SHORT COMMUNICATION

The plant homologue of MAP kinase is expressed in a cell cycle-dependent and organ-specific manner

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Summary

In animals, MAP kinase plays a key role in growth factor-stimulated signalling and in mitosis. The isolation of a *Medicago sativa* cDNA clone *MsK7* which shows 52% identity to animal MAP kinases is reported. The deduced protein sequence shows all the important structural features of MAP kinases and also contains the highly conserved Thr-183 and Tyr-185 residues. Northern analysis of synchronized alfalfa cells showed that the *MsK7* kinase gene is expressed at low levels in G1 phase but at higher levels in S and G2 phases of the cell cycle. In the plant, only stems and roots were found to contain MAP kinase *MsK7* mRNA. Southern and PCR analyses indicated that alfalfa contains at least four highly related MAP kinase genes.

Introduction

Animal MAP kinases are serine/threonine kinases whose activation and phosphorylation on tyrosine and threonine residues are rapidly induced by a variety of mitogens (Gotoh et al., 1990a, 1990b, 1991; Hoshi et al., 1988, 1989; Ray and Sturgill, 1987, 1988; Rossonando et al., 1989). This class of kinases is characterized by their ability to phosphorylate microtubule associated protein 2 (MAP2) and myelin basic protein (MBP). MAP kinase was also found to phosphorylate and partially reactivate S6 kinase II (Ahn et al., 1991; Sturgill et al., 1988) and the transcription factors c-myc and c-jun (Alvarez et al., 1991; Pulverer et al., 1991). In *Xenopus* oocytes, MAP kinase becomes activated during meiotic maturation and in M phase during the following mitotic cycles (Ferrell et al., 1991; Gotoh et al., 1990a; Posada et al., 1991). MPF (a cdc2 kinase-cyclin complex) is a central element in the G2-M transition (for review see Nurse, 1990) and acts as an upstream activator of MAP kinase (Gotoh et al., 1991). Microinjection of MPF into immature oocytes leads to activation of MAP kinase (Gotoh et al., 1991) and S6 kinase II (Haccard et al., 1990).

Two forms of MAP kinase have recently been isolated and designated according to their molecular weights p42mapk and p44mapk (Sturgill and Wu, 1991). In p42mapk the phosphorylation sites have been mapped to Thr-183 and Tyr-185 (Payne et al., 1991). Boulton et al. (1990, 1991) have cloned several MAP kinases and provided evidence for the existence of a MAP kinase gene family of more than three members.

Two very similar kinases (FUS3 and KSS1) have been cloned from budding yeast. They play a pivotal role in the mating pathway. In the presence of a mating factor, the pathway is activated and leads to arrest of cell division. KSS1 was shown to activate CLN3, a G1-cyclin. Furthermore, KSS1 can only arrest cells in G1 when CLN3 is expressed. Therefore, KSS1 and CLN3 act along the same pathway (Courchesne et al., 1989). FUS3 appears to antagonize the action of KSS1 on CLN3 (Elion et al., 1990). KSS1 and FUS3 both contain the conserved Thr-183 and Tyr-185 phosphorylation sites found in p42mapk.

We report here the isolation of a *Medicago sativa* cDNA clone which putatively encodes the first MAP kinase homologue from plants. The plant protein kinase shows 52% identity to animal MAP kinases and contains the conserved Thr-183 and Tyr-185 residues. Northern analysis of synchronized cells revealed cell cycle-dependent expression of the plant MAP kinase. *In planta*, an organ-specific transcription pattern of the MAP kinase gene was observed.

Results

Isolation of alfalfa cDNA clone *MsK7*

A *M. sativa* cDNA library prepared from somatic embryos (Páy et al., 1993) was screened with a radioactively labelled probe that contained the kinase domain of the plant homologue to *shaggy*, a segment-polarity gene from *Drosophila* (Páy et al., 1993). The weakly hybridizing plaques were picked and rescreened at lower plating density. After *in vivo* excision of clone *MsK7*, the cDNA insert was determined to be approximately 1.5 kb. Sequencing the cDNA insert from both strands revealed an open reading frame of 1161 nucleotides (Figure 1) corresponding to a protein with a length of 387 amino acids.

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Figure 1. Nucleotide and deduced amino acid sequence (single letter code) of MsK7 cDNA.
MsK7 encodes a homologue to MAP kinase

Comparison of the alfalfa MsK7 protein with current protein sequence libraries revealed highest homologies to rat ERK1 (52%) and yeast FUS3 and KSS1 (50 and 49%, respectively). Alignment to the three sequences (Figure 2) shows all four proteins to have a similar structural arrangement of the 11 conserved kinase domains. With the exception of the first 20 amino acids, the sequences are highly similar including the very carboxy terminus. Considerable homology of the MsK7 kinase was also observed to the different homologues of the fission yeast cdc2 kinase (30–40%). Interestingly, the MAP kinases including MsK7 and the yeast FUS3 and KSS1 kinases look like amino and carboxy terminally extended versions of the cdc2 kinases. At each end, 30–40 amino acids are extending the basic cdc2 kinase structure.

MsK7 is expressed in a cell cycle-dependent manner

A cell suspension culture of the closely related alfalfa species, *Medicago varia* that consisted mostly of single cells, was cell cycle-arrested by addition of aphidicolin. After washing out the drug, the cells were followed through a synchronous division cycle. At different time points, aliquots were removed and analysed by flow cytometry (Figure 3). In asynchronous cultures (Figure 3.0) approximately 70% of the cells were in G1 phase. After treatment with aphidicolin for 24 h, 85% of the cells accumulated at the G1/S transition point (Figure 3.1). When the drug was washed out and cells were allowed to grow in fresh medium, nearly 60% of the cells passed through S phase synchronously (Figure 3.3) and entered G2 phase after 6.5 h (Figure 3.4). After 10 h, according to microscopic examination, about 30% of the cells were in mitosis. After 15 h, more than 60% of the cells were in G1 again. At the indicated time points in Figure 3 (0 to 8), aliquots were removed and analysed by Northern hybridization with a 32P-labelled MsK7 fragment (Figure 4, upper panel). In asynchronously growing and aphidicolin-arrested G1/S cells the kinase transcript levels were hardly detectable (Figure 4, upper panel, lanes 0 and 1, respectively). However, after release from the aphidicolin block for 5 h, when cells had entered S phase, MsK7 kinase transcript levels had accumulated (Figure 4, upper panel, lane 3). Throughout S and G2 phases, considerable amounts of MsK7 transcripts were observed (Figure 4, upper panel, lanes 3 to 6). When cells entered G1 phase of the next cell cycle (Figure 4, lane 7), MsK7 kinase mRNA levels had decreased to undetectable levels. As a control, the same set of RNAs were hybridized to the alfalfa homologue of the human translationally controlled tumour protein (Péy et al., 1992), which is denoted as Msc27 (Figure 4, bottom panel). Although there are minor variations in Msc27 transcript levels, the changes are clearly not occurring in parallel to those of the MsK7 kinase and indicate that the pattern of the MsK7 transcript levels represents true cell cycle-dependent fluctuation.

Stem- and root-specific expression of MsK7

To investigate the expression pattern of the MsK7 kinase in different organs, poly(A)+ RNA was prepared from the following alfalfa organs: young and mature roots, stems, etc.

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**Figure 2.** Alignment of the predicted amino acid sequences of alfalfa MsK7 with rat ERK1 and the yeast FUS3 and KSS1 kinases. Single dots indicate identical amino acids. Deletions were introduced to give maximal homology and are shown by dashes.

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Figure 3. Synchronization of an alfalfa suspension culture.
Flow cytometric analysis of cell synchrony. (0) Asynchronous cells; (1) cells after 24 h of aphidicolin arrest; (3 to 8) synchronized cells after 2.5, 5, 6.5, 8.5, 10.5, 15 and 24 h after release from the aphidicolin block. Samples were taken at the indicated time points and analysed by flow cytometry. The profiles represent the distribution of the cells in the different phases: the left peak constitutes cells with 2C levels of DNA, the right peak constitutes cells with 4C levels; the area between 2C and 4C peaks represents cells with an intermediate DNA content.

Figure 4. Cell cycle-dependent MsK7 transcript levels.
Cells were synchronized by aphidicolin for 24 h and then released from the block. Aliquots were taken after 2.5, 5, 6.5, 8.5, 10.5, 15 and 24 h. From 100 μg total RNA poly(A)⁺ RNA was selected and analysed by Northern hybridization with MsK7 (upper panel) or Msc27 (bottom panel). Lanes represent the following phases: 0, asynchronous cells; 1, aphidicolin-arrested G1/S cells; 2 and 3, S phase; 4 and 5, S2 phase; 6, M phase; 7 and 8, G1 phase.

nodes, young and mature leaves, flowers and suspension cultured cells. Northern hybridization with a ³²P-labelled MsK7 fragment (Figure 5, upper panel) showed high transcript levels in suspension cultured cells (Figure 5, upper panel, lane 8) and low levels in old roots and stems only (Figure 5, upper panel, lanes 2 and 3, respectively), but not in nodes, leaves or flowers (Figure 5, upper panel, lanes 4 to 7, respectively). The same filter was also hybridized with a ³²P-labelled Msc27 fragment as a control (Figure 5, bottom panel). Clearly, transcript levels of Msc27 are not constant in the different organs. Compared with the transcript levels found in suspension cultured cells (Figure 5, bottom panel, lane 8), relatively high Msc27 mRNA levels were detected in most organs (Figure 5, bottom panel, lanes 1 to 7, respectively) indicating that the organ-specific expression pattern of the MsK7 gene was not due to different amounts of loaded RNA.
cycle phase-specific transcript levels were observed. This disagrees with data reported from *Xenopus*, where no change of MAP kinase transcript levels was observed during mitotic cycles (Gotoh et al., 1991). However, since it is now clear that a family of MAP kinase genes exists in higher eukaryotes, it is likely that they should have different functions. It is expected that the different members would show different expression patterns in the cell cycle. Furthermore, since kinases are known to be regulated at both the transcriptional and the post-translational level, further complexity was anticipated. For this purpose, we are presently preparing antibodies against the MsK7 kinase to study the activity of the kinase during the cell cycle.

When different vegetative organs and flowers were analysed, only roots and stems were found to synthesize detectable MsK7 mRNA levels. Boulton et al. (1991) have isolated three MAP kinases (called ERK-1, -2 and -3) from rats and showed them to be expressed in a tissue- and stage-specific developmental manner. Southern analysis of alfalfa indicated the existence of a number of MAP kinases (data not shown) that may perform unique signalling roles in particular tissues or developmental programmes. Corroborative evidence comes from the isolation of PCR fragments that indicate the existence of at least three additional MAP kinases in alfalfa (Páy, unpublished results). Present work in our laboratory focusses on the question whether different members of this gene family are expressed in a tissue-specific or cell-cycle phase-specific manner.

**Experimental procedures**

**Library screening and sequence analysis**

Poly(A)⁺ RNA was isolated from somatic embryos of *M. sativa* suspension cultured cells (György et al., 1991). A lambda ZAP-cDNA library was prepared according to the manufacturer (Stratagene, California, USA). Approximately 300 000 colonies were hybridized with a ³²P-labelled alfalfa MsK1 kinase gene fragment encoding the homologue to the *Drosophila* shaggy kinase gene (Páy et al., 1993). One positive clone was isolated and sequenced from both directions with a T7 polymerase sequencing kit (Pharmacia LKB, Uppsala, Sweden).

**Plant tissue culture**

A suspension culture of *M. varia* (line A2) was used. The suspension culture was derived from callus tissue which originated from stem segments and was grown in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid and 0.2 mg l⁻¹ kinetin.

**Synchronization of alfalfa cells**

Synchronization of *M. varia* suspension cultured cells was performed by treating the cells with 20 µg ml⁻¹ aphidicolin (Sigma).
for 24 h. After washing out the drug, the cells were allowed to continue the cell cycle for 24 h. During this time, aliquots of the culture were taken for RNA preparation and for flow cytometry, which was performed as described by Pfoerster (1989).

Southern analysis

DNA (10 μg) of M. sativa was digested with HindIII and separated on 0.7% agarose gels. After blotting, the nylon filters were hybridized to the coding sequence of the MsK7 clone. Washing of the filter was done with 2 × SSC, 0.1% SDS for 30 min at room temperature for low stringency. After exposure of the filter, it was washed with 0.1 × SSC, 0.1% SDS for 30 min at 60°C for high stringency.

RNA extraction and Northern analysis of transcript levels

Suspension cultured cells or plant tissue was frozen in liquid nitrogen and extracted by grinding in a mortar as described (Györgey et al., 1991). Total RNA was quantified by gel electrophoresis and ethidium bromide staining and by spectroscopy. Of each sample 100 μg of total RNA were poly(A)+-purified by Dynabeads according to the manufacturer’s instructions (Dynal, Oslo, Norway). Poly(A)+ RNAs were separated on denaturing agarose gels, blotted to nylon filters and hybridized with a 32P-labelled MsK7 cDNA fragment according to standard procedures.

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References


EMBL Data Library, GenBank and DDBJ databases accession number X66469.