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MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function

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Abstract Mitogen-activated protein (MAP) kinases are serine/threonine protein kinases that are activated in response to a variety of stimuli. Here we report the isolation of an alfalfa cDNA encoding a functional MAP kinase, termed *MMK2*. The predicted amino acid sequence of *MMK2* shares 65% identity with a previously identified alfalfa MAP kinase, termed *MMK1*. Both alfalfa cDNA clones encode functional kinases when expressed in bacteria, undergoing autophosphorylation and activation to phosphorylate myelin basic protein in vitro. However, only MMK2 was able to phosphorylate a 39 kDa protein from the detergent-resistant cytoskeleton of carrot cells. The distinctiveness of *MMK2* was further shown by complementation analysis of three different MAP kinase-dependent yeast pathways; this revealed a highly specific replacement of the yeast *MPK1*(*SLT2*) kinase by *MMK2*, which was found to be dependent on activation by the upstream regulators of the pathway. These results establish the existence of MAP kinases with different characteristics in higher plants, suggesting the possibility that they could mediate different cellular responses.

Key words Serine/threonine protein kinase · MAP kinase · Signal transduction · Alfalfa · MPK1

Introduction

Mitogen-activated protein (MAP) kinases comprise a family of serine/threonine kinases that are activated in specialized cell types in response to mitogenic stimuli (Hoshi et al. 1988; Ray and Sturgill, 1987; Rossomondo et al. 1992), but also during meiosis (Gotoh et al. 1991; Posada et al. 1991), differentiation (Boulton et al. 1991; Gotoh et al. 1990) or various stresses (Ely et al. 1990; Galcheva-Gargova et al. 1994; Han et al. 1994; Stratton et al. 1991). MAP kinase activation requires phosphorylation on tyrosine and threonine residues (Anderson et al. 1990; Posada et al. 1991) and is mediated by a single dual-specificity activator protein kinase. MAP kinase kinase (Alessandrini et al. 1992; Crews and Erikson 1992; Matsuda et al. 1992). Activation of the MAP kinase activator occurs by phosphorylation on threonine residues by other protein kinases, Raf-1 and MAP kinase kinase kinases (Hattori et al. 1992; Kyriakis et al. 1992; Lange-Carter et al. 1993). This set of three functionally interlinked protein kinases has been identified in animals, yeast and plants and appears to be conserved in modular form in diverse signal transduction pathways in all eukaryotes (for review, see Jonak et al. 1994).

Several MAP kinase-based signal transduction pathways have been identified in budding yeast. The FUS3 (Elion et al. 1991) and KSS1 (Couchesne et al. 1989) MAP kinases are mediators of the pheromone signaling pathway. When cells are treated with pheromone, FUS3 and KSS1 become activated and are directly responsible for cell cycle arrest and differentiation, through activation of the transcription factor STE12. Whereas both kinases are required for the differentiation program, FUS3 is able to mediate cell cycle arrest through phosphorylation of FAR1. The HOG1 MAP kinase is involved in osmoregulation (Brewster et al. 1993). *S. cerevisiae* normally responds to increase in external osmolarity by increasing glycerol synthesis and decreasing glycerol permeability, leading to accumulation of cytoplasmic glycerol in concentrations in the molar range. HOG1 mediates this response. The MPK1 (SLT2) (Lee et al. 1993; Mazzoni et al. 1993; Torres et al. 1991) MAP kinase participates in a protein

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kinase cascade mediated by protein kinase C (Lee et al. 1993) and leads to a temperature-dependent cell lysis phenotype.

Plant MAP kinases have been identified from alfalfa, *Arabidopsis*, pea and tobacco (Duerr et al. 1993; Jonak et al. 1993; Mizoguchi et al. 1993; Stafstrom et al. 1993; Wilson et al. 1993). Tobacco genes with homology to MAP kinase kinases (Shibata et al. 1995) and MAP kinase kinase kinases (Banno et al. 1993) have also been isolated and indicate that plants contain MAP kinase signal transduction modules similar to those that have been identified in animals and yeasts. The function of the majority of the plant MAP kinases is largely unclear, but preliminary evidence indicates a role for MAP kinases in plant horomone signaling, such as auxin-induced cell division (Mizoguchi et al. 1994) or ethylene responses (Kieber et al. 1993).

We report here the isolation of an alfalfa cDNA that encodes a MAP kinase, MMK2, that can functionally replace the yeast MPK1 (SLT2) kinase and is dependent on functional upstream regulators of the MPK1 signal transduction pathway for activity.

Materials and methods

Gene isolation, cloning and sequencing

Two redundant synthetic oligonucleotides, MAP1: 5′-TTGAATTCGGNAAC/TGCNTTC/TGAC/TAA-3′ and MAP2: 5′-TTTCTAGANGTNACNACA/GTAC/TTCNGT-3′, were prepared (N stands for all four nucleotides), corresponding to the GNAFDN and TEYVVT motifs which are relatively specific for MAP kinases. At the 5′ MAP1 contains *Eco*RI site, whereas MAP2 carries an *Xba*I site at the 3′. Plasmid DNA from an alfalfa cDNA library prepared from somatic embryos (Pay et al. 1993) was used as template for PCR amplification. Reaction conditions were as described (Gould et al. 1989). A 300 bp PCR reaction product was isolated from a 2% agarose gel, restricted with *Eco*RI + *Xba*I and cloned into pBluescript SK(−) vectors (Stratagene, LaJolla, Calif.). Positive clones were identified by DNA sequence analysis with a T7 sequencing kit (Pharmuan, Uppsala, Sweden) ADNA fragment corresponding to a novel class of MAP kinases was radiolabeled by random priming and used as a probe to screen an alfalfa cDNA library which was prepared from suspension cultures of alfalfa (*Medicago varia* A2) cells. From 5 μg of poly $(A)^+$ RNA, cDNA was generated and cloned into λZAPII according to the manufacturer's recommendations (Stratagene). Of six positive cDNA clones, two contained sequences identical to the previously isolated alfalfa MAP kinase, hereafter denoted by MMK1 (Jonak et al. 1993). The other four clones contained DNA sequences that were identical to the sequence which was used as screening probe. The longest clone contained a cDNA insert of 1600 bp and was sequenced completely. It identified a novel putative alfalfa MAP kinase and was therefore termed MMK2.

Expression of alfalfa MMK1 and MMK2 in bacteria

To study the MMK1 and MMK2 kinase activities, the alfalfa MAP kinases were cloned into the pGEX-3x vector (Pharmacia). *Bam*HI sites were introduced before and after the open reading frames of pmmk1 and pmmk2 via PCR amplification with the following primers: for pmmk1, the 5′ and 3′ primers were: 5′-TTTTGGATCCGAGAAACAACAATGGAAG-3′ and 5′-ATATGGATCCACTACTGCTGGTACTCAG-3′; for pmmk2, the 5′ and 3′ primers were: 5′-TTTTGGATCCACATGTCTGTTGAATCAGC-3′ and 5′-ATATGGATCCGAGCACCGTCATAACG-3′. After transforming *Escherichia coli* with these constructs, positive clones containing the fragments in the right orientation were isolated and sequenced to verify that no mutations had occurred in the open reading frames. Preparation of the glutathione-S-transferase (GST) fusion proteins and affinity purification was done as described (Ausubel et al. 1991). Protein concentration was determined with a BioRad detection system (BioRad, Richmond, Calif). Proteins were collected in 50 μl aliquots, frozen in liquid nitrogen and stored at −80° C. The quality of the purification was checked on denaturing 10% polyacrylamide gels.

Preparation of detergent-resistant proteins from carrot cells

Cytoskeletons were prepared from carrot cells by extracting protoplasts with NP40 and then with Triton X-100, essentially as described by Xu et al. (1992). These preparations contain the nucleus, microtubules, actin filaments and fibrillar bundles (Hussey et al. 1987). They were extracted in 0.6 M NaCl, 3 mM Ca^{2+}, 25 mM PIPES, 1 mM dithiothreitol, 1 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 1 mg/ml DNase (DN-EPSigma), containing several protease inhibitors, pH 6.9 at 4° C for 45 min. This was centrifuged at 38000 g for 20 min at 4° C. The supernatant was diluted to 0.15 M NaCl, brought to 5 mM EGTA, 100 mM PIPES, 1 mM GTP, 50 mg/ml RNase, 5%(v/v)DMSO, 10 μM taxol, pH 6.9 and incubated at 30° C for 20 min before centrifugation. The pellet was re-extracted in Ca^{2+}/NaCl and the solubilized proteins treated with taxol/EGTA as before. The final pellet was stored at −20° C. This fraction has endogenous kinase activity and was therefore heat inactivated for 10 min at 80° C before use as a substrate for the bacterially expressed kinases. Monospecific antibodies to the 39 kDa protein were purified by the method of Olmsted (1986) from a rat antiserum raised against the cytoskeletal fraction. Because microtubule-associated proteins are well known substrates of MAP kinases, particular attention was paid to possible staining of the cytoplasmic microtubules. In controls, the tubulin antibody YOL1/34 could be eluted from nitrocellulose-bound tubulin in identical conditions and still stained microtubules in immunofluorescence experiments. Antibodies to the 39 kDa protein did not stain any cytoplasmic component of the cytoskeleton but clearly stained the nucleus.

In vitro phosporylation assays

To test whether the GST-MMK1 and GST-MMK2 fusion proteins were able (i) to undergo autophosphorylation and (ii) to phosphorylate other substrates, in vitro phosphorylation assays were carried out in 30 mM HEPES pH 7.5, 40 mM KCl, 4 mM $MgCl_2$, 5.2% glycerol, 0.06 mM ATP and 4 μCi γ-^{32}P-ATP at 30° C for 10 min. One microgram of myelin basic protein (Sigma) or 5 μg heat-treated (10 min, 80° C) carrot detergent-resistant proteins were used as substrates. Control reactions were also performed with (i) substrate alone, (ii) substrate plus glutathione-S-transferase, and (iii) glutathione-S-transferase alone. Samples were either frozen at −20° C or analyzed directly on denaturing 15% polyacrylamide gels before autoradiography.

Yeast strains, growth conditions, transformation, and nucleic acid manipulations

The yeast strains used in this study are listed in Table 1. Yeast cultures were grown in YPD (1% yeast extract, 2% bactopeptone and 2% glucose). Synthetic minimal medium (Sherman et al. 1979), supplemented with the appropriate nutrients, was employed to

Table 1 Yeast strains used

Strain	Genotype	Source or reference
DL376	*MAT***a**, *pck1Δ::LEU2, leu 2-3, 112, ura3-52, trp1-1, his4, can1*	Levin and Bartlett-Heubusch (1992)
DL456	*MAT***a**/*MATα*, *mpk1Δ::TRP1/mpk1Δ:: TRP1, leu2-3/leu2-3, 112, ura3-52/ura3-52, trp1-1/trp1-1, his4/his4, can1*^{r}/*can1*^{r}	Lee et al. (1993)
DL1059	*MAT***a**/*MATα*, W303, *bck1Δ::HIS3/bck1Δ::HIS3*	D. E. Levin
JBY10	*MAT***a**, *hog1Δ::TRP1, ura3, leu2, lys2, ade2, his3, trp1*	Brewster et al. (1993)
K217	*MATα*, *his1*	Gartner et al. (1993)
K2381	*MAT***a**, *bar1::HISG, fus1-PacZ-URA3, hoΔ, HML***a**, *HMR***a**, *ho::lacZ, ura2-52, ade2-1, can1-100, his3, leu2-3, 112, trp1-1, met*	Gartner et al. (1993)
K2702	*MAT***a**, *bar1::HISG, fus3::LEU2, kss1::URA3, fus1-lacZ-URA3, hoΔ, HML***a**, *HMR***a**, *ho::lacZ, ura2-52, ade2-1, can1-100, his3, leu2-3, 112, trp1-1, met*	Gartner et al. (1993)
RC11	*MAT***a**/*MATα*, *pkc1*^{ts}, *his3, leu2, trp1, ura3, met3*	D. E. Levin
YPH499	*MAT***a**, *ura3, leu2, lys2, ade2, his3, trp1*	Brewster et al. (1993)

select for plasmid maintenance. Yeast transformation was done by the lithium acetate method (Ito et al. 1983).

Complementation of yeast mutants

To assay for complementation of yeast MAP kinases, PCR-amplified ORFs of *MMK1* and *MMK2* were generated as described above and subcloned as *Bam*HI fragments into pSP72 (Promega, Madison, Wis.). Sequence analysis confirmed the integrity of the *MMK1* and *MMK2* coding regions. Expression of the alfalfa kinases in the respective yeast transformants was quantified by Western blot analysis.

To test for complementation of the *FUS3* and *KSS1* gene functions, the *MMK1* and *MMK2* ORFs were isolated from pSP72 plasmids as *Eco*RI–*Hin*dIII fragments and cloned into the yeast expression vector pGA1840 (Gartner et al. 1992) containing the *TRP1* selection marker gene. Expression of the *MMK1* and *MMK2* genes was under the control of the triosephosphate isomerase (*TPI1*) promoter. K2702, a yeast strain with deletions in both the *FUS3* and the *KSS1* genes (Gartner et al. 1992), was transformed with pGA1840-*MMK1* or pGA1840-*MMK2*. As a positive control, an isogenic strain (K2381) containing the wild-type *FUS3* and *KSS1* genes was used. Complementation was tested by the ability to (i) mate with a K217 tester strain (Hartwell 1980), (ii) arrest growth after treatment with pheromone (Sprague 1991), or (iii) induce β-galactosidase activity from an integrated copy of a *FUS1* promoter-*lacZ* fusion gene (Breeden and Nasmyth 1987).

To test for replacement of the *HOG1* gene function, the *MMK1* and *MMK2* ORFs were cut out from pSP72 plasmids as *Xho*I–*Eco*RI fragments and cloned into the yeast expression vector pFL60. pFL60 contains the constitutive phosphoglycerate kinase promoter and the *URA3* gene as selection marker (Minet and Lacroute 1990). JBY10 is an osmoregulation-defective yeast strain deleted for *HOG1* and fails to grow on high-osmolarity medium (Brewster et al. 1993). By transforming JBY10 with pFL60-*MMK1* and pFL60-*MMK2* we tested whether these plant MAP kinases were able to complement the mutation. As a positive control, the isogenic yeast strain YPH499, containing the intact *HOG1* gene, was used. Two different strategies were followed: (i) After transformation, single colonies were streaked out on plates containing different concentrations of NaCl (0.3–0.7 M) and tested for growth at 28° C; (ii) catalase assays were performed as described (Schüller et al. 1994).

DL456, a yeast strain which is deleted for the *MPK1*(*SLT2*) gene, can grow on rich medium at 28° C but not at 37° C (Lee et al. 1993). The cells are able to grow at 37° C in a medium that is enriched with 1 M sorbitol as an osmotic stabilizing agent DL456 cells were transformed with pFL60-*MMK1* and pFL60-*MMK2* or with pFL60 alone as a negative control. As a positive control a 3 kb genomic fragment carrying the yeast *MPK1*(*SLT2*) gene (a kind gift of G. Ammerer) was cloned into the *E. coli*/yeast shuttle vector pRS316, which is a *CEN*-based vector that carries the *URA3* gene as selection marker (Sikorski and Hieter 1989). Transformed cells were selected on uracil-free medium plates in the presence of 1 M sorbitol. Transformants were visible after 2 days at 28° C. To test for complementation, single colonies were streaked out onto YPD plates and incubated at 28° C or at 37° C.

RC11 is a yeast strain with a temperature-sensitive *PKC1* allele (Lee and Levin 1992) and can grow at 28° C but not at 37° C in the absence of osmotic stabilizers. To test for suppression of this phenotype by alfalfa MAP kinases, RC11 cells were transformed with pFL60-*MMK1* and pFL60-*MMK2* or pFL60 alone, and selected on uracil-free medium in the presence of 1 M sorbitol. Transformants were incubated at 28° C or 37° C on 1 M sorbitol medium plates. Strains DL376 and DL1059, in which the *PKC1* and *BCK1* genes, respectively are deleted (Lee and Levin 1992), can grow at 28° C on medium containing 1 M sorbitol. Both strains were transformed with pFL60-*MMK1* and pFL60-*MMK2*, or with pFL60 alone, and selected at 28° C on uracil-free medium in the presence of 1 M sorbitol. Transformants were tested for suppression by incubation at 28° C on medium containing no sorbitol.

Results

Isolation of alfalfa *MMK2* cDNA

Synthetic oligonucleotides were prepared corresponding to two conserved sequences preferentially found in MAP kinases. PCR amplification using an alfalfa cDNA library as template yielded fragments with a length of 300 nucleotides that were cloned and analyzed by sequencing. Two different classes of clones were obtained. One class of clones corresponded to a MAP kinase cDNA that had previously been isolated and is hereafter denoted as *MMK1* (Jonak et al. 1993). The other class of clones showed several differences from *MMK1* in the predicted amino acid sequences and was used as a probe to screen the alfalfa cDNA library. Out of six cDNAs isolated, two corresponded to *MMK1* and four to the novel *MMK2* class. The longest cDNA of this class had a length of approximately 1600 nucleotides, which corresponded closely to the size of the transcript detected by Northern blot analysis

(data not shown). Sequence analysis revealed an ORF of 1113 nucleotides, potentially encoding a 371 amino acid polypetide. Several in-frame stop codons upstream of the ATG at position 283 indicate that this is the correct translational initiation codon and that the transcript possesses a relatively long (282 nucleotides) untranslated 5′ region. In contrast, the translational stop codon is followed by an unusually short 3′ non-translated region (144 nucleotides) containing a poly A tail of 26 nucleotides.

MMK2 encodes a novel alfalfa MAP kinase

Comparison of the predicted MMK2 protein sequence with current data banks revealed up to 46% identity to various animal MAP kinases and 42–48% identity to budding yeast kinases. MMK2 showed 65% identity to MMK1, a previously identified alfalfa MAP kinase (Duerr et al. 1993; Jonak et al. 1993); 52–74% identity was obtained when MMK2 was compared to the MAP kinases reported from other plant species (Mizoguchi et al. 1993; Stafstrom et al. 1993; Wilson et al. 1993).

Alignment of the MMK2 protein sequence with those of alfalfa MMK1 (Jonak et al. 1993), rat ERK1 (Boulton et al. 1991) and the yeast FUS3 (Elion et al. 1991), MPK1(SLT2) (Torres et al. 1991) and HOG1 (Brewster et al. 1993) sequences (Fig. 1) shows that the most conserved amino acids lie in a central region that is involved in catalysis of the phosphotransfer reaction. The threonine and tyrosine residues that are required for activation of animal, yeast and plant MAP kinases

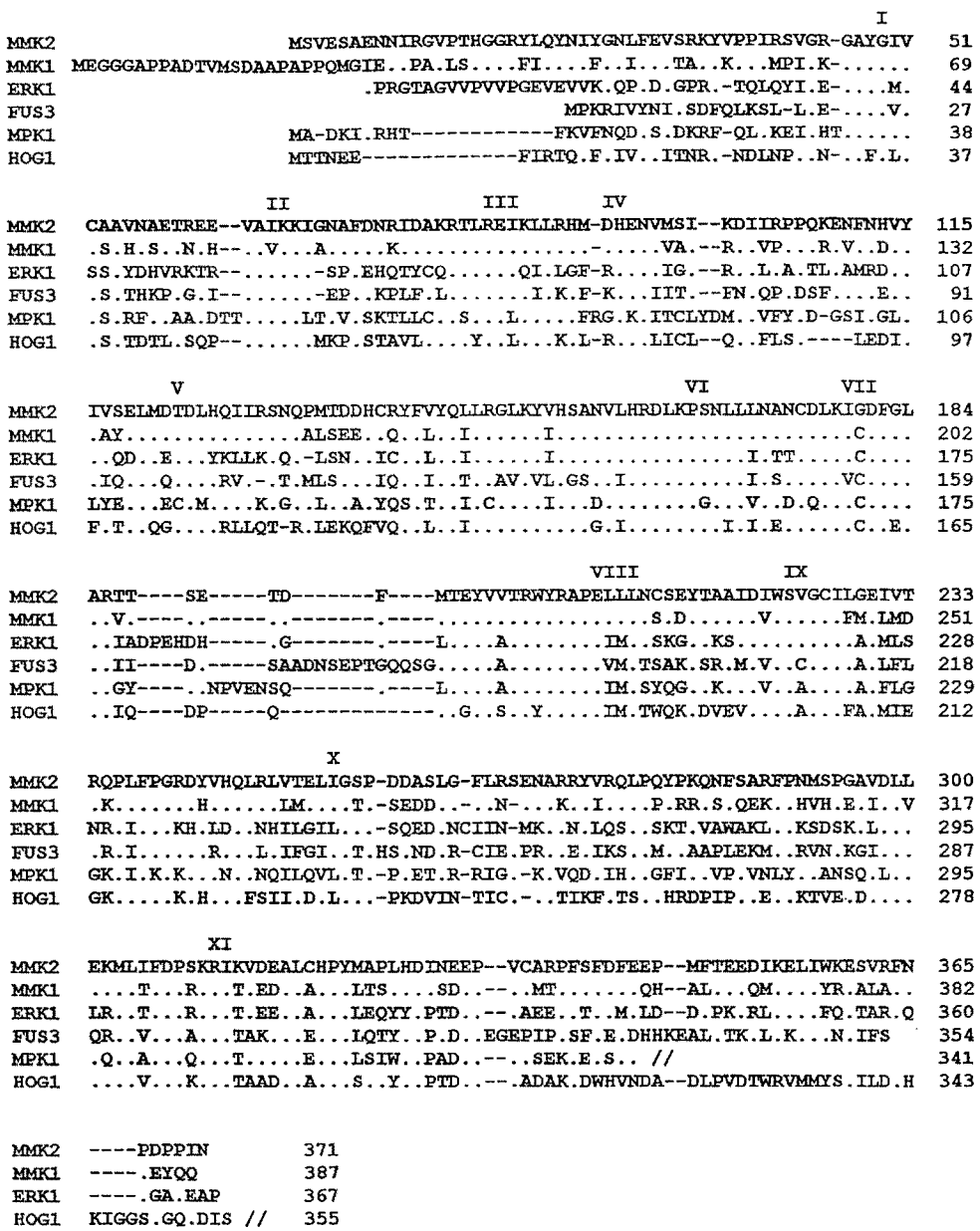

Fig. 1 Alignment of the deduced protein sequences of alfalfa MMK1 (Jonak et al. 1993) and MMK2, rat ERK1 (Boulton et al. 1991), and yeast FUS3 (Elion et al. 1991), MPK1(SLT2) (Torres et al. 1991) and HOG1 (Brewster et al. 1993). Amino acids are shown in the single-letter code. Identical amino acids in all sequences are indicated by *single dots*. For optimal alignment, gaps were introduced into the sequences and are shown by *dashes*. Diagonals indicate the presence of additional amino acids that are not shown in the alignment. The eleven domains that define the catalytic region of all serine/threonine protein kinases are represented by Roman numerals above the respective regions. The nucleotide and predicted protein sequence of MMK2 is available in the GSDB, DDBJ, EMBL and NCBI data bases under the accession number X82268

are also found in the MMK2 kinase at positions 195 and 197. In contrast, the N-terminal and C-terminal regions diverge considerably and can be expected to be responsible for substrate specificities or interaction with different upstream regulators.

MMK2 encodes a functional MAP kinase

To determine whether the alfalfa *MMK1* and *MMK2* cDNAs encode functional MAP kinases, the genes were expressed as glutathione-S-transferase fusion proteins in *E. coli* and affinity purified. Both GST-MMK1 and GST-MMK2 kinases showed autophosphorylation after in vitro incubation with ^{32}P-γ-ATP (Fig. 2, lanes 1 and 3, respectively). When myelin basic protein was added as external substrate, both kinases were able to transfer ^{32}P-label onto the substrate (20 kDa band in Fig. 2, lanes 2 and 4). Whereas myelin basic protein was phosphorylated to very high levels by GST-MMK2 (Fig. 2, lane 4), much less label was transferred by the GST-MMK1 kinase under the same conditions (Fig. 2, lane 2). These data indicate that both the *MMK1* and the *MMK2* genes encode functional MAP kinases but show different phosphorylation rates on myelin basic protein as substrate.

MMK2 phosphorylates a 39 kDa protein in the detergent-resistant skeleton of carrot cells

Since cytoskeletal proteins have been shown to be targets of metazoan MAP kinases, we tested both MMK1 and MMK2 for kinase activity using detergent-resistant skeletal proteins as substrates. When these preparations were incubated with [γ-^{32}P] ATP alone (Fig. 3A, lane 1), no phosphorylation was detected (Fig. 3B, lane 1). Addition of GST-MMK1 (arrowhead in Fig. 3A, lane 2) only resulted in autophosphorylation of the GST-MMK1 protein (Fig. 3B, lane 2). However, when GST-MMK2 kinase was incubated with the preparation (arrowhead in Fig 3A, lane 3), a 39 kDa protein was specifically phosphorylated (arrow in Fig. 3B, lane 3) in addition to the autophosphorylated kinase (arrowhead in Fig. 3B, lane 3). These results show that MMK kinases have different substrate specificities in vitro and suggest that the two alfalfa MAP kinases might also have different targets in vivo.

To determine whether the 39 kDa protein – a target of MMK2 – was located in one of the various cytoskeletal filaments or the nuclear skeleton, an immune serum was raised in rats against the final pellet. This was then affinity purified using the 39 kDa protein following transfer to nitrocellulose by Western blotting. By immunofluorescence, the affinity-purified antibodies, but not the preimmune serum, were found to stain nuclei in detergent-resistant cytoskeletons (data not shown).

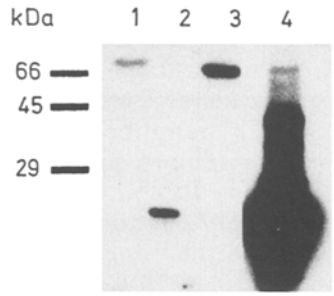

Fig. 2 In vitro kinase activity of MMK1 and MMK2. One microgram of affinity purified GST-MMK1 and GST-MMK2 were incubated with [γ-^{32}P] ATP alone (lanes 1 and 3, respectively) or together with 1 µg myelin basic protein as substrate (lanes 2 and 4, respectively). After separation of the samples on 15% denaturing polyacrylamide gels, autoradiography was performed for 1 h. The additional bands in lane 4 derive from contaminant proteins in the myelin basic protein preparation

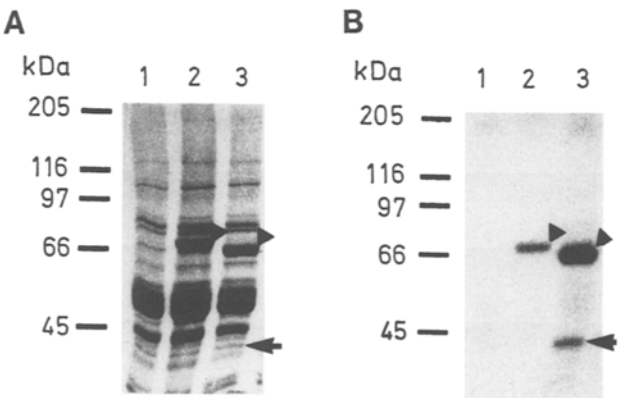

Fig. 3A, B MMK2 specifically phosphorylates a 39 kDa protein in detergent-resistant cytoskeleton preparations from carrot cells. Five micrograms of cytoskeletal protein was incubated with [γ-^{32}P] ATP alone (lanes 1) or together with 1 µg of affinity-purified GST-MMK1 or GST-MMK2 (lanes 2 or 3, respectively) and analyzed on 15% SDS polyacrylamide gels. *Arrowheads* in lanes 2 and 3 indicate the GST-MMK1 and GST-MMK2 protein respectively. The *arrow* indicates the 39 kDa protein that is specifically phosphorylated by MMK2. **A** Coomassie-stained gel. **B** Autoradiogram of the same gel as shown in **A**

MMK2 specifically complements mutations in the yeast *MPK1* (*SLT2*) gene

To test whether the function of the MMK1 and MMK2 kinases is evolutionarily conserved, three different yeast signal transduction pathways were investigated by complementation analysis with the two alfalfa kinases.

Deletion of the *MPK1*(*SLT2*) gene, encoding a yeast MAP kinase acting downstream of the yeast protein kinase C homologue *PKC1*, results in a temperature-dependent cell lysis defect. Strain DL456, in which the *MPK1*(*SLT2*) gene is deleted, can grow at 28° C

on rich medium. To permit cell growth at 37° C, the medium has to be supplemented with 1 M sorbitol. To test whether alfalfa MMK1 or MMK2 can functionally substitute for the yeast MPK1 (SLT2) kinase, DL456 cells were transformed with pFL60-*MMK1* and pFL60-*MMK2* plasmids and grown at 37° C without osmotic stabilizers. In contrast to *MMK2*-expressing cells, which could grow at 37° C, *MMK1*-containing cells were unable to grow under these conditions (Fig. 4). Whereas DL456 cells containing the empty pFL60 vector could only grow at 28° C in the absence of osmotic stabilizers (control in Fig. 4), DL456 cells transformed with the pRS316 vector carrying the intact *MPK1*(*SLT2*) gene could grow equally well at 37° C (Fig. 4). To exclude the possibility that the inability of the *MMK1* gene to complement the *mpk1*(*slt2*) deletion mutant was due to low-level expression, the amounts of MMK1 and MMK2 protein in DL456 cells were quantified by Western analysis with antibodies directed against the MMK proteins. Both MMK1 and MMK2 proteins were present in cell extracts, but the levels of MMK1 were approximately two to three times higher than MMK2 levels, indicating that lack of MMK1 protein was not the cause of the complementation deficiency (data not shown). These results show that MMK2 can replace the function of the yeast *MPK1*(*SLT2*) gene in a highly specific way.

MMK1 and *MMK2* cannot complement mutations in yeast *FUS3, KSS1* or *HOG1* genes

Yeast cells prepare for mating by arresting in the G1 phase of the cell cycle undergoing a specific differentiation program, called shmooing. Haploid yeast cells secrete specific pheromone peptides that bind to receptors and inititate these processes in cells of the opposite mating types. A signal transduction cascade, including a MAP kinase module, transduces the pheromone signal intracellularly and is one of the best understood signal transduction mechanisms in all eukaryotes. Cells of K2702, a yeast strain in which both the *FUS3* and the *KSS1* MAP kinase genes of this pathway have been deleted, are viable but unable to arrest or mate on pheromone-containing medium. Expression of either alfalfa MAP kinase in K2702 cells in the presence of pheromone did not result in increased mating capability or cell cycle arrest. Activation of FUS3/KSS1 also leads to transcription of the *FUS1* gene. *FUS1* promoter-*lacZ* fusions can therefore be used as an indicator of FUS3/KSS1 activity. When β-galactosidase activity in mutants bearing either alfalfa gene was monitored in a colony colour assay in the presence and absence of pheromone, no increase in activity was observed (data not shown), indicating that the *FUS1* promoter was not induced. Taken together, these data indicate that neither the FUS3 nor the KSS1 functions can be substituted by MMK1 or MMK2.

Adaptation to hyperosmotic conditions is a highly conserved process in yeast and animals and is mediated by a MAP kinase module. To determine whether MMK1 or MMK2 can replace the function of the yeast HOG1 MAP kinase that is responsible for mediating the adaptive response to hyperosmotic challenge, the alfalfa MAP kinases were expressed in JBY10 cells, which fail to grow on high osmolarity medium, due to deletion of the *HOG1* gene. Expression of both alfalfa MAP kinases in these cells was confirmed by Western blotting but did not increase cell viability even under moderate salt stress conditions. Since the above analysis might not have detected partial replacement of the kinase functions, another assay involving measurement of catalase activity which is also known to be regulated by the HOG1 pathway (Schüller et al. 1994) was performed with the same strains. However, increased catalase activity could only be obtained with cells containing an intact *HOG1* gene. Therefore, neither MMK1 nor MMK2 can functionally substitute for the *HOG1* gene in yeast.

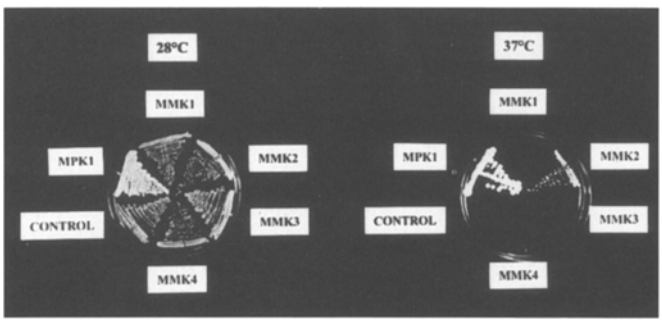

Fig. 4 Expression of *MMK2* specifically suppresses the temperature-sensitive growth defect of yeast cells deleted for *MPK1* (*SLT2*). DL456 cells that are transformed with vector alone (Control) or with an *MMK1* expression vector fail to grow at high temperatures (37° C). In contrast, expression of wild-type *MPK1* or alfalfa *MMK2* restores the capacity to grow at 37° C

MMK2 cannot rescue defective upstream components of the *MPK1* pathway

MPK1 lies on the yeast PKC1 signal transduction pathway (Lee et al. 1993) and is regulated through phosphorylation by the MKK1 and MKK2 kinases (Irie et al. 1993). The BCK1 protein kinase is an activator of the MKK1 and MKK2 kinases and is itself regulated by PKC1 (Lee and Levin 1992). Mutant alleles of the *BCK1* gene can suppress the cell lysis defect of a *pkc1* deletion mutant (Lee and Levin 1992), indicating that the dominant downstream components can suppress a defective upstream regulator of the pathway. We have found that the alfalfa MMK2 can complement the function of the MPK1 kinase,

indicating that the MMK2 kinase recognizes and phosphorylates the substrate(s) of the yeast MPK1 kinase. This ability could depend on activation of MMK2 via the upstream regulators of the MPK1 pathway. Alternatively, since bacterially produced MMK2 kinase shows autoactivation in vitro, it could be imagined that the kinase acts independently of the pathway. To distinguish between these possiblities, the RC11 yeast strain carrying a temperature-sensitive *PKC1* allele (Levin and Bartlett-Heubusch 1992) was transformed with an *MMK2* expression vector. RC11 cells can grow at 28° C but not at 37° C in the absence of osmotic stabilizers. Expression of the *MMK2* gene did not suppress this phenotype. Similar results were obtained with DL376 cells, in which the *PKC1* gene was deleted (Levin and Barlett-Heubusch 1992). Growth of DL376 cells at 28° C was restricted to medium containing 1 M sorbitol. Expression of the *MMK2* gene did not lead to growth on medium lacking the osmotic stabilizer. DL1059 cells, which are disrupted in the *BCK1* gene (Lange-Carter et al. 1993), could also not be suppressed by expression of the *MMK2* gene. These results indicate that mutant upstream components of the MPK1 pathway can not be rescued by MMK2.

Discussion

MAP kinases have been reported from animals, yeasts and plants and appear to be involved in a multitude of signal transduction processes in all eukaryotic cells. Different responses imply regulation of specific proteins. One intriguing question is how so many different substrates can be regulated by just a few MAP kinases. One possibility is to regulate the access of the MAP kinases to different substrates. This actually appears to be the case in several cell types after mitogen stimulation where MAP kinases become compartmentally relocated (Gonzalez et al. 1993; Lenormand et al. 1993). Another possibility is to use different MAP kinases for different sets of substrates in the same cell. Evidence for this comes from the identification of several MAP kinases in budding yeast (Brewster et al. 1993; Couchesne et al. 1989; Elion et al. 1991; Lee et al. 1993; Mazzoni et al. 1993). These kinases belong to a diverged family of MAP kinases and function in separate signalling pathways and appear to have distinct substrate specificities. A third possibility is to restrict the expression of particular MAP kinases to particular cells within a multicellular organism. The expression of the three related mammalian MAP kinases *ERK1*, *ERK2* and *ERK3* has been shown to occur in a developmental stage- and tissue-specific way (Boulton et al. 1991) and was suggested to mediate responses in different cell types and developmental stages, or to different stimuli. Recently, another MAP kinase (Jun kinase) has been cloned (Kyriakis et al. 1994) and was shown to respond to a restricted set of stimuli (e.g. stress factors but not to growth factors). Therefore, it is expected that, as for yeast, higher eukaryotes also use separate MAP kinases for different signalling pathways. In order to analyze the situation in plants, we decided to isolate cDNAs coding for different MAP kinases and compare their properties. We report here the isolation of a cDNA that encodes a novel type of alfalfa MAP kinase, MMK2, and show that its expression in bacteria yields an active protein kinase. Comparison of the abilities of the MMK2 kinase and a previously isolated alfalfa MAP kinase, MMK1, to phosphorylate myelin basic protein reveals that the kinases differ in their capacity to phosphorylate this artificial substrate, suggesting that their physiological substrates might also be distinct. In a search for more appropriate substrates we employed detergent-extracted carrot cytoskeletons composed of microtubules, actin filaments, intermediate filament-related fibrillar bundles and the nuclear skeleton (Hussey et al. 1987; Xu et al. 1992). MMK2, but not MMK1, specifically phosphorylated a 39 kDa protein in these preparations. Monospecific antibodies to the 39 kDa protein did not stain any cytoplasmic component of the cytoskeleton but clearly stained the nucleus. This nuclear protein is the subject of further investigation. Nevertheless, regardless of the exact identity of this protein, the different specificities of MMK1 and MMK2 with respect to myelin basic protein and cytoskeletal substrates clearly establish that the kinases have different properties and might therefore mediate different cellular responses.

To test the possibility that different MAP kinases have retained functional specificities over large evolutionary distances in eukaryotes, we expressed the alfalfa *MMK1* and *MMK2* genes in different yeast strains in which the endogenous MAP kinase genes were deleted. Out of four different yeast MAP kinases, only the yeast MPK1(SLT2) kinase could be functionally replaced by the alfalfa MMK2 kinase. Several lines of evidence indicate that the ability of the MMK2 kinase to complement the MPK1(SLT2) MAP kinase is highly specific and suggests functional conservation of the pathway between yeast and higher plants. A closely related alfalfa MAP kinase, MMK1, could not functionally substitute for the yeast MPK1(SLT2) kinase. Since alfalfa MMK2 has severalfold higher in vitro kinase activity against myelin basic protein than alfalfa MMK1, it could be argued that MMK1 activity might not suffice to restore the function of the deleted yeast MAP kinase. However, another alfalfa MAP kinase that has recently been isolated shows severalfold higher kinase activity towards myelin basic protein in vitro but still cannot complement the yeast *mpk1*(*slt2*) mutant (our unpublished results). Therefore, complementation of the yeast MPK1(SLT2) function by a heterologous MAP kinase does not correlate with the in vitro kinase activity against myelin basic protein but requires substrate

specificities highly similar to the MPK1(SLT2) kinase. Functional conservation of MAP kinase function in yeast and metazoans has been shown for two mammalian MAP kinases that are activated in response to osmotic stress (Galcheva-Gargova et al. 1994; Han et al. 1994) and which can complement the HOG1 kinase that is involved in the transduction of hyperosmolarity signals in yeast (Brewster et al. 1993).

We also investigated whether MMK2 could suppress upstream mutants of the MPK1(SLT2) pathway. Previous studies showed that dominant alleles of downstream components of the MPK1 pathway can suppress the lytic phenotype of *pkc1* deletion mutant (Lee and Levin 1992). If MMK2 activity were independent of activation of the upstream regulators of the MPK1(SLT2) pathway, *MMK2* expression would be expected to restore the growth defect of the upstream regulator under restrictive conditions. Analysis with several upstream mutants of the MPK1 pathway showed that MMK2 was unable to suppress the growth defects under these conditions. Although these results suggest that MMK2 activity depends on activation by the PKC1 signal transduction cascade, and could be most easily explained by the necessity for phosphorylation of MMK2 by the yeast MKK1 and MKK2 MAP kinase kinases (Irie et al. 1993), more direct evidence is required to prove this possibility.

Assuming that alfalfa MMK2 and the yeast MPK1(SLT2) kinase perform homologous functions, it is worth considering the function of MPK1(SLT2) in yeast. A defect in the *MPK1(SLT2)* gene causes a temperature-dependent lytic phenotype that can be rescued by the addition of osmotic stabilizers (Lee et al. 1993). Another report (Mazzoni et al. 1993), describing the same gene, found *slt2(mpk1)* mutants to have aberrant vesicular transport and cisternal membrane accumulation indicating a defect in targeting and fusion of secretory vesicles. Arguing that similar phenotypes are known for a variety of cytoskeletal mutants, the authors suggested that the lytic phenotype could be a secondary consequence of the inability to build up a proper cytoskeleton. A possible role for MPK1 in cytoskeleton-associated processes is suggested by the observation that a *Xenopus* MAP kinase, which is activated during oocyte maturation and is involved in germinal vesicle breakdown (Gotoh et al. 1991), can specifically replace the function of the yeast *MPK1(SLT2)* protein but not of the FUS3 or KSS1 kinase (Lee et al. 1993). In summary, several lines of evidence suggest the involvement of cytoskeletal components in the action of MPK1(SLT2). With respect to these studies, the MMK2 kinase-specific phosphorylation of a 39 kDa protein that is part of, or tightly associated with, the nuclear cytoskeleton of plants is intriguing. However, given that the exact function of MPK1(SLT2) is still largely unclear, it is premature to speculate on the significance of this finding. Deeper insights must await further studies.

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