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Interaction of nucleoside diphosphate kinase and catalases for stress and light responses in *Neurospora crassa*

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Abstract ***N*ucleoside *di*phosphate *k*inase (NDK) is an ubiquitous enzyme with the function of a signal transducer. In *Neurospora crassa*, an *ndk-1*^{P72H} mutant carrying the point mutation Pro72His was isolated. We found that *ndk-1*^{P72H} showed hypersensitivity to oxidative and heat stress and a decrease in the levels of catalase (Cat)-1 and -3 induced by oxidative, heat stress and illumination compared with wild type (Wt). We found, by conducting a yeast two-hybrid assay, that Cat-1 interacted with NDK-1. NDK-1 was suggested to control Cat-1 and Cat-3 at the post-transcriptional level in response to heat, oxidative stress and light.**

Keywords: Nucleoside diphosphate kinase; Oxidative stress; Heat stress; Light reaction; *Neurospora*

1. Introduction

*N*ucleoside *di*phosphate *k*inase (NDK; EC 2.7.4.6), catalyzing the transfer of the terminal phosphate from NTPs to NDPs, is an ubiquitous enzyme in prokaryotes and eukaryotes [1]. One critical role of NDK is to maintain a pool of NTPs regulated by the K_m of NDK for each NTP. However, evidence from genetic analyses indicates that this enzyme is multifunctional, regulating various biological processes such as tumor metastasis, cellular proliferation, differentiation and tumor promotion in mammals and photo-signaling in plants [2–4]. In molecular analyses, NDKs have been demonstrated to interact with various proteins constituting signaling pathways such as GTP-binding protein [5] and phosphatase [6]. These observations revealed that NDK has the capacity to regulate a wide range of signal transduction pathways.

In *Neurospora crassa*, we isolated NDK-1 as a candidate light-signal transducer and a mutant, *ndk-1*^{P72H}, with a point mutation causing the amino acid proline to be replaced by histidine at position 72 [7,8]. The P72H substitution in NDK-1 was responsible for the decrease in two enzymatic activities, autophosphorylation and protein kinase activity [8]. *ndk-1*^{P72H} was defective in the light-induced response of perithecial polarity. NDK-1 was suggested to play a crucial role in an unidentified pathway including NDK-1 in the response of perithecia to light. However, the molecular mechanisms involved remain unknown. In our laboratory, catalase was isolated as a protein interacting with *Arabidopsis thaliana* NDK1 (AtNDK1) using the yeast two-hybrid system, and bleaching in the leaves was suppressed in *AtNDK1*-overexpressing transgenic plants after methyl viologen (MV) treatment [9]. To obtain a better understanding of ROS- and light-signal transduction via NDK-1, we investigated the interrelationship of NDK-1 and oxidative stress or light. *ndk-1*^{P72H} showed hypersensitivity on media containing reagents releasing reactive oxygen species or under heat treatment. Catalase levels inducible by oxidative stress and illumination showed a decrease in *ndk-1*^{P72H}, compared to the Wt.

2. Materials and methods

2.1. Strains of N. crassa

The *N. crassa*, wild type (Wt); 74-OR23-1A (FGSC #987) and a Cu, Zn superoxide dismutase (SOD-1) null mutant, sod-1 (FGSC #7437; sod-1 mutant obtained after crossing twice with the Wt) were obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, KS). ndk-1^{P72H} (FGSC #8416) in which proline 72 of ndk-1 is changed to histidine and a complementary strain (PCN1) transformed with extra ndk-1 cDNA were isolated previously [8]. An ndk-1^{P72H} cDNA-transformed strain (DN; ndk-1^{+}/ndk-1^{P72H}) was obtained as follows. Wt; 74-OR23-1A was transformed with a plasmid containing the plasmid for ectopic expression of ndk-1^{P72H} cDNA (pTHNDK-1^{P72H}).

2.2. Assays of sensitivity to heat and oxidative stress

Conidia (200 conidia/plate) were germinated for 2 h in Vogel's minimal medium before being plated onto sorbose medium containing 20, 50 and 100 μM MV (Sigma) or 0.5, 1 and 1.5 mM H_2O_2 (Mistubishi Gas Chemical Co.). After the plates were incubated for 3 days at 25 °C, rates of survival among colonies were determined. In heat treatment, conidia (100 conidia/tube) were inoculated onto glycerol complete solid medium in race tubes. After the tubes were incubated at 30, 37 and 42 °C for 3 days, the growth of mycelia from the inoculation point was measured at 24-h intervals.

2.3. Assays for catalase activity

Conidia (10^5 conidia/ml) were inoculated into 200 ml of Vogel's minimal medium with 2% sucrose in 500-ml Erlenmeyer flasks. After cultures were shaken (200 rpm) for 24 h at 25 °C, cultures were refreshed with 200 ml of Vogel's minimal medium. Mycelia were treated with 1 mM MV or at 37 and 42 °C for 3 h, and then the mycelia were harvested. In illumination experiments, mycelia cultured in darkness were harvested, and mycelial mats were exposed to light (20 μE m^{-2} s^{-1}) for 2 h. The mycelia were powdered with a pestle and mortar in liquid nitrogen. Catalase activity was determined by in-gel activity assay [10]. The relative values for catalase were calculated from the strength of catalase after electrophoresis using the NIH image program.

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2.4. Yeast two-hybrid assay

The Yeast two-hybrid assay was performed as described (Match Maker Two-hybrid system 3; Clontech, CA, USA) Oligo-DNA primers for *ndk-1*, *ndk-1TH3*-F: 5'-gaattcttgtccaaccaggagcagac-3' and *ndk-1TH3*-R: 5'-gtcgacttactcgaagatccaggcag-3', were designed to introduce into the *Eco*RI/*sal*I site in pGBKT7 (pGBKT7-NDK-1). For construction of the *ndk-1* N-terminal, the pGBKT7-NDK-1 fragment cut by *Nco*I was ligated into the *Nco*I site in pGADT7 (pGBKT7-NDK-1N). For the C-terminal, pGBKT7-NDK-1 cut by *Nco*I was self-ligated (pGBKT7-NDK-1C). Oligo-DNA primers for *cat-1* and *-3*, *Cat-1TH3*-F: 5'-gaattcttgtccaacatcatcagc-3' plus *Cat-1TH3*-R: 5'-ctcgagttagtacgcaatcatgga-3', and *Cat-3TH3*-F: 5'-gaattcttgcgtgtcaacgctcct-3' plus *Cat-3TH3*-R: atcgatttactcctcatcatcgcc-3', were designed to introduce into the *Eco*RI or *Eco*RI/*Cla*I site in pGADT7 (pGADT7-CAT1 and pGADT7-CAT3). AH109 cells were transformed with these vectors and grown on SD-Trp, -Leu (SD-WL) and SD-Trp, -Leu, -His, -Ade medium (SD-WLHA).

2.5. Immunoprecipitation assay

Affinity purified NDK-1 antibody was incubated with 20 μl of protein A Sepharose (Amersham Pharmacia Biotech) for 3 h at 4 °C in 1 ml of IP buffer (50 mM potassium phosphate buffer, pH 7.0, and 10 mM NaCl). The soluble fraction from mycelia was incubated with the antibody-protein A Separose for 6 h at 4 °C and centrifuged. The pellet was washed five times with 1 ml of IP buffer. The final pellet was loaded onto the native PAGE gel, and catalase activity was determined by an in-gel assay as described above. NDK-1 was detected by Western blot analysis [11].

In all cases, averages for three independent experiments with standard error bars are presented.

3. Results

3.1. The sensitivity in ndk-1^{P72H} to oxidative and heat stress

First, we examined the influence of oxidative stress (MV and H_2O_2) on the viability of conidia. After germination, the rate of survival was determined on each medium. *ndk-1^{P72H}* was hypersensitive to both of these treatments (Fig. 1A and B). The complementary strain PCN-1 did not show a complete reversal of phenotype (Fig. 1C). The Wt strain transformed with *ndk-1^{P72H}*cDNA (DN; *ndk-1^{+}/ndk-1^{P72H}*) showed a decrease in survival compared with the untransformed Wt on the medium containing 100 μM MV (Fig. 1C, DN). Because the phenotype sensitive to MV was dominant in the heterozygote, *ndk-1^{P72H}* was revealed to be a dominant-negative mutant. These results indicated that the phenotype in these strains depended greatly on the influence of NDK-1^{P72H} protein, reported to have a dominant negative effect.

Next, we examined the effect of temperature. *ndk-1^{P72H}* was hypersensitive to heat stress at 37 and 42 °C in terms of mycelial growth (Fig. 2). The complementary strain completely recovered, being similar in phenotype to the Wt (Fig. 2). These results indicate that the phenotype of *ndk-1^{P72H}* is caused by the P72H substitution in NDK-1. The *sod-1* mutant was used as a strain sensitive (positive control) to oxidative and heat stress.

3.2. The level of stress-induced cat-3 in ndk-1^{P72H} in the in-gel activity assay

It was reported that the activity of Cat-3 and the expression of *cat-3* were induced by H_2O_2, MV and heat shock [12]. In the present study, the level of catalases in the Wt increased 5-fold in response to MV and 8-fold in response to heat treatment, as determined using the in-gel activity assay. However, the level increased about 50% in *ndk-1^{P72H}* compared with the Wt (Fig. 3). *N. crassa* has three types of catalases, Cat-1, -2 and -3 [12]. Because the positions of Cat-1c (oxidized catalase) and Cat-3 were the same in native-PAGE, we separated these catalases by native isoelectrofocusing. The catalase whose expression was enhanced by stress in *ndk-1^{P72H}* was Cat-3 (data not shown).

3.3. The level of cat-1a in the ndk-1^{P72H} in the in-gel activity assay

The activity of Cat-1 and the expression of *cat-1* have been reported to be induced by illumination [13]. In *N. crassa*, Cat-1 shows different oxidative patterns (Cat-1a, b, c, d and e) in

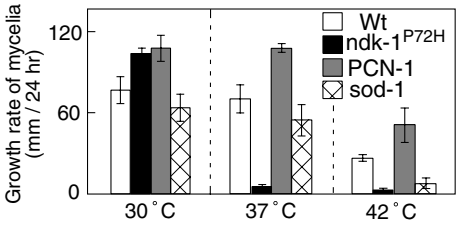

Fig. 2. Effects of heat treatment on the growth rate of mycelia in the *ndk-1^{P72H}* mutant. Growth of mycelia in the Wt, *ndk-1^{P72H}*, *sod-1* and PCN-1 at 30, 37 and 42 °C in race tubes. The growth rate of mycelia in the race tube was examined by measuring the mycelial growth extension per 24 h after inoculation.

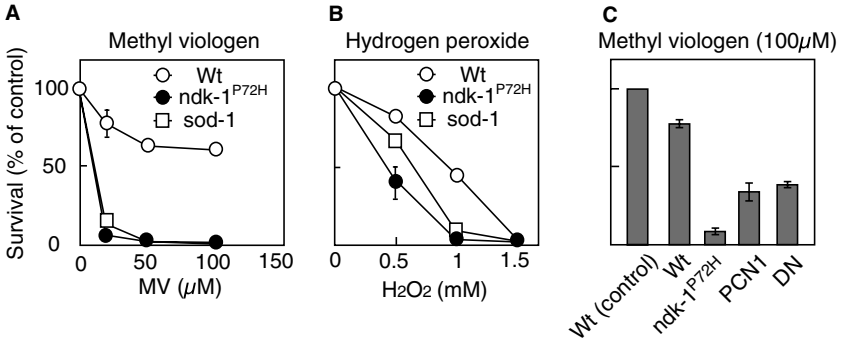

Fig. 1. Effects of oxidative stress on the *ndk-1^{P72H}* mutant. (A) Sensitivity of the Wt and *ndk-1^{P72H}* to MV. Germinated conidia were plated on sorbose-agar media containing 20, 50 and 100 μM MV. The *sod-1* mutant was used as a positive control. (B) Sensitivity of the Wt and *ndk-1^{P72H}* to H_2O_2. Mycelia were cultured on sorbose agar media containing 0.5, 1 and 1.5 mM H_2O_2. (C) Sensitivity of the Wt, *ndk-1^{P72H}*, PCN-1 and DN (Wt lines with *ndk-1^{P72H}* cDNA) on media containing 100 μM MV.