). Cells were inoculated from TAP (Harris, 1989) agar plates into Erlenmeyer flasks and grown at 23°C under continuous white light (14 W/m^2) with shaking as described previously (Treier et al., 1989; Pan et al., 1996).

Reagents

The pharmacological compounds were purchased from the sources listed previously and the stock solutions were prepared as described before (Pan et al., 1996).

Gametogenesis

Different gametogenesis protocols were used. For mating partner CF14, gametes were generated by resuspending vegetative cells in nitrogen-free TAP medium (Harris, 1989) at a density of 1.2×10^7 cells/mL and incubated under continuous light for 16 to 24 h. For the generation of pregametes from strain CC-124, liquid cultures of vegetative cells were centrifuged and resuspended in nitrogen-free TAP medium at a density of 1.0×10^7 cells/mL and incubated for 16 h in the dark. Gametes were obtained either from pregametes by exposing them to light for 2 h or by incubation of vegetative cells in nitrogen-free TAP medium with continuous irradiation for 18 h. Dark-inactivated gametes were generated by dark incubation of gametes produced by the two-step protocol, i.e. involving pregametes. The effects of agonists/antagonists were tested by the protocol described previously (Pan et al., 1996). In dark experiments care was taken to avoid any exposure of the cells to light. For this purpose, cultures were wrapped and placed in a lightproof black box.

Determination of Mating Competence

We followed the protocol described previously (Pan et al., 1996). Briefly, equal volumes of cells from opposite mating types were mixed together. The mating was allowed to proceed in the dark for 30 min and stopped by adding glutaraldehyde (final concentration 0.5%). The percentage of quadriflagellate cells was microscopically recorded and the mating percentage was calculated as described by Beck and Acker (1992).

Illumination Protocol Using Different Wavelengths

The light source used was a 500-W lamp (110 V, 500 W, Type 285, Osram, Germany) mounted in a slide projector. The light beam was collimated by a lens before it passed through an interference filter. A mirror was used to direct the light to the cell suspensions in the beakers. The bandwidths (full width at half-maximum) of the interference filters with maximal transmission at 450.9, 573, and 655 nm (Jenaer Glaswerk, Schott and Genossen, Mainz, Germany) were 18.1, 20.0, and 18.1 nm, respectively. The fluence rates applied to the algal suspensions at different wavelengths were the same. Applied fluence rates were measured with a digital photometer (model J16, Tektronix Inc., Beaverton, OR).

Preparation of Agglutinin and Test for Its Activity

A modification of the procedure of Hunnicutt et al. (1990) was employed. For deflagellation, the pH of vigorously shaking cultures was lowered rapidly to pH 4.2 by the dropwise addition of 5 M acetic acid. After 30 s, the pH was raised again rapidly to pH 7.2 to 7.4 by the dropwise addition of 2 M NaOH. To harvest the cell bodies, the cells were pelleted at 2,000*g* for 5 min at 4°C. The supernatant, containing the flagella, was centrifuged at 37,000*g* for 20 min at 4°C. The pellets of cell bodies and flagella were resuspended in 10 mM Tris-HCl, pH 7.5, containing 1 mM PMSF, frozen immediately in liquid nitrogen, and stored at −20°C. Typically, flagella and cell bodies from a 20-mL culture at a density of 1×10^7 cells/mL were finally dissolved in 0.2 mL and 2 mL of Tris buffer, respectively. For the preparation of agglutinins, the samples of flagella or cell bodies were first thawed at room temperature and then placed immediately on ice. Flagellar agglutinins were prepared by disrupting flagellar samples by sonication. These samples were frozen in liquid nitrogen and stored at −20°C until assayed for activity. Agglutinins from cell bodies were prepared by disrupting the samples either by sonication or by using a French pressure cell at 500 to 800 psi (American Instrument Co., Silver Spring, MD). These samples were then centrifuged at 100,000 rpm for 20 min at 4°C (TL-100 ultracentrifuge, rotor TLA-100.2, Beckman). The supernatants again were frozen in liquid nitrogen and stored at −20°C until they were assayed for activity.

The dried-spot bioassay of Adair et al. (1982) was used to assay the agglutinin levels of cell body and flagellar extracts. Samples were serially diluted 2-fold in distilled water and 2-μL samples were dried onto clean glass microscope slides. Twenty microliters of *mt*+ gametes was applied to the dried spot and then covered with a coverslip. After several minutes, the agglutinin activity of the sample was determined visually under the microscope and the most diluted sample to which the gametes were able to

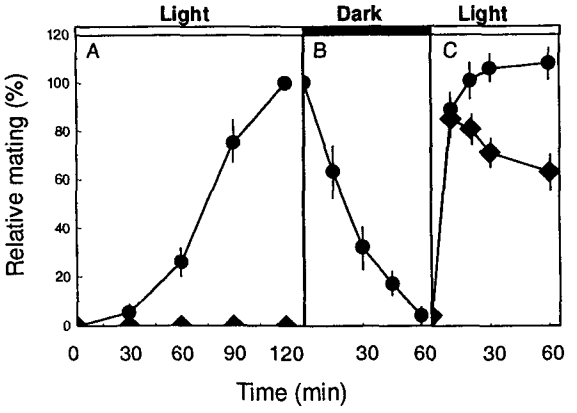

Figure 1. Time course of pregamete-to-gamete conversion in the light (A), inactivation of gametes in the dark (B), and reactivation of dark-inactivated gametes upon illumination (C). Pregametes were generated by incubation in TAP-N medium for 16 h in the dark. The relative percentage of mating was determined using cells incubated in the absence (●) and presence (♦) of 12.5 μM cycloheximide. Maximal mating of pregametes irradiated for 2 h was set as 100%.

adhere was noted. The reciprocal of this dilution multiplied by the original volume of the sample in milliliters represents the agglutinin activity for that sample (agglutinin units).

Assay of Gamete Lytic Enzyme

A modification of the protocol used by Matsuda et al. (1987) was employed. The supernatant of extracts from cell bodies that had been passed through a French pressure cell was used as crude preparation of gamete lytic enzyme. The crude preparation was used directly to digest zoosporangia to estimate the activity of gamete lytic enzyme. Zoosporangia were generated by synchronizing vegetative cells (strain CC-124) in a 15-h light/9-h dark cycle at 23°C in TMP medium (Harris, 1989). Two hours prior to the dark period in the fourth cycle, cells were fixed with glutaraldehyde (final concentration 0.25%), washed three times with sterile distilled water by centrifugation (2000*g*, 5 min at 4°C), resuspended in sterile distilled water at a final cell density of 5×10^7 cells/mL, and stored at 4°C until use.

The activity assay was carried out by a protocol adapted from Tamaki et al. (1981). In a total volume of 250 μL, the reaction mixtures contained 10 mM Tris-HCl (pH 7.5), 2 mg/mL BSA, 5×10^5 zoosporangia, and different volumes of crude gamete lytic enzyme preparations. The reaction was allowed to proceed at 35°C for 30 min (tapping the reaction tube by hand every 10 min) and then stopped by addition of 10 μL of 0.5 M EDTA, pH 8.0. The samples were vortexed for 20 s and zoosporangia were scored with a hematocytometer using a phase contrast microscope. One unit of enzyme was defined as the amount that reduced the number of zoosporangia by 50%.

RNA Isolation and Northern-Blot Hybridization

Total RNA was isolated and processed for blotting and hybridization as described previously (Wegener and Beck, 1991). The *GLE* gene probe (Kinoshita et al., 1992) and the *CBLP* gene, encoding a *Chlamydomonas* Gβ-like polypeptide (von Kampen et al., 1994), were kindly provided by Dr. Matsuda and Dr. Wettern, respectively.

RESULTS

Control of Mating Competence by Light

The kinetics of the conversion of pregametes to gametes in the light, the loss of mating competence upon incubation of these cells in the dark, and their reconversion into gametes upon reexposure to light are shown in Figure 1. Gametic cells that have lost their mating ability after dark treatment were named dark-inactivated gametes. Dark-inactivated gametes exhibited two traits that set them apart from pregametes. First, upon exposure to light, they were converted into gametes without a lag phase and with rapid kinetics (Fig. 1C). Second, as shown by the addition of cycloheximide, this increase in mating-competent cells was independent of protein synthesis. In contrast, pregamete-to-gamete conversion was completely inhibited by cycloheximide (Fig. 1A). To test whether these changes in mating ability in response to dark–light treatments were a property shared by other *C. reinhardtii* strains, we also assayed wild-type strain C-9 (Harris, 1989). The responses in mating ability observed were identical to those seen with strain CC-124 (data not shown).

A moderate decrease was observed in the number of gametes obtained upon exposure to light of dark-inactivated gametes in the presence of cycloheximide (Fig. 1C). This suggested that factor(s) with a rather short half-life are required for mating. In experiments in which cycloheximide was added to gametes kept in the light, about 50% of the cells lost their mating competence after an incubation for 1.3 h (Fig. 2A). This disappearance of mating ability may be caused by a loss of agglutination activity. Agglutinins are localized on flagella (flagellar agglutinin) and the cell body (cell body agglutinin) (Saito et al., 1985; Musgrave et al., 1986; Hunnicutt et al., 1990). After an incubation of gametes with cycloheximide for 6 h, active

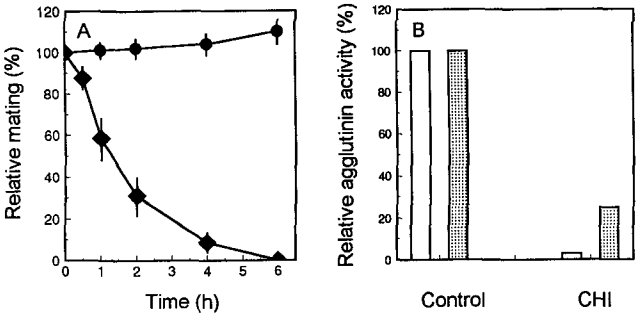

Figure 2. Continuous protein synthesis is required for the maintenance of mating competence. A, Gametes obtained from pregametes exposed to light for 2 h were incubated with illumination either in the absence (●) or presence of 12.5 μM cycloheximide (♦). B, The activities of flagellar agglutinin (open boxes) and cell body agglutinin (stippled boxes) upon incubation with cycloheximide (CHI) were determined at the beginning (control) and after 6 h (CHI).

flagellar agglutinin disappeared, whereas a significant amount of cell body agglutinin remained (Fig. 2B).

Changes in Gametic Markers

To learn more about the regulation of mating competence by light, we analyzed the parameters that determine successful mating. Well-established gametic markers are the activities of gamete lytic enzyme and agglutinin (Matsuda et al., 1987; Hunnicutt and Snell, 1991). Gamete lytic enzyme activity was not detectable in vegetative cells (Matsuda et al., 1987, and data not shown). It accumulated during the differentiation of vegetative cells to pregametes. During pregamete-to-gamete conversion, its activity more than doubled and then remained constant during subsequent light/dark changes (Fig. 3).

In pregametes flagellar agglutinin activity remained undetectable. The titer of flagellar agglutinin only rose during light-induced pregamete-to-gamete conversion, roughly proportional to the increase in mating activity (Figs. 1 and 3). Subsequent dark treatment resulted in a loss of flagellar agglutinin activity and reillumination of dark-inactivated gametes in a rapid increase in its activity (Fig. 3). In contrast, cell body agglutinin, which was absent in vegetative cells but accumulated during development into pregametes, remained at approximately the same level during subsequent dark/light treatments. This indicates that flagellar agglutinins are a main target of light regulation during gametogenesis.

Blue Light Reactivates Dark-Inactivated Gametes

We have shown previously that the conversion of pregametes to gametes is induced by blue light (Weissig and Beck, 1991). To gain information on the photoreceptor that controls the reactivation of dark-inactivated gametes, light of different wavelengths was tested. Clearly, blue light was most effective in restoring the mating ability of dark-inactivated gametes (Fig. 4). Since light of longer wavelengths to a certain extent also was effective, we assayed the reactivation of dark-inactivated gametes using the same filters but different light intensities. In all tests blue light was most effective (data not shown). From these results no information on the molecular nature of the photoreceptor involved may be deduced. However, the preference of the reactivation reaction for blue light suggests that a blue light photoreceptor is involved.

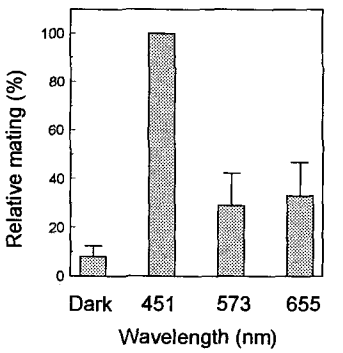

Figure 4. Reactivation of dark-inactivated gametes by irradiation with light of different wavelengths. Cells were irradiated using the wavelengths indicated with a fluence rate of 0.2 μmol m^{-2} s^{-1} for 30 min.

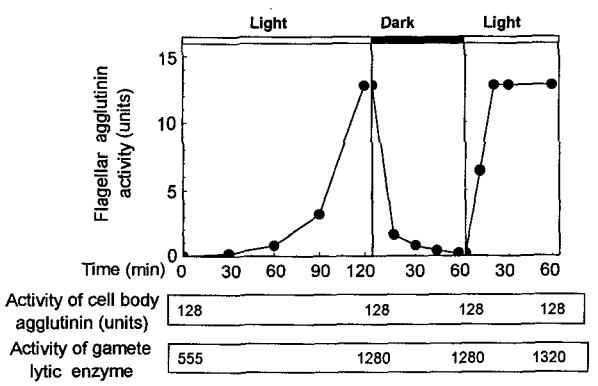

Figure 3. Activities of flagellar agglutinin, cell body agglutinin, and gamete lytic enzyme in pregametes, gametes, dark-inactivated gametes, and reactivated gametes. The activity of gamete lytic enzyme was up-regulated only during the conversion of pregametes to gametes and then remained constant. Cell body agglutinin activity was unaffected by dark/light treatments. Flagellar agglutinin activity increased during pregamete-to-gamete conversion and was inactivated upon shift of the gametes in the dark. Gamete lytic enzyme activity is given per 1×10^9 cells.

Effect of Agonists/Antagonists of Signal Transduction on the Expression of *GLE*, a Marker Gene for Pregamete-to-Gamete Conversion

We have demonstrated previously specific effects of several pharmacological agents on the signal transduction chain by which blue light mediates pregamete-to-gamete conversion (Pan et al., 1996). Since gene expression is essential for this conversion (Treier et al., 1989), we investigated whether treatments with these drugs influenced the expression of *GLE*. This gene, which encodes gamete lytic enzyme (Kinoshita et al., 1992), has been demonstrated to be induced upon exposure of pregametes to light (von Gromoff and Beck, 1993). Three compounds that inhibited pregamete-to-gamete conversion—papaverine, a phosphodiesterase inhibitor that also has other targets in the cell (Sgaragli et al., 1993), genistein, a protein Tyr kinase inhibitor, and SC-10, an activator of PKC—also prevented light-induced expression of the *GLE* gene (Fig. 5).

In the case of SC-10 and papaverine, even a reduction in the basal level of *GLE* mRNA was observed. Staurosporine, a specific inhibitor of PKC at low concentrations, induced a further increase in *GLE* mRNA when added to pregametes in the light. The inducing effect of staurosporine was more prominent when the compound was added to pregametes that were kept in the dark (Fig. 5). This result is consistent with our observation that staurosporine activates pregamete-to-gamete conversion in the dark. Apparently, all pharmacological agents tested previously influenced the level of gamete-specific gene expression and the

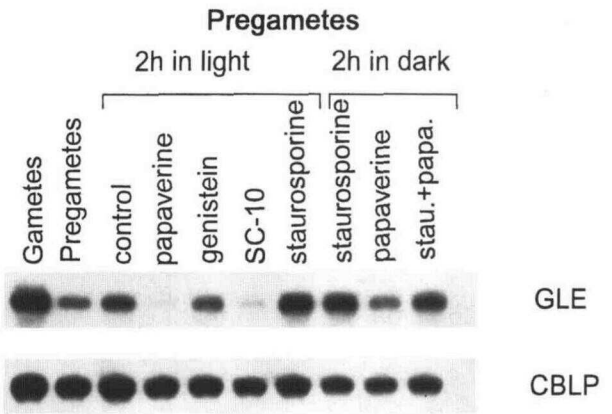

Figure 5. Effects of various pharmacological compounds on the expression of *GLE*. Pregametes and gametes were generated as described in "Materials and Methods." Gametes generated from pregametes by 2 h of light incubation are labeled "control." Drugs were added to pregametes 10 min prior to light exposure for 2 h or to pregametes kept in the dark for an additional 2 h. The concentrations applied were 50 and 100 μM for papaverine in the dark and in the light, respectively, 300 μM for genistein, 100 μM for SC-10, and 100 nM for staurosporine. The RNA gel blot was hybridized with *GLE* and, as a loading control, with a probe of a gene encoding a Gβ-like polypeptide (CBLP).

changes observed were coincident with those seen when mating was assayed (Pan et al., 1996).

Inhibition/Activation of Dark-Inactivated Gametes by Pharmacological Compounds

To gain information on the signal transduction chain by which blue light controls the activation of dark-inactivated gametes, we tested whether compounds that affected the light-signaling pathway controlling pregamete-to-gamete conversion also had an effect on the reactivation of dark-inactivated gametes by light. The compounds used in these experiments have been demonstrated neither to affect the mating reaction itself nor to disable our assay system by causing deflagellation (Pan et al., 1996). Compounds that inhibited light-induced pregamete-to-gamete conversion were added to dark-inactivated gametes 10 min prior to light exposure and samples were taken after an incubation for 1 h in the light. Genistein did not significantly reduce the percentage of gametes (Fig. 6). Also, db-cAMP had no inhibitory effect on the reactivation of dark-inactivated gametes. Rather, this compound appeared to activate mating. These compounds at the concentrations employed have been shown to severely inhibit pregamete-to-gamete conversion (Fig. 6). Two other compounds tested, papaverine and the PKC activator SC-10, inhibited the light activation of dark-inactivated gametes (Fig. 6), as well as the light activation of pregametes.

We then tested various compounds for an activating effect on dark-inactivated gametes in the dark. Addition of cypermethrine, a phosphatase 2B inhibitor (Enan and Matsumura, 1992), and okadaic acid, an inhibitor of type 1 and type 2A protein phosphatases (Cohen, 1989; MacKintosh and MacKintosh, 1994), promoted gamete formation in the dark in a concentration-dependent manner (Fig. 7). Neither compound influenced pregamete-to-gamete conversion (Pan et al., 1996). The protein kinase inhibitor staurosporine and the phosphodiesterase inhibitor papaverine had no effect on dark-inactivated gametes (Fig. 8), which contrasts with their activating effects on pregametes kept in the dark. Activation of mating competence was most strongly promoted by the addition of db-cAMP. This positive effect of db-cAMP on dark-inactivated gametes was corroborated by the observation that this cyclic nucleotide prevented the loss of mating competence when added to gametes prior to the dark shift (data not shown).

Since SC-10 and papaverine inhibited the activation of dark-inactivated gametes in the light, combined treatments of SC-10 and papaverine with the activating agents were performed (Fig. 8). SC-10 completely blocked the activation induced by okadaic acid and cypermethrine treatments. The activation by db-cAMP, though, was not affected. Papaverine also reduced the activation caused by okadaic acid and cypermethrine. In agreement with its function as a phosphodiesterase inhibitor, papaverine promoted the activation caused by db-cAMP treatment. Together, these results suggest that the light-signaling pathway controlling the activation of dark-inactivated gametes is different from that controlling pregamete-to-gamete conversion.

Mechanisms by Which Light May Restore Flagellar Agglutination Activity

Upon illumination of dark-inactivated gametes, recovery of flagellar agglutination activity and mating competence were shown to take place with similar kinetics (Figs. 1 and 3). There are two possibilities for the restoration of flagellar agglutination activity and mating ability of dark-inactivated gametes upon illumination in the absence of protein synthesis. First, an in situ reactivation of dark-inactivated flagellar agglutination activity, and, second, a

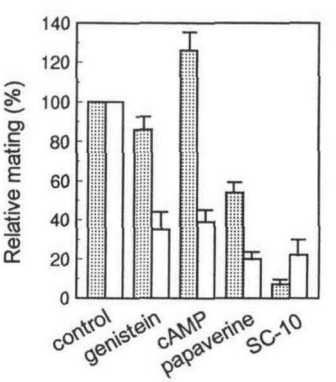

Figure 6. Effects of various pharmacological compounds on the light-induced activation of dark-inactivated gametes (stippled bars). Compounds were added to dark-inactivated gametes 10 min prior to start of illumination. The concentrations used were 300 μM for genistein, 100 μM for SC-10, 20 mM for db-cAMP, and 100 μM for papaverine. For comparison, the effects of the compounds at the same concentrations on light-induced pregamete-to-gamete conversion are shown (open bars).

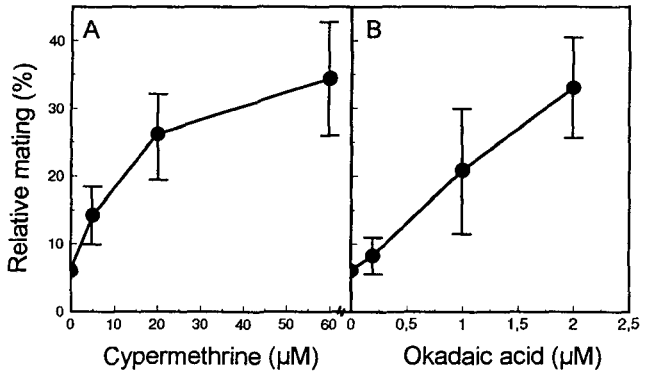

Figure 7. Effect of cypermethrine (A) and okadaic acid (B) on dark-inactivated gametes in the dark. Compounds were added to dark-inactivated gametes, which were kept in the dark for another hour before assaying for mating.

recruitment of cell body agglutinin to the flagella. To distinguish between these two possibilities, we made use of gametes that were incubated in darkness for 6 h. These cells differed from gametes inactivated by 1-h dark treatment since upon reillumination in the presence of cycloheximide, their mating activity was not restored (Fig. 9). Thus, they differ from the dark-inactivated gametes defined above (Fig. 1) that were obtained by dark incubation for 1 h. The level of cell body agglutinin in these cells did not change during this incubation (data not shown). This suggested that recruitment of cell body agglutinin to the flagella could not be induced by light. To confirm that such recruitment is possible in these cells, we treated them with db-cAMP, which is known to induce recruitment (Pasquale and Goodenough, 1987; Goodenough, 1989, 1993; Kooijman et al., 1990). The combined addition of cycloheximide and db-cAMP to these cells in the light caused restoration of mating ability (20% of the nontreated control, Fig. 9). (Papaverine in these experiments enhanced the activating effect of db-cAMP but db-cAMP alone was also effective.) Thus, in contrast to light, db-cAMP in these cells could restore mating ability. This confirms that recruitment of cell body agglutinin to the flagella is possible in these cells, although light will not induce this recruitment.

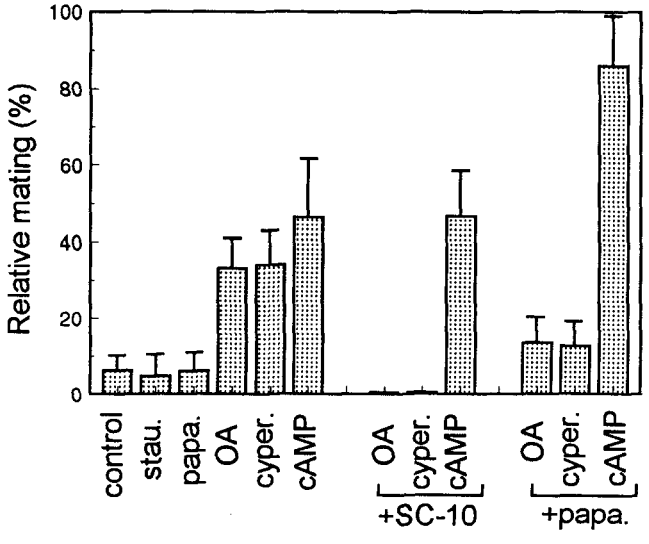

Figure 8. Combined treatments of dark-inactivated gametes with activating and inhibiting compounds. Compounds were added to dark-inactivated gametes and incubation was continued for 1 h in the dark. The compounds used were: staurosporine, 50 nM; papaverine, 100 μM; okadaic acid (OA), 2 μM; cypermethrine 60 μM; db-cAMP, 20 mM; and SC-10, 100 μM.

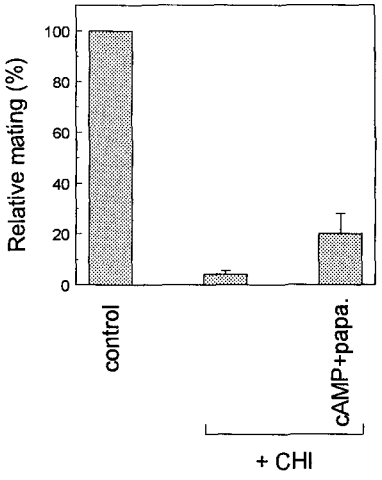

Figure 9. Effect of the combined addition of cycloheximide and db-cAMP together with papaverine on the light-induced activation of gametes that were incubated in the dark for 6 h. Compounds were added 10 min prior to start of illumination, which lasted for 1 h. The concentrations used were 12.5 μM for cycloheximide, 20 mM for db-cAMP, and 100 μM for papaverine.

DISCUSSION

Effects of Light, Darkness, and Protein-Synthesis Inhibitors on Mating Competence

Light plays a key role in the sexual development of *C. reinhardtii*. Not only is light essential for gametogenesis to be completed, it is also required for the maintenance of mating competence. As shown here, gametes generated from pregametes rapidly lost mating competence when placed in the dark (Fig. 1). Re-exposure of dark-inactivated gametes to light caused a fast recovery of mating ability: 50% of the cells were converted into activated gametes within 5 min. This fast response to light contrasts with the kinetics of pregamete-to-gamete conversion: after 75 min 50% of the pregametes were converted into mature gametes. A clue to the difference between these two events is the observation that light activation of dark-inactivated gametes proceeded normally in the presence of a protein synthesis inhibitor (cycloheximide), whereas the conversion of pregametes to gametes was completely inhibited (Fig. 1). Still, protein synthesis is required to keep gametes in an active state, even if they are illuminated (Fig. 2). In the light, complete inactivation of gametes by inhibition of protein synthesis is achieved after 6 h, whereas a dark treatment was effective within 1 h. Summarizing these

data, we can state that gametes need light and protein synthesis to maintain mating competence.

There are several explanations for the loss of mating competence upon a shift of gametes into the dark and the regaining of this activity upon incubation in the light: (a) inactivation of the flagellar agglutinin complex and its in situ reactivation by light, (b) loss of agglutinin molecules from the flagella during dark incubation and a light-induced recruitment of agglutinins to the flagella, and (c) loss of gamete lytic enzyme activity in the dark and its recovery in the light. Our data show that only flagellar agglutinin activity reflects all changes in gametic competence, whereas gametic lytic enzyme activity was affected only by pregamete-to-gamete conversion and cell body agglutinin was not affected at all (Fig. 3). If the loss of agglutinin activity was caused by the loss of agglutinin complexes from the flagella, one could expect that recruitment of new agglutinins from the cell body pool would be one way by which light restores mating competence. Previously, such recruitment has been shown to be induced by db-cAMP (Pasquale and Goodenough, 1987; Goodenough, 1989, 1993; Kooijman et al., 1990) and we confirmed that db-cAMP in the presence of cycloheximide may restore mating (Fig. 9). However, as shown also with cycloheximide-treated cells, light treatment, in contrast to db-cAMP, could not activate the recruitment of cell body agglutinin to the flagella (Fig. 9), ruling out an involvement of cell body agglutinin in the light-activated restoration of flagellar agglutinin activity. Rather, we speculate that light reactivates agglutinin molecules on the flagella by some chemical modification, e.g. (de)phosphorylation. Previously, a light-dependent activation of flagellar agglutinins involving as-yet-undefined changes of the agglutinin molecules has been suggested for *Chlamydomonas eugametos*, a distantly related species (Kooijman et al., 1986, 1988). Our data indicate that a similar regulatory process also controls flagellar agglutination activity in *C. reinhardtii*.

A Model for the Light-Signaling Pathways That Control Sexual Competence of *C. reinhardtii* Gametes

A model that summarizes our data on the light signal transduction cascade that controls gamete formation and the reactivation of dark-inactivated gametes is presented in Figure 10. We propose that the conversion of pregametes to gametes and the reactivation of dark-inactivated gametes are controlled by two independent light-signaling pathways. An important part of the pathway that mediates light control of pregamete-to-gamete conversion is dedicated to the regulation of gamete-specific genes. This is illustrated by the fact that pharmacological compounds that were found to influence pregamete-to-gamete conversion had similar effects on the expression of *GLE* (Fig. 5). This would suggest that they regulate the gene-expression branch.

Because the pathway that controls the reactivation of dark-inactivated gametes does not require protein synthesis, it could be expected that some of the pharmacological compounds that affected the light activation of pregametes would have no or different effects. This holds true for genistein, which inhibits pregamete-to-gamete conversion but has no significant effect on dark-inactivated gametes (Fig. 6). Interestingly, protein phosphatase inhibitors such as okadaic acid and cypermethrine can activate dark-inactivated gametes in the absence of light, although they have no influence on pregamete-to-gamete conversion (Fig. 7). We assume that the protein phosphatase(s) attenuates basal signal flux in the dark, preventing the activation of dark-inactivated gametes (Bowler and Chua, 1994). A role

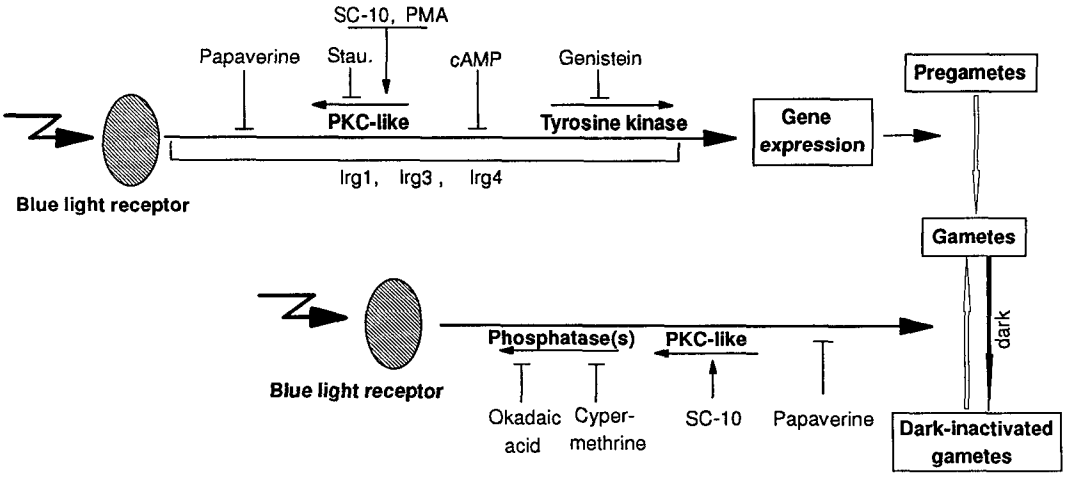

Figure 10. A model for the signaling pathways by which blue light controls pregamete-to-gamete conversion and the reactivation of dark-inactivated gametes. It is proposed that the pathway controlling pregamete-to-gamete conversion affects gene expression. Mutations in genes *Irg1*, *Irg3*, and *Irg4*, resulting in light independence of pregamete-to-gamete conversion, also result in the constitutive activation of gamete-specific genes. The pathway by which dark-inactivated gametes are activated by light may involve the activation of target molecules by posttranslational modifications. The order of targets of SC-10 and papaverine are not known. A basal flux through these light-activated signal pathways is indicated by the long lines with arrowheads. Arrows indicate an activating effect of a pharmacological compound, T-lines (T) indicate an inhibiting effect.

for protein phosphatases in light-signal transduction has also been found in higher plants (Sheen, 1993).

We also observed that some compounds affected both pathways. For instance, SC-10, a specific activator of PKC (Ito et al., 1986), is a very potent inhibitor of the light-induced activation of dark-inactivated gametes (Fig. 6), suggesting that a PKC-like kinase is involved in this signal pathway. Also, papaverine significantly inhibited the activation of dark-inactivated gametes in the light (Fig. 6). The same effects of these drugs on both signal pathways suggests that the pathways partially employ similar components. Both the PKC activator SC-10 and papaverine inhibited the reactivating effects of the phosphatase inhibitors (Fig. 8). Assuming a linear signal pathway, we suggest that SC-10 and papaverine act on targets downstream from the phosphatase(s).

As illustrated in Figure 10, we view dark-inactivated gametes as derivatives of gametes rather than as intermediates between pregametes and gametes. The principal argument for the sequence pregamete–gamete–dark-inactivated gamete rests on the activation of pregametes in the dark by staurosporine (Pan et al., 1996) and on the absence of such an activation in dark-inactivated gametes (Fig. 8). If dark-inactivated gametes were an intermediate between pregametes and gametes, staurosporine treatment of dark-inactivated gametes would have resulted in gametes.

Chlamydomonas spp. mutants with the capacity to form gametes in the dark have been identified (*lrg1*, *lrg3*, and *lrg4*; Buerkle et al., 1993; Gloeckner and Beck, 1995). These *lrg* mutants apparently have overcome the inactivation problem. Because these light-independent strains all have an up-regulated and light-independent expression of gametic genes (Buerkle et al., 1993; Gloeckner and Beck, 1995), we propose that they continuously generate new gametic proteins that are synthesized in an active form. The dark-inactivation process on the flagella is not fast enough to remove all activity.

The outline of blue light-signaling pathways described here provides a basis for the further characterization of these *lrg* mutants and new mutants generated by insertional mutagenesis (G. Dame and C.F. Beck, unpublished data). Using these mutants, a molecular identification of individual signaling pathway components will become possible.

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