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Overexpression of Human Myotonic Dystrophy Protein Kinase in *Schizosaccharomyces pombe* Induces an Abnormal Polarized and Swollen Cell Morphology

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We expressed human myotonic dystrophy protein kinase (DMPK) in the fission yeast *Schizosaccharomyces pombe*, in which the overexpression of human DMPK affects cell growth and cell shape. The human DMPK protein has a leucine-rich domain at the N-terminus, a serine/threonine kinase domain in the middle, and a hydrophobic region at the C-terminus. C-Terminus–deleted DMPK produced a middle-swollen phenotype (lemon-like shape), indicating an abnormality in cell division. On the other hand, when both the kinase domain and C-terminus were present, the expression of DMPK resulted in polarized cell growth and multinucleated/branched cells. The lemon-like phenotype seen with the C-terminus-deleted DMPK disappeared when the ATP binding site of DMPK was disrupted by replacing the lysine at amino acid 100 with arginine (K100R mutant). However, polarized and/or multinucleated cells lacking the DMPK N-terminus were not rescued by the K100R mutation. Therefore, we conclude that the N-terminus of DMPK plays an important role in DMPK kinase activity, and that the C-terminus of DMPK determines the intracellular localization of the protein.

Key words: ATP binding domain, fission yeast *Schizosaccharomyces pombe*, multinucleated, myotonic dystrophy protein kinase.

Abbreviations: DM, myotonic dystrophy; DMPK, DM protein kinase.

Myotonic Dystrophy (dystrophia myotonica, commonly abbreviated as DM) is a dominantly inherited multisystemic disorder characterized by myotonia, cataracts, mental retardation, and so on (*1*). The molecular basis of DM is regarded as expansion of CTG triplet repeats in the 3′-untranslated region of the myotonic dystrophy protein kinase (DM protein kinase, DMPK) gene (*2–5*). The human DMPK protein has a leucine-rich domain at the N-terminus, a serine/threonine kinase domain in the middle, and a hydrophobic region at the C-terminus. To determine the functions of these domains, we designed several deletion constructs for the expression of human DMPK in fission yeast *Schizosaccharomyces pombe*. In our study, the overexpression of human DMPK affected both the cell growth and morphology of *S. pombe*. The fission yeast showed different morphologies when N-terminus– and/or C-terminus–deleted DMPK was expressed. The morphological changes indicate that the N-terminus functions as the regulator of DMPK activity. In addition, the C-terminus of DMPK was also shown to be important for the intracellular localization of the protein.

MATERIALS AND METHODS

Yeast Strain and DMPK Constructs—The *S. pombe* strain used in this study was *leu1-32h*$^-$. Plasmid pREP1, which has an *nmt1* promoter, was used in the experiments. Full-length human DMPK was obtained according to Sasagawa *et al*. (*6*). PCR primers were designed to amplify deletion constructs of DMPK (Fig. 1). *Pfu* polymerase (STRATAGENE) was used in the experiments. Yeast transformation was achieved by the standard method using lithium acetate, and positive transformants were selected on standard minimal medium (MM) plates (without leucine) with 2 μM thiamine.

Introducing Point Mutations into DMPK—To abolish DMPK activity, we introduced a point mutation (*7*) to convert lysine to arginine in the ATP-binding consensus sequence (K100R mutant). We designed PCR primers with mutations that anneal the DMPK cDNA sequence of the ATP binding site encoding lysine (primer A, 5′-CTTGTTCATGATgcgCATGGCATACAC-3′; and primer B, 5′-GTGTATGCCATGcgcATCATGAACAAG-3′; small letters represent the mutations). These primers were also designed to produce a restriction enzyme *Fsp*I site when the mutation was successfully introduced. Primer A was applied to amplify the N-terminus of DMPK with primer 1 (product A), and primer B was used to amplify the C-terminus with primer 5 (product B). Products A and B were then mixed, and PCR was performed again (without primers) to produce the full-length K100R mutant. Sequence analysis was carried out to confirm the mutation.

Induction of Recombinant DMPK—Cells were preincubated on MM plates (+thiamine), and the growing cells were seeded onto MM plates (-thiamine). The plates were incubated at 30°C for 5 d. Cell shapes and sizes were analyzed with the Scion Image software (Scion Corporation).

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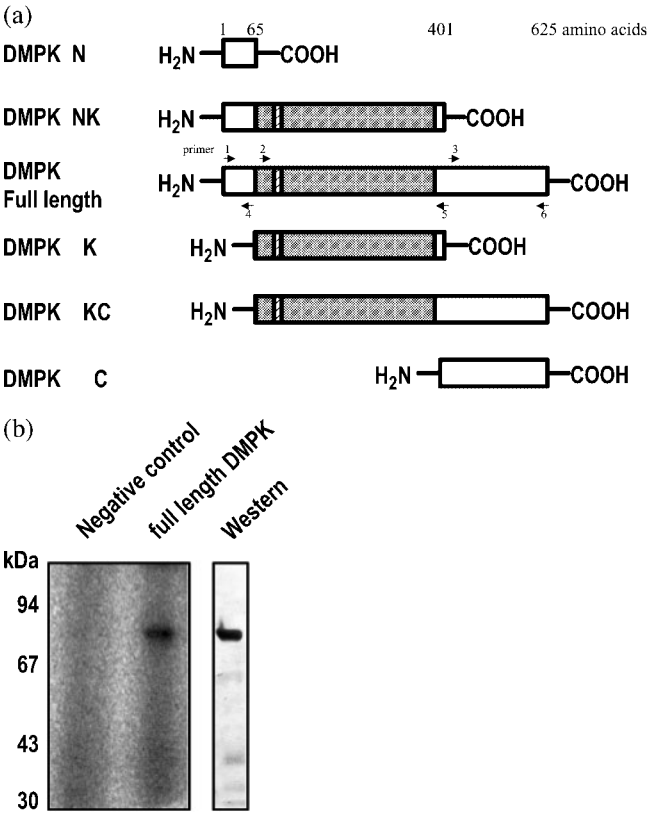

Fig. 1. **(a) Schematic structures of human myotonic dystrophy protein kinase (DMPK) and the deletion constructs used in these experiments.** The arrows indicate the primers used to prepare PCR fragments of each construct. The hatched boxes indicate ATP binding sites and the gray boxes indicate serine/threonine kinase domains. **(b) Autophosphorylation of full-length DMPK.** The recombinant human DMPK was expressed in *Pichia pastoris* and purified using Ni-NTA resin. The transformant, which expresses β-galactosidase, was used as a negative control. A His-probe was used to visualize the DMPK protein on Western blot analysis.

Analysis of Expressed Recombinant DMPK Protein—*S.pombe* transformants were incubated at 30°C for 2 d in MM liquid medium without thiamine, and the cells were harvested by centrifugation at 1,000 ×*g*. Then, the cells were incubated in spheroplast buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 15 mM β-mercaptoethanol, 1 M sorbitol) with 1 mg/ml Zymolyase 20 T (Seikagaku) at 30°C for 1 h. The cells were washed three times with spheroplast buffer, and then gently homogenized in a Potter's glass homogenizer. The broken cells were centrifuged at 10,000 ×*g* for 5 min, and the separated supernatant and pellet were subjected to SDS–polyacrylamide gel electorophoresis and immunoblotting. The two DMPK antibodies used for immunoblot analysis are shown in Fig. 5.

Nuclear Staining with DAPI—We stained the nuclear chromosomal DNA of living cells with 4′,6-diamidino-2-phenylindole (DAPI). DMPK-expressing cells were collected, and washed in 1× phosphate-buffered saline (PBS), and then an equal volume of DAPI (6.25 μg/ml) was mixed directly into the cell suspension. Cells were observed under a microscope within 30 min of DAPI staining.

Kinase Activity of Full-Length DMPK—We introduced a restriction enzyme *Bgl*II site at the start methionine position of the DMPK cDNA by *in vitro* mutagenesis. This cDNA was subcloned into pET16b using *Bam*HI, to fuse a 6× histidine tag to the N-terminus of DMPK. The DMPK/his-tag construct was then subcloned into pPIC3.5, and then transformed into yeast *Pichia pastoris* GS115. The addition of methanol to the medium induced the expression of the DMPK protein. The cells were harvested, incubated with Zymolyase 20 T, and then disrupted by voltexing with glass beads. The expressed DMPK protein was recovered from the soluble fraction and the expressed DMPK was purified with Ni-NTA resin. Autophosphorylation of DMPK was performed by incubating the protein in 30 mM Tris/HCl (pH 7.5), 25 mM $MgCl_2$, 100 μM ATP, 0.37 MBq [γ-^{32}P]ATP, at 37°C for 30 min. After SDS-PAGE, we used a Bas-Mac image analyzer (Fuji Film) to analyze the results.

RESULTS

Cell Growth of DMPK-Overexpressing Cells—Figure 1a shows a schematic representation of DMPK and the deletion constructs used in these experiments. DMPK has a serine-threonine kinase domain in the middle (amino acids 66–401). An ATP-binding site is located from amino acid number 77 to 100. The hydrophobic region is at the C-terminus, and exhibits homology to those of cytoskeletal proteins. A leucine-rich region is present at the N-terminus. We constructed five deletion mutants, as shown in Fig. 1a. The kinase activity of the full-length DMPK was observed as DMPK autophosphorylation (Fig. 1b).

The overexpression of human DMPK in fission yeast affected cell growth (Fig. 2). After 5 d induction with the thiamine promoter, a decrease in colony size was observed. Although the colonies in the presence of thiamine reached 2 mm in diameter, when thiamine was removed from the medium, the colonies reached only 1 mm in diameter, *i.e.* about half the control size.

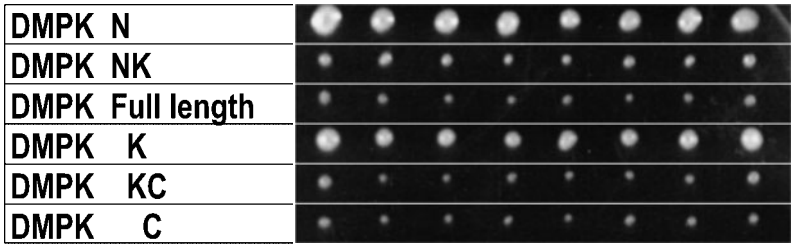

Fig. 2. **The overexpression of human DMPK deletion constructs resulted in a decrease in colony size.** Cells were pre-incubated on minimal medium (MM) plates containing thiamine, and then transferred to MM plates lacking thiamine. Eight colonies for each construct were incubated. The decrease in colony size appeared 5 d to 1 w after the removal of thiamine.

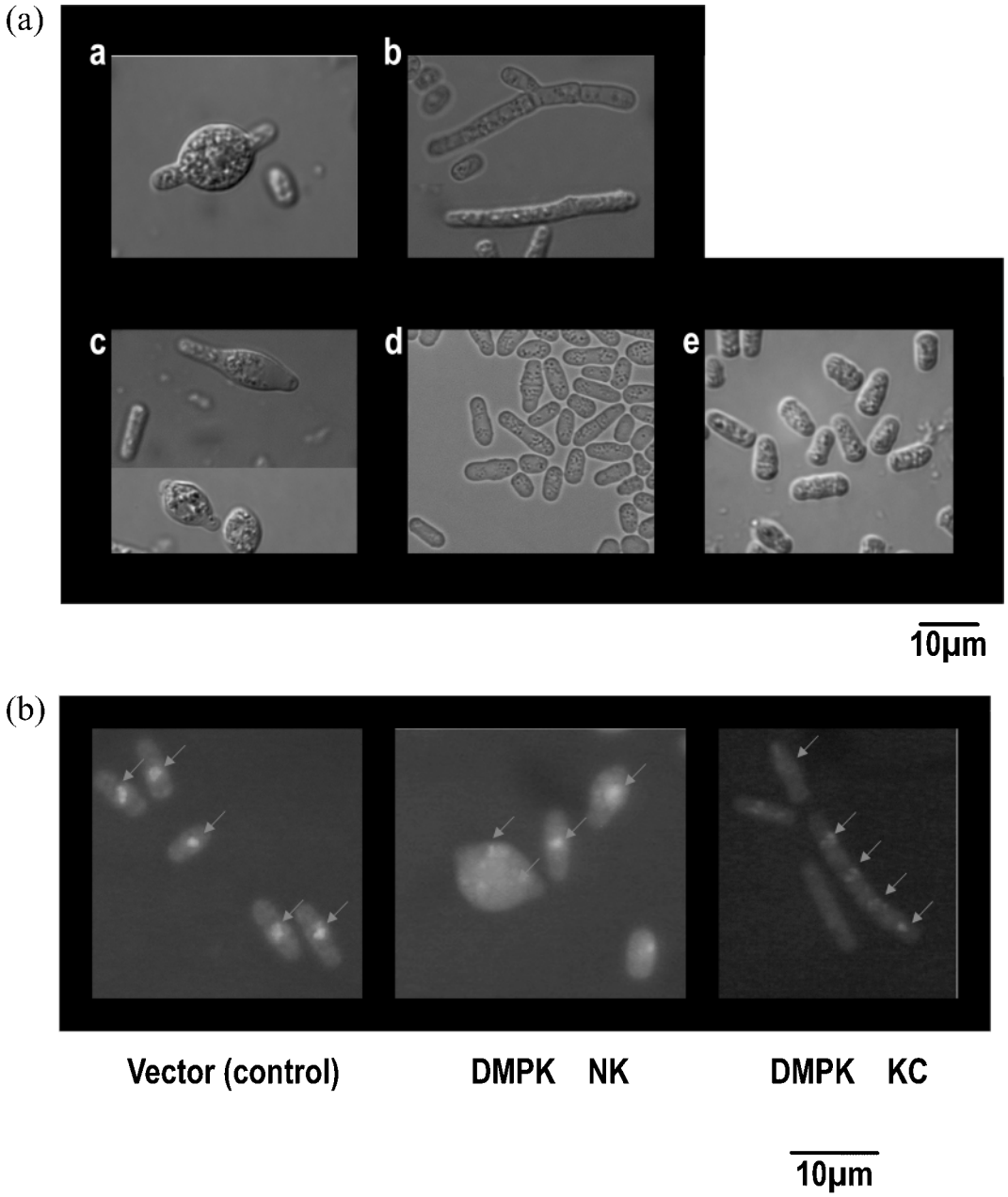

Fig. 3. **(a) Morphological changes in fission yeast cells overexpressing human DMPK constructs.** a, DMPK NK (lacking the C-terminus); b, DMPK KC (lacking the N-terminus); c, full-length DMPK; d, DMPK-C (only the C-terminus); e, pREP1 vector only (negative control). **(b) Nuclear staining with DAPI.** The arrows indicate chromosomal DNA.

Changes in Cell Shape on Overexpression of DMPK—The overexpression of DMPK induced a change in cell shape (Fig. 3a). When DMPK-NK (lacking the C-terminus) was expressed, the cells assumed a round, swollen, nonpolarized shape. On the other hand, the overexpression of DMPK lacking the N-terminus (DMPK-KC) resulted in a morphological change to a polarized and/or branched cell type. The full-length DMPK induced an intermediate shape, swollen but elongated. These results indicate that DMPK affects the cell cycle or cytoskeletal reorganization of *S. pombe*. The overexpression of the N-terminus, kinase domain, or C-terminus alone did not produce any morphological changes.

The *nmt* promoter initiates gene expression 15 h after the removal of thiamine. On the other hand, the morphological changes induced by DMPK constructs were observed 3–5 d after the removal of thiamine (data not shown). This indicates that DMPK has a mild effect on cell growth.

Nuclear staining with DAPI revealed diffusion of the nuclear DNA in the swollen cells. On the other hand, the elongated cells exhibited a multinucleated phenotype (Fig. 3-b).

Inactivation of the ATP Binding Site—We introduced a point mutation into the ATP binding site of DMPK (K100R mutation) (Fig. 4a). The lysine residue 100 amino

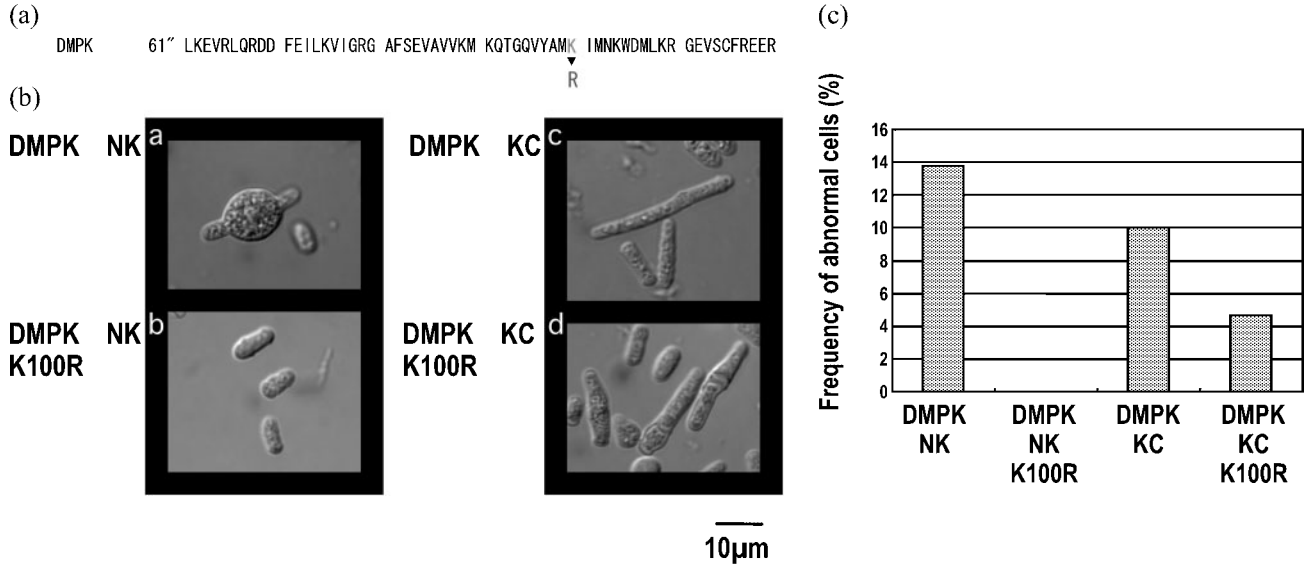

Fig. 4. **(a) A point mutation was introduced to convert the catalytic lysine to arginine, which abolishes the kinase activity. (b) Morphological changes induced on overexpression of DMPK with the K100R mutation.** The swollen cell type produced by the overexpression of DMPK-NK (lacking the C-terminus) reverted to the normal cell shape when the K100R mutant was expressed. On the other hand, the cell shape seen for cells expressing DMPK-KC (lacking the N-terminus) was not completely prevented by the mutation. (c) The frequencies of abnormal cells were determined using Scion Image, an image analyzing software program.

acids from the N-terminus, a widely conserved ATP binding site among serine/threonine protein kinases, was converted to an arginine. The swollen cell shape produced by DMPK-NK (lacking the C-terminus) reverted to a normal cell shape with the mutation (Fig. 4b). On the other hand, the multinucleated/multiseptated shape produced by DMPK-KC did not revert completely with the K100R mutation. The long, polarized cells became short and fat, but the multiseptate phenotype remained.

Figure 4c shows the results of statistical analysis of the frequencies of abnormal cell types. The lengths of 100–250 cells were measured. A normal cell is 3–4 μm in width and 12–15 μm in length at the time of cell division. In this study, cells of over 6 μm in width (DMPK NK) or over 20 μm in length (DMPK KC) were counted as abnormal cells. The overexpression of DMPK constructs induced morphological changes in 10–14% of total cells. The full-length DMPK also produced morphological changes at almost the same frequencies (7% long cells and 22% fat cells; 5% both long and fat; data not shown). These percentages suggest that only high expression levels of DMPK induce morphological changes.

When the K100R mutation was introduced, the swollen shape produced by DMPK-NK disappeared completely. On the other hand, the introduction of the K100R mutation into DMPK-KC decreased the number of abnormal cells, although about 5% remained fat and long. These results indicate that DMPK is a multifunctional protein with a kinase function and/or an unidentified function without enzymatic activity.

Solubility of the DMPK Protein—We collected recombinant DMPK proteins from transformants. Because the cell wall of fission yeast becomes very hard with long incubation, we collected the cells 2 d after the removal of thiamine. Cells were incubated with Zymolyase 20 T, mildly homogenized in a Potter's glass homogenizer, and then centrifuged at 10,000 ×*g*. The DMPK protein with the C-terminus were recovered as insoluble proteins after centrifugation, while the expression product lacking the C-terminus was recovered in the soluble fraction (Fig. 5). The cell wall of fission yeast becomes harder after 2–3 d of incubation; therefore, Fig. 5 indicates that not all cells were broken.

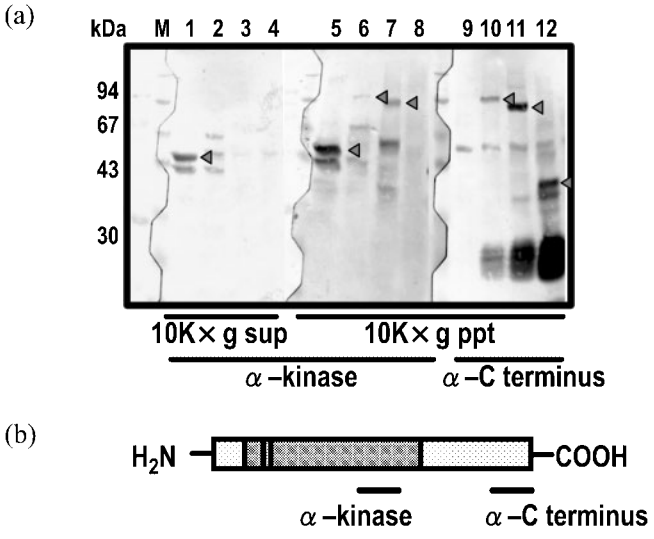

Fig. 5. **(a) Western analysis of recombinant human DMPK proteins.** M: molecular markers; lanes 1, 5, 9: DMPK-NK (lacking the C-terminus); lanes 2, 6, 10: full-length DMPK; lanes 3, 7, 11: DMPK-KC (lacking the N-terminus); lanes 4, 8, 12: DMPK-C (the C-terminus of DMPK). Arrowheads indicate the expressed DMPK proteins. Only DMPK-NK (lacking the C-terminus) was recovered from the supernatant. The antibodies used and their recognition sites are shown in (b).

DISCUSSION

Schizosaccharomyces pombe is a eukaryotic organism, which makes it possible to express mammalian proteins with physiological functions (*8*). We can expect that expressed proteins in *S. pombe* are post-translationally modified, as in insect cells and mammalian expression systems. Moreover, the cell cycle pathway and mutants showing morphological changes have been widely investigated in *S. pombe*. Therefore, to investigate the physiological function of each domain of DMPK and to test the possibility that DMPK is involved in the cell cycle pathway, we expressed human DMPK in *S. pombe*.

DMPK is a member of the serine/threonine protein kinase family, and thought to be a member of the "myotonic dystrophy family of protein kinases" (MDFPK) (*9*). Several MDFPK members have been shown to interact with small GTPases. So far, many spliced forms of human DMPK have been reported (*10*), which are classified mainly into two subgroups, the DMPK-A and B group, and the DMPK-C and D group. All isoforms have the same kinase domain, the main differences lying in the C-terminus. Both groups have hydrophobic C-termini, but there is no homology between them. The DMPK-A and B group has a higher hydrophobic amino acid content than the DMPK-C and D group. In this study, we used DMPK-D, originally cloned by us. Although the relationship between the expression of DMPK isoforms and the symptoms of DM is unclear, the C-terminus of each DMPK isoform might have an important physiological function.

The orb6 gene of *S. pombe* exhibits homology to that of human DMPK. The overexpression of orb6 results in long polarized cells (*11*), although they seem to have a single nucleus. CBK1p is a homolog of *orb6* in *Saccharomyces cerevisiae* whose binding affinity for transcription factor Ace2p has been reported. It has also been demonstrated that CBK1p bound to Ace2p mediates the transcription of chitinase (*12*). The homology between orb6/CBK1 and human DMPK lies mainly in the kinase domain, and they show no homology in the C-terminus.

In this study, we found that the overexpression of DMPK constructs resulted mainly in two different morphological changes. Many cell division-deficient mutants of fission yeast show abnormal cell shapes. Moreover, polarized cell growth is regulated during the cell cycle (*13*). Consistent with the data presented in Fig. 2, the possibility that human DMPK disturbs the cell cycle/cell division of *S. pombe* has been suggested. The DMPK clone lacking the C-terminus caused the cells to assume a swollen shape. The S phase might be repeated by skipping the M phase in DMPK-NK transformants. The swollen shape is also seen in fission yeast on the overexpression of Pob1p. Pob1p is a protein of 871 amino acids with SH, PH and SAM domains, but does not contain a catalytic site. Although the precise physiological function of Pob1p is not completely understood, the association of Pob1p with actin has been indicated (*14*). The presence of Boi1p/Boi2p, counterparts of Pob1p, has also been reported in *Saccharomyces cerevisiae* (*15*, *16*). Boi1 and Boi2 interact with Bem1p, which is necessary for cell polarization (*17*). Boi1p/Boi2p also have SH and PH domains. The overexpression of Boi1p/Boi2p in *Saccharomyces cerevisiae* results in growth arrest and a morphological change to a round shape. Moreover, the morphological change seen in Boi1p/Boi2p-overexpressing cells is suppressed by the overexpression of Cdc42p (*16*), a member of the Ras superfamily of small GTP binding proteins that have a function in cell polarity (*18*).

DMPK lacking the N-terminus produced an elongated shape and the cells became multinucleated. This indicates that the C-terminus of DMPK also has an important role in the activity of DMPK, including its intracellular localization (Fig. 5). It was found that rho-1 affects septum formation in both disruption and overexpression experiments in *S. pombe* (*19*).

The swollen shape depends on DMPK activity. The overexpression of the inactive K100R mutant produced no morphological changes. Considering that no morphological change is caused by the expression of DMPK-K, the above results indicate that the N-terminus of DMPK plays an important role in the enzymatic activity of DMPK. On the other hand, the multinucleated shape produced by DMPK-KC was not completely prevented by the K100R mutation. It is widely known that, as in the case of p21-activated kinase 1 (*20*), several serine/threonine kinases have multi-physiological functions that are carried out in both kinase-dependent and independent manners. Our results indicate that human DMPK has another physiological function unrelated to its kinase activity.

It has been reported that the overexpression of DMPK-A and B, different spliced isoforms with a different C-termini to the DMPK-D in our study, induces apoptotic-like blebbing of the plasma membrane in human and rabbit lens cells (*21*). Moreover, the C-terminus of DMPK-A and B seems to be associated with Rac (*22*), despite its lack of a significant G-protein binding domain. However, this apoptotic-like blebbing is also induced by the overexpression of DMPK lacking the C-terminus, indicating that the C-terminus is not responsible for this morphological change.

Although we tried to purify recombinant DMPK proteins from *Schizosaccharomyces pombe*, we have not yet succeeded. Significant expression of the recombinant proteins is observed 2–3 d after thiamine removal. However, it is very difficult to break the cells after 2–3 d, indicating that the cell wall has become very hard. Therefore, we could not purify the recombinant DMPK proteins for an activity assay.

When we expressed human DMPK in *Pichia pastoris*, the full-length DMPK was recovered as a soluble protein and purified using Ni-NTA resin (Fig. 1b). This suggests that DMPK itself is a soluble protein, and might associate with the membrane or cytoskeletal components in fission yeast. The C-terminus of DMPK contains hydrophobic peptides and exhibits homology to several cytoskeletal proteins. Therefore, we conclude that the C-terminus of DMPK might be a key factor in the intracellular localization of DMPK.

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