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Light signals are transduced to the phosphorylation of 15 kDa proteins in *Neurospora crassa*

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Abstract

A microsomal fraction prepared from the mycelia of the *band* (*bd*) strain of *Neurospora crassa* showed enhanced phosphorylation of two small proteins with molecular masses of around 15 kDa (ps15) by the irradiation of the reaction mixture containing [γ-^{32}P]ATP at 0 °C for 1 s with blue light (450 nm, 6 μmol/m^2/s or 420 nm, 80 μmol/m^2/s). The reaction was stopped at 5 s of incubation at 0°C after blue light irradiation. The light effect could not be detected in ps15, when a microsomal fraction from a blind mutant, *wc-1* or *wc-2* was used. The mixing followed by homogenization of the microsomal fractions from *wc-1* and *wc-2* restored the activity to stimulate the phosphorylation of ps15 by blue light. The phosphorylated amino acid residue of ps15 was unstable when the proteins on a nylon membrane were exposed to an acid or alkaline solution, suggesting that the phosphorylated residue was aspartic acid. The other phosphorylated protein with a molecular mass of 70 kDa (p70) showed no light effect in the phosphorylation and the phosphorylated residue was estimated to be histidine, since it was stable in alkaline solution.

Key words: Blue light; Microsomal fraction; *band*; *wc-1*; *wc-2*; Two-component system

1. Introduction

In *Neurospora crassa*, a UV-A or blue light signal can change the phase of conidiation rhythm in band (*bd*) strain [1], suppress the circadian conidiation under constant light [1], induce the synthesis of carotenoids in the mycelia [2], stimulate the expression of the genes called blue light inducible (*bli*) genes [3], enhance the morphogenesis of protoperithecia [4], and induce the bending of the beak of perithecia [5]. However, two blind mutants, *wc-1* and *wc-2*, showed no light response to this light-regulated syndrome [6].

We have already reported the stimulation of the binding of [α-^{32}P]ATP and [α-^{32}P]GTP to four proteins in the microsomal fraction by UV-A light irradiation [7]. However, these reactions were analyzed after 60 min of incubation at 25°C. These reactions may not be rapid reactions, but rather may be the consequence of several events occurring immediately after the UV-A light irradiation of the reaction mixture containing the microsomal fraction.

The bacterial two-component signal transducing systems have been studied extensively. The systems included sensors to external stimuli, such as nitrogen deprivation, phosphorous deprivation, chemotaxis and ionic stress and also signal transducers [8]. Sensor showed that phosphorylation of His-residue and the phosphoryl group was transferred to the Asp-residue of a transducer.

To analyze the molecular basis of light signal perception, the establishment of an in vitro system is essential and the results must be consistent with the results observed in several photobiological phenomena in the mutants. Herein, we describe the blue-light-induced phosphorylation of 15 kDa proteins (ps15) from [γ-^{32}P]ATP. Such a phenomenon could be detected by incubation of the reaction mixture containing the microsomal fraction for 5 s at 0 °C after blue light irradiation. These findings were consistent with several light responses observed in *wc-1* and *wc-2*.

2. Materials and methods

2.1. *Strains of* Neurospora crassa

Band (*bd*)a, strain used as wild type was our stock, which was obtained by crossing *bda* (FGSC#1859) into standard wild type, 74-OR23–1A (FGSC#987). Blind mutants, *wc-1* (FGSC#4395 and FGSC#3628) and *wc-2* (FGSC#4407) were provided by the Fungal Genetics Stock Center (University of Kansas Medical Center, USA).

2.2. *Preparation of a microsomal fraction from mycelia*

Conidia of *wc-1*, *wc-2* and *bd* strains of *Neurospora crassa* grown for 7 days on glycerol complete slant medium [9] were suspended in sterilized water. One ml of conidial suspension (1×10^6 cells) was inoculated into 100 ml of Fries minimal medium in 1 l Roux flasks [10]. Ten flasks were incubated at 25 °C for 34 h in complete darkness. From the mycelia the microsomal fraction was prepared as described in a previous report [7]. The amount of protein in the microsomal fraction was measured by a modification of the method of Lowry using SDS [11]. The protein concentration of the microsomal fraction was adjusted to 700 μg/ml.

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Abbreviations: [α-^{32}P]ATP, [α-^{32}P]adenosine 5'-triphosphate; [α-^{32}P]GTP, [α-^{32}P]guanosine 5'-triphosphate; [γ-^{32}P]ATP, [γ-^{32}P]adenosine 5'-triphosphate; *bd, band;* PAGE, polyacrylamide gel electrophoresis; PIPES, Piperazine-*N*,*N*'-bis(2-ethane sulfonic acid); PMSF, phenylmethylsulfonyl fluoride; *wc, white collar.*

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2.3. Phosphorylation of proteins by irradiation with a flash of blue light

Phosphorylation reactions were performed as follows, except where otherwise noted. A fivefold-concentrated reaction mixture (5 × RMγ) contained 0.1 M PIPES-NaOH (pH 6.3), 0.5 mM EDTA, 0.5 M NaCl, 7.5 mM $MgCl_2$, and 2.5 μCi/4 μl of [γ-^{32}P]ATP (3,000 Ci/mmol, NEG-002A, NEN Research Products, USA). The standard reaction mixture (total volume of 20 μl) contained 4 μl of 5 × RMγ, 2 μl of 1% Triton X-100, 2 μl of 10^{-4} M NAD and 2 μl of 10^{-5} M riboflavin. The reaction mixture was kept in ice water and the reaction was started by the addition of 10 μl of the microsomal fraction. At 24 s, the reaction mixture was irradiated with blue light at 450 nm by use of BPB-45 (Fuji Photo Film Co., Japan) at 6 μmol/m^2/s or at 420 nm by an interference filter (P10–420-S, Hoya Co., Japan) at 80 μmol/m^2/s for 1 s. At 5 s after a blue light flash, 20 μl of SDS-PAGE sample buffer [12] was added to terminate the reaction. To reduce the fluence rate, ND filters (Fuji Photo Film Co.) were used.

2.4. ATP-binding assay

[γ-^{32}P]ATP in 5 × RMγ was substituted by 2.5 μCi/4 μl of [α-^{32}P]ATP (3,000 Ci/mmol, NEG003H, NEN). The subsequent procedure was the same as those described in the phosphorylation of protein.

2.5. Time course of incubation

The reaction mixture (final volume of 240 μl) contained the same components as described in the phosphorylation of proteins in the microsomal fraction. The reaction mixture was kept in ice water. The reaction was started by the addition of 120 μl of the microsomal fraction to the reaction mixture and subsequently the mixture was divided into two parts. One part (120 μl) was kept in darkness (D) and the other (120 μl) was irradiated with blue light (420 nm, 80 μmol/m^2/s) at 24 s after mixing with the microsomal fraction for 1 s (L). After the flash, both mixtures were incubated for 5, 15, 30, 60 and 120 s in ice water and subsequently 20 μl of the reaction mixtures from D and L was sucked up from each of the pools and added to each of 20 μl of SDS-PAGE sample buffer.

2.6. In vitro complementation test of phosphorylation

The microsomal fractions independently prepared from mycelia of *wc-1* (FGSC#4395) and *wc-2* were mixed and homogenized by three strokes of a Teflon–glass homogenizer. Then the reactions were allowed to proceed as described above.

2.7. Electrophoresis and autoradiography

The samples were loaded onto a 5–20% SDS-gel [7]. Except otherwise noted, the proteins in the gel were blotted onto a nitrocellulose membrane (0.2 μm; Schleicher & Schüell, Germany) at 4 V/cm for 40 h [13]. The membrane was exposed to Kodak X-Omat AR film [7]. The amount of protein on the filter was estimated by staining with amido black [14].

2.8. Acid or alkaline treatment of phosphorylated proteins on a nylon membrane

After the phosphorylation reaction and SDS-PAGE as described above, the proteins on the gel were blotted onto a nylon membrane (NEF-976, NEN). The radioactivity of the protein band was measured by autoradiography on Kodak X-Omat AR film before the following treatments. Then the membrane was cut into four parts containing a pair of lanes and three of them were shaken either in 0.5 N HCl, 0.5 M Tris-HCl (pH8.8), or 0.5 N NaOH at 65 °C for 60 min. After these treatments, the radioactivity on the proteins was measured by autoradiography.

3. Results

Blue light stimulated phosphorylation of the proteins in the microsomal fraction from mycelia. Using [γ-^{32}P]ATP at 4×10^{-8} M, radioactivity was observed in the proteins with molecular weights of around 15 kDa (Fig. 1a). At least two radioactive protein bands were detected at 15 kDa and the amount of phosphorylation of these two proteins in response to blue light was equivalent. Hence we designated these proteins to be 15 kDa proteins (ps15). Another protein band that was rapidly phosphorylated was a 70 kDa protein (p70). The capacity of p70 to be phosphorylated changed depending on the preparation of the microsomal fraction. To examine their relationship, ps15 and p70 were shown on the same gel (Fig. 1 b).

To examine the background level of the ATP-binding reaction in ^{32}P-radioactivity on ps15 by [γ-^{32}P]ATP, we examined the binding of [γ-^{32}P]ATP by substituting [α-^{32}P]ATP for [γ-^{32}P]ATP in the reaction mixture. As Fig. 1b shows, the phosphorylation reaction by our procedure was confirmed to include only phosphorylation of proteins by [γ-^{32}P]ATP and not to include the binding of [γ-^{32}P]ATP, which was tested by the use of [α-^{32}P]ATP.

The effect of incubation time at 0°C on the phosphorylation of proteins after blue light flash was examined (Fig. 2). Only 5 s of incubation at 0 °C was required to detect the stimulation of the phosphorylation of ps15. The best conditions for incubation time to show a maximum difference between the dark control (D) and light irradiated sample (L) was 15–30 s. Longer than 30 s of incubation rather reduced the difference between these two samples.

The effect of the intensity of blue light on the stimulation of the phosphorylation of ps15 was examined. After 1 s of irradiation of the reaction mixture with 0, 6 or 60 μmol/m^2/s blue light (450 nm) at 0°C, the reaction kept at 0°C was stopped by the addition of SDS-sample buffer (data not shown). A light intensity between 6 and

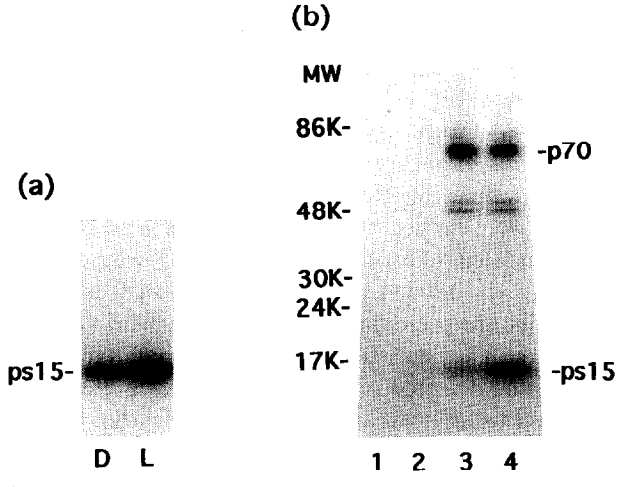

Fig. 1. (a) Effect of blue light on the phosphorylation of proteins in the microsomal fraction prepared from the mycelia of *bd*. D, dark control; L, blue light (420 nm) irradiated for 1 s at a fluence rate of 80 μmol/m^2/s. The reaction mixture contained [γ-^{32}P]ATP. (b) Effect of blue light on ATP-binding and phosphorylation of proteins in the microsomal fraction. Lanes 1 and 2, the reaction mixture contained [α-^{32}P]ATP and proteins were labeled under darkness (lane 1) and labeled with blue light irradiation (lane 2); lanes 3 and 4, the reaction mixture contained [γ-^{32}P]ATP and proteins were labeled under darkness (lane 3) and labeled with blue light irradiation (lane 4).

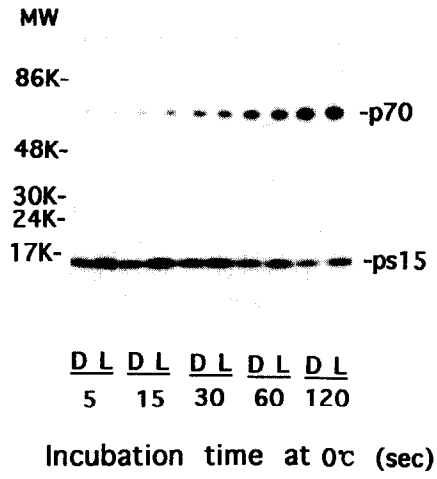

Fig. 2. Effect of incubation time after a blue light flash on the phosphorylation of proteins in the microsomal fraction prepared from the mycelia of *bd*. Numbers indicate the incubation time (s) at 0°C. D, dark control; L, blue light (420 nm) irradiated for 1 s at a fluence rate of 80 μmol/m^2/s.

60 μmol/m^2/s was sufficient to detect the stimulation of the phosphorylation of ps15. In the following experiment, blue light at a wavelength of 420 nm at 80 μmol/m^2/s or that at 450 nm at 6 μmol/m^2/s was used.

To examine whether the blue-light-stimulated phosphorylation of ps15 was dependent on the *wc* gene products, microsomal fractions from the mycelia of these mutants were prepared. Their mixture was tested for the capacity to show stimulated phosphorylation of ps15 by blue light (Fig. 3a). The blue light effect could not be detected with the microsomal fraction either from *wc-1* alone, or from *wc-2* alone. In the former case, the blue light effect was rather inhibitory for the phosphorylation of ps15. However, when the microsomal fractions from *wc-1* and *wc-2* were mixed at a ratio of 1 to 2, the blue-light-stimulated phosphorylation of ps15 was restored to the level comparable to that of *bd*. These complementation tests were carried out 13 times and in most cases the light-stimulated phosphorylation of ps15 was detected maximally at a mixing ratio of *wc-1* and *wc-2* of 1 to 2. The results are presented in a histogram with standard error bars. The statistical hypothesis that light effect could not be detected in *bd* was rejected at a 5% significance level using *t*-test and that in a 1/2 mixture of *wc-1*/*wc-2* was rejected at 10%. The level of significance in the latter case was a little high. In these experiments, the process to homogenize the microsomal fractions from *wc* mutants with a Teflon–glass homogenizer was essential. No restoration of the light effect could be detected by the mixture of these microsomal fractions without using the Teflon–glass homogenizer. These experimental difficulties might make the results fluctuating. Therefore, we further analyzed several allelic mutants of *wc-1* and *wc-2*, and detected a *wc-1* mutant (FGSC#3628) with no capacity to phosphorylate ps15 in the soluble and the microsomal fractions (data not shown).

To identify the phosphorylated amino acid residues of several radioactive protein bands including ps15 and p70, we tested the stability of the [^{32}P]phosphoryl group on these against acid and/or alkaline treatment (Fig. 4).

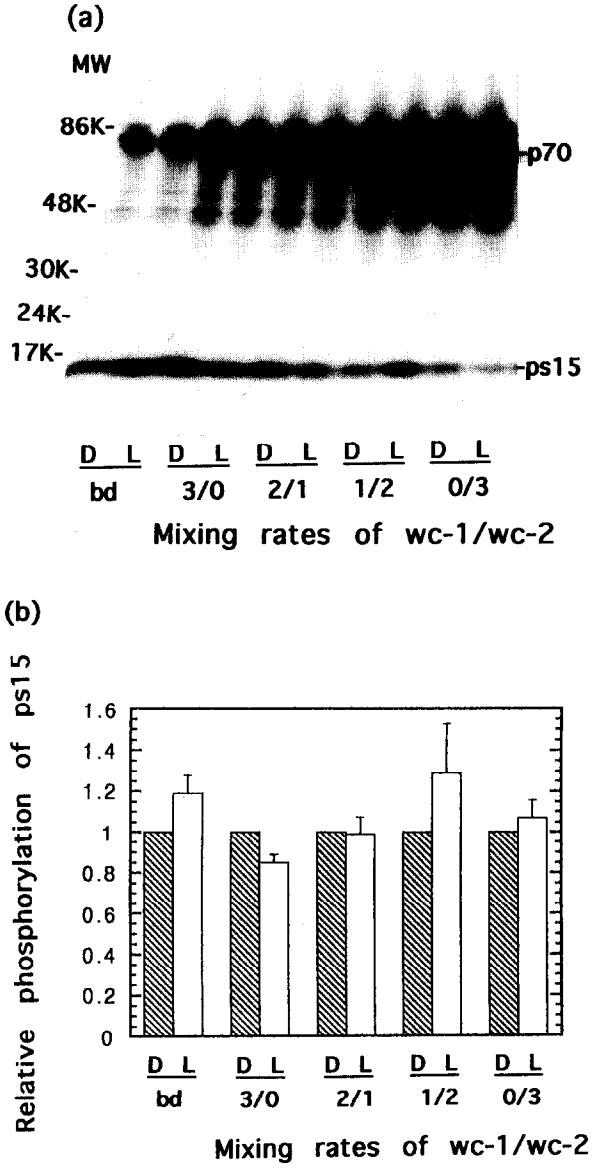

Fig. 3. Effect of blue light on the phosphorylation of proteins in the microsomal fractions prepared from the mycelia of *bd* and those of blind mutants, *wc-1* and *wc-2*. The microsomal fraction from *bd* and those from *wc-1* and *wc-2*, and their homogenates were tested for their capacity to stimulate the phosphorylation of ps15 by 1 s of irradiation with blue light (450 nm) at a fluence rate of 6 μmol/m^2/s at 0°C. D, dark control; L, blue light irradiated; bd, the microsomal fraction from *bd*; wc-1/wc-2, a mixed ratio of the microsomal fractions independently prepared from *wc-1* and *wc-2*. (a) An autoradiogram of one of the 13 experiments. (b) A histogram showing averages of 13 relative amounts of ^{32}P-phosphorylation of ps15 measured by a densitometer. Each of the phosphorylations of ps15 under light was normalized on the basis of the phosphorylation under darkness after subtraction of the background noise from each experimental value. Bars indicate standard errors.

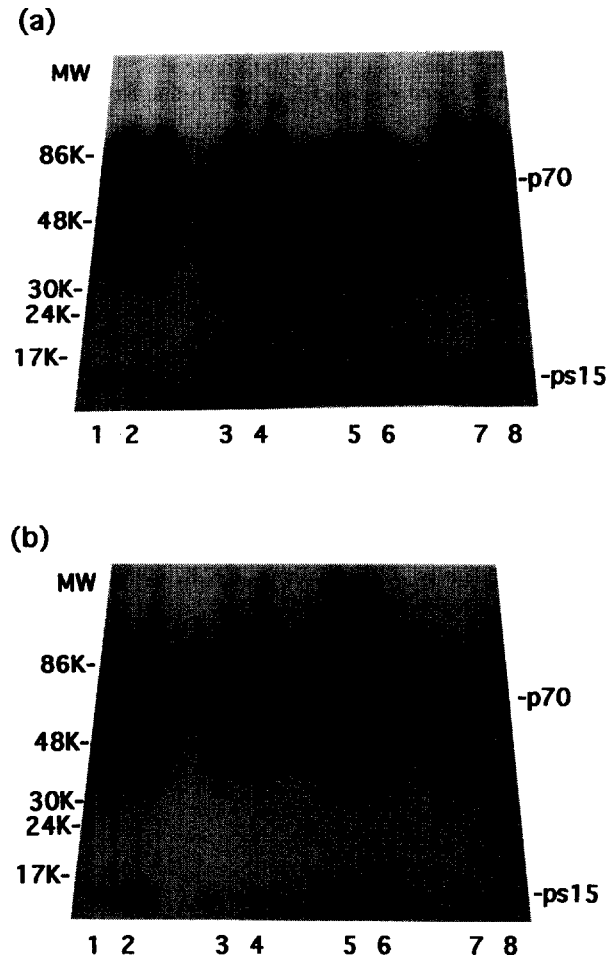

Fig. 4. Effect of the treatment with acid or alkaline condition on the phosphorylated radioactive protein bands. After the phosphorylation of the proteins in the microsomal fraction, the radioactive proteins were separated by SDS-PAGE, and the proteins were blotted onto a nylon membrane. The light source was set at 420 nm at a fluence rate of 80 μmol/m^2/s. (a) and (b) are the same membranes before each treatment (a) and after each treatment (b). Lanes 1 and 2, the proteins were phosphorylated under darkness (lane 1) and with blue light (lane 2) without any treatment; lanes 3 and 4, the proteins labeled under darkness (lane 3) and with blue light (lane 4) were treated with a 0.5 N HCl solution; lanes 5 and 6, those labeled under darkness (lane 5) and with blue light (lane 6) were treated with a 0.5 M Tris-HCl (pH 8.8) buffer solution; lanes 7 and 8, those labeled under darkness (lane 7) and with blue light (lane 8) were treated with a 0.5 N NaOH solution.

Several sets of phosphorylated microsomal fractions under darkness and a sample obtained after blue light irradiation were subjected to SDS-PAGE. The radioactive proteins were electroblotted to a nylon membrane. Fig. 4a and b shows the radioactive protein bands obtained after the autoradiography of the nylon membranes before and after treatment of the membrane, respectively. The results indicate that the phosphorylated amino acid residue in ps15 was unstable both under acidic and alkaline conditions, whereas, that in p70 was stable under alkaline conditions but was unstable under acidic conditions.

4. Discussion

The data presented in Figs. 1 and 2 show that we established the in vitro assay system to analyze the blue light effect on the phosphorylation of proteins in the microsomal fraction. Although the results of the in vitro complementation test were partially significant (Fig. 3), the results obtained in this assay system were parallel with the several blind phenotypes of *wc-1* and *wc-2*. These findings suggest that the protein complex consisting of the *wc-1* and *wc-2* products and/or ps15 and/or photoreceptor was rearranged by the mixing of the microsomal fractions and the light-specific activity was restored, and that ps15 are either the gene product of *wc-1* or *wc-2*, or activities of ps15 are controlled by the product of these genes.

The microsomal fraction of *wc-1* showed decreased rate of phosphorylation of the 15 kDa protein by blue light. We could not explain the underlying mechanism in the present state.

The present system of light stimulated phosphorylation was compared with other signal transmission systems. The phosphorylation of CheY, a bacterial chemotaxis protein, showed saturation of the phosphorylation after 10–60 s of incubation at 22 °C in a reconstructed in vitro system [15,16] and GTP-binding to transducin saturated after 10 min at 37 °C [17]. The data presented in Fig. 2 required 5–30 s at 0°C and therefore show that the light-stimulated phosphorylation of ps15 is rapid enough, suggesting that it is an early step in light-stimulated phenomena.

The radioactive protein band, ps15, included at least two proteins (Fig. 1 a). Blue light stimulated-phosphorylation of these two proteins showed equivalent behaviour. This suggested that ps15 is a member of the protein family or differently processed products from a single gene.

ps15 and p70 could also be detected in the soluble fraction, but ps15 in the soluble fraction showed only slight stimulation of phosphorylation by blue light (unpublished). Mixture of the microsomal fractions from *wc-1* and *wc-2* with a Teflon–glass homogenizer was an essential procedure to achieve in vitro complementation (Fig. 3a and b). These findings suggest that ps15 is functional at the peripheral part of the membrane and may form a protein complex, or ps15 may be located in the cytosol and can function by interacting with membrane components.

Acyl phosphates are known to be labile at both pH extremes and phosphoamidates are extremely acid-labile [18]. As shown in Fig. 4, the phosphorylated residues of ps15 and p70 may be acidic amino acids and alkaline amino acids, respectively. These findings suggested that p70 is phosphorylated at the His residue and that ps15 is phosphorylated at the Asp residue. The results were similar in the phosphorylated amino acid residues to

those of bacterial signal transducing systems, where sensor proteins are phosphorylated at the His residue and transducer proteins are phosphorylated at the Asp residue.

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