A Point Mutation in Nucleoside Diphosphate Kinase Results in a Deficient Light Response for Perithecial Polarity in Neurospora crassa*

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In Neurospora crassa, the phosphorylation of nucleoside diphosphate kinase (NDK)-1 is rapidly enhanced after blue light irradiation. We have investigated the function of NDK-1 in the blue light signal transduction pathway. A mutant called psp (phosphorylation of small protein) shows undetectable phosphorylation of NDK-1 and is defective in light-responsive regulation of perithecial polarity. Sequencing analysis of ndk-1 cDNA by reverse transcription-polymerase chain reaction revealed that proline 72 of ndk-1 was replaced with histidine in psp. The mutation ndk-1P72H resulted in accumulation of normal levels of mRNA and of about 25% of NDK-1P72H protein compared with that of wild type as determined by Western blot analysis. The ectopic expression of cDNA and introduction of genomic DNA of wild type ndk-1 in psp (ndk-1P72H) suppressed the reduction in accumulation and phosphorylation of NDK-1 and the light-insensitive phenotype. These findings demonstrated that the phenotype of psp was caused by the ndk-1P72H mutation. Biochemical analysis using recombinant NDK-1 and NDK-1P72H indicated that the P72H substitution in NDK-1 was responsible for the decrease in phototransfer activities, 5% of autophosphorylation activity, and 2% of V_max for protein kinase activity phosphorylating myelin basic protein, compared with those of wild type NDK-1, respectively.

Development and morphogenesis during the life cycles of fungi, as well as plants, are greatly affected by environmental stimuli such as light (1–3). Recently, the nature of the photoceptors in fungi and plants has been investigated. In Arabidopsis thaliana, the photoreceptors (4, 5) are phytochromes (6), cryptochromes (7, 8), phototropin (7, 9), and zeaxanthin (7). However, the light signal transduction downstream of the photoreceptors has remained to be elucidated unambiguously (10).

The filamentous fungus Neurospora crassa shows several biological responses to blue light, such as a mobilization of ions in the mycelia within minutes after illumination (11, 12), initiation of carotenoid synthesis in the mycelium (13), promotion of conidial and protoperithecial development (14, 15), positive phototropism of perithecial beaks (16), regulation of perithecial polarity (the position of the beak formed on the peritheium) (17), and resetting of the circadian rhythm of conidiation (18). The N. crassa mutants white collar-1 (wc-1) and white collar-2 (wc-2) lack all of these blue light responses (19, 20). Isolation and characterization of wc-1 and wc-2 genes revealed that both of them have a zinc finger DNA-binding motif, a PAS PERIOD, ARNT, and SIM dimerization domain, and a glutamine-rich transcriptional activation domain (21, 22). WC proteins are proposed to form heterodimers through their PAS domain (23). Furthermore, WC-1 has the LOV (light, oxygen, or voltage) domain found in blue light receptor phototropin (7). These results suggest that WC proteins perceive blue light and regulate the transcription of the blue light-responsive genes. However, it is difficult to explain ion mobilization by WC proteins (12). Furthermore, phosphorylation of WC-1 is increased after blue light irradiation (24), which appears to be an important part of this pathway. However, except for protein kinase C, no candidate for a protein kinase mediating the Neurospora blue light signaling has been suggested (25).

We developed a system to analyze the light-responsive phosphorylation of proteins in vitro utilizing the mycelial membrane fraction. The phosphorylation of a 15-kDa protein increased specifically after blue light irradiation in the wild type, but not in either wc-1 or wc-2. However, mixing the membrane fractions from wc-1 and wc-2 restored the increase in the phosphorylation. These results suggested that the 15-kDa protein was involved in blue light signal transduction downstream of WC proteins (26). This protein was purified and identified as nucleoside diphosphate kinase (NDK) (27). The characteristics of an N. crassa mutant known as psp suggested that it is a mutant allele of ndk-1. Phosphorylation of NDK-1 is undetectable in psp (see Fig. 1A), and the psp mutant is known to be defective in light-responsive regulation of perithecial polarity (17), suggesting that phosphorylation of NDK-1 is critical for the photoreponse.

NDK is a ubiquitous enzyme that catalyzes the transfer of the γ-phosphate group of nucleoside 5′-triphosphate to nucleoside 5′-diphosphate to form nucleoside 5′-triphosphate (28). Recent studies suggest that NDK is involved in various signal transduction pathways such as suppression of tumor metastasis (29), modulation of muscarinic K+ channels (30), transcriptional activation of proto-oncogenes (31), development of the wing disc cells in Drosophila melanogaster (32), and sexual development in Schizosaccharomyces pombe (33). In plants, the phosphorylation of NDK in pea, PNDK1, was stimulated by red light, and the stimulation was reversed by subsequent irradia-
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The present study, we have investigated the roles of NDK-1 in the light signal transduction pathway through the γ-phosphotransfer activity from nucleoside 5′-triphosphate to nucleoside 5′-diphosphate and the phosphotransfer activity to other proteins.

**EXPERIMENTAL PROCEDURES**

**N. crassa Strains and Growth Conditions**—The *N. crassa* wild type strains 74OR23–1A (FGSC987) and 74OR8–1a (FGSC988) were obtained from the Fungal Genetics Stock Center (Kansas City, KS). The mutants *psp A* and *psp a* (renamed *ndk-1P72H* in this paper) were isolated previously (17). Culture was carried out in Vogel’s minimal medium with 2% sucrose at 25 °C. Protoperithecia of 74OR8–1a, *ndk-1P72H a*, and the transformants were developed in darkness at 25 °C on synthetic crossing medium for 5 days and fertilized with the 74OR23–1A conidia. After 3 days in darkness, they were placed in a box with a slit 4-cm wide and illuminated from the side under a 12-h light/12-h dark regime for 2 weeks. Perithecia were observed and photographed through dissecting microscopy.

**Northern Blot Analysis**— *N. crassa* wild type 74OR23–1A and *ndk-1P72H* were grown for 2 days. The mycelia were harvested by filtration and powdered with a pestle and mortar in liquid nitrogen. Total RNA was prepared by the small scale method of RNA extraction (41). 5 μg of total RNA was separated by glyoxal gel electrophoresis and transferred to a piece of Gene Screen Plus membrane (PerkinElmer Life Science). Hybridization with ^32^P-labeled *ndk-1* probe was carried out overnight at 42 °C in hybridization buffer containing 50% formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl. Radioisotopic signals were visualized by autoradiography using X-Omat AR film (Eastman Kodak Co.).

**Trichothecene Production**— *N. crassa* strain CHC 1077 was grown in a 10-μl reaction mixture (50 mM KCl, 0.15 mM MgCl₂, 0.05 mM ATP, 0.1 mM NAD, 0.1 mM GTP, 0.05 mM MgCl₂, 0.1 mM dATP, 10 mM dGTP, 10 mM dCTP, and 0.5% glucose) for 4 h at 30 °C. The reaction mixture was extracted with toluene, and the extracts were assayed for trichothecene production by thin layer chromatography.

**Quantitative Analysis**— The amounts of trichothecenes in the extracts were determined by high pressure liquid chromatography (HPLC) using a CAPCELL PAK C18 column (Shiseido) and a Waters 510 HPLC pump with a Waters 486 UV detector.
structed a plasmid pTREB, which carried bar for selection and the promoter and terminator regions of Aspergillus nidulans trpC for ectopic expression (44). This plasmid has a multiconティング site between the trpC promoter and terminator for Smal, EcoRI, EcoRV, and ClaI. The ndk-1 fragment digested with EcoRI and ClaI, ~600 bp, was ligated into pTREB to construct pTRNDK-1. The transformants were selected on sorbose-BASTA medium (Vogel’s salt mixture without NH4NO3, 0.5% proline, 2% sucrose, 2% sorbose, 1.5% agar, 0.005% BASTA (for basal medium) and 1% sorbitol (for top medium)).

**Expression and Purification of Recombinant Proteins**—Two oligoDNA primers, ESTndk1–5t (5'-AGGATCCACAGAAAAGGACCA-3'), which was designed to have a BamHI site at the 5’ terminus and to delete the start codon (ATG) and Ndk-1ER (5'-AGGATCCACAGAAAAGGACCA-3'), were used for PCR amplification of ndk-1 and ndk-1P72H. PCR products were subcloned into the EcoRV site of pBluescriptISK . ndk-1 and ndk-1P72H DNA were cut out by BamHI and Smal double digestion and were ligated into pEEST-1 (Stratagene), which contained a glutathione S-transferase (GST) gene in the 5’-region of a multicloning site (pESTNDK-1 and pEPTSTP72H). S. pombe SP-Q01 (Stratagene) was transformed with pESTNDK-1 or pEPTSTP72H. Transformants were cultured in Edinburgh minimal medium (45) for 48 h at 30 °C. Cells were centrifuged, and the pellet was resuspended in an equal volume of phosphate-buffered saline (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, and 2.7 mM KCl). An equal volume of glass beads was added, and the cells were crushed by 20 cycles of a set of 1 min of vortexing and 1 min of chilling on ice. After centrifugation, the supernatant was mixed with GST-Sepharose (Amersham Pharmacia Biotech) and stood for 30 min at room temperature. The resin was washed 10 times with PBS. GST fusion protein was eluted with an equal volume of elution buffer containing 50 mM Tris-HCl, pH 9.6, and 10 mM reduced glutathione. The protein concentration was calculated from the strength of Coomassie Brilliant Blue R-250 staining after electrophoresis by the NIH image program.

**Analysis of Enzymatic Activities**—The kinetic constants for NDV activity were determined by a coupled pyruvate kinase-lactate dehydrogenase assay essentially according to Agarwal et al. (46) at 25 °C. In this assay, 10 ng of purified recombinant protein was used. The concentrations of dTDP used were 0.1, 0.2, 0.3, 0.4, and 0.5 mM. The kinetic constants were calculated from the double reciprocal plots. The activity of GST-NDK-1 and GST-NDK-1P72H to phosphorylate myelin basic protein (MBP, lot number HBDOB1; Life Technologies, Inc.) was analyzed essentially according to a previous study (27). The mixture, containing 10 ng of GST-NDK-1 or GST-NDK-1P72H, MBP (1.35, 2.7, 5.4, 8.1, or 10.8 μM), 50 mM Heps, pH 7.5, 1 mM dithiothreitol, and 10 mM MgCl2, was incubated for 1 min at 25 °C after addition of 0.2 MBq of γ-32P]ATP (110 MBq/mmol) at a final concentration of 0.04 μCi. After the electrophoresis, a part of the gel containing MBP was cut out. The radioisotopic activity of this piece of gel was measured by the Cerenkov ray method with a liquid scintillation counter (Alola). The autophosphorylation activity of GST-NDK-1 or GST-NDK-1P72H was assayed by use of 10 ng of the protein in the above reaction mixture. The reaction (20 μl) was started by adding 0.1 nM γ-32P]ATP on ice, and after 10 s the reaction was stopped by adding SDS sample buffer. The following procedure was the same as described above.

**RESULTS**

**Proline 72 of NDK-1 Is Replaced with Histidine in psp**—No phosphorylation of NDK-1 was detected in the crude extracts from the mutant psp (ndk-1P72H) (see Fig. 1A and Ref. 17). This result suggested that the sequence of ndk-1 was changed to reduce the accumulation of transcript or to reduce the protein activity. We first examined the levels of ndk-1 mRNA and NDK-1 protein in psp (ndk-1P72H). Northern blot analysis showed no significant difference in the accumulation of ndk-1 mRNA between psp (ndk-1P72H) and wild type (Fig. 1B). In contrast, Western blot analysis using anti-NDK-1 antiserum indicated that about 25% of NDK-1 protein was accumulated in psp (ndk-1P72H) compared with that in wild type (Fig. 1C) from three independent experiments. The amounts of NDK-1 proteins from wild type and mutant were estimated by the densitometric assay of the Western blot. From these results, we speculated that an amino acid substitution in NDK-1 itself affected its stability or activity. To examine this hypothesis, the nucleotide sequence of ndk-1 cDNA, which was amplified from psp (ndk-1P72H) by RT-PCR, was determined. Nucleotide sequences in the 5'-3' untranslated regions of ndk-1 cDNA were used as primers for RT-PCR. A fragment whose length corresponded to the predicted ndk-1 cDNA, ~580 bp, was amplified (Fig. 1D). Ten clones were isolated from each strain, wild type and psp (ndk-1P72H) A and a. In the psp (ndk-1P72H), there were three nucleotide replacements in ndk-1. The first was in the 3'-untranslated region, the second was silent, and the third was a replacement of cytosine with adenosine, causing the replacement of proline 72 with histidine, whereas the sequence of cDNA from wild type was identical to that of ndk-1.

A 360-bp fragment was also amplified by RT-PCR (Fig. 1D). The nucleotide sequence of this fragment was identical to that of ndk-1 except that the fragment lacked 219 nucleotides, from the
28th to 246th base from the putative translation start site (data not shown). The deduced amino acid sequence of this fragment was identical to that of ndk-1 except that it lacked 73 amino acids in the N-terminal region. This transcript was designated as Tnk-1 (Truncated ndk-1). The fact that Tnk-1 of the psp (ndk-1P72H) mutant contains two nucleotide replacements (data not shown) suggested that Tnk-1 was the alternative transcript of ndk-1. The physiological role of this transcript, however, remains unclear, because the amount of Tnk-1 transcript was quite low compared with that of ndk-1 (Fig. 1D), and no translation product was detectable by Western blot analysis.

The Phenotype of psp (ndk-1 P72H) Is Complemented by Wild Type NDK-1—To examine whether the point mutation in ndk-1 (ndk-1 cDNA was fused with the promotor of trpC) caused the phenotype of psp (ndk-1P72H), a genomic DNA fragment containing wild type ndk-1 or the trpC-promoter::ndk-1 chimeric gene for cDNA ectopic expression was introduced into psp (ndk-1P72H). Homokaryotic transformants were isolated by more than seven rounds of colony selection on a medium containing BASTA. The integration of fragments into the transformants was confirmed by Southern blot analysis (data not shown). Two independent lines containing the extra genomic DNA fragment of ndk-1 (PGN-1 and 2) and three independent lines containing the trpC::ndk-1 chimeric construct (PCN-1, 2, and 3) were isolated. The abundance of NDK-1 in the transformants was examined by Western blot analysis. The transformants were able to accumulate as much NDK-1 as the wild type did (Fig. 2A). Phosphorylation of NDK-1 was also examined. The ability to phosphorylate NDK-1 itself was recovered in all of the transformants (Fig. 2B). In PGN-2, more NDK-1 was recovered than in psp (ndk-1P72H), but less than in other transformants and the wild type (Fig. 2A, lane 4). These results and the results in Fig. 1, A and C, showed that the point mutation in ndk-1 caused less NDK-1 accumulation of about 25% and almost no activity of NDK-1 phosphorylation in psp (ndk-1P72H) compared with those in wild type (Fig. 2, lane 2).

The perithecial polarities of the ndk-1 transformants were observed. The probabilities that the beaks would form at the top or at some other position on the perithecia are shown in Fig. 3. In darkness, the positioning of the perithecial beak was nondirectional when any of the strains were used as a protoperithecial parent. When white light was illuminated from one direction parallel to the surface of the solid medium, the beaks were formed preferentially at the top of the perithecia in the wild type. In psp (ndk-1P72H), the positioning of the beaks was nondirectional even when white light was illuminated from one direction, as in darkness. In psp (ndk-1P72H) transformants carrying wild type ndk-1, beaks formed at the top of the perithecia under unidirectional light. Fig. 4 shows typical perithecia of wild type, psp (ndk-1P72H), and PGN-2 strains grown in darkness or under unidirectional light. These results indicate that the phenotype of the psp mutant is caused by the ndk-1P72H mutation and that NDK-1 plays a role in the light-responsive regulation of perithecial polarity. Here the psp mutation was determined to be ndk-1P72H.

**TABLE I**

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>HisNDK-1</td>
<td>0.28 ± 0.04</td>
<td>0.10 ± 0.09</td>
</tr>
<tr>
<td>HisNDK-1P72H</td>
<td>0.29 ± 0.12</td>
<td>0.12 ± 0.10</td>
</tr>
<tr>
<td>GST-NDK-1</td>
<td>0.31 ± 0.02</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>GST-NDK-1P72H</td>
<td>0.45 ± 0.06</td>
<td>0.36 ± 0.02</td>
</tr>
</tbody>
</table>

$K_m$ and $V_{max}$ values of fusion proteins of NDK-1 and NDK-1P72H for $\gamma$-phosphotransferring activity for dTDP.
**1**P72H Are Reduced—Kinetic constants for NDK activity and protein-phosphorylation activity of NDK-1 or NDK-1P72H were determined using purified recombinant proteins. HisNDK-1 and HisNDK-1P72H showed comparable γ-phosphotransferring activity for CDP, GDP, UDP, dCDP, dGDP, and dTDP in the thin-layer chromatography assay (data not shown). Therefore dTDP was used as a typical phosphate acceptor of γ-phosphotransferring activity to determine the kinetic constants for the production of dTTP. As shown in Table I, neither the Michaelis constant (Km) that referred to dTDP as a substrate nor Vmax differed significantly between HisNDK-1 and HisNDK-1P72H, the Km and Vmax values were 0.28 ± 0.04 mM (dTDP) and 0.10 ± 0.09 nmol dTTP/min/mg of protein with HisNDK-1 and 0.29 ± 0.12 nmol (dTDP) and 0.12 ± 0.10 nmol dTTP/min/mg of protein with HisNDK-1P72H, respectively. Similar results were also obtained by use of GST-NDK-1 and GST-NDK-1P72H. These findings indicated that the nucleotide-phosphorylation activity of NDK-1 was not affected by the P72H substitution. In contrast, the autophosphorylation and protein kinase activities of GST-NDK-1 and GST-NDK-1P72H showed comparable activity for MBP. As shown in Table II, the Km and Vmax values were 7.3 ± 2.6 μM (MBP) and 0.48 ± 0.18 nmol/min/mg of protein (MBP) for GST-NDK-1 and 4.9 ± 0.1 μM (MBP) and 0.01 ± 0.00 nmol/min/mg of protein (MBP) with GST-NDK-1P72H, respectively. The Vmax value with GST-NDK-1P72H was about 2% of that with GST-NDK-1. These results revealed that the P72H mutation affected the activities of NDK-1 to phosphorylate both itself and MBP.

**DISCUSSION**

We report here that the P72H substitution in NDK-1 causes partial reduction in accumulation of NDK-1 and significant loss of the activity to phosphorylate itself and MBP. This proline residue is highly conserved among the NDK genes reported to date (27) and is thought to be located between the α2-helix and the β3-sheet from x-ray structural analyses of NDK in Dictyostelium discoideum (47, 48). These effects result in light insensitivity for perithecial polarity. In transformants of either construct containing wild type ndk-1 genomic DNA or cDNA, NDK-1 was adequately accumulated...
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and phosphorylated as in the wild type, although in the
PGN-2 strain, NDK-1 was accumulated less than in the wild
type or other transformants (Fig. 2). However, the amount
of NDK-1 in PGN-2 was sufficient, because there was no signif-
cient difference in the phosphorylation of NDK-1 compared
with the wild type and other transformants (Fig. 2). This
difference may be caused by the position of the integrated
transgene in the N. crassa chromosome. The recovery of
NDK-1 accumulation and phosphorylation in the transform-
ants, even in PGN-2, results in the normal light response of
perithecial polarity that is defective in psp (ndk-1P72H). This
shows that the psp mutation was an allele of the ndk-1 locus
and indicated that NDK-1 played a role in the regulation of
perithecial polarity in response to light.

WC-1 and WC-2 are the key components in the blue light
signaling pathway in N. crassa (21, 22). They are putative
transcription factors and form a heterocomplex that was sugges-
ted to function as a signal transducer (23). The WC com-
plex is also proposed to be a photoreceptor based on its
sequence similarity in the LOV domain with Arabidopsis blue
light receptor phototropin (NPH1) (7). WC-1 is phosphory-
lated, and this phosphorylation increases in response to light
treatment (24). This fact suggests the involvement of a pro-
tein phosphorylation in the blue light signaling pathway.
However, the protein kinases that mediate the light signaling
have not been identified in N. crassa, though protein kinase
c is suspected to play a role (25). Because the increase in the
level of NDK-1 phosphorylation after blue light irradiation is
very rapid, but not detected in wc mutants, the protein-
phosphorylation activity of NDK-1 would contribute to the
early steps of the light signaling downstream of WC proteins.
Light response of perithecial polarity was also defective in
wc-1 and wc-2, whereas other light-insensitive phenotypes
observed in wc mutants, such as phototropism of perithecial
beak, were not observed in ndk-1P72H. This fact suggests that
various light-response phenomena were regulated by differ-
te pathways downstream of WC proteins and that NDK-1
was involved in one such pathway regulating perithecial
polarity.

There was no significant difference in the level of ndk-1
mRNA accumulation between wild type and ndk-1P72H
mutant, but Western analysis revealed that about 25% of
NDK-1P72H protein was accumulated in the crude extract when
compared with that of wild type. This result suggests that some
post-translational regulation could play an essential role for NDK-1
accumulation. In a fish hepatocyte cell line, PLHC-1, NDK
was co-purified with molecular chaperone HSC70 as an accessory
protein (39). It could be possible that the region around the
proline 72 is responsible for the contact with proteins such as
molecular chaperons regulating the stability or folding of
NDK-1. We are now underway on isolating proteins interacting
with NDK-1 by affinity purification and by two-hybrid
screening.

The reduction in V_{\text{max}} for the phosphorylation of MBP by
GST-NDK-1P72H shows that the P72H substitution affects not
only autophosphorylation but also protein kinase activities
of NDK-1. This result indicates that autophosphorylation and
protein kinase activities occur via different mechanisms from
that of nucleotide-phosphorylating activity of NDK-1. There-
fore, the region around the proline 72 is proposed to contribute
to the proper access to the substrate protein. Thus, the total
decrease in the NDK-1 protein kinase activity, caused syner-
gistically by less protein accumulation (25%) and less V_{\text{max}} (2%)
in the activity, could lead Neurospora to the loss of response to
light.

We speculated that NDK-1 plays a crucial role in an uniden-
tified pathway including autophosphorylation and protein ki-

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