The NPK1 mitogen-activated protein kinase kinase kinase contains a functional nuclear localization signal at the binding site for the NACK1 kinesin-like protein

Masaki Ishikawa, Takashi Soyano, Ryuichi Nishihama and Yasunori Machida
Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

Received 5 July 2002; revised 19 August 2002; accepted 27 August 2002.
For correspondence (fax +81 52 789 2966; e-mail yas@bio.nagoya-u.ac.jp).
*Present address: Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA.
**Present address: Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA.

Summary

The tobacco mitogen-activated protein kinase kinase kinase NPK1 localizes to the equatorial region of phragmoplasts by interacting with kinesin-like protein NACK1. This leads to activation of NPK1 kinase at late M phase, which is necessary for cell plate formation. Until now, its localization during interphase has not been reported. We investigated the subcellular localization of NPK1 in tobacco-cultured BY-2 cells at interphase using indirect immunofluorescence microscopy and fusion to green fluorescent protein (GFP). Fluorescence of anti-NPK1 antibodies and GFP-fused NPK1 were detected only in the nuclei of BY-2 cells at interphase. Examination of the amino acid sequence of NPK1 showed that at the carboxyl-terminal region in the regulatory domain, which contains the binding site of NACK1, NPK1 contained a cluster of basic amino acids that resemble a bipartite nuclear localization signal (NLS). Amino acid substitution mutations in the critical residues in putative NLS caused a marked reduction in nuclear localization of NPK1 in BY-2 cells, indicating that this sequence is functional in tobacco BY-2 cells. We also found that the 64-amino acid sequence at the carboxyl terminus that contains NLS sequence is essential for interaction with NACK1, and that mutations in the NLS sequence prevented NPK1 from interacting with NACK1. Thus, the amino acid sequence at the carboxyl-terminal region of NPK1 has dual functions for nuclear localization during interphase and binding NACK1 in M phase.

Keywords: nuclear localization signal, BY-2 cell, coiled-coil structure, MAPKKK, NPK1 protein kinase, NACK1 kinesin-like protein.

Introduction

In eukaryotes, the nucleus is surrounded by the nuclear envelope that provides a physical barrier regulating the movement of substances into and out of the nucleus. Transport of nuclear proteins containing nuclear localization signals (NLSs), such as transcription factors and cell cycle regulators, into the nucleus across the nuclear envelope is mediated by importin α/β (Jans et al., 2000). A typical NLS is a short stretch of basic amino acids, such as the monopartite NLS of simian virus 40 (SV40) large tumor (T) antigen (Kalderon et al., 1984), or two clusters of basic amino acid residues separated by a spacer of several amino acids, such as the bipartite NLS of Xenopus nucleoplasmin (Robbins et al., 1991). Some proteins that contain NLSs are not always transported into the nucleus immediately, but are translocated into the nucleus by various types of mechanisms such as phosphorylation and proteintein interaction, in response to the appropriate environmental or chemical stimuli (Cyert, 2001). These mechanisms allow the cell to tightly control the activity of cell cycle regulators and signaling proteins, such as Cdc25C protein phosphatase which regulates G2/M transition (Takizawa and Morgan, 2000), and NF-AT4 transcription factor which plays a key role in activating gene expression in mammalian T lymphocytes (Zhu and McKeon, 1999).

Mitogen-activated protein kinase (MAPK) signaling cascades play important roles in signal transduction pathways, such as those controlling cell proliferation and cell death (Herskowitz, 1995; Kyriakis and Avruch, 2001). The cascades
are composed of members of three protein kinase families: the MAPK kinase kinase family (MAPKKK), the MAPK kinase (MAPKK) family, and the MAPK family. These kinases are sequentially activated through phosphorylation by respective upstream protein kinases in response to an extraordinarily diverse array of stimuli, such as growth factors, irradiation, and osmolarity. Phosphorylation of the terminal component of the cascade, MAPK, by MAPKK also stimulates translocation of the MAPK from cytoplasm to nucleus, where activated MAPK then phosphorylates and activates the target nuclear proteins, including transcription factors in the nucleus (Treisman, 1996). On the other hand, known MAPKKs and MAPKKKs normally remain in the cytoplasm to transmit signals from the plasma membrane to MAPK while it is in the cytoplasm. These protein kinases also change their intracellular localization in response to various stimuli. For example, Raf MAPKKK is normally cytosolic, but it is recruited from the cytoplasm to the plasma membrane by small GTP-binding protein Ras in response to growth factors (Leevers et al., 1994; Stokoe et al., 1994), resulting in activation of the MAPK-signaling cascade.

Recently, it has been shown that components of MAPK-signaling cascades are localized to the spindle midbody in animal cells and to the equatorial region of phragmoplasts in plant cells, suggesting that MAPK-signaling cascades also regulate cytokinesis (Bögre et al., 1999; Calderini et al., 1998; Nishihama et al., 2001; Shapiro et al., 1998; Zecevic et al., 1998). Thus, knowledge of the regulation of subcellular localization of components in the MAPK-signaling cascades is important for understanding the cellular functions of the individual cascades.

The NPK1 gene from tobacco, which is transcribed in all meristematic tissues of the plant, encodes a protein kinase related to MAPKKK, and the gene product consists of two major domains: the kinase domain at the amino (N)-terminal half, and the regulatory domain at the carboxyl (C)-terminal half which negatively regulates the kinase activity (Banno et al., 1993; Nakashima et al., 1998; Nishihama et al., 2002). Recently, it has been demonstrated that NPK1 is necessary for the formation of cell plate during cytokinesis of plant cells (Nishihama et al., 2001), which progresses from the center to the periphery of the cell with the formation of the phragmoplast (Nishihama and Machida, 2001; Staehelin and Hepler, 1996). The four lines of evidence are: (i) the activation of NPK1 occurs at late M phase; (ii) NPK1 is localized to the equatorial region of phragmoplasts; (iii) overexpression of the kinase-negative form of NPK1 results in the generation of multinucleate cells; and (iv) this inhibits the lateral expansion of the phragmoplast and the formation of cell plate. In addition, we also isolated two cDNAs for activators of NPK1 and anticipate that these cDNAs encode for kinesin-like proteins (designated NACK1 and NACK2), which are essential for cytokinesis and transport of NPK1 to the equatorial region of phragmoplasts (Nishihama et al., 2002).

During the course of our previous experiments on NPK1, we noticed that NPK1 was localized to the nucleus during interphase (Nishihama et al., 2001). Here, we report direct evidence for nuclear localization of NPK1 during interphase. This is facilitated by a functional NLS at the C-terminus of NPK1. In addition, the NLS sequence is present at the binding site for kinesin-like protein NACK1 that is responsible for activation and phragmoplast localization of NPK1. Thus, the C-terminal region of NPK1 is bifunctional for NPK1 subcellular localization and NACK1 binding in M phase.

Results

NPK1 is localized to the nucleus of BY-2 cells

Tobacco-cultured BY-2 cells at the logarithmic phase were fixed with 3.7% formaldehyde and treated with rabbit polyclonal antibodies against NPK1 (P557-2; Nishihama et al., 2001). NPK1 was visualized with the rhodamine-conjugated second antibodies against rabbit IgG. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). As shown in Figure 1(a), the signal due to fluorescence of NPK1 was detected within the nuclei of BY-2 cells at interphase.

To further analyze the subcellular localization of NPK1 in detail, we performed experiments with the construct of modified green fluorescent protein (sGFP; Chiu et al., 1996)-fused NPK1, designated sGFP-NPK1 (Figure 1b). We found that sGFP-NPK1, like wild-type NPK1 (Nishihama et al., 2002), was activated by interaction with NACK1 in yeast cells (data not shown), thus providing evidence that the sGFP-NPK1 fusion is functional. To examine the subcellular localization of sGFP-NPK1 at interphase, we transiently introduced plasmids, which carried sGFP cDNA alone, NLS::sGFP cDNA (which encodes SV40 NLS-fused sGFP) and sGFP::NPK1 cDNA driven by the cauliflower mosaic virus 35S promoter, into BY-2 cells at the logarithmic phase by using the bombardment method. The BY-2 cells transformed with these plasmids were cultured on LSD medium (solidified with 0.25% gelum gum) for 12 h at 28°C. The cells were then fixed with 2% paraformaldehyde, stained with DAPI, and the fluorescence from sGFP and DAPI was examined. Figure 1c shows that signals due to fluorescence of sGFP-NPK1 were detected exclusively within the nuclei of the BY-2 cells, which was similar to the distribution of signals due to NLS-sGFP (Figure 1c, NLS-sGFP). We also found that sGFP-NPK1 was distributed inside the nucleus, using confocal laser microscopy (data not shown). Signals due to fluorescence of sGFP alone were detected in both the nuclei and the cytoplasm of BY-2 cells (Figure 1c, sGFP).
The C-terminal region of NPK1 is necessary for its nuclear localization

As shown in Figure 2(a), a computer-based analysis using the COILS program (Lupas et al., 1991) indicated that amino acid residues 631–670 at the C-terminus of NPK1 were predicted to form a coiled-coil structure. Interestingly, an examination of the amino acid sequence of NPK1 indicates that it contains a short stretch of amino acid residues 645–659 which resemble the consensus sequence of bipartite nuclear localization signal (NLS) in the deduced coiled-coil structure (Figure 2a,b). To examine whether this putative NLS is necessary for the nuclear localization of NPK1, we constructed three types of deletion mutants of sGFP-NPK1:sGFP-NPK1:1–627 that contained amino acid residues 1–627, sGFP-NPK1:627–690 that contained amino acid residues 627–690, and NPK1:627–690-sGFP (Figure 2c). These fusion constructs were transiently introduced into...
BY-2 cells and the distributions of fluorescence signals were examined. Figure 2(d) shows that sGFP-NPK1:1–627, which lacked the 64 amino acid residues of the C-terminus, including the putative NLS, exhibited signals due to the fluorescence of sGFP in both the nuclei and the cytoplasm of BY-2 cells, which was similar to the distribution of sGFP alone. In contrast, signals due to fluorescence of both sGFP-NPK1:627–690 and NPK1:627–690-sGFP were detected exclusively within the nuclei of BY-2 cells (Figure 2d, middle and bottom). These results indicate that the 64-amino acid residue region of the C-terminus is sufficient for the nuclear localization of NPK1.

Basic amino acid residues in the regulatory domain function as a bipartite NLS in BY-2 cells

Next, to examine whether the two basic clusters depicted in Figure 2(b) function as a bipartite NLS in plant cells, we introduced base substitution mutations into codons for NPK1 corresponding to basic amino acid residues (R645, R646, K648, R657, K658, and R659) in the sequence (Figure 3a). These mutant NPK1 alleles, designated NPK1m1, NPK1m2, NPK1m3, and NPK1m4, were expressed as fusion proteins to sGFP in BY-2 cells that grew at logarithmic phase, and the distribution of these fusion proteins (designated sGFP-NPK1m1, sGFP-NPK1m2, sGFP-NPK1m3, and sGFP-NPK1m4) was investigated by fluorescence microscopy. As shown in Figure 3(b), fluorescence due to sGFP-NPK1m1 (R645S and R646S) was detected within nuclei, which had the same distribution as that of sGFP-NPK1. Fluorescence signals due to sGFP-NPK1m2 (R645S, R646S, and K648T) and sGFP-NPK1m3 (R657S, K658T, and R659S) were detected not only within the nuclei but also in the cytoplasm of BY-2 cells, although the intensity of the signals due to fluorescence of sGFP-NPK1m2 and sGFP-NPK1m3 in the cytoplasm seemed to be weaker than that of sGFP alone (see also Figure 1c). GFP fluorescence of sGFP-NPK1m4 (R645S, R646S, K648T, R657S, K658T, and R659S), with introduced amino acid changes at all the basic amino acid residues in this sequence, was mostly detected in the cytoplasm. Thus, mutations of these basic amino acid residues caused a drastic reduction in the nuclear localization of NPK1, indicating that the basic clusters in the regulatory domain function co-operatively as a bipartite NLS in BY-2 cells.

The C-terminal region of NPK1 interacts with the stalk region of NACK1 kinesin-like protein

Next, we identified the regions required for the interactions between NPK1 and NACK1 using a yeast two-hybrid system. We fused various segments of NACK1 cDNA to the LexA DNA-binding domain and examined their interactions with full-length NPK1 that was fused to the VP16 transactivation domain (Figure 4a). Unexpectedly, full-length NACK1 did not appear to interact with NPK1 in the two-hybrid system, despite the fact that it was co-immunoprecipitated with NPK1 (Nishihama et al., 2002). The motor domain of NACK1, which is thought to be responsible for binding to microtubules, may have prevented the full-length NACK1 from localizing to nuclei. We did find, however, that segments of NACK1 cDNA were useful for our proposed binding analysis. This analysis revealed that residues 685–756 of NACK1, a region that corresponded to the fifth putative coiled-coil region (Nishihama et al., 2002), was sufficient for the interaction (Figure 4a).
We also investigated the NACK1-binding site in NPK1 by examining the interactions of various segments of NPK1, fused to the VP16 activation domain, with residues 685–959 of NACK1, which were fused to LexA DNA-binding domain. Figure 4(b) shows that the C-terminal 64 amino acids of NPK1 (residues 627–690) interacted to a significant extent with NACK1. Interestingly, the NACK1-binding site contained the coiled-coil structure and the NLS (see also Figure 2a,b).

Mutations in NLS affect the interaction with NACK1

We then examined whether the point mutations in the NLS sequence that were described above affect NPK1 interaction with NACK1, by using the yeast two-hybrid system (Figure 5a). sGFP-NPK1, sGFP-NPK1m1, sGFP-NPK1m2, sGFP-NPK1m3, and sGFP-NPK1m4 were, respectively, fused to the VP16 transactivation domain, and interaction with the NPK1-binding site in NACK1 (amino acid residues 685–959) fused to the LexA DNA-binding domain was tested.
sGFP-NPK1m2 (Figure 5a, 4), and sGFP-NPK1m3 (Figure 5a, 5) were able to interact with NACK1 in yeast cells, whereas sGFP-NPK1m4 (Figure 5a, 6) was not.

Analysis with the COIL program (Lupas et al., 1991) led to the prediction that NPK1m1, NPK1m2, and NPK1m3 could form the coiled-coil structure with significant probabilities, but NPK1m4 could not (Figures 5b–f).

**Discussion**

**NPK1 contains a functional bipartite NLS in the NACK1-binding site**

In the present study, we demonstrated that NPK1 was localized to the nucleus during interphase (Figure 1). We also identified a single bipartite NLS that consists of two clusters of basic amino acids in the regulatory domain. The clusters are very similar to those of many other bipartite NLSs that have been identified previously (Jans et al., 2000; Raikhel, 1992; Figure 2b). Because mutations in each of the two basic clusters reduced the NLS activity, this region is a functionally bipartite NLS in BY-2 cells.

To our knowledge, NPK1 is the only member of the MAPKKK family that contains the functional NLS. MEKK1, which encodes for a member of the mammalian MAPKKK family, exists in both the nucleus and the cytoplasm of mammalian cells and is activated by DNA damage agents (Fanger et al., 1997), but no obvious NLS is present. It is suggested that MEKK1 could function, in part, as a stress sensor for DNA damaging agents in the nucleus. A downstream process that may be controlled by MEKK1, however, has yet to be found.

With regards to the nuclear localization of NPK1, we demonstrated that the region containing the C-terminal 64 amino acid residues in the regulatory domain of NPK1 also includes a binding site of NACK1 (Figure 4b), which is necessary for its transport to the equatorial region of phragmoplasts by NACK1 (Nishihama et al., 2002). Mutations in either of the two basic clusters in the NLS (NPK1m2 and NPK1m3) had no effect on the interaction between NPK1 and NACK1 in yeast cells (Figure 5a). The NPK1m2 and NPK1m3 mutants, however, exhibited reduced but detectable nuclear localization (summarized in Table 1), suggesting that the NLS function and the NACK1 binding are at least partially separated. This activity was negated by...
Table 1 Summary of subcellular localization, interaction with NACK1, and prediction of coiled-coil structure

<table>
<thead>
<tr>
<th>Protein(^a)</th>
<th>Localization(^b)</th>
<th>Interaction with NACK1(^c)</th>
<th>Coiled-coil structure(^d)</th>
</tr>
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<tbody>
<tr>
<td>NPK1</td>
<td>N</td>
<td>+</td>
<td>High</td>
</tr>
<tr>
<td>NPK1m1</td>
<td>N</td>
<td>+</td>
<td>High</td>
</tr>
<tr>
<td>NPK1m2</td>
<td>N/C</td>
<td>+</td>
<td>High</td>
</tr>
<tr>
<td>NPK1m3</td>
<td>N/C</td>
<td>+</td>
<td>Low</td>
</tr>
<tr>
<td>NPK1m4</td>
<td>C</td>
<td>-</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^a\)Refer to text for the makeup of mutant NPK1 alleles.
\(^b\)Subcellular localization of NPK1 and mutant NPK1 alleles, as summarized from Figure 3(b). N and C represent nuclear and cytoplasmic localization, respectively. N/C denotes localization to nucleus and cytoplasm just outside of the nucleus.
\(^c\)Interaction of NPK1 and mutant NPK1 alleles with NACK1 using the yeast two-hybrid system, as summarized from Figure 5(a). + and – represent growth and no growth, respectively, on the medium without exogenous histidine.
\(^d\)Prediction of coiled-coil structure of mutant NPK1 alleles with the COILS program (Lupas et al., 1991), as summarized from Figure 5(b-f). High, Low, and No represent high, low, and no potential for a coiled-coil structure at the carboxyl-terminal region, respectively.

mutations of all the basic residues in NPK1m4 (Figure 3b). Furthermore, the mutation in NPK1m4, which disrupted the association of NPK1 with NACK1, markedly reduces the probability of a coiled-coil structure in the regulatory domain (Figure 5; Table 1). These results suggest that the coiled-coil structure at the NACK1-binding site is necessary for association with NACK1, and that the amino acids of the NLS region structurally overlap with NACK1-binding site to a certain extent.

The significance of the nuclear localization of NPK1

Nuclear localization of NPK1 may be important for properly exerting the cellular role of this protein kinase. We have already reported that although NPK1 is present from the G1–S phase to the end of the M phase, it is activated only after metaphase in the cell cycle by association with M phase-specific kinesin-like protein NACK1 (Ito et al., 1998; Nishihama et al., 2002). It also plays an essential role in the formation of the cell plate (Nishihama et al., 2002). Considering the cytokinin control exerted by NPK1, it may also be worth noting the subcellular localizations of MAPKK and MAPK that are placed downstream of NPK1. Recently, we have identified a MAPKK (NQK1) and a MAPK (NRK1) as putative factors that act downstream of NPK1 (Machida et al., 1998; Nishihama and Machida, 2000; unpublished observations by Soyano and Machida). Examination of the subcellular localization of these downstream factors showed that both NQK1 and NRK1 proteins are present throughout the BY-2 cell cycle and the majority of NQK1 and NRK1 molecules are present in the cytoplasm but not in the nuclei during interphase (unpublished observation by Soyano and Machida). Thus, localization of the downstream factors is in sharp contrast to the nuclear localization of the MAPKK, NPK1. Such spatially separate subcellular localization of the components of MAP kinase cascade (NPK1, NQK1, and NRK1) might be important for precise control of the activation of this kinase cascade.

It can also be presumed that the nuclear localization may be important for proper localization to spindle microtubules after breakdown of the nuclear membrane. Previous data (Nishihama et al., 2001) showed that NPK1 is localized in the nucleus before breakdown of the nuclear membrane, present as dot-like structures around spindle microtubules from prophase to metaphase, and localized to the midzone of spindle microtubules and the equatorial zone of phragmoplasts from late anaphase to telophase when NPK1 is activated. The nuclear localization of NPK1 may be the key for its proper movement during M-phase progression.

The nuclear localization of NPK1 may also be related to an aspect of the cell cycle progression which is different from cytokinesis. Since the NPK1 kinase has some level of activity in interphase (unpublished data by Nishihama and Machida), it might phosphorylate a nuclear protein(s). NPK1 might control the cell cycle progression during interphase or the G2–M transition through such protein phosphorylation. This type of cell cycle control has been reported for the positive regulation by the Polo-like protein kinase at G2–M of cyclin B activity, which is also functional at M phase (Glover et al., 1998; Toyoshima-Morimoto et al., 2001). Regardless of such speculation, the nuclear localization of NPK1 during interphase is probably crucial for proper cell cycle progression. The cellular significance of such localization of NPK1 must be further examined.

Recently, it has been reported that overexpression of truncated NPK1 lacking the regulatory domain results in suppression of auxin-induced gene expression and in the activation of oxidative stress signaling (Kovtun et al., 1998, 2000). They proposed that NPK1 would negatively control auxin-inducible expression of the gene and positively regulate expression of the genes involving the stress response. The present results, however, showed that the regulatory domain of NPK1 contains bifunctional sequences that determine subcellular localization of NPK1 during cell cycle progression. It must be further examined whether phenotypes generated by overexpression of such a truncated form would be related to cellular functions of the full-length NPK1.

Most recently, two groups have reported phenotypes of Nicotiana plants in which NPK1 expression was abrogated by a virus-induced gene-silencing system (Jin et al., 2002) and in mutants of Arabidopsis homologs of NPK1, ANP1, ANP2, and ANP3 (Krysan et al., 2002; Nishihama et al., 1997). These plants displayed upregulation of pathogen- and stress-related genes as well as defective cell division.
and growth, which is consistent with our previous results (Nishihama et al., 2001). On the basis of their observations, it was proposed that NPK1 would negatively regulate the expression of stress-related genes, which appears to be inconsistent with the previous proposal by Kovtun et al. (2000). It may be intriguing to elucidate how subcellular localization of NPK1 described in the present study would be involved in processes of disease resistance and stress response.

**Experimental procedures**

**Cultures of BY-2 cells**

Tobacco suspension-cultured BY-2 cell lines were maintained in modified Linsmaier and Skoog medium (LSD medium) according to a previously reported method (Banno et al., 1993).

**Plasmid constructs**

Plasmid DNAs were constructed using PCR amplification and standard cloning techniques. The coding regions of NPK1, NPK1:1–627, and NPK1:627–690 were fused to the 3′ end of the coding region of GFP-S65T (sGFP) derived from pTH-2 (Chiu et al., 1996) to generate sGFP-NPK1, sGFP-NPK1:1–627, and sGFP-NPK1:627–690, respectively. The restriction fragments corresponding to the amino acid residues 1–627 and 627–690 were generated by restriction digestion of DNAs. NPK1:627–690-sGFP was generated as follows: the DNA fragment corresponding to amino acid residues 627–690 was amplified by PCR and the amplified fragment was inserted into cleavage sites of SalI and NcoI restriction enzymes in pTH-2. The base substitution mutations at codons 645, 646, 648, 657, 658, and 659 in the basic clusters at the C-terminus were generated by PCR with mutagenic primers to codons 645, 646, 648, 657, 658, and 659 in the basic clusters at the amino acid residues 627–690, respectively. The restriction fragments corresponding to the amino acid residues 1–627 and 627–690 were generated by restriction digestion of DNAs. NPK1:627–690-sGFP was generated as follows: the DNA fragment corresponding to amino acid residues 627–690 was amplified by PCR and the amplified fragment was inserted into cleavage sites of SalI and NcoI restriction enzymes in pTH-2. The base substitution mutations at codons 645, 646, 648, 657, 658, and 659 in the basic clusters at the C-terminus were generated by PCR with mutagenic primers to yield NPK1m1, NPK1m2, NPK1m3, and NPK1m4. These DNA fragments were fused to the 3′ end of the sGFP sequence in pTH-2 to give sGFP-NPK1m1, sGFP-NPK1m2, sGFP-NPK1m3, and sGFP-NPK1m4, respectively. All the sGFP fusion constructs contained the DNA insert encoding amino acid residues, LEQFPGI, between the sGFP and NPK1 sequences. The DNA fragments corresponding to various segments of NPK1 and sGFP-NPK1 mutant alleles or various segments of NACK1 were inserted into the cloning site of yeast expression vector pVP16 or pBTM116, respectively, for expression of stress-related genes, which appears to be inconsistent with the previous proposal by Kovtun et al. (2000). It may be intriguing to elucidate how subcellular localization of NPK1 described in the present study would be involved in processes of disease resistance and stress response.

**Purification of recombinant protein from E. coli**

E. coli BL21 (DE3) cells harboring pET28-NPK1:RD or pSBETH-NACK1:ST were cultured overnight. One ml of the overnight culture was transferred to 100 ml of fresh LB medium and cultured for a further 2 h. Expression of proteins was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside for 3 h. Cells were collected and re-suspended in 2 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 20 mM imidazole) and disrupted by sonication. Each cleared lysate was passed through a 1-ml HiTrap Chelating™ column (Amersham Pharmacia Biotech., Buckinghamshire, UK) that had been preloaded with 0.1 mM NiSO₄. The column was washed with 6 ml of wash buffer (lysis buffer plus 50 mM imidazole). Recombinant proteins (HisT7-NPK1:RD and His-NACK1:ST, respectively) were eluted with 2.5 ml of elution buffer (lysis buffer plus 250 mM imidazole) and the eluate was dialyzed five times with 200 ml of Tris buffered saline (TBS) for 1 h at 4 °C. Two-thirds volume of glycerol was added to each solution of protein, which was then stored at –20°C.

**Binding assay in vitro**

We incubated 0.5 μg of His-NACK1:ST with or without 0.5 μg of HisT7-NPK1:RD in 50 μl of TBS that contained 0.1% Triton X-100 (TBSX) for 1 h on ice. The mixture was then incubated with 1 μg of T7 epitope-specific mouse monoclonal antibodies (Novagen, Madison, WI, USA) for 2 h on ice. Immunoprecipitation was performed by incubation of the mixture with 20 μl of a 50% slurry of Protein A-Sepharose (Amersham Pharmacia Biotech.) in 300 μl of TBSX for 1 h at 4 °C, followed by five washes of the resin with TBSX. Precipitates were suspended in 40 μl of 1× sample buffer for SDS-PAGE and boiled. Samples (10 and 20 μl for detection of NPK1 and NACK1, respectively) were analyzed by SDS-PAGE and Western blotting.

**Fluorescence microscopy**

DNA staining was performed using our previous procedure (Nishihama et al., 2001). Bombarded BY-2 cells were suspended in 2% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 8.10 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄ (pH 7.4)) and kept for 10 min at room temperature. After the cells were washed twice with PBS, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) at a concentration of 1 μg ml⁻¹ for 10 min at room temperature. Immuno-fluorescence microscopy was performed following standard methods using anti-NPK1 polyclonal antibodies as described previously (Nishihama et al., 2001). The fluorescence images from staining or from sGFP were observed with a fluorescence microscope (Axioplan2, Carl Zeiss, Oberkochen, Germany). After photographs were taken using a cooled-CCD camera system (Photometrics, Tucson, AZ, USA), the images were deconvoluted.
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