An *in vitro* system to analyze light signal transduction in *Neurospora crassa*

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Background

In *Neurospora crassa*, various processes in morphogenesis during the life cycle are controlled by light. The light-induced morphogenesis includes the following phenomena:

1) carotenoid accumulation in mycelia
2) development of aerial hyphae from mycelia leading to the formation of conidia
3) phase shift of the circadian rhythm of conidial formation
4) suppression of the circadian formation of conidia showing continuous conidiation under strong light
5) protoperithecial formation under nitrogen-limited conditions
6) bending of the perithecial beak toward the direction of light
7) development of the lateral side of the perithecial beak towards the light.

In *Neurospora*, WC-1 is a blue light photoreceptor that functions in a complex with WC-2 (the WCC complex). The WCC controls the expression of genes involved in the synthesis of carotenoids, including *al-1*, *al-2* and *al-3*, by binding to the upstream promoters of these genes.

To investigate the molecular mechanisms underlying these developmental phenomena, an *in vitro* system was devised to detect phosphorylated proteins in response to blue light treatment. Dark-grown mycelia from wild type and *bd* (*band*) strains were used to prepare a crude membrane fraction (Procedure 1). After 1 sec of illumination of the membrane fraction with blue light in the presence of (γ-^{32}P) ATP in ice-water, the reaction was stopped by adding SDS sample buffer. The proteins were separated by SDS-PAGE, and a 15kDa and 70kDa phosphorylated protein were detected by autoradiography (Oda and Hasunuma, 1994). The 15kDa protein was purified and identified as nucleoside diphosphate kinase, and hence designated NDK-1 (Ogura et al. 1999). A mutant with no phosphorylation activity was isolated and identified as *ndk-1*^{P72H}. The *ndk-1*^{P72H} strain showed a lack of light-induced perithecial polarity (#7 above) (Oda and Hasunuma 1997, Ogura et al. 2001).

The *ndk-1* gene was knocked out using RIP (Repeat induced point mutation). The *ndk-1*$^{RIP-1}$ and *ndk-1*$^{RIP-2}$ mutants exhibited multiple morphological defects including defects in processes 1, 2 and 6 listed above. The amount of carotenoid accumulated under illumination in the mutants was less than half that in the wild type (Lee et al. 2005). The superoxide dismutase (SOD) knockout mutant, *sod-1,* showed enhanced accumulation of carotenoids under illumination, suggesting that for the accumulation of carotenoid the presence of reactive oxygen species (ROS) may be required (Yoshida and Hasunuma 2004). The presence of oxygen gas in place of air stimulated the accumulation of not only carotenoids, but also mRNA for the carotenoid synthesis-related gene, *al-1,* under illumination. These data suggested that ROS may enhance the process of light signal transduction to gene expression (Iigusa et al. 2005). Furthermore, the molecular association of AtNDK-1 with three species of catalases, AtCat-1, AtCat-2 and AtCat-3, in *Arabidopsis* suggested that one of the functional forms of NDK may be NDK in association with catalases (Fukamatsu et al. 2003). In *N. crassa,* catalase-1 (CAT-1) has the capacity to bind singlet oxygen and CAT-1c bound to two molecules of singlet oxygen appears in the mycelia of wild type strains after illumination. Longer illumination of mycelia stimulated the appearance of CAT-1a with no binding of singlet oxygen. However, in the *ndk-1*^{P72H} mutant, CAT-1a did not appear under illumination (Yoshida et al. 2006). To analyze the molecular mechanisms behind the light-stimulated phenomena described above, the establishment of an *in vitro* system was required.

Procedure

To obtain reproducible results for the light-stimulated enhancement of NDK-1 phosphorylation, the preparation of the membrane fraction need to be carried out carefully. Stock cultures of wild type and the *band* strains frozen at –20 ˚C were inoculated onto slants of solid glycerol complete media. The conidia formed after incubation at 25 ˚C for 7 days were re-inoculated onto slants and incubated for 5 days. The conidia were dissolved in 10 ml of sterilized water, filtered through a double layer of cheese cloth and the cell number was adjusted to 10^6 cells /ml. One milliliter of conidial suspension was inoculated into 100 ml of Fries minimal liquid medium containing 1.5 g/L of sucrose in a 1 L Roux flask in darkness. All procedures after the conidial inoculation were conducted in darkness under a red safe light or using an

infrared safe light visualized with a Noctovision System (NVR2015; Nihon Electric Co., Ltd.). After incubation of the standing culture at 25 ˚C for 34 h, the mycelia were harvested on nylon mesh on a Buchner's funnel, and then washed with 1L of deionized water. The mycelia were wrapped in aluminum foil and immersed in liquid nitrogen. The mycelial pad was kept at –80 ˚C to be used within 1 week.

All subsequent procedures were performed at 0~4˚C. To 2 g of mycelia (wet weight) in a mortar, 10 ml of ice-cold homogenization buffer (Table 1) was added, and the mycelia in the mortar on ice were well ground by thirty sets (10 strokes each) of grinding with a pestle. The macerated mycelia were homogenized three times with a Potter's homogenizer. The homogenate was filtered through nylon mesh with a pore size of 30 µm or filtered through nylon cloth on a funnel, and the homogenate was collected in a centrifuge tube. The filtrate was centrifuged at 9,000 x g for 10 min at 4 ˚C. The resulting supernatant was further centrifuged at 105,000 x g for 30 min at 4 ˚C. The supernatant is the **soluble fraction**; a 0.5 ml aliquot of which was delivered to a plastic tube wrapped with aluminum foil. The supernatant can be stored at –80 ˚C for at least 1 week with a good response to light. To the precipitate, 10 ml of extraction buffer was added. The precipitate was well dissolved by stirring with a glass rod, and then homogenized gently by three strokes of a Potter's homogenizer. A 0.3 ml aliquot was transferred to a plastic tube, wrapped with aluminum foil, and stored at –80 ˚C until usage. This is the **crude membrane fraction**. The crude membrane fraction was kept at –80 ˚C for 2 weeks with good responses in terms of light-induced phosphorylation of NDK-1. The procedures used to prepare the crude membrane fractions and the soluble fractions are summarized in Fig 1.

Table 2 describes the components of the reaction mixture used for the light-stimulated phosphorylation of the 15kDa protein (NDK-1). The protein concentrations of both fractions were measured by the modified method of Lowry using SDS (Markwell et al 1981) or by the method of Bradford and adjusted to 700 µg/ml.

To 10 µl of reaction mixture in ice water, 10 µl of the crude membrane fraction was added. At 24 sec after mixing by pumping, the reaction mixture was illuminated with blue light at 450 nm using a BPB-45 (Fuji Photo Film Co., Japan) at 6 µmoles/m^2/sec or at 420 nm by an interference filter (P10-420-S, Hoya Co., Japan) at 80 µmoles/m^2/sec for 1 sec. At 5 sec after the blue light flash, 20 µl of SDS-PAGE sample buffer was added to stop the reaction. To reduce the fluence rate, ND filters (Fuji Photo Film Co.)

were used.

The samples were loaded onto a 5-20 % SDS-gel or 12.5 % SDS-gel. The proteins in the gel were blotted onto a nitrocellulose membrane (0.2 μm; Schleicher & Schuell, Germany) at 4 V/cm for 40 h, or after electrophoresis the gels were stained with Coomasie Brilliant Blue and destained. After destaining, the gels were dried on a Whatman No3 MM filter. The radioactivity on the membrane filter or in the gel dried on filter paper was visualized by exposure to Kodak X-Omat AR film.

As an alternative method of detecting the effect of light, mycelial shaking culture in Vogel's medium was illuminated by white fluorescent light for 10 min, and a shaking culture grown in darkness was used as a control. After collection of the mycelial pad by passage through filter paper on Buchner's funnel in darkness under a red safe light, mycelia were wrapped in aluminum foil, immersed in liquid N_2, and stored at –80 ˚C. The membrane fraction and the soluble fraction prepared by the above method were used for the direct phosphorylation experiment under a red safe light. With this method, we could achieve the enhanced phosphorylation of NDK-1 through the illumination of mycelial cultures (unpublished results by Bumkyu Lee, Yusuke Yoshida and Kohji Hasunuma).

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Table 1

Constituents of the extraction buffer;
25 mM PIPES-NaOH, pH 6.3
0.25 mM EDTA
0.1 M NaCl
1 mM $MgCl_2$
0.25 M sucrose
0.01 mM pepstatin

0.01 mM leupeptin

0.5 mM phenylmethylsulfonyl fluoride

Table 2

Five-fold-concentrated reaction mixture (4 µl, final volume of RM was 20 µl);

0.1M PIPES-NaOH, pH 6.3

0.5 mM EDTA

0.5 M NaCl

7.5 mM $MgCl_2$

92.5 KBq [γ-^{32}P] ATP; 4 µl of 110TBq/mmole (NEG-002A, NEN Research Products)

0.1% Triton X-100

10 µM NADH

1 µM riboflavin

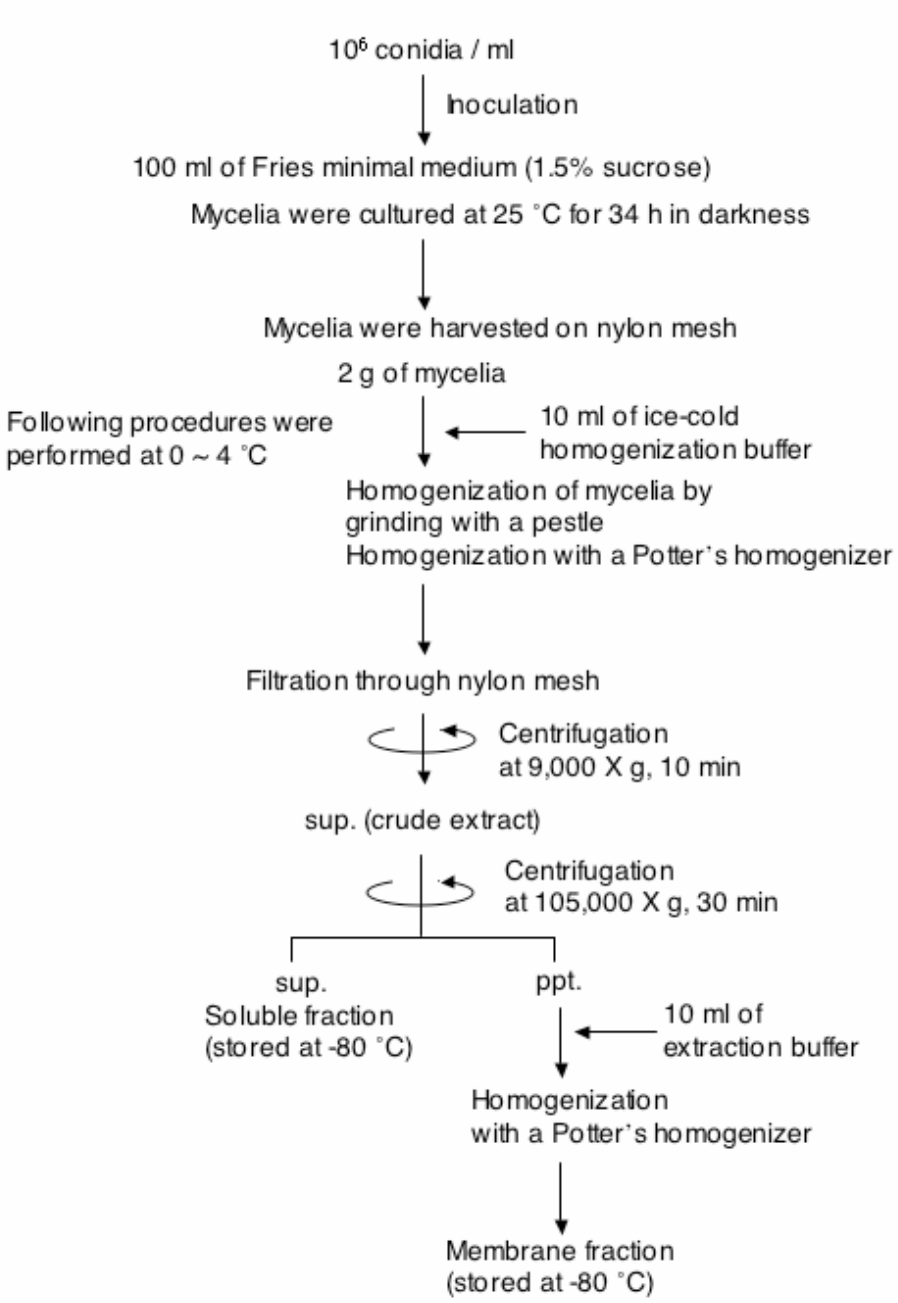

Fig 1. Preparation of the crude membrane and soluble fractions