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Involvement of protein kinase C in the response of *Neurospora crassa* to blue light

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Abstract As a first step towards understanding the process of blue light perception, and the signal transduction mechanisms involved, in *Neurospora crassa* we have used a pharmacological approach to screen a wide range of second messengers and chemical compounds known to interfere with the activity of well-known signal transducing molecules in vivo. We tested the influence of these compounds on the induction of the *al-3* gene, a key step in light-induced carotenoid biosynthesis. This approach has implicated protein kinase C (PKC) as a component of the light transduction machinery. The conclusion is based on the effects of specific inhibitors (calphostin C and chelerythrine chloride) and activators of PKC (1,2-dihexanoyl-*sn*-glycerol). During vegetative growth PKC may be responsible for desensitization to light because inhibitors of the enzyme cause an increase in the total amount of mRNA transcribed after illumination. PKC is therefore proposed here to be an important regulator of transduction of the blue light signal, and may act through modification of the protein White Collar-1, which we show to be a substrate for PKC in *N. crassa*.

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Introduction

Organisms are able to sense and respond to fluctuations in environmental conditions via complex mechanisms in which exogenous stimuli are transformed into signals generated and transmitted in the form of biochemical changes in the cell. Light signals are especially important for plants, and many other organisms and microorganisms are specifically dependent on this kind of stimulus for their growth and development. The ascomycete *Neurospora crassa* is able to sense light in the blue region of the spectrum, and this triggers diverse developmental, morphological and physiological responses (reviewed by Degli Innocenti and Russo 1984; Linden et al. 1997a). During conidiation, blue light stimulates the formation of the conidial structures and influences the number of conidia produced; during the sexual cycle it influences development of protoperithecia and the number of perithecia formed, and it stimulates positive phototropism of the perithecial beaks. During vegetative growth, light induces the production of carotenoids and acts to entrain a circadian rhythm.

There has recently been a consolidated effort to elucidate the blue-light signal transduction pathways in *N. crassa*. The possibility that cAMP participates in transduction of the blue-light signal has been explored (Shaw and Harding 1987; Kallies et al. 1996), but different studies have led to conflicting results, so that the involvement of cAMP remains questionable. Both the *N. crassa* adenylate cyclase (Kore-eda et al., 1991) and a member of the cAMP-dependent protein kinase family have been cloned (Yarden et al. 1992), but no information suggesting a possible role of these enzymes in blue light signalling has been published. A possible function of inositol phosphates in light signalling has also been examined. However, no changes in the levels of inositol

phosphates were detected following illumination (Lakin-Thomas 1993; Kallies et al. 1996).

On the other hand, the two molecular intermediates encoded by the genes *wc-1* and *wc-2* have been isolated and characterized (Ballario et al. 1996; Linden and Macino 1997). Few regulatory mutants involved in light signalling are known in *N. crassa* (Harding and Shropshire 1980; Paietta and Sargent 1981, 1983) and only *white-collar 1* (*wc-1*) and *white-collar 2* (*wc-2*) are blind; i.e. mutations in these genes block almost all known photoresponses (reviewed in Lauter 1996; Ballario and Macino 1997; Linden et al. 1997a). A new selection system has also been developed for the isolation of regulatory mutants affected in light signal transduction (Carattoli et al. 1995). An exhaustive screen for mutants that are completely blocked in light signal transduction permitted the isolation of only *wc-1* and *wc-2* and failed to identify additional *wc* genes (Linden et al. 1997b). This approach, based on the hypothesis that blindness is not lethal, applies rigorous selection for blind mutants. However, it excludes the possibility of isolating additional components of the light transduction pathway whose functions are essential for growth. Recently Oda and Hasunuma (1997) reported the isolation of a new mutant (*psp*) that is defective for phosphorylation of a 15-kDaprotein – a modification which is induced in crude membrane fractions upon illumination of *N. crassa* mycelia. This report shows that the *wc-1* gene is epistatic to *psp* in the light signal transduction pathway that controls phototropism and perithecial polarity (Oda and Hasunuma 1997).

In higher plants, a biochemical approach has proven to be very successful in determining the mechanisms involved in phytochrome signalling (Neuhaus et al. 1993, 1997 Bowler and Chua 1994; Bowler et al. 1994; Christie and Jenkins 1996). Similarly, we have designed pharmacological experiments to interfere with light signal transduction in *N. crassa* by utilizing agonists and antagonists either to induce or to block signal transduction. We focused our attention on an early light response elicited during vegetative growth of the fungus: namely, the light-regulated expression of the carotenoid biosynthetic gene *albino-3* (*al-3*). In *N. crassa* the biosynthesis of carotenoids is induced by light in the mycelium, but is constitutive in the conidia. The carotenoid biosynthetic genes *al-1*, *al-2* and *al-3* are induced coordinately and transiently at an early stage following light exposure in the mycelium (Baima et al. 1991; Schmidhauser et al. 1990, 1994). The response is transient, irrespective of whether illumination is continuous or interrupted, and this indicates that a photosensory adaptation mechanism is operating that leads to temporary insensitivity of the system. In our laboratory the gene *al-3* has been cloned and its expression has been extensively characterized, both during vegetative growth and conidiation (Baima et al. 1991; Arpaia et al. 1995b). The *al-3* gene is an ideal probe with which to study the effects of light on gene expression and we used it as a reporter to investigate the effects of chemical compounds on light signal transduction.

In the present study we have investigated the potential role of protein kinase C (PKC) in mediating light signal transduction. This was of interest because PKC is involved in light signalling in vertebrates (Udovichenko et al. 1996; Wood et al. 1997), *Drosophila* (Hardie et al. 1993; Scott and Zuker 1997; Tsunoda et al. 1997; Xu et al. 1998) and plants (Raghuram and Sopory 1995). We tested the effect of inhibition of PKC activity on light-induced accumulation of *al-3* mRNA in mycelium by using calphostin C, a potent PKC inhibitor which binds to the regulatory domain of the protein in animal cells (Bruns et al. 1991) and chelerythrine chloride, a second specific PKC inhibitor that binds to the catalytic domain of the kinase (Herbert et al. 1990). In order to confirm the involvement of PKC in the regulation of light-dependent *al-3* mRNA synthesis, we used 1,2-diexanoyl-*sn*-glycerol (DAG), a membrane-permeable PKC agonist. The results of our study indicate that PKC is involved in the regulation of the blue light photoreceptor-mediated induction of the *al-3* gene. We applied these compounds to light-adapted *Neurospora* mycelia to investigate which step in the light-response was affected by PKC, and we found that the enzyme may be responsible for the process of desensitization to light. Furthermore, in searching for possible PKC substrates, we have studied the phosphorylation of two central regulators of blue-light responses: the proteins WC-1 and WC-2.

Materials and methods

Strains of *N. crassa*

N. crassa Oak Ridge wild-type74-OR23A (FGSC No. 987) used in this work was obtained from the Fungal Genetic Stock Center (FGSC; University of Kansas, Kansas City, Kan.).

Chemicals

The compounds 1,2-dihexanoyl-*sn*-glycerol (diacylglycerol, here referred to as DAG), chelerythrine chloride, 4-bromo-calcium ionophore A23187, and ionomycin were purchased from Sigma (Milan, Italy). Calphostin C was obtained from Biomol (Plymouth Meeting, Penn.). The compounds were tested at the following concentrations: 1,2-dihexanoyl-*sn*-glycerol (1,2-DGG), 25 μM and 100 μM (dissolved in 100% ethanol); chelerythrine chloride, 0.6 μM, 6 μM and 25 μM [dissolved in 10% dimethyl sulfoxide (DMSO)]; calphostin C, 0.1 μM, 1 μM and 10 μM (dissolved in 100% DMSO); and A23187 and ionomycin,10 μM and 100 μM (dissolved in 100% DMSO). Dimethyl sulfoxide, used to dissolve the compounds, was present in the media at dilutions of 1 in 10^4–10^5.

Other compounds utilized to investigate transduction of the blue light signal in Neurospora, which gave negative results in this study (not shown), were the following: 8-bromo-cAMP (a membrane-permeable cAMP analog); 8-bromo-cGMP (a membrane-permeable cGMP analog); cholera toxin (an activator of the α-subunit of G proteins); cyclosporin A and FK506 (calcineurin inhibitors); genistein (an inhibitor of tyrosine phosphorylation); H89 (a protein kinase A inhibitor); KN-62 (a Ca^{2+}/calmodulin kinase II inhibitor); melittin (an activator of phospholypase A2); nifedipine [which blocks voltage-sensitive (L-type) calcium

channels]; okadaic acid (a protein phosphatase inhibitor); pertussis toxin (a G protein inhibitor) and trifluoperazine (a cell-permeable cytosolic calcium modulator):

Photoinduction and culture conditions

For photoinduction studies, 1×10^7 conidia were inoculated into 100 ml of Vogel's minimal medium and shaken at 150 rpm in the dark at 28° C. After 18 h, cultures were divided as necessary under safe red light conditions. Calphostin C, chelerythrine chloride, A23187 and ionomycin were added 30 min before illumination of the cultures, and 1,2-DGG was added 1 min before illumination. Equivalent amounts of DMSO and ethanol were added to the controls 30 min and 1 min before illumination, respectively. Photoinduction was elicited by treatment of the cultures with saturating white light (energy fluence rate ≥1.7 W/m^2 in the blue region). Light intensity was measured with a photometer (EL150, International Light, Newburyport, Mass.).

RNA extraction and Northern blot hybridization

RNA extraction and Northern analysis were done as described in Arpaia et al. (1995b). Hybridization was carried out with a 5′ end-labelled oligonucleotide (5′-ACGGCCATGGTGACGTGTTC-CATTTCC-3′) specific for *al-3* (Baima et al. 1991) or a cDNA probe for a non-light-inducible *N. crassa* ribosomal protein gene (Tarawneh et al. 1990) that was used to normalize transcript levels. The latter probe was labelled with ^{32}P using the random-primer method (Feinberg and Vogelstein 1983) and was also used to determine whether general cellular processes were affected by the addition of the chemical compounds used in this study. Quantitative analysis of the radioactive bands was carried out with an Instant Imager (Electronic Autoradiography; Packard, Meriden Conn.).

Preparation of cell extracts and partial purification of PKC

N. crassa mycelia, grown in the light, were homogenized in extraction buffer (20 mM TRIS-HCl pH 7.5, 0.5% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 25 μg/ml aprotinin and leupeptin, 1 mM PMSF) with 20–30 pestle strokes in a tight-fitting Dounce homogenizer. Lysates were kept on ice for30 min, centrifuged 2 min at 13,000 ×*g* and the supernatant was collected. Partial purification was achieved by chromatography on DEAE cellulose (Whatman DE52) and the protein was eluted with 0.2 M NaCl.

Phosphorylation assay and immunoprecipitation procedures

PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrates. The reaction contained 5 μl of protein extract, 20 mM TRIS-HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 50 μM peptide substrate PSP (RKGSVRQRKE, purchased from Tan Laboratories, Houston, Tex.), 20 μM ATP, and 4 μCi/ml [γ-^{32}P]ATP in a total volume of 50 μl. When necessary, a 10- or 100-fold excess of pseudosubstrate inhibitory peptide PIP (RKGAVRQRKE, Tan Laboratories), was pre-incubated in the mix for 20 min at room temperature. After addition of extract, samples were incubated for 15 min at 30° C. To determine the PKC activity, 25-μl aliquots of the mix were spotted on P81-phosphocellulose paper (Whatman), rinsed twice with 10 ml/l H_3PO_4 for 5 min, and once with water, and the radioactivity quantified with a Beckman LS1801.

WC-1- and WC-2-GST fusion proteins were prepared as described by Ballario et al. (1996) and Linden and Macino (1997). When GST fusion proteins were assayed, the reaction was performed as above, except that 5 μM GST fusion protein substrates and a 100-fold excess pseudosubstrate inhibitory peptide PIP were used. After incubation at 30° C, samples were diluted to 160 μl with buffer A (100 mM TRIS-HCl pH 7.5, 150 mM NaCl; 2 mM EDTA) and immunoprecipitations were preceded by a clearing step with 40 μl of protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) for 1 h at 4° C. Samples were centrifuged for 3 min at 5000 ×*g* and the supernatant was recovered; anti-GST antibody (Sigma G1160) was added (at a final dilution of 1:1500) and the reaction was incubated at 4° C for 1 h. After addition of another 40 μl of protein A-Sepharose and further incubation for 1 h at 4° C, immunocomplexes were recovered, washed three times with 1 ml of buffer A, denatured by heating for 3 min at 95° C in reducing Laemmli buffer and resolved by SDS-PAGE on a 9% polyacrylamide gel. Phosphorylated proteins were visualized by autoradiography of the dried gel.

Results

PKC activity regulates light-directed *albino-3* gene expression in mycelia

We tested a variety of known pharmacological agonists and specific inhibitors which may either induce or block the light signal transduction pathway during the mycelial growth of *N. crassa*. Although the involvement of many different signal transduction molecules were investigated (e.g. the second messengers calcium, cAMP and cGMP; G-proteins,calmodulin, protein kinases and phosphatases), we only obtained consistently reproducible effects when the activity of PKC was altered by the use of specific inhibitors or activators.

Protein kinase C inhibitors

We tested the effect of inhibition of PKC activity on light-induced accumulation of *al-3* mRNA in mycelia using calphostin C. A second specific PKC inhibitor, chelerythrine chloride, was used to confirm PKC involvement and to test the effect of inhibitors in the dark. This step was necessary since full activation of calphostin C is light dependent and it was therefore not tested in the dark. Mycelia were grown for 18 h in the dark and either calphostin C nor chelerythrine chloride was added. Cultures were then incubated for 30 min in darkness and then illuminated with continuous light for up to 90 min. The effect of each compound was tested at different concentrations and the lowest fully effective concentration was used in further experiments.

Results are shown in Fig. 1. Figure 1A shows the effect of calphostin C on light-mediated induction of *al-3*. The timecourse of light-regulated induction of the *al-3* gene in mycelia has been characterized previously (Baima et al. 1991), and the gene was found to be transiently expressed both following a pulse of light and during continuous illumination. Calphostin C had no effect on the first phase of the response curve until 20 min had passed (corresponding to the time of maximal expression). However, the decrease in steady-state levels of the mRNA normally observed in controls did not occur in the presence of calphostin C, and at 60 and

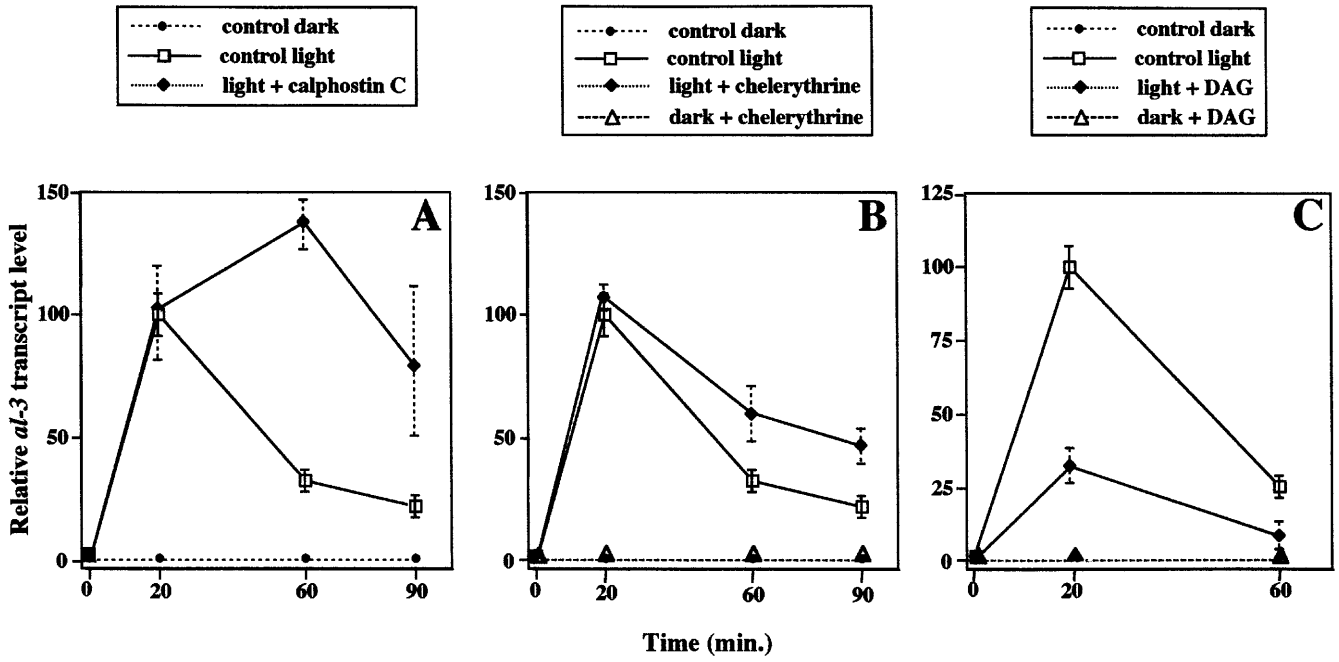

Fig. 1A–C Effects of protein kinase C-specific inhibitors and activators on levels of *al-3* transcripts in mycelia. The plots show relative levels of *al-3* mRNA in cultures incubated with or without 1 μM calphostin C (**A**); 25 μM chelerythrine chloride (**B**) or 100 μM diacylglycerol (**C**). Cultures in **A** and **B** were preincubated with the inhibitors for 30 min in the dark, whereas the cultures in **C** were preincubated with the agonist for 1 min in the dark. All cultures were then illuminated for 20, 60 or 90 min and harvested immediately . Error bars indicate standard errors for three independent experiments in **A**, four in **B** and three in **C**. The amount of *al-3* transcript in cultures illuminated continuously for 20 min was taken as 100

90 min, transcript levels were still very high compared to controls. The curve was modified in such a way that the peak of expression was shifted and increased, transcript levels decreased slowly and the total amount of mRNA transcribed in the presence of the inhibitor was increased by about two fold. Figure 1B shows the results of similar experiments performed with chelerythrine chloride. As with calphostin C, in the presence of this compound expression of *al-3* mRNA reached a higher level than in the controls and persisted for at least 90 min. The effect of chelerythrine chloride was weaker than that of calphostin C, resulting in a 30% increase in total *al-3* mRNA against a two-fold increment with calphostin C (values were calculated on the basis of three experiments using calphostin C and four experiments using chelerythrine chloride).

Effects of agonists

To confirm the involvement of PKC in the regulation of the light-dependent accumulation of *al-3* mRNA, we used the membrane-permeable PKC agonist DAG. DAG acts as a second messenger to activate PKC. We explored the possibility that *N. crassa* PKC is activated upon binding of diacylglycerol by administering different concentrations of DAG to the cells immediately before exposure to light. In the experiment shown in Fig. 1C we applied 100 μM DAG to cells grown in the dark, immediately before illuminating the cultures for 20 or 60 min. Stimulation of PKC was predicted to cause inhibition of *al-3* gene expression in illuminated cultures. Figure 1C shows that administration of the agonist resulted in a four-fold reduction in *al-3* expression at 20 min, and the overall level of expression was reduced to about one-third. These results confirmed that PKC blocks the light-induced transcription of the *al-3* gene and hence influences the photoadaptation process.

In animal systems the activation of some isotypes of PKC is dependent on both lipids and Ca^{2+} (Mellor and Parker 1998). In *N. crassa*, alteration of the intracellular Ca^{2+} level through the administration of Ca ionophores (A23188 or ionomycin) in the presence of high concentrations of external Ca^{2+} (0.3 to 30 mM) did not affect the light-regulated expression of the *al-3* gene, when applied either alone or in the presence of diacylglycerol (data not shown). Assuming that these ionophores can function in *N. crassa*, these experiments indicate that the isotype of PKC that acts in the transduction of the blue-light signal might be independent of external calcium.

Light adaptation

The kinetics of *al-3* mRNA induction in different light and dark regimes indicated that a photosensory adaptation mechanism was operating. At a given light intensity, *al-3* gene expression in mycelia is transient even in continuous light, and no mRNA is detectable after

100 min (Macino et al. 1993). The data discussed above suggested that PKC acts at the level of this photoadaptation.

In order to determine whether the temporary insensitivity of the system was due to a desensitization phenomenon, we performed photobiological studies designed to provide more information on the adaptive response at the molecular level (Fig. 2A). This approach has been extensively used to characterize light adaptation in fungi and plants (Corrochano et al. 1995; reviewed by Galland 1989, 1991) Mycelia were incubated under different light regimes as indicated in the legend to Fig. 2. Lane 2 shows that 40 min after the first light pulse, *N. crassa* was almost totally unresponsive to the application of a second light pulse. As dark incubation time increased, light responsiveness was gradually restored (lanes 3 and 4), but even after 70 min in the dark full responsiveness had not been recovered (compare lanes 5 and 1). In lane 6 a dark control shows that the transcription of the gene is strictly light-regulated in mycelia. The experiment suggests that after a first pulse of light *al-3* transcription is temporarily blocked and the system is insensitive to a second pulse, in agreement with the expectations based on the results reported by Schrott (1981) for carotenoid accumulation in response to light.

In the second experiment, we tested the responsiveness of mycelia exposed to continuous light and increasing light intensities (Fig. 2B). The *al-3* mRNA accumulated transiently upon illumination, a peak of expression was observed after 20 min and the RNA was almost undetectable at later times, both after a pulse of light or under continuous illumination (lanes 1, 2 and 3). Nevertheless, when adapted to a given light intensity, the fungus was still able to respond to increasing light intensities (lane 4). If we compare lanes 2 and 4 it is evident that photoadaptation is a dynamic process; insensitivity is therefore not due to complete loss of the capacity to respond to a light stimulus, but to desensitization. A six-fold increase in light intensity was not able to induce a full response, but the steady-state level of *al-3* mRNA was reduced to about one-third of that obtained in non-adapted cultures after 20 min of illumination (compare lanes 3 and 4).

The general picture that emerges from these experiments suggests that PKC may be involved in the adaptation to light i.e. in desensitization. In this scenario, PKC would be part of an active mechanism to counterbalance the input signal emanating from the receptor. To investigate this possibility we adapted cultures to light for 2 h, after which PKC was inhibited by adding 1 μm calphostin C to the treated cultures, while DMSO was added to controls. Cultures were incubated in the light for an additional 60 or 90 min and harvested. RNA was extracted and a Northern blot was probed with an oligonucleotide specific for the *al-3* gene.

As shown in Fig. 3, the level of *al-3* mRNA increased in the cultures incubated with calphostin C (lanes 4,5) compared to untreated cultures (lanes 2, 3). These data suggest that PKC action might be required to block signalling after exposure to light: the system is capable of responding again if PKC activity is inhibited.

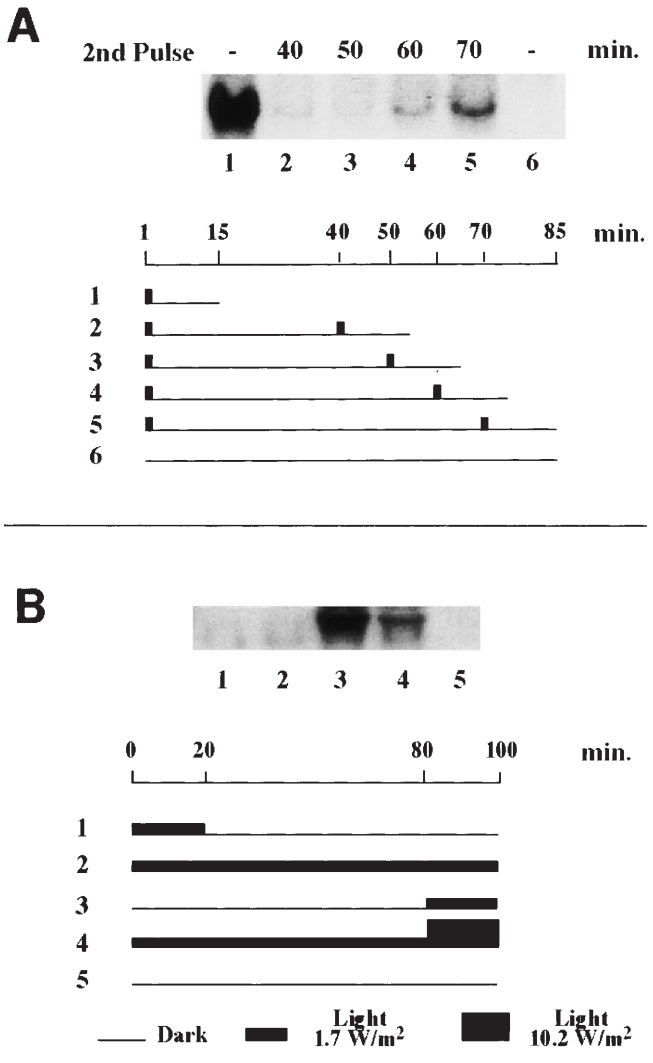

Fig. 2A, B Modulation of the expression of the *al-3* gene in response to light. Mycelia were grown for 18 h in the dark, incubated in the light as indicated in the schemes, harvested in liquid nitrogen and RNA was extracted. **A** A refractory period in the expression of the *al-3* gene in response to light. Samples were illuminated with a 1-min pulse of light. Mycelia were harvested at the peak of mRNA accumulation, i.e. at 15 min (lane 1), or incubated in the dark for periods varying from 40 to 70 min. A second 1-min light pulse was then given and mycelia were harvested after an additional 15 min in the dark, as shown (lanes 2–5). Lane 6 represents a control kept in continuous darkness. **B** Adaptation of *al-3* gene expression to high light intensity. The culture used as the source for the sample in lane 1 was illuminated for 20 min and harvested after an additional 80-min incubation in the dark. Lane 2 contained mRNA extracted from a culture that was illuminated for 100 min before harvesting, while the mycelia for lane 3 were exposed to light for 20 min and harvested immediately. Lane 4 contained RNA from a culture that was exposed to continuous light for 80 min, followed by a 20-min exposure to a six-fold higher light intensity. Lane 5 is a dark control. *Filled bars* represent light periods, *lines* represent dark periods. In all samples the light intensity was 1.7 W/m^2 except for sample 4 in **B**; here the intensity was increased to 10.2 W/m^2 for the last 20 min of the experiment

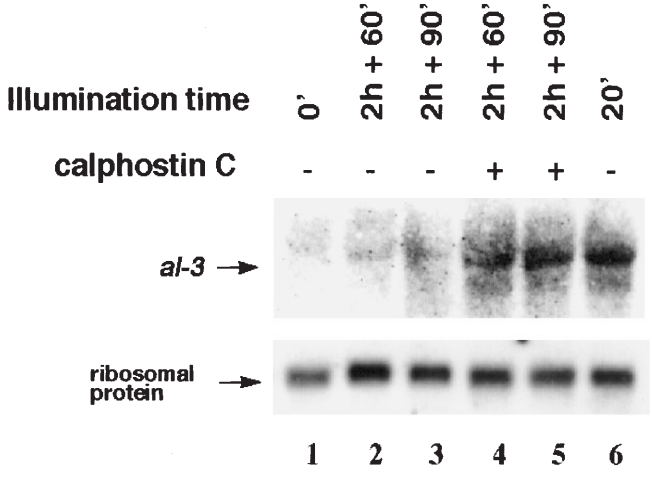

Fig. 3 Inhibition of PKC restores light responsiveness in light-adapted cultures. Northern analysis of total RNA extracted from cultures grown for 18 h in the dark and adapted to light for 2 h. DMSO was added to the control cultures (lanes 1–3) while in others calphostin C was added at 1 μM final concentration (lanes 4 and 5) and the cultures were kept in the light for an additional 60–90 min. Lane 1 represents a culture maintained in the dark. The RNA in lane 6 was extracted from a culture kept in continuous light for 20 min. The results shown are representative of those found in three independent experiments

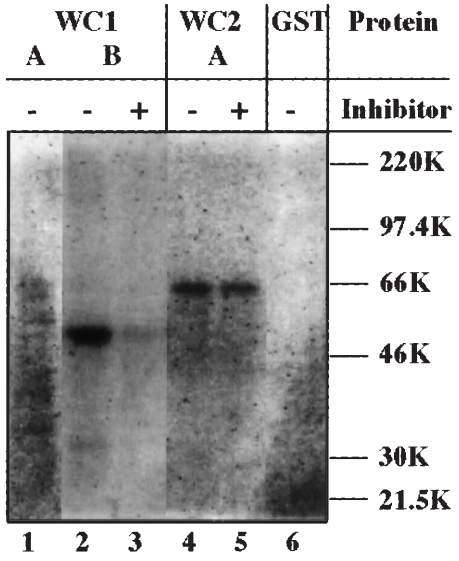

Fig. 4 WC-1 can be phosphorylated by protein kinase C in vitro. Bacterially expressed GST-WC fusion proteins WC-1/A (aa 354–818), WC-1/B (aa 889–1000), WC-2/A (aa 169–530) and GST alone were incubated with *N. crassa* extracts in the presence or absence of a 100-fold excess of PIP inhibitory peptide (inhibitor), and a kinase assay was performed. Following immunoprecipitation with anti-GST antibody, samples were loaded on a 9% polyacrylamide gel. Phosphorylated proteins were visualized by autoradiography of the dried gel. One of three independent experiments is shown

WC-1 protein is a substrate for phosphorylation by PKC

Protein kinase C may exert its action through modification of target substrates representing effectors of the blue light pathway. Light-regulated expression of almost all *N. crassa* light-induced genes, with few exceptions such as the *wc-2*, *ccg-1* and *frq* genes in a *wc-2* background (Arpaia et al. 1995a; Crosthwaite et al. 1997; Linden and Macino 1997b), seems dependent on the WC-1 and WC-2 proteins which are involved in transcriptional activation. These two proteins, therefore, represent good candidates for targets of the action of PKC.

We set up a kinase assay in *N. crassa* extracts. Extracts from illuminated mycelia were partially purified by ion-exchange chromatography on DE52-cellulose columns. PKC activity in the extracts was first tested by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to a synthetic decapeptide (PSP), derived from the *N. crassa* PKC pseudosubstrate sequence (G. Arpaia, O. Yarden, O. Shlomo, C. Catalanotto and G. Macino, manuscript in preparation) and in which alanine was replaced by a serine (RKGAVRQRKE) (data not shown). PKC specificity was tested by using an inhibitor peptide (PIP) bearing the intact *N. crassa* PKC pseudosubstrate sequence (RKGSVRQRKE) (peptides with similar characteristics have been shown to be excellent PKC substrates and inhibitors, respectively; see House and Kemp 1987). The WC-1 and WC-2 proteins were expressed as GST fusion proteins from the pGEX vector in *E. coli* (Ballario et al. 1996; Linden and Macino 1997), purified and used as substrates in the assay. Proteins were then immunoprecipitated with anti-GST antibodies and the complexes were denatured and subjected to electrophoresis. As shown in Fig. 4, both WC-1 and WC-2 fusion proteins (lanes 2 and 4), but not the glutathione S-transferase protein alone (lane 6), were phosphorylated in *N. crassa* extracts. Phosphorylation occurred in the C-terminal portion of WC-1, since we only detected a labelled band with a WC-1-GST fusion carrying residues 889–1000 (lane 2) and not with a fusion polypeptide containing amino acids 354–818 of WC-1 (lane 1). When phosphorylation was competed with the PIP inhibitory peptide, however, labelling of WC-1 was severely reduced while the level of WC-2 phosphorylation remained unchanged (lanes 3–5). Therefore, in in vitro experiments, WC-1 but not WC-2 represents a substrate for a PKC in *N. crassa*.

Discussion

The data presented here support a potential role for protein kinase C in the blue-light transduction pathway in *N. crassa* by showing that PKC may be responsible for the desensitization to the light stimulus that occurs in the mycelium.

A wide variety of long- and short-term effects of blue light have been described in *N. crassa*. A clear example of a short-term early response is the light-dependent induction of carotenoid biosynthesis in mycelia (Har-

ding et al. 1969; Harding and Shropshire 1980; Harding and Turner 1981). In a study of the dependence of photoinduced carotenoid biosynthesis and subsequent pigment accumulation on light intensity, Schrott described a temporary insensitivity to light after an initial saturating light pulse (Schrott 1981). A period of 2 h after a first illumination was described as necessary to restore maximum competence for a second light induction. Our study confirms this phenomenon at the molecular level and reveals that a PKC may be the molecular intermediate responsible for the negative control of light signal transduction in the mycelium, determining desensitization to the stimulus itself. The kinetics of light-induced expression of a light-regulated gene such as *al-3* is modified by the action of PKC inhibitors in such a way that the period of light sensitivity and responsiveness is prolonged. We can deduce that the enzyme should act at around the time when the light-regulated peak of expression is maximal, since higher *al-3* expression levels were reached under the same light conditions when inhibitors were used. Activation of the kinase with DAG prior to light induction results instead in a significant reduction in the amount of *al-3* mRNA accumulated in response to the light stimulus. Schrott (1981), suggesting that the photoreceptor and/or elements of the signal transduction relay become depleted during the first illumination. However, we show here that adaptation to the light stimulus and the consequent insensitivity seem to be due to an active mechanism determined by PKC activity rather than to simple depletion of transducing elements. In fact inhibition of the enzyme in light-adapted cultures re-establishes light responsiveness. At a given fluence rate, a steady-state level of input and counteracting signals should be reached to avoid unnecessary accumulation of carotenoids. If the external conditions change, however, it is necessary for the organism to stimulate new synthesis of protective pigments, and in fact the system is able to respond to an increasing light stimulus that eventually alters the pre-existing equilibrium.

A characteristic of the response of organisms to an external stimulus is their ability to coordinate complex developmental changes and to sense and respond to fluctuations in their surroundings with high sensitivity. Negative control mechanisms are necessary to allow changes in sensitivity to a stimulus, permitting a signalling pathway to work over a broad range of stimulus intensities and to terminate a response even if the stimulus remains. Based on the present study PKC appears to play such a role, although the existence of other effector proteins involved in the quantitative regulation of the response cannot be excluded. As occurs in *Drosophila* vision, additional effector proteins might act in the termination of the activated state of the receptor and downstream signalling molecules (Dolph et al. 1993; Hardie et al. 1993; Scott and Zuker 1997).

For several light-responsive genes, gene expression and promoter studies suggest that different stimuli address distinct regulatory *cis* elements in promoters (Arpaia et al. 1993, 1995a; Bell-Pedersen et al. 1996), but the overall response due to the interplay of the different stimuli is strictly coordinated and finely tuned. Our study opens up an interesting field of investigation, in raising the possibility that within the promoters, the components that bind at light-regulatory *cis* elements are regulated by PKC action. The reason for this stems from the fact that light induction of almost all light-induced genes in *N. crassa* is dependent on the products of the regulatory genes *wc-1* and *wc-2*, which show DNA-binding ability and contain regions that match putative transcriptional activation domains. In this work we have also shown that the WC-1 protein is a substrate for PKC in vitro. The portion of an *E. coli*-expressed WC-1 protein corresponding to the zinc-finger region (residues 726–1000), which includes several putative PKC phosphorylation sites, is phosphorylated in vitro, while a polypeptide corresponding to amino acids 354–818, also containing putative PKC phosphorylation sites, is not. The WC-1 protein comprises several distinct domains that make it a candidate for a crucial role in blue light perception, transduction and regulation (Ballario et al. 1996).Work by Crosthwaite et al. (1997) has shown that *wc-1* is also a clock-associated gene, and is required to sustain the rhythm of the circadian clock in the dark and to drive phase resetting of the *N. crassa* oscillator. Therefore, the WC-1 protein may be more than simply a light-linked effector required for the regulation of all light-inducible genes in *N. crassa*, it may well be the connecting link between several pathways, most probably acting in concert with WC-2, which, besides its role in light regulation, has also been shown to be a component of the *N. crassa* clock (Crosthwaite et al. 1997). In a recent study, Ballario et al. (1998) demonstrated that WC-1 is able to homodimerize through its LOV domain and heterodimerize with WC-2 through the PAS dimerization domain present in both factors. Interestingly, we have shown that WC-2 is phosphorylated in *Neurospora* extracts, but not by PKC. Another kinase must be involved in the post-translational modification of WC-2 but nothing is yet known about the importance of this event in the transduction of the light signal. The interplay between the two components WC-1 and WC-2 and their post-translational modification should generate different light responses and control circadian rhythmicity. In this scenario PKC might be important for regulating the action of downstream effectors. The findings presented here therefore suggest a way to regulate the action of WC-1 and WC-2 as effectors of the blue-light response, both singly and together.

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References

Arpaia G, Loros JJ, Dunlap JC, Morelli G, Macino G (1993) The interplay of light and circadian clock. Independent dual regulation of clock-controlled gene *ccg-2 (eas)*. Plant Physiol 102:1299–1305

Arpaia G, Loros JJ, Dunlap JC, Morelli G, Macino G (1995a) Light induction of the clock-controlled gene *ccg-1* is not transduced through the circadian clock in *Neurospora crassa*. Mol Gen Genet 247:157–163

Arpaia G, Carattoli A, Macino G (1995b) Light and development regulate the expression of the *albino-3* gene in *Neurospora crassa*. Dev Biol 170:626–635

Baima S, Macino G, Morelli G (1991) Photoregulation of the *albino-3* gene in *Neurospora crassa*. J Photochem Photobiol 11:107–115

Ballario P, Macino G (1997) White collar proteins: PASsing the light signal in *Neurospora crassa*. Trends Microbiol 5:458–462

Ballario P, Vittorioso P, Magrelli A, Talora C, Cabibbo A, Macino G (1996) White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. EMBO J 15:1650–1657

Ballario P, Talora C, Galli D, Linden H, Macino G (1998) Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. Mol Microbiol 29:719–729

Bell-Pedersen D, Dunlap JC, Loros JJ (1996) Distinct *cis*-acting elements mediate clock, light, and developmental regulation of the *Neurospora crassa eas (ccg-2)* gene. Mol Cell Biol 16:513–521

Bowler C, Chua NH (1994) Emerging themes of plant signal transduction. Plant Cell 6:1529–1541

Bowler C, Yamagata H, Neuhaus G, Chua NH (1994) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes Dev 8:2188–2202

Bruns RF, Miller FD, Merriman RL (1991) Inhibition of protein kinase C by calphostin C is light-dependent. Biochem Biophys Res Commun 176:288–293

Carattoli A, Kato E, Rodriguez-Franco M, Stuart WD, Macino G (1995) A chimeric light-regulated amino acid transport system allows the isolation of blue light regulator *(blr)* mutants of *Neurospora crassa*. Proc Natl Acad Sci USA 92:6612–6616

Christie JM, Jenkins GI (1996) Distinct UV-B and UV-A/blue light signal transduction pathways induce chalcone synthase gene expression in Arabidopsis cells. Plant Cell 8:1555–1567

Corrochano LM, Lauter FR, Ebbole DJ, Yanofsky C (1995) Light and developmental regulation of the gene *con-10* of *Neurospora crassa*. Dev Biol 167:190–200

Crosthwaite SK, Dunlap JC, Loros JJ (1997) *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. Science 276:763–769

Degli Innocenti F, Russo VEA (1984) Genetic analysis of blue light-induced responses in *Neurospora crassa*. In: Senger H (ed) Blue light effects in biological systems. Springer Verlag, Berlin

Dolph PJ, Ranganathan R, Colley NJ, Hardy RW, Sokolic M, Zuker CS (1993) Arrestin function in inactivation of G protein-coupled receptor rhodopsin in vivo. Science 260:1910–1916

Feinberg A.P, Vogelstein B (1983) Random oligonucleotide priming of DNA for labeling. Anal Biochem 132:6–13

Galland P (1989) Photosensory adaptation in plants. Botanica Acta 102:11–20

Galland P (1991) Photosensory adaptation in aneural organisms. Photochem Photobiol 54:1119–1134

Hardie RC, Peretz A, Suss-Toby E, Rom-Glas A, Bishop SA, Selinger Z, Minke B (1993) Protein kinase C is required for light adaptation in *Drosophila* photoreceptors. Nature 363:634–637

Harding RW, Shropshire W (1980) Photocontrol of carotenoid biosynthesis. Annu Rev Plant Physiol 31:217–238

Harding RW, Turner RV (1981) Photoregulation of the carotenoid biosynthetic pathway in albino and white collar mutants of *Neurospora crassa*. Plant Physiol 68:745–749

Harding RW, Huang PC, Mitchell HK (1969) Photochemical studies of the carotenoid biosynthetic pathway in *Neurospora crassa*. Arch Biochem Biophys129:696–707

Herbert JM, Augereau JM, Gleye J, Maffrand JP (1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 172:993–999

House C, Kemp BE (1987) Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. Science 238:1726–1728

Kallies A, Gebauer G, Rensing L (1996) Light effects on cyclic nucleotide levels and phase shifting of the circadian clock in *Neurospora crassa*. Photochem Photobiol 63:336–343

Kore-eda S, Murayama T, Uno I (1991) Isolation and characterization of the adenylate cyclase structural gene of *Neurospora crassa*. Jpn J Genet 66:317–334

Lakin-Thomas PL (1993) Evidence against a direct role for inositol phosphate metabolism in the circadian oscillator and the blue-light signal transduction pathway in *Neurospora*. Biochem J 292:813–818

Lauter FR (1996) Molecular genetics of fungal photobiology. J Genet 75:375–386

Linden H, Macino G (1997) White collar 2, a partner in blue light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J 16:98–109

Linden H, Ballario P, Macino G (1997a) Blue light regulation in *Neurospora crassa*. Fungal Genet Biol 22:141–150

Linden H, Rodriguez-Franco M, Macino G (1997b) Mutants of *Neurospora crassa* defective in regulation of blue light perception. Mol Gen Genet 254:111–118

Macino G, Baima S, Carattoli A, Morelli G, Valle EM (1993) Blue light-regulated expression of geranylgeranylpyrophosphate synthetase *(albino-3)* gene in *Neurospora crassa*. In: Maresca B, Kobayashi GS, Yamaguchi H (eds) Molecular biology and its application to medical mycology (Nato ASI Series, vol H69). Springer-Verlag, Berlin-Heidelberg, pp 117–124

Mellor H, Parker PJ (1998) The extended protein kinase C superfamily. Biochem J 332:281–292

Neuhaus G, Bowler C, Kern R, Chua NH (1993) Calcium/calmodulin-dependent and -independent phythochrome signal transduction pathways. Cell 73:937–952

Neuhaus G, Bowler C, Hiratsuka K, Yamagata H, Chua NH (1997) Phytochrome-regulated repression of gene expression requires calcium and cGMP. EMBO J 16:2554–2564

Oda K, Hasunuma K (1997) Genetic analysis of signal transduction through light-induced protein phosphorylation in *Neurospora crassa* perithecia. Mol Gen Gen 256:593–601

Paietta J, Sargent ML (1981) Photoreception in *Neurospora crassa*: correlation of reduced light sensitivity with flavin deficiency. Proc Natl Acad Sci USA 78:5573–5577

Paietta J, Sargent ML (1983) Isolation and characterization of light-insensitive mutants of *Neurospora crassa*. Genetics 164:11–21

Raghuram N, Sopory SK (1995) Evidence for some common signal transduction events for opposite regulation of nitrate reductase and phytochrome-I gene expression by light. Plant Mol Biol 29:25–35

Schmidhauser TJ, Lauter FR, Russo VEA, Yanofsky C (1990) Cloning, sequence, and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. Mol Cell Biol 10:5064–5070

Schmidhauser TJ, Lauter FR, Schumacher M, Zhou W, Russo VEA, Yanofsky C (1994) Characterization of *al-2*, the phytoene synthase gene of *Neurospora crassa*. J Biol Chem 269:12060–12066

Schrott EL (1981) The biphasic fluence response of carotenogenesis in *Neurospora crassa*: temporary insensitivity of the photoreceptor system. Planta 151:371–374

Scott K, Zuker C (1997) Lights out: deactivation of the phototransduction cascade. Trends Biochem Sci 22:350–354

Shaw NM, Harding RW (1987) Intracellular and extracellular cyclic nucleotides in wild-type and white collar mutant strains of *Neurospora crassa*. Plant Physiol 83:377–383

Tarawneh KA, Wang Z, Free SJ (1990) Nucleotide sequence of a *Neurospora crassa* ribosomal protein gene. Nucleic Acids Res 18:7445

Tsunoda S, Sierralta J, Sun Y, BodnerR, Suzuki E, Becker A, Socolich M, Zuker CS (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388:243–249

Udovichenko IP, Cunnick J, Gonzalez K, Yakhnin A, Takemoto DJ (1996) Protein kinase C in rod outer segments: effects of phosphorylation of the phosphodiesterase inhibitory subunit. Biochem J 317:291–295

Wood JP, McCord RJ, Osborne NN (1997) Retinal protein kinase C. Neurochem Int 30:119–136

Xu XZ, Choudhury A, Li X, Montell C (1998) Coordination of an array of signaling proteins through homo-and heteromeric interactions between PDZ domains and target proteins. J Cell Biol 142:545–555

Yarden O, Plamann M, Ebbole DJ, Yanofsky C (1992) *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J 11:2159–2166