

# Role of a white collar-1–white collar-2 complex in blue-light signal transduction

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**Mutations in either white collar-1 (*wc-1*) or white collar-2 (*wc-2*) lead to a loss of most blue-light-induced phenomena in *Neurospora crassa*. Sequence analysis and *in vitro* experiments show that WC-1 and WC-2 are transcription factors regulating the expression of light-induced genes. The WC proteins form homo- and heterodimers *in vitro*; this interaction could represent a fundamental step in the control of their activity. We demonstrate *in vivo* that the WC proteins are assembled in a white collar complex (WCC) and that WC-1 undergoes a change in mobility due to light-induced phosphorylation events. The phosphorylation level increases progressively upon light exposure, producing a hyperphosphorylated form that is degraded and apparently replaced in the complex by a newly synthesized WC-1. WC-2 is unmodified and also does not change quantitatively in the time frame examined. Light-dependent phosphorylation of WC-1 also occurs in a *wc-2* mutant, suggesting that a functional WC-2 is dispensable for this light-specific event. These results suggest that light-induced phosphorylation and degradation of WC-1 could play a role in the transient expression of blue-light-regulated genes. Our findings suggest a mechanism by which WC-1 and WC-2 mediate light responses in *Neurospora*.**

**Keywords:** blue light/dimerization/phosphorylation/signal transduction

## Introduction

Blue light is perceived in a wide variety of organisms from bacteria to plants, where it regulates fundamental processes (Short and Briggs, 1994; Cashmore *et al.*, 1999). In blue light signal transduction studies, two aspects are considered crucial: (i) how is light perceived; and (ii) how is the light signal transduced to elicit the appropriate responses? Progress on the first issue has been made in the past few years by the identification of two blue-light photoreceptors in *Arabidopsis thaliana*: the DNA photolyase-like CRY1 and CRY2 proteins (Ahmad and Cashmore, 1993; Lin *et al.*, 1995, 1998), which mediate

many blue-light-dependent responses, and NPH1, which has serine-threonine kinase activity and is implicated in phototropic responses (Huala *et al.*, 1997). In spite of the identification of blue-light photoreceptors, there remains a severe lack of information regarding second messengers involved in blue-light signal transduction or specific transcription factors involved in blue-light-activated transcription in plants (Mustilli and Bowler, 1997; Frohnmeyer *et al.*, 1998).

The filamentous fungus *Neurospora crassa* is responsive only to blue light. *Neurospora* is an important model for the study of blue-light signal transduction, because of genetic dissections of blue-light responses and the diversity of the downstream blue-light-stimulated phenotypes. Most light-induced phenomena in *Neurospora*, such as mycelial carotenogenesis, conidiation and phototropism of perithecial beaks, are abolished in *wc-1* and *wc-2* single mutants (Lauter, 1996; Ballario and Macino, 1997; Linden *et al.*, 1997). *wc-1* and *wc-2* mutants are only defective in blue-light-induced processes, but not in their growth behavior or in the expression of non-light-regulated genes. For this reason, their gene products are thought to be specifically dedicated to blue-light signal transduction.

*wc-1* and *wc-2* genes have been cloned (Ballario *et al.*, 1996; Linden and Macino, 1997), and sequence analysis indicates the presence of GATA-like zinc finger DNA-binding domains (Orkin, 1992) in both proteins, suggesting that they function as transcription factors. Consistent with this, WC-1 and WC-2 (transcription factors regulating the expression of light-induced genes) are both able to bind *in vitro* to the same region (GATA) of the light-regulated promoter of the *albino-3* gene (*al-3*) (Ballario *et al.*, 1996; Linden and Macino, 1997; Feng and Marzluf, 1998). *Neurospora* light-regulated processes are grouped in early and late responses. Light induction of the mRNA of early light-regulated genes can be detected within 5 min after a light pulse (Sommer *et al.*, 1989). *al-3* is transiently expressed in the mycelium upon light induction: its mRNA level starts decreasing after 20 min of constant light, reaching the dark level after 1 h. This phenomenon, where light-induced genes are not transcribed even in the presence of continuous light, is called adaptation (Baima *et al.*, 1991). However, some light-regulated genes are under delayed transcriptional control and expressed several hours after light induction (Sommer *et al.*, 1989; Arpaia *et al.*, 1993, 1995a,b).

WC-1 and WC-2 sequences indicate that both proteins have multifunctional PAS domains, which are also found in eubacteria, archaeobacteria and eukaryotes (Ponting and Aravid, 1997). In animals, PAS domains are mainly involved in protein–protein interaction, and they are widely distributed in proteins able to sense environmental changes (Lindebro *et al.*, 1995; Zhulin *et al.*, 1997). This motif may also be a conserved molecular signature of proteins

involved in the circadian clock, a light-dependent phenomenon (Ponting and Aravid, 1997; Rutila *et al.*, 1998). In *Drosophila*, the circadian rhythmicity appears to be mainly based on an autoregulatory feedback loop where a protein containing a PAS domain positively and negatively controls circadian gene expression (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998; Suri *et al.*, 1999).

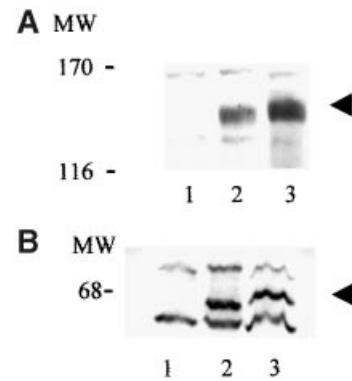
A degenerated PAS domain called LOV (for Light, Oxygen and Voltage) or S-box (Zhulin *et al.*, 1997) has been identified in prokaryotic proteins (like PYP, Bat and NifL) and in the plant blue-light photoreceptor NPH1 (Huala *et al.*, 1997). In the case of NPH1, the LOV domain seems to act as a versatile ligand-binding domain, non-covalently binding flavin mononucleotide, a likely chromophore for light-dependent autophosphorylation (Christie *et al.*, 1998). On the basis of classical photobiology studies, a flavin is also the predicted chromophore of the *Neurospora* photoreceptor (Paietta and Sargent, 1983). Furthermore, a LOV domain is present in WC-1 and not in WC-2. These last observations, together with the fact that to date only *wc-1* mutants show a completely 'blind' phenotype (absence of any blue-light-induced phenomena), have led to the intriguing suggestion that this protein could participate in the first step of light signal transduction, notably light perception (Ballario and Macino, 1997).

The WC proteins form hetero- and homodimers *in vitro*, thus suggesting a model for blue-light signal transduction through the formation of light-specific WC complexes which lead to light-dependent activation of transcription (Ballario *et al.*, 1998). Although WC-1 and WC-2 are essential components for the blue-light responses, and although several other studies have addressed the role of these proteins in different light-induced phenomena, there is as yet no information about the nature of post-translational modifications with respect to WC protein function. Analysis of their regulation and interaction should provide significant insight into the mechanism that underlies blue-light signal transduction. We report the immunochemical analysis of WC-1 and WC-2 proteins in dark and light growth conditions. Light-induced post-translational modifications of the WC proteins have been investigated in wild-type *Neurospora* and in *wc-2* and *wc-1* backgrounds. Here we show that WC-1 undergoes a progressive, light-induced phosphorylation, even in a *wc-2* genetic background. WC-1 and WC-2 are assembled in a complex (WCC) in both the absence and presence of light. Furthermore, we show that the hyperphosphorylated WC-1 is degraded, suggesting that a new WCC is formed between the stable WC-2 and a newly synthesized WC-1. A new model of *Neurospora* blue-light signal transduction and adaptive regulation, based on WCC phosphorylation and turnover, is presented.

## Results

### Characterization of WC-1 and WC-2 antibodies

We were interested in the analysis of light-induced molecular events with respect to WC-1 and WC-2. To this end, we generated antisera against WC-1 and WC-2 expressed in *Escherichia coli*. Figure 1 shows Western blots with WC-1 immunopurified antibodies (Figure 1A) and with

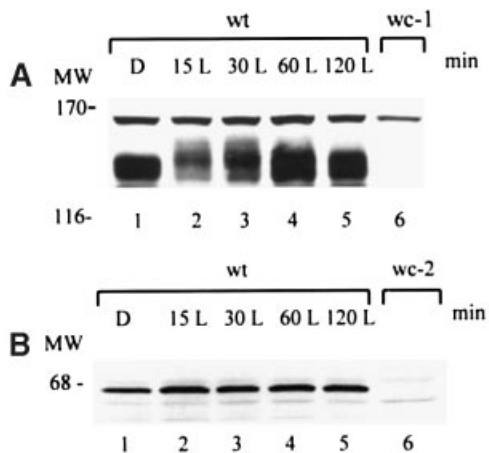


**Fig. 1.** Characterization of antibodies: Western blots of *Neurospora* extracts. (A) WC-1 immunopurified antibodies were used to probe: lane 1, *N.crassa wc-1* null mutant; lane 2, *N.crassa* wild-type strain; lane 3, *N.crassa wc-1* null mutant expressing a recombinant WC-1. (B) WC-2 antiserum was used to probe: lane 1, *234w (wc-2)* mutant; lane 2, *N.crassa* wild-type strain; lane 3, *N.crassa 234w* allele expressing a recombinant WC-2. Arrows indicate the specific signals.

WC-2 antiserum (Figure 1B). Specific immunoreactive bands (indicated by arrows) were detected, migrating on SDS-PAGE as 150 and 68 kDa for WC-1 and WC-2, respectively, in wild-type strains (Figure 1A and B, lanes 2). The molecular weight estimated on the gel for both proteins was 10–15 kDa more than the predicted size, possibly due to the acidic charge of the two proteins. *wc-1* and *wc-2* mutant strains were used to control for correct assignment of WC-1 and WC-2 signals. WC-1 antibodies did not recognize any signal (Figure 1A, lane 1) in the extract from a *wc-1* null mutant (see Materials and methods). Similarly, no specific 68 kDa WC-2 band was present in a *wc-2* mutant (Figure 1B, lane 1) (see Materials and methods). In lane 3 of both Figure 1A and B, the antibodies detected a specific band in the extracts from the *wc-1* or *wc-2* mutants transformed with a plasmid that overexpresses WC-1 or WC-2 under the inducible *qa-2* promoter (Baum and Giles, 1985). The slower electrophoretic mobility of the WC-2 bands in lane 3 corresponds to the increase in molecular weight of the recombinant proteins due to an artificial tag (see Materials and methods). No cross-reaction of WC-1 antibodies against WC-2, or vice versa, has been observed (data not shown). Non-specific bands recognized by the antibodies were used as an internal control for protein loading.

### Light-regulated post-translational modification of WC-1 and WC-2 in wild-type strains

Light-induced transcription of the *al-3* gene (Carattoli *et al.*, 1994), and of other early light-dependent genes, takes place rapidly (specific mRNAs are detected after 1 min of irradiation), and is observed even in the presence of cycloheximide (Baima *et al.*, 1991). Based on these observations, the molecular model for the blue-light response in *N.crassa* involves transcription of these light-activated genes as a result of light-mediated activation of pre-existing WC-1 and WC-2 proteins (Ballario and Macino, 1997). To test this hypothesis, liquid cultures of a *Neurospora* were grown in the dark, switched to constant light and harvested at intervals. Western blot analysis of mycelial extracts showed that, as expected, both WC-1 and WC-2 proteins were already present in



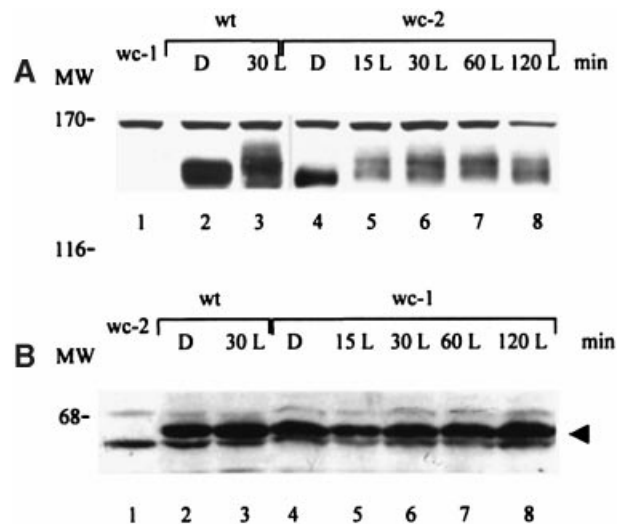
**Fig. 2.** Light-regulated post-translational modification of WC-1 and WC-2 in wild-type strains. (A) Western analysis of WC-1 in mycelial extracts from wild-type *Neurospora* grown in the dark (D) or induced by light (L). Lane 1, wild-type *Neurospora* dark; lanes 2–5, wild-type *Neurospora* collected after 15, 30, 60 or 120 min of illumination; lane 6, *wc-1 null* mutant. (B) The same as in (A), but probed with anti-WC-2 antibodies. Lanes 1–5 as in (A); lane 6, *234w* allele (*wc-2* mutant).

the dark (Figure 2A and B, lanes 1). The WC-1 signal appeared as a broad band in the dark (Figure 2A, lane 1). Upon light irradiation, characteristics of the band changed: after 15 min, the WC-1 signal decreased in intensity and was reduced in mobility (Figure 2A, lane 2); at 30 min, the mobility was further reduced, but the intensity of the signal increased and reached a maximum at 1 h. After 2 h of irradiation, the signal was comparable in intensity and mobility to dark levels (Figure 2A, lane 5). The light-induced changes in mobility suggest that WC-1 is subject to transient post-translational modification. In contrast, the WC-2-specific signal in the dark (Figure 2B, lane 1), and at all time points of continuous light that were examined, was constituted by a sharp band, and its amount was stable. As a control for signal specificity, extracts from *wc* mutants were used (Figure 2A and B, lane 6).

#### Light-regulated post-translational modification of WC-1 and WC-2 in mutant strains

In order to understand whether the absence of one functional WC protein influences the presence, light response or stability of the other, experiments on light response kinetics were repeated in *wc* mutated genetic backgrounds. In Figure 3A, lane 1 represents the negative-control *wc-1 null* mutant, whereas lanes 2 and 3 represent the wild-type signal in dark or light conditions. This figure shows that in a *wc-2* mutant, as well as in the wild type, WC-1 is present in the dark (lane 2 and 4) and that a post-translational modification takes place after light induction (lanes 5–8). However, the WC-1 light-dependent post-translational modification observed in this genetic background does not have the same pattern as observed in wild type (Figure 2A). We see a slight decrease in WC-1 protein levels and degree of post-translation modification, as well as an absence of the transience of the modification, compared with wild type.

Light-induced transcription of *wc-2* is very limited (Linden and Macino, 1997), and the WC-2 protein levels and gel migration pattern in a WC-1 *null* strain are

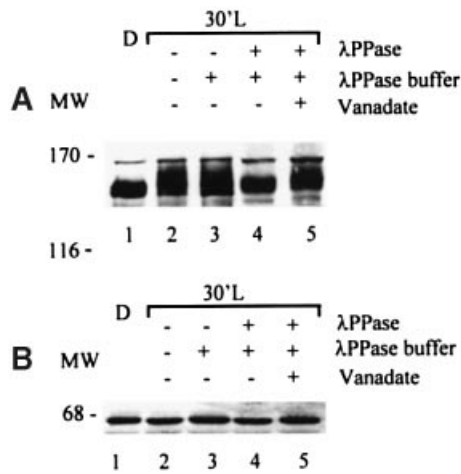


**Fig. 3.** Light-regulated post-translational modification of WC-1 and WC-2 in mutant strains. (A) Western analysis of WC-1 in mycelial extracts from *234w Neurospora* mutant grown in the dark (D) or induced by light (L). Lane 1, *wc-1 null* mutant; lane 2, wild-type *Neurospora* dark-grown culture; lane 3, wild-type *Neurospora* after 30 min of light; lanes 4–8, *234w* mutant (*234w* allele): lane 4, dark; lanes 5–8, *234w* irradiated for 15, 30, 60 or 120 min. (B) Western analysis of WC-2 in mycelial extracts from a *wc-1 null Neurospora* mutant grown in the dark or induced by light. Lanes are the same as in (A), but with *wc-1* instead of *wc-2* mutant (*234w* allele) in lanes 4–8.

not visibly different from wild type (Figure 3B). This experiment confirms that WC-2 synthesis is constitutive and that it is independent both of light (lanes 2 and 3) and of the presence of a functional WC-1 (lanes 4–8). The results of Figure 3 suggest that a functional WC-2 is not required for a post-translational modification of WC-1, but that it could influence the degree of this modification and the stability of WC-1.

#### WC-1 is phosphorylated in response to light

Changes in mobility and complexity of the WC-1 band on SDS-PAGE after light induction (Figure 2A) could be due to the progressive addition of phosphate residues, generating a series of isoforms of increasing molecular weight. Furthermore, sequence analysis of WC-1 indicates the presence of several putative phosphorylation sites. Therefore, extracts obtained from light-induced mycelia were treated with lambda protein phosphatase ( $\lambda$ PPase), an enzyme which removes phosphates from serine, threonine, tyrosine and histidine residues. The samples were analyzed by Western blot (Figure 4A and B). Dark-grown and light-irradiated mycelial extracts were used as controls (lanes 1 and 2). Treatment with  $\lambda$ PPase buffer only (lane 3 compared with lane 2) showed no changes in mobility. However, upon  $\lambda$ PPase treatment of extracts from light-grown mycelia, the mobility of WC-1 increased, and the complexity of the bands reverted to dark levels, indicating that WC-1 was present in light-treated cultures as multiply phosphorylated forms (Figure 4A, lane 4). The change in mobility of  $\lambda$ PPase-treated WC-1 was fully inhibited by vanadate, excluding the possibility that contaminating proteases are responsible for the effect (lane 5). Figure 4B shows the electrophoretic behavior of WC-2 upon  $\lambda$ PPase treatment; no changes in the treated samples were detected,



**Fig. 4.** WC-1 is phosphorylated in response to light. (A) Anti-WC-1 antibody was probed on wild-type *Neurospora*. Lane 1, dark; lane 2, 30 min of light induction; lane 3, extract as in lane 2 incubated with the phosphatase buffer alone; lane 4, the same as in lane 3 treated by  $\lambda$ PPase; lane 5, the same as in lane 3 treated by  $\lambda$ PPase in the presence of vanadate. (B) The same samples as in (A) probed with anti-WC-2 antiserum.

confirming the previous results that light has no apparent effect on WC-2.

#### Light-induced phosphorylation of WC-1 is correlated with protein turnover

In order to elucidate the role of WC-1 post-translational modification in the blue-light response, this aspect has been examined in further detail. We previously described that *wc-1* transcription is strongly enhanced upon light induction (Ballario *et al.*, 1996). Thus, it is reasonable to expect an increase in WC-1 protein levels in response to irradiation. On the contrary, the amount of WC-1 upon irradiation is relatively constant for 2 h, although it shows a clear decrease at 15 min (Figure 2A, lane 2) and an increased level at 60 min before returning to the dark level. It can be hypothesized that the amount of WC-1 protein following irradiation is the product of two opposing events: the degradation of pre-existing WC-1 and subsequent *de novo* synthesis. WC-2 is apparently stable throughout (Figure 2B).

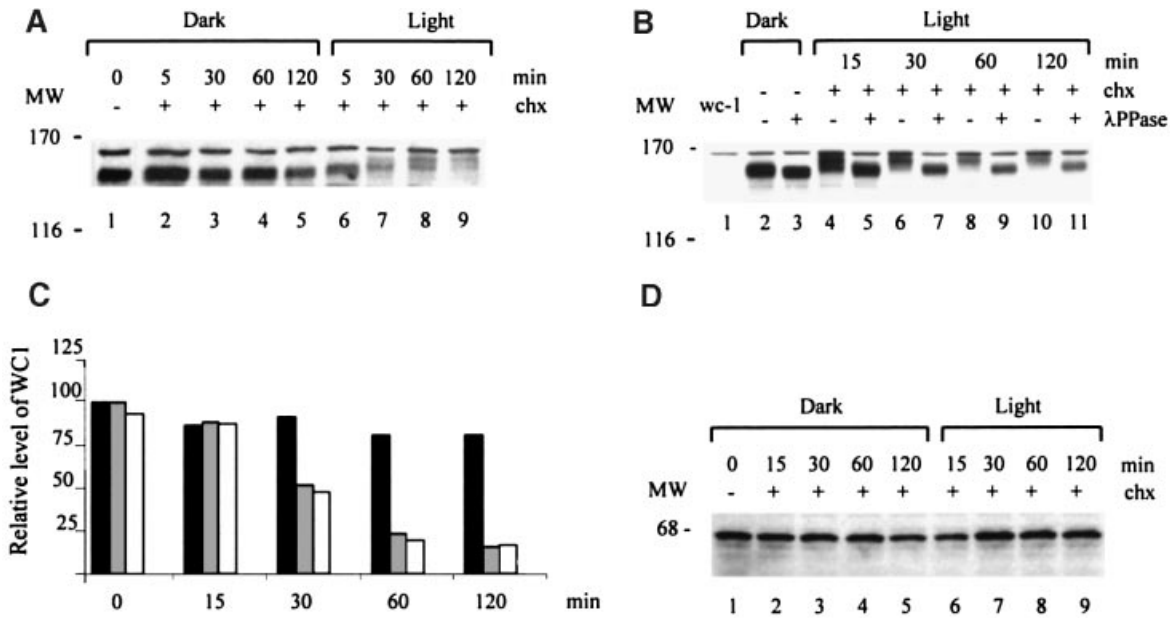
Previous studies (such as with I $\kappa$ B $\alpha$ ; Beg *et al.*, 1993; Deshaies, 1997) indicate a role for phosphorylation in signal-induced protein turnover, where this post-translational modification targets the protein for degradation. We therefore investigated whether light-induced phosphorylation of WC-1 has a role in its degradation. To understand whether the steady-state level of WC-1 is a result of a continuous turnover, we analyzed the protein levels in extract obtained from mycelia treated with the protein synthesis inhibitor cycloheximide (Figure 5). Mycelia grown in the dark or irradiated with light were collected at different time intervals (from 5 min to 2 h) after cycloheximide treatment. Figure 5A shows that in the presence of cycloheximide, WC-1 protein is stable in the dark for at least 1 h (lanes 1–4), with a slight reduction of protein levels after 2 h of treatment (lane 5). However, following irradiation with light, there is a continuous increase in WC-1 phosphorylation and a concurrent decrease in the quantity of WC-1 (Figure 5A, lanes 6–9).

In the absence of the newly synthesized WC-1 (Figure 5A, lane 7; Figure 5B, lane 6), the majority of WC-1 is already phosphorylated at 30 min. At 1 and 2 h (Figure 5A, lanes 8 and 9; Figure 5B, lanes 8 and 10), WC-1 is completely phosphorylated, with no residual unphosphorylated band.

In order to measure the amount of phosphorylated WC-1 accurately, the same samples from the cycloheximide-treated light-induced cultures shown in Figure 5A were incubated with  $\lambda$ PPase (Figure 5B). The  $\lambda$ PPase-treated samples (lanes 3, 5, 7, 9 and 11) showed a continuous reduction in the quantity of WC-1, corresponding to an increase in irradiation length. Figure 5C shows the histogram derived by densitometric scanning of the signals in Figure 5 (A, lanes 1–5; B, lanes 2–11). Corresponding WC proteins bands were normalized against the relative intensity of the contaminating bands (shown in Figure 1A). There is a clear difference in stability of WC-1 in the dark versus the light, suggesting a more rapid turnover of the protein when it is phosphorylated. Figure 5D shows the Western analysis for WC-2 carried out on the same cycloheximide-treated samples of Figure 5A. These results clearly indicate that WC-2 is stable both in dark and light conditions.

#### A WC-1–WC-2 complex (WCC) is present both in dark and light conditions

Both WC proteins have a PAS dimerization domain. The ability of WC-1 and WC-2 to form dimers *in vitro* was tested previously (Ballario *et al.*, 1998), and it was shown that these PAS domains enable homo- and heterodimer formation by WC proteins. In order to investigate whether WC-1–WC-2 interaction occurs *in vivo*, and to understand how light regulates this interaction, co-immunoprecipitation assays were performed (Figure 6). WC-2 was immunoprecipitated with anti-WC-2 antiserum, and the membrane was probed with an antibody to WC-1. Dimerization was tested in mycelia grown in the dark, as well as after 1, 5, 30, 60 and 120 min of constant saturating light exposure. Figure 6 shows the signals obtained on untreated (odd lanes) or  $\lambda$ PPase-treated (even lanes) co-immunoprecipitated samples probed with anti-WC-1 antibody (Figure 6A) and with anti-WC-2 antiserum (Figure 6B). The co-immunoprecipitation of WC-1 and WC-2 in both dark and light growth conditions suggests that a complex of WC-1 and WC-2 occurs *in vivo*, here called white collar complex (WCC). Figure 6A shows that, following light irradiation, the complex-associated WC-1 protein is progressively hyperphosphorylated (see lanes 5, 7 and 9). After 2 h of light treatment, however, the level of phosphorylation of complexed WC-1 is reduced to a level comparable to that observed in the dark (lanes 11). The kinetics of WC-1 light-dependent phosphorylation in the complex parallels that observed by Western analysis on total mycelial extract (Figure 2A). However, the less pronounced reduction in electrophoretic mobility of the co-immunoprecipitated WC-1 after 1 and 2 h suggests that the hyperphosphorylated form of WC-1 is released from the complex and degraded. The immunoprecipitated WC-2 samples in Figure 6B were clearly unaffected by  $\lambda$ PPase treatment, confirming once more that WC-2 is not phosphorylated. The  $\lambda$ PPase-treated samples (even lanes) were used for scanning measurements



**Fig. 5.** Light-induced phosphorylation of WC-1 is correlated with turnover. (A) Anti-WC-1 antibody was used to probe cycloheximide-treated cultures. Lanes 1–5: extracts from *Neurospora* wild type grown in the dark and collected after the addition of cycloheximide for 5, 30, 60 or 120 min. Lanes 6–9: extracts from *Neurospora* wild type grown in the light and collected after the addition of cycloheximide for 5, 30, 60 or 120 min. (B) Anti-WC-1 antibodies used to probe cycloheximide-treated wild-type samples grown in the light untreated (lanes 2, 4, 6, 8 and 10) or treated by  $\lambda$ PPase (lanes 3, 5, 7, 9 and 11); lane 1: *wc-1* null mutant. (C) Histogram representing the relative amount of WC-1 at different time points of light irradiation: Black, dark with cycloheximide; gray, light induced with cycloheximide; white, light induced with cycloheximide and incubated with  $\lambda$ PPase. The WC-1 signal in (A) and (B) was traced densitometrically, and the data were plotted as a function of time. The WC-1 signal detected in the dark was set to 100% and used to normalize the signal at subsequent time points. (D) The same as (A), but probed with anti-WC-2 antiserum.

of the amount of WC-1 present in the complex. Figure 6C shows the ratio between WC-1 and WC-2 in the complex as a function of the time of irradiation. These data are derived from three independent experiments, identical to those in Figure 6A and B, and they show that the ratio of the two components in the complex remains stable.

## Discussion

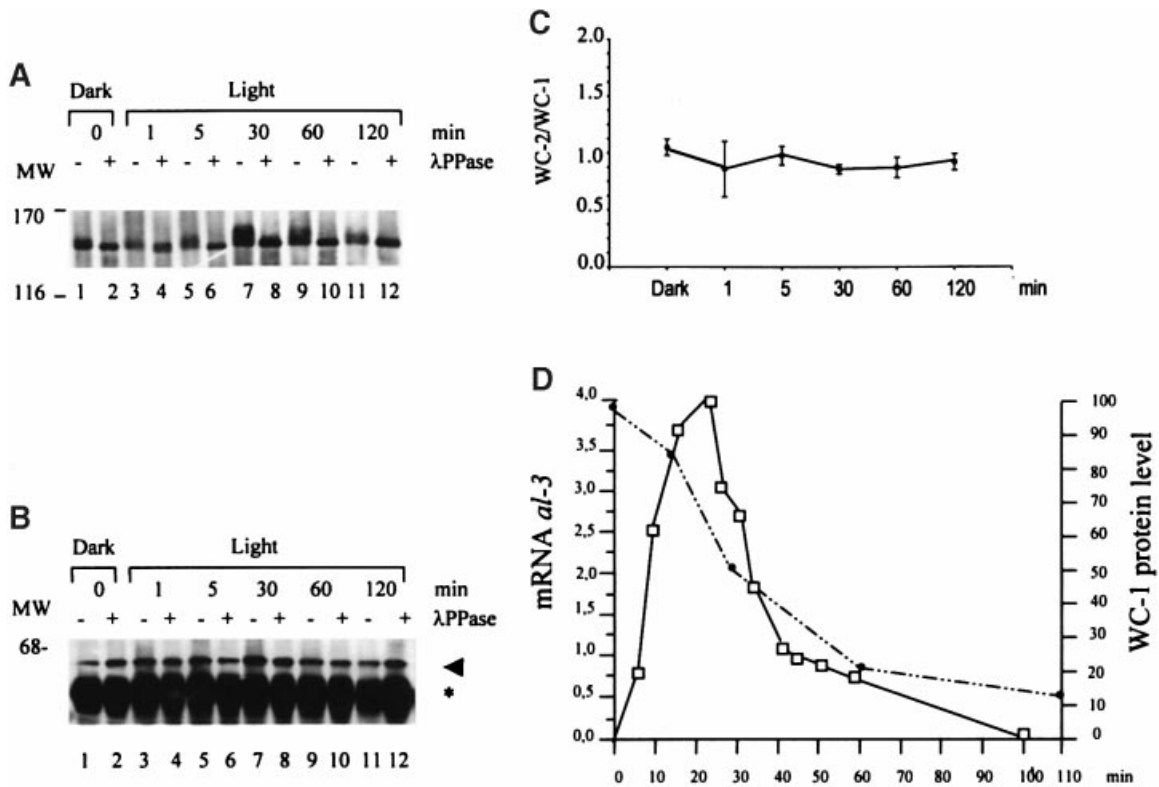
*wc-1* and *wc-2* *Neurospora* mutants are phenotypically indistinguishable. Previously, we described the isolation and characterization of the *wc* genes, and showed that WC-1 and WC-2 encode zinc finger GATA factors (Ballario *et al.*, 1996; Linden and Macino, 1997) involved in light-induced gene expression. The presence of additional functional domains (LOV and PAS) and genetic evidence, however, suggests that they could also be directly involved in light signal transduction (Ballario and Macino, 1997).

Recently, we reported that the PAS domain of both WC-1 and WC-2 allows their interaction *in vitro*, leading to the formation of homo- and heterodimers (Ballario *et al.*, 1998). This observation led to the hypothesis that WC-1 and WC-2 both participate in light signal transduction through the formation of light ‘responding’ complexes (Ballario *et al.*, 1998). The existence of such a WCC has been demonstrated *in vivo* in this study. WCC has been detected in mycelia grown in both dark and light conditions (Figure 6). However, this does not exclude participation of WC-1 and WC-2 monomers and homodimers in the light response.

The WCC is always present; however, it is subject to a light-dependent modification of one of its subunits.

WC-1, but not WC-2, becomes progressively and transiently phosphorylated in response to light. WC-1 phosphorylation is already present at very low levels in the dark and it increases significantly upon light induction, reaching a maximum at 30 min. Experiments carried out in the presence of cycloheximide have shown that at 15 min WC-1 is heavily phosphorylated and, at 30 min, while the phosphorylation increases, WC-1 is degraded. These data show that hyperphosphorylation and degradation of WC-1 are events that take place exclusively in light conditions. The histogram in Figure 5C shows that increasing light exposure corresponds to a progressive decrease in WC-1 protein level. These experiments cannot indicate whether WC-1 phosphorylation is directly involved in activation of the WCC, in WC-1 degradation, or in both events. However, they establish a correlation between light and phosphorylation of WC-1, and between phosphorylation and WC-1 degradation.

The stoichiometry of the complex remains stable, independent of the presence or absence of light. The ratio between WC-1 and WC-2 in the complex is constant even with the light-induced degradation of WC-1. This suggests that the newly synthesized WC-1 deriving from the light-induced transcription of *wc-1* participates in the formation of the WCC. Figure 6D shows the transient expression of *al-3* mRNA (derived from Baima *et al.*, 1991), compared with the amount of WC-1 measured in the presence of a protein synthesis inhibitor. This diagram shows that a decrease in WC-1 protein level corresponds to a decrease in *al-3* light-induced expression. This result suggests that WC-1 degradation is involved in the downregulation of light induction of *al-3*, and that the complex between the newly synthesized WC-1 and WC-2 does not sustain light-



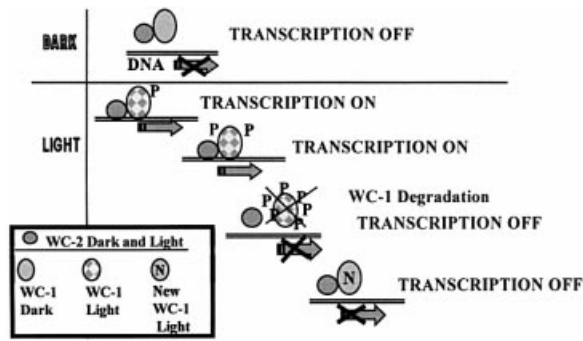
**Fig. 6.** White collar complex (WCC) is present in dark and light growth conditions. WC-2 protein was immunoprecipitated with an anti-WC-2 antiserum from extracts of wild-type *Neurospora* grown in dark or light conditions. The immunoprecipitate was resuspended in Laemmli buffer and identical aliquots were loaded on different SDS-PAGE [(A) 5% and (B) 10%]. The Western blots were probed with (A) anti-WC-1 affinity-purified antibodies and (B) anti-WC-2 antiserum. (A) and (B): lane 1, dark; odd lanes, wild type at different intervals of light induction (1, 5, 30, 60 and 120 min); even lanes, the same samples treated by λPPase. (C) This diagram represents a densitometric analysis of the ratio between WC-1 and WC-2 co-immunoprecipitates. The curve shows the average of three independent experiments (the vertical bar represents the SD). (D) The kinetics of *al-3* mRNA from Baima *et al.* (1991) is compared with the decrement in the amount of WC-1 in the absence of *de novo* synthesis of proteins. The continuous line represents the *al-3* relative transcription levels; the dashed line represents the WC-1 signal detected in cycloheximide-treated mycelia incubated with λ-PPase (as in Figure 5B).

dependent transcription in continuous light. It is hard to predict the reason for the light insensitivity of the new WCC under continuous light irradiation. We can only suggest that it could be due to the lack of the correct redox state of the chromophore or to the absence of some additional component of the light complex. However, after a short dark period, or following irradiation with a higher intensity of light, transcription resumes (Arpaia *et al.*, 1999), indicating that the WCC is again responsive, and 'adapted' to the light condition. WC-1 sequence analysis has revealed the presence of several putative phosphorylation sites throughout the molecule, and WC-1 is a substrate for the *Neurospora* protein kinase C (PKC) *in vitro* (Arpaia *et al.*, 1999). PKC inhibition *in vivo* modifies the transient light-induced expression of *al-3*, suggesting a role of this enzyme in light adaptation of the fungus (Arpaia *et al.*, 1999).

In contrast to WC-1, WC-2 is unmodified and stable after light induction. Interestingly, a functional WC-2 appears to be dispensable for the light-induced modification of WC-1, which also takes place in a *wc-2* mutant. In this genetic background, however, we observed a decrease in WC-1 protein levels and degree of phosphorylation, and an absence of the transiency of the phosphorylation compared with wild type. These results suggest that a functional WC-2 is not required for post-translational modification of WC-1, but is necessary to sustain both

the transiency and magnitude of WC-1 modification. These data suggest that WC-2 directly or indirectly plays a role in these processes. The reduced amount of WC-1 observed in the *wc-2* mutant could be the result of the absence of the *wc-1* light-induced transcription that is known to be dependent on *wc-1* and *wc-2*. This is another example of the responsiveness of *Neurospora* to light in the absence of WC-2, together with the previously reported data showing that *cgc-1* and *frq* gene expression are still light inducible in a *wc-2* mutant (Arpaia *et al.*, 1995; Crosthwaite *et al.*, 1997).

The data reported suggest that WC-1 and WC-2 play distinct roles in transducing a light signal. Previously, a functional difference was described for the role of WC-1 and WC-2 in the circadian rhythm of conidiation in *Neurospora* (Crosthwaite *et al.*, 1997). Analysis of the *frq* transcript and of its protein product FRQ has shown that rhythmicity occurs via a transcription-translation autoregulatory loop. WC-1 and WC-2 are both required for maintenance of the circadian rhythm in *Neurospora*, but apparently with different functions. WC-1 is essential for light resetting of the circadian cycle, by the induction of *frq* transcription upon a pulse of blue light. By contrast, WC-2 is not required for light-induced transcription of *frq*, but is proposed to be a new component of the circadian clock, acting as a positive transcription factor necessary for maintaining circadian cycling. This could be facilitated



**Fig. 7.** The model. In the dark, the WCC complex is inactive. Upon light irradiation, the WCC activates the transcription of light-dependent genes. With the addition of phosphate groups to WC-1, the factor enters into a degradation pathway and is substituted, in the complex, by the newly synthesized WC-1 (NWC-1). However, the new WCC is not able to promote light-specific transcription. WC-2 has a turnover that is longer than the interval of 2 h examined and, therefore, it is hypothesized that it is recycling.

by an interaction of WC-2 with FRQ (Ballario and Macino, 1997; Dunlap, 1999).

On the basis of these experiments, a new mechanism describing the WC-1- and WC-2-mediated light response can be proposed (Figure 7). In this model, the WCC is already present in the dark, when the transcription of the light-regulated genes is off and WC-1 is minimally phosphorylated. Upon light exposure, WC-1 is further phosphorylated and light-regulated gene transcription starts. As the time of light exposure increases, WC-1 phosphorylation also increases and when WC-1 becomes hyperphosphorylated it is degraded. Subsequently, newly synthesized WC-1 is substituted for the degraded molecule, leading to reconstitution of the complex, which does not, however, promote light-induced transcription. During this time, WC-2 is stably maintained and unmodified. Since the WCC is present even after light induction, the same pleiotropic effects obtained in either *wc-1* or *wc-2* mutants may reflect a requirement for both WC-1 and WC-2 participation in light transduction.

Importantly, there is still no clear evidence as to the identity of the *Neurospora* blue-light photoreceptor. That the blue-light photoreceptor NPH1 contains a flavin-binding LOV domain suggests that the WCC activity could be directly controlled by light, via the WC-1 LOV domain. In support of this, single amino acid substitutions in the LOV domain of WC-1 have been shown to produce *white collar-1* phenotypes *in vivo*, while they did not affect the *in vitro* ability of the LOV domain to self-dimerize (Ballario *et al.*, 1998). This suggests that a function different from protein-protein interaction (i.e. flavin binding) is present in WC-1 and altered in the mutants (Ballario *et al.*, 1998).

The model presented here does not exclude the participation of other factors in blue-light signal transduction. For instance, light induction of late response genes could be dependent on the activity of other factors whose expression is regulated earlier by the WCC. In conclusion, we report the identification and characterization of WC-1 post-translational modification in response to light. This phosphorylation and subsequent turnover are precisely correlated with an established pattern of gene expression. Thus, the biochemical features of WC-1 in the WCC

indicate that this complex of transcription factors could be a central element, namely, a photoreceptor with direct capability of gene activation, in blue-light signal transduction.

## Materials and methods

### Strains and growth condition

The wild-type strain 74OR23-1a (FGSC 988) and the mutant *wc-2*, allele *234w* (FGSC 3817), were obtained from the Fungal Genetics Stock Center (Kansas City, KS). The *null* strain of *wc-1* was obtained by the RIPing technique (Selker, 1990). A *wc-1* subfragment of 450 bp (*Xba*I-*Xho*I) from the putative ATG of the gene was cloned in SKqa vector. This construct was used to transform *Neurospora* spheroplasts of the strain 3957 (*qa-2*, *aro-9A*). Independent transformants were isolated and crossed with the *74a* strain (wild type). Resulting ascospores were examined, selected for *wc* phenotype and subjected to nucleotide sequence analysis. Seventeen stop codons were introduced into the N-terminal of the *wc-1 null* mutant used in this work and the absence of WC-1 protein was confirmed by Western blot analysis. This *null* strain was used as a recipient for the expression of WC-1 tagged protein. The inducible *qa-2pWC-1* construct was obtained by cloning the entire WC-1 coding sequence under control of the quinic acid-inducible *qa-2* promoter (Baum and Giles, 1985). The general growth medium was comprised of Vogel's minimal medium supplemented with 2% sucrose. After 24 h of growth in the dark, mycelia were collected by filtration either directly or after light induction (constant saturating light, 10 W/m<sup>2</sup>) and frozen in liquid nitrogen. For the analysis of the stability of WC proteins, minimal medium was supplemented with cycloheximide (100 µg/ml) for the time indicated in the figure legends. The *234w* strain used as a WC-2-negative control in the experiments has a point mutation that generates a stop codon, creating a truncated protein of 356 amino acids (Linden and Macino, 1997).

### Antisera against WC-1 and WC-2

GST-WC-1 and GST-WC-2 fusion proteins (residues 354–838 for WC-1 and residues 168–530 for WC-2) were expressed in the BL21 *E. coli* strain and purified to homogeneity by glutathione-agarose beads (Pharmacia) as described (Ballario *et al.*, 1996). The WC-1 and WC-2 antisera were generated by immunizing rabbits with purified GST-WC-1 and GST-WC-2 according to standard protocols. A histidine-tagged WC-1 protein (residues 354–838) was purified to homogeneity by nickel-agarose affinity chromatography and used to purify anti-WC-1 IgG fractions by CNBr affinity chromatography.

### Western blot analysis of protein

Western blot analysis was performed as previously described (Crosthwaite *et al.*, 1997). Tissue was ground in liquid nitrogen with a mortar and pestle, and suspended in ice-cold lysis buffer [50 mM HEPES pH 7.4, 137 mM KCl, 10% glycerol containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 1 mM leupeptin, 1 mM pepstatin A], at a ratio of 0.5 ml of buffer to 0.1 g of tissue. Extracts were homogenized by three strokes of a Teflon/glass homogenizer, and cellular debris was removed by centrifugation at 10 000 g. The protein concentration of the supernatant was determined with the Bio-Rad reagent according to their manual. Equal quantities of proteins (100 µg) for different extracts were denatured in Laemmli sample buffer and separated by 5% SDS-PAGE for WC-1 analysis and 10% SDS-PAGE for WC-2 analysis. As a control for protein loading and transfer, we used the intensity of the signals due to the non-specific bands (not shown in the figures, except Figure 1). After transfer to nitrocellulose (Amersham), blots were incubated with either WC-2 antiserum or WC-1 affinity-purified IgG. Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody (Bio-Rad) and Western blots were developed using chemiluminescence (ECL, Amersham).

### Immunoprecipitation assay

Affinity-purified WC-1 antibody or WC-2 antiserum was incubated with 20 µl of protein A-Sepharose (Pharmacia) for 1 h at 4°C in 500 µl of lysis buffer (50 mM HEPES pH 7.4, 137 mM KCl, 10% glycerol containing 1 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 1 mM pepstatin A). Total cell extracts (600 µg), prepared as described above, were pre-incubated with protein A-Sepharose for 1 h at 4°C and centrifuged. The supernatant was added to the pellet composed of protein A-Sepharose bound to antibodies, previously washed twice with

lysis buffer, and incubation was continued for another 2 h at 4°C with mixing. The immunoprecipitate was washed five times with 500 µl of lysis buffer containing 0.05% Triton X-100.

### Phosphatase treatment

The immunoprecipitated complex or 50 µg of total protein were diluted in 1× phosphatase buffer (New England Biolabs), 1000 U of λPPase were added, and the reaction was incubated at 30°C for 30 min in the presence or absence of 20 mM sodium vanadate. The Laemmli sample buffer was added to the reaction, and the sample subjected to SDS-PAGE and Western blot analysis.

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