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MsERK1: A Mitogen-Activated Protein Kinase from a Flowering Plant

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The induction of proliferation and differentiation in cultured mammalian cells is mediated by a cascade of protein phosphorylations. A key enzyme in this signaling pathway is mitogen-activated protein (MAP) kinase (or ERK, extracellular signal–regulated kinase). We report the recovery of a full-length cDNA clone encoding a MAP kinase from alfalfa. We have named the 44-kD protein encoded by this clone MsERK1. Recombinant MsERK1 (rMsERK1), when overexpressed in *Escherichia coli*, is recognized by antibodies raised against MAP kinases from rat, Xenopus, and sea star and by anti-phosphotyrosine antibodies. Site-directed mutagenesis of MsERK1 demonstrated that Tyr-215 is either directly or indirectly responsible for recognition of the protein by anti-phosphotyrosine antibodies. Semipurified rMsERK1 phosphorylated itself and a model substrate, myelin basic protein, in vitro, but the Tyr-215 mutant did neither. Genomic DNA gel blot analysis suggested that the gene that encodes MsERK1 is either a member of a small multigene family or a member of a polymorphic allelic series in alfalfa. Because MAP kinase activation has been associated with mitotic stimulation in animal systems, such an enzyme may play a role in the mitogenic induction of symbiotic root nodules on alfalfa by *Rhizobium* signal molecules.

INTRODUCTION

The transitions between quiescence, differentiation, and the mitotic state in eukaryotic cells are promoted and accompanied by a variety of cellular changes. Among the most studied of these is the alteration in the pattern of protein phosphorylations. Treatment of cultured mammalian cells with growth factors, insulin, mitogenic phorbol esters, or transforming proteins of oncogenic viruses results in serine phosphorylation of the 40S ribosomal subunit S6 (Pelech et al., 1990). This post-translational modification leads to an enhancement in protein synthesis presumably required for cells to negotiate a new developmental pathway.

The S6 ribosomal subunit is phosphorylated by either of two protein kinases, S6 kinase II (pp90^{rsk}, 90-kD ribosomal protein S6 kinase phosphoprotein) or the 70-kD S6 kinase (pp70^{S6k}, 70-kD S6 kinase phosphoprotein) (Ballou et al., 1991). Each of these enzymes is, in turn, regulated by phosphorylation. The upstream kinase responsible for phosphorylation of S6 kinase II has several designations, including p42, mitogen-activated protein (MAP) kinase, or extracellular signal–regulated kinase (ERK). Additional substrates for MAP kinase include the protooncogene transcription factor c-*jun* (Pulverer et al., 1991), the epidermal growth factor receptor (Takishima et al., 1991), myelin basic protein (MBP) (Cicirelli et al., 1988), and microtubule-associated protein-2 (Ray and Sturgill, 1987). MAP kinase homologs have been identified in rat (Boulton et al., 1990, 1991), mouse (Ray and Sturgill, 1987), chick (Cooper et al., 1984), human (Hoshi et al., 1988), Xenopus (Ferrell et al., 1991; Posada et al., 1991), budding yeast (Courchesne et al., 1989), fission yeast (Toda et al., 1991), and starfish (Posada et al., 1991).

Whereas the elements downstream from MAP kinase in the signal transduction pathway have been relatively well characterized in animal cells, neither the multiplicity nor the exact nature of the upstream signaling steps between binding of ligand to a cell surface receptor and MAP kinase activation is well understood. MAP kinases do not appear to be direct substrates for receptor kinases themselves (Seger et al., 1991; Sturgill and Wu, 1991). Identification of MAP kinase activators has been complicated by MAP kinase's ability to autophosphorylate in vitro on threonine and tyrosine residues (Boulton et al., 1991; Crews et al., 1991; Seger et al., 1991; Wu et al., 1991). Therefore, it is possible that, in some systems, the MAP kinase activator may be a noncatalytic regulatory subunit that provides or enhances the enzyme's auto-activation capacity in vivo (Ahn et al., 1991). However, several candidates for upstream kinases that can phosphorylate, and thus activate, MAP kinase in vivo have been reported recently (Adams and Parker, 1991; Gomez and Cohen, 1991; Ettehadieh et al., 1992; L'Allemain et al., 1992). The development of phosphorylatable, kinase-dead MAP kinase variants by site-directed mutagenesis has facilitated the identification of what now appear to be authentic MAP kinase kinases (L'Allemain et al., 1992). The identification and cloning of a mouse MAP kinase kinase has

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recently been reported (Alessandrini et al., 1992; Crews et al., 1992a; Crews and Erickson, 1992).

Whereas developmental arrest is a feature of the cell differentiation process in both the plant and animal kingdoms, plants ubiquitously employ a return from proliferative quiescence during the course of development. Examples include germination in dormant seeds, sprouting in bulbs and tubers, "breaking" of axillary buds, and the mitotic stimulation of the root cortex by rhizobia. As a consequence of our pursuit of the mechanism of mitotic induction in the latter system, we report here the cloning and partial characterization of a MAP kinase homolog from alfalfa, a legume in the roots of which *Rhizobium meliloti* induces an organogenic mitotic response. We also report the apparent tyrosine autophosphorylation of this protein when expressed in *Escherichia coli.*

RESULTS

Cloning and Sequence Analysis of an Alfalfa MAP Kinase

We used a pea *cdc2* homolog cDNA (Feiler and Jacobs, 1990, 1991) to screen an alfalfa root cDNA library for a MAP kinase clone. *cdc2* gene products and MAP kinases are members of the same protein kinase subfamily based on primary amino acid sequence homologies (Boulton et al., 1990). Of three positive clones obtained, the clone carrying the longest insert (~1.5 kb) was sequenced in its entirety, as shown in Figure 1. This clone contains an open reading frame of 387 amino acids encoding a polypeptide with a predicted molecular mass of 44,373 D. Of the three potential translation initiation sites (located at nucleotides 79, 115, and 145; Figure 1), the nucleotide sequence context surrounding the first ATG, at nucleotide 79, conforms best to the published consensus for eukaryotic translation initiation (Kozak, 1989). Specifically, the ATG at nucleotide 79 (Figure 1) is both preceded by a purine residue at position −3 and followed by a G residue at position +4 (Kozak, 1989), one of which is lacking from the alternative start sites at nucleotides 115 or 145.

A FASTA search (Pearson and Lipman, 1988) of the Gen/Pept data base with the derived amino acid sequence revealed greatest identities with the following polypeptides, as shown in Figure 2: rat ERK2 (52%) (Boulton et al., 1991), Xenopus p42 (52%) (Posada et al., 1991), rat ERK1 (52%) (Boulton et al., 1990), budding yeast *KSS1* (49%) (Courchesne et al., 1989), rat ERK3 (40%) (Boulton et al., 1991), and maize $p34^{cdc2}$ (37%) (Colasanti et al., 1991). The first five of these are members of the ERK or MAP kinase family of serine/threonine protein kinases, whereas the last reflects the similarity between $p34^{cdc2}$ and MAP kinases.

We have named the protein encoded by the alfalfa MAP kinase clone MsERK1. Its amino acid sequence contains all of the consensus amino acids and oligopeptide motifs characteristic of the 11 stereotypical serine/threonine protein kinase subdomains (Hanks et al., 1988) (Figure 2). In particular, the MsERK1 cDNA sequence encodes the DLKPSN variation of the DLxxxN motif in subdomain VI and the TxYxxTxxYxAxE version of subdomain VIII, both characteristic of, if not unique to, the ERK family of serine/threonine protein kinases. Conserving the latter (subdomain VIII) motif in this plant gene is significant because the TxY tripeptide represents the target for stimulatory threonine and tyrosine phosphorylations in a mouse MAP kinase (Payne et al., 1991), and protein tyrosine phosphorylation in higher plants has yet to be unequivocally demonstrated. The derived amino acid sequence of MsERK1

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GTTAGGGAGAGATAGTACAGATCTGTTTGACCAAAAGCATTCACAACA    48
CAAATCAGAGCTTGAAGAAGAGAAACAACAATGGAAGGAGGAGGAGCT    96
                              M  E  G  G  G  A       6
CCACCAGCTGACACCGTGATGTCAGATGCTGCACCGGCGCCGCCGCAG   144
 P  P  A  D  T  V  M  S  D  A  A  P  A  P  P  Q    22
ATGGGGATCGAGAATATTCCGGCAGTCCTTAGCCACGGTGGTAGGTTC   192
 M  G  I  E  N  I  P  A  V  L  S  H  G  G  R  F    38
ATTCAATACAACATTTTCGGCAACATTTTTGAAGTTACTGCAAAATAC   240
 I  Q  Y  N  I  F  G  N  I  F  E  V  T  A  K  Y    54
AAACCACCCATCATGCCAATCGGTAAAGGCGCTTATGGTATTGTTTGC   288
 K  P  P  I  M  P  I  G  K  G  A  Y  G  I  V  C    70
TCTGCTCATAATTCGGAGACGAATGAACATGTCGCGGTTAAGAAGATT   336
 S  A  H  N  S  E  T  N  E  H  V  A  V  K  K  I    86
GCAAATGCATTTGACAACAAGATTGATGCCAAGAGGACTCTCCGTGAA   384
 A  N  A  F  D  N  K  I  D  A  K  R  T  L  R  E   102
ATCAAGCTGCTTCGCCATATGGATCATGAAAACGTGGTTGCAATCAGG   432
 I  K  L  L  R  H  M  D  H  E  N  V  V  A  I  R   118
GATATAGTGCCACCACCTCAGAGGGAGGTATTCAATGATGTTTACATT   480
 D  I  V  P  P  P  Q  R  E  V  F  N  D  V  Y  I   134
GCATATGAGCTAATGGACACCGACCTTCACCAAATCATTCGATCAAAT   528
 A  Y  E  L  M  D  T  D  L  H  Q  I  I  R  S  N   150
CAAGCACTATCGGAGGAGCACTGTCAGTACTTTCTGTACCAAATCCTT   576
 Q  A  L  S  E  E  H  C  Q  Y  F  L  Y  Q  I  L   166
CGTGGTTTGAAGTACATACATTCTGCAAATGTTCTGCATAGGGACTTA   624
 R  G  L  K  Y  I  H  S  A  N  V  L  H  R  D  L   182
AAACCCAGCAACCTTCTCCTGAACGCCAACTGTGACTTAAAGATTTGT   672
 K  P  S  N  L  L  L  N  A  N  C  D  L  K  I  C   198
GATTTTGGACTGGCCCGTGTAACCTCTGAAACCGATTTTATGACTGAA   720
 D  F  G  L  A  R  V  T  S  E  T  D  F  M  T  E   214
TATGTTGTTACTAGATGGTACCGTGCACCAGAGCTTCTGTTAAACTCT   768
 Y  V  V  T  R  W  Y  R  A  P  E  L  L  L  N  S   230
TCTGATTATACAGCAGCAATTGATGTATGGTCTGTTGGTTGTATTTTC   816
 S  D  Y  T  A  A  I  D  V  W  S  V  G  C  I  F   246
ATGGAACTGATGGATCGAAAGCCTTTGTTTCCTGGCAGAGATCATGTG   864
 M  E  L  M  D  R  K  P  L  F  P  G  R  D  H  V   262
CATCAGCTGCGTCTACTCATGGAGTTGATCGGTACCCCATCAGAAGAT   912
 H  Q  L  R  L  L  M  E  L  I  G  T  P  S  E  D   278
GATTTGGGGTTTCTGAATGAAAATGCTAAGAGATACATTAGGCAACTA   960
 D  L  G  F  L  N  E  N  A  K  R  Y  I  R  Q  L   294
CCTCCTTACCGCCGTCAATCTTTCCAAGAAAAGTTTCCGCATGTCCAT  1008
 P  P  Y  R  R  Q  S  F  Q  E  K  F  P  H  V  H   310
CCTGAAGCTATAGATCTTGTTGAAAAAATGTTAACTTTTGATCCTAGA  1056
 P  E  A  I  D  L  V  E  K  M  L  T  F  D  P  R   326
AAAAGAATTACTGTTGAAGATGCACTGGCACACCCCTACTTGACATCT  1104
 K  R  I  T  V  E  D  A  L  A  H  P  Y  L  T  S   342
CTGCATGACATCAGTGATGAACCTGTGTGCATGACTCCCTTCAGCTTT  1152
 L  H  D  I  S  D  E  P  V  C  M  T  P  F  S  F   358
GACTTTGAACAGCATGCATTGACCGAGGAACAGATGAAAGAACTGATA  1200
 D  F  E  Q  H  A  L  T  E  E  Q  M  K  E  L  I   374
TACAGAGAGGCTCTAGCATTTAACCCTGAGTACCAGCAGTAGTGTAGT  1248
 Y  R  E  A  L  A  F  N  P  E  Y  Q  Q             387
AGTGATAATGATGGTAATGTTGTCATTGGATTTGGAATTTGTTATGTT  1296
TGTTTCCCATAGGTCAAGAGATTTATGTGTATATATATCATCATATCT  1344
ATCATCTGAAACCAGATGACCTTGGAATTTAATTATTTTGTTATAGGC  1392
TTGATTGGTAGACTCACTGGAAAACAATCATAAGGTGTTTTTGTATTT  1440
TGTATTGACTTAATTGTTTCAAATTATTCTCCTATTCATTGGCTTCAG  1488
CTTCAGCCAACTTCTGATAAAAAAAAAAAAAAAAAAA             1526
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Figure 1. Nucleotide and Derived Amino Acid Sequence (Single Letter Code) of the MsERK1 Clone.

Numbers at right indicate the number of final nucleotide or amino acid residues in that row. The cDNA sequence has been submitted to GenBank and has been given accession number L07042.

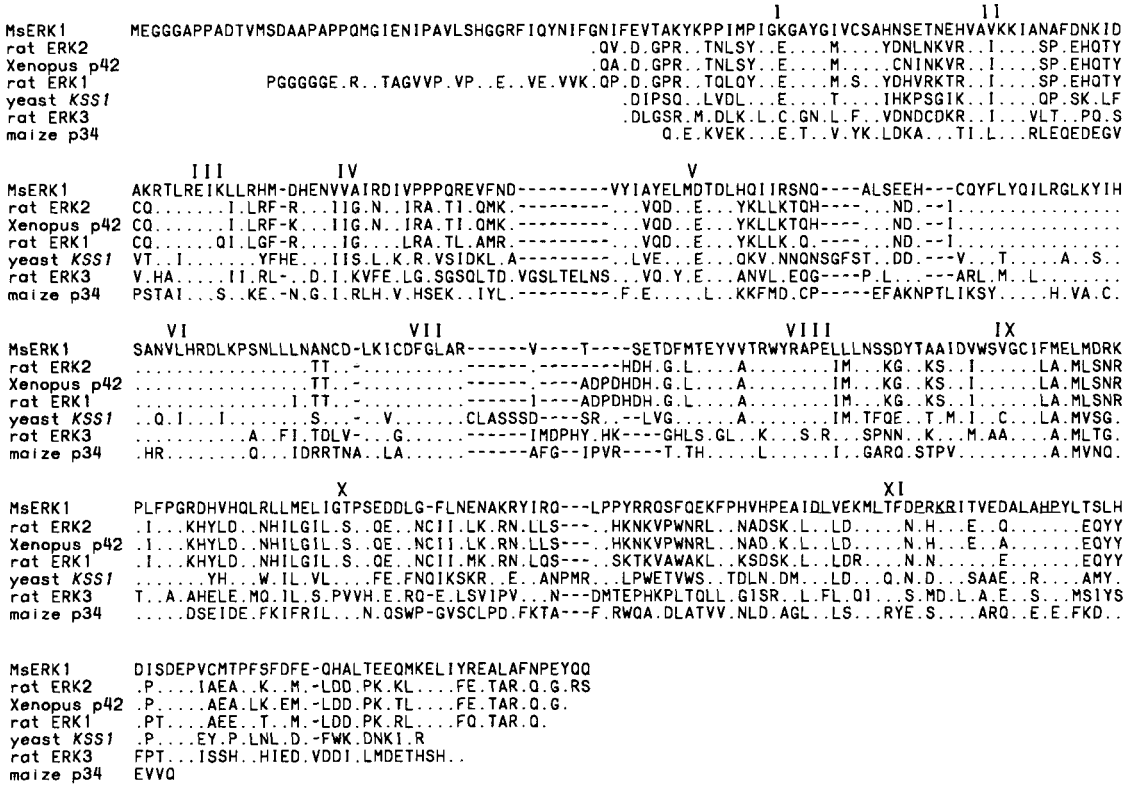

Figure 2. Comparison of Amino Acid Sequences of MsERK1 and Other Closely Related Protein Kinases.

A hyphen indicates that a gap has been introduced to optimize alignment, whereas a period represents identity with MsERK1. Serine/threonine protein kinase consensus subdomains (Hanks et al., 1988) are numbered with Roman numerals. Nine highly conserved residues, characteristic of tyrosine-autophosphorylating protein kinases, are underlined in subdomain XI (Seger et al., 1991).

contains nine of the 11 conserved tyrosines and 10 of the 11 conserved prolines identified among ERK homologs in rat, Xenopus, and the yeasts (Crews et al., 1992b). The MsERK1 sequence also includes, in the consensus catalytic segment of protein kinase subdomain XI (Figure 2), all nine residues recently identified to be highly conserved among tyrosine-autophosphorylating protein kinases (Seger et al., 1991). That this alfalfa gene is not a member of the *cdc2* subgroup is evidenced by its lack of the signature PSTAIR oligopeptide motif in subdomain III, characteristic of all $p34^{cdc2}$ kinases.

Genomic Organization of MsERK1 in Alfalfa Cultivar Iroquois

In rat, multiple MAP kinase genes constitute a small multigene family (Boulton et al., 1991). Genomic DNA gel blot analysis provides an initial view of such genetic structural features. A gel blot of digested alfalfa genomic DNAs was prepared and hybridized with a probe consisting of the entire MsERK1 cDNA clone, as illustrated in Figure 3. The sums of the lengths of the fragments detected in all but the HindIII lane are greater than the length of the probe (1.5 kb). This result suggests that either MsERK1 is a member of a small gene family or an allelic series, or simply that the MsERK1 genomic coding sequence is interrupted by large introns. Alfalfa is an autotetraploid that does not naturally self-pollinate. The DNA that was blotted in this experiment was prepared from pooled leaf tissue obtained from more than 100 Iroquois alfalfa plants. Given the genetic structure of alfalfa, such a genomic DNA preparation might be expected to display considerable polymorphism at any given locus.

Expression of MsERK1 in *E. coli* and Alfalfa

While the primary amino acid sequence of MsERK1 suggests that it is a MAP kinase homolog, we sought more biochemical confirmation of its membership in this broad family of protein kinases. The MsERK1 coding sequence was ligated into an *E. coli* expression vector, where it was translationally fused to a strong bacteriophage T7 promoter (Studier et al., 1990). To determine whether the MsERK1 clone encoded a protein with higher order (than primary) structural similarity to reported

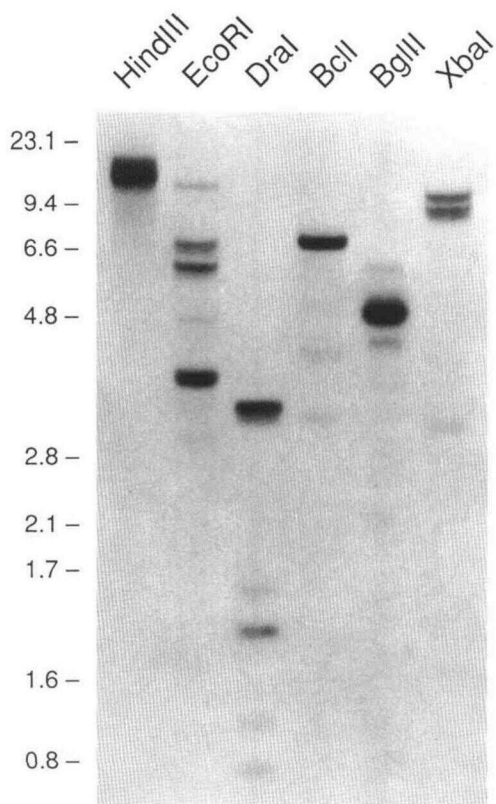

Figure 3. Genomic DNA Gel Blots of Alfalfa DNA Probed with the MsERK1 cDNA.

Autoradiogram of cultivar Iroquois alfalfa DNA that was digested with the six indicated restriction endonucleases, electrophoresed, blotted, and probed with the full-length MsERK1 cDNA. Numbers at left are length markers in kilobases.

MAP kinases, we surveyed a number of anti-MAP kinase antibodies obtained from other laboratories for cross-reactivity with the alfalfa gene product. Antisera raised against MAP kinases from sea star, shown in Figure 4, lane 2, and rat, shown in Figure 4, lanes 4, 6, and 8, detected an ~44-kD protein present in *E. coli* carrying the fusion construct, but not in strains carrying the vector alone. The anti–rat ERK2 antibody 691 yielded a particularly strong signal (Figure 4, lane 8), probably owing to the high homology (16 of 23 amino acids) between the peptide against which it was raised (KRITVEEALAHPYLEQYYDPTDE; Boulton and Cobb, 1991) and the corresponding sequence in MsERK1 (subdomain XI; Figure 2).

Rat ERK1 and ERK2 and mouse Erk-1 MAP kinases expressed in *E. coli* autophosphorylate on threonine, serine (mouse Erk-1), and tyrosine residues (Boulton et al., 1991; Crews et al., 1991; Seger et al., 1991; Wu et al., 1991). We tested whether MsERK1 expresses a tyrosine autophosphorylation activity in *E. coli* by probing immunoblots of recombinant MsERK1 (rMsERK1) with the monoclonal anti-phosphotyrosine antibody 4G10 (Drucker et al., 1989). This antibody detects a polypeptide, shown in Figure 5, lane 5, with an electrophoretic mobility corresponding to the slowest migrating species in the ~44-kD family of signals recognized by the anti-ERK antibody 691 (Figure 5, lanes 1 to 4). Phosphorylated proteins generally run slower in SDS gels than their unphosphorylated counterparts. A comparison of the intensities of immunoblot signals obtained with anti-phosphotyrosine 4G10 versus anti-ERK 691 antibodies suggests that only a small fraction of the MsERK1 produced in *E. coli* may be tyrosine phosphorylated. Alternatively, the difference in signal intensity obtained with the two antibody probes may be partially attributable to differential affinity of the antibodies for their respective sites on MsERK1.

That the signal detected by antibody 4G10 is attributable to its specific binding to phosphotyrosine was confirmed by incubating the anti-phosphotyrosine antibody with phosphotyrosine, phosphoserine, and phosphothreonine prior to probing the rMsERK1 immunoblot (Figure 5, lanes 6, 7, and 8, respectively). Complete inhibition of the 44-kD signal by phosphotyrosine, but not by phosphoserine or phosphothreonine, strongly suggests that rMsERK1 is tyrosine phosphorylated in *E. coli*, presumably by an autophosphorylation mechanism, because protein tyrosine phosphorylation is not otherwise known to occur in *E. coli.*

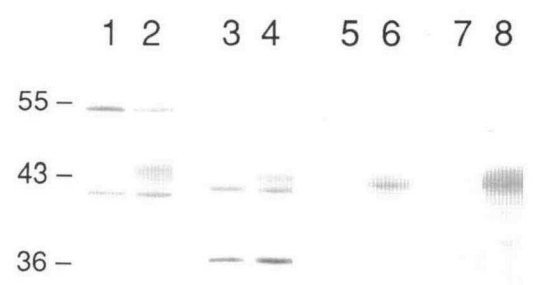

Figure 4. Recognition of *E. coli* Overexpressed MsERK1 by Anti-MAP Kinase Antibodies.

The antibody probes are given as follows: lanes 1 and 2 contain affinity-purified rabbit polyclonal antibody raised against entire purified sea star p44^{mpk} (Sanghere et al., 1991); lanes 3 and 4, affinity-purified rabbit polyclonal antibody raised against subdomain III region of rat ERK1 (PFEHQTYCQRTLREIQILLGFRHENVIGIRDILRAP); lanes 5 and 6, affinity-purified rabbit polyclonal antibody raised against the carboxy terminus of rat ERK1 (PFTFDMELDDLPKERLKELIFQETARFQPGPEAP); lanes 7 and 8, affinity-purified rabbit polyclonal antibody 691 raised against a peptide from subdomain XI of rat ERK1 (KRITVEEALAHPYLEQYYDPTDE) (Boulton and Cobb, 1991). Lanes 1, 3, 5, and 7 contain total *E. coli* lysate of BL21(DE3) carrying vector pET11d without insert. Lanes 2, 4, 6, and 8 contain total *E. coli* lysate of BL21(DE3) carrying vector pET11d with MsERK1 insert. Molecular mass standards are given at left in kilodaltons.

Figure 5. Anti-Phosphotyrosine–Probed Immunoblot of MsERK1 Protein Overexpressed in *E. coli*.

Each lane contains whole cell lysate from *E. coli* overexpressing MsERK1. Antibody probes were anti-ERK 691 (lanes 1 to 4) and anti-phosphotyrosine 4G10 (lanes 5 to 8). Prior to probing the blot, primary antisera were incubated with buffer alone (lanes 1 and 5), phosphotyrosine (lanes 2 and 6), phosphothreonine (lanes 3 and 7), or phosphoserine (lanes 4 and 8). Molecular mass standards are given at left in kilodaltons.

At this initial stage of its characterization, it was important to verify that rMsERK1 has an authentic counterpart in alfalfa cells. Polyclonal antibodies were raised against a synthetic peptide corresponding to the carboxy-terminal 17 residues of the derived amino acid sequence of MsERK1. When this antiserum was used to probe a protein blot carrying whole cell protein extracts from alfalfa roots and shoots, a single band was detected at ~44-kD, as shown in Figure 6, supporting the notion that the gene we have cloned is indeed expressed in Iroquois alfalfa.

The MsERK1 clone detects an RNA of ~1.7 kb in RNA gel blot hybridizations with alfalfa total and poly(A)$^+$ RNA (data not shown), suggesting that the 1.5-kb cDNA clone described above may be missing 100 to 200 bp of 5′-untranslated RNA present in the authentic MsERK1 transcript. Preliminary results suggest that there is little or no variation in the level of MsERK1 mRNA among alfalfa tissues and between mock-inoculated roots and those inoculated with *Rhizobium* (data not shown).

Site-Directed Mutational Analysis of MsERK1 Phosphorylation in *E. coli*

Activation of mouse MAP kinase's enzymatic activity is associated with phosphorylation of a threonine and a tyrosine corresponding to Thr-213 and Tyr-215 in MsERK1 (Payne et al., 1991). We hypothesized that tyrosine autophosphorylation of MsERK1 in *E. coli* is dependent upon these residues and therefore changed them to the nonphosphorylatable amino acids valine and phenylalanine, respectively, in two separate mutant proteins. Each of these mutants was overexpressed in *E. coli* and lysates were analyzed by immunoblotting. The tyrosine→phenylalanine substitution at position 215 (Y215F), which is shown in Figure 7, abolished recognition of the protein by the anti-phosphotyrosine antiserum 4G10, whereas the threonine→valine mutation at position 213 (T213V; Figure 7) had no detectable effect. The simplest interpretation of this result is that Tyr-215 is the sole phosphorylated tyrosine in the protein, when expressed in *E. coli*, and that a substitution of phenylalanine at this position eliminates the only autophosphorylated tyrosine in rMsERK1. However, it is also possible that phosphorylation at Tyr-215 is required for acquisition of an enzymatic activity which results in autophosphorylation at other tyrosine residues. The lack of effect of the T213V mutation suggests that phosphorylation at this residue is not essential for rMsERK1 autophosphorylation in *E. coli*, but leaves open the possibility that this residue is indeed modified during, and required for, the enzyme's activation in the plant.

Protein Kinase Activity of rMsERK1

We assayed the protein kinase activity of wild-type and mutant rMsERK1. As shown in Figure 8, wild-type rMsERK1 phosphorylates both itself and the model substrate, MBP (~17 kD). The T213V mutant showed reduced activity relative to wild-type rMsERK1, and the Y215F mutant lacked both autophosphorylation and MBP kinase activity, further confirming that enzymatic activity requires a tyrosine, and presumably a phosphotyrosine, at position 215.

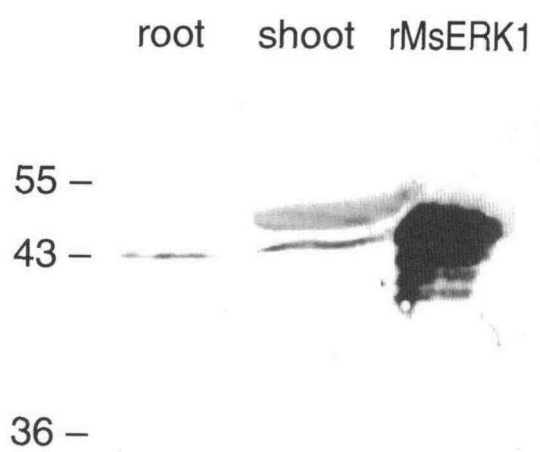

Figure 6. Immunoblot of Cultivar Iroquois Alfalfa Proteins and Wild-Type rMsERK1 Probed with Antibodies Raised against the Carboxy-Terminal Peptide of MsERK1.

The band at ~50 kD in the shoot lane corresponds to the large subunit of rubisco, the most abundant protein in shoot tissues, to which either the primary or secondary antibody bound nonspecificially. Molecular mass standards are given at left in kilodaltons.

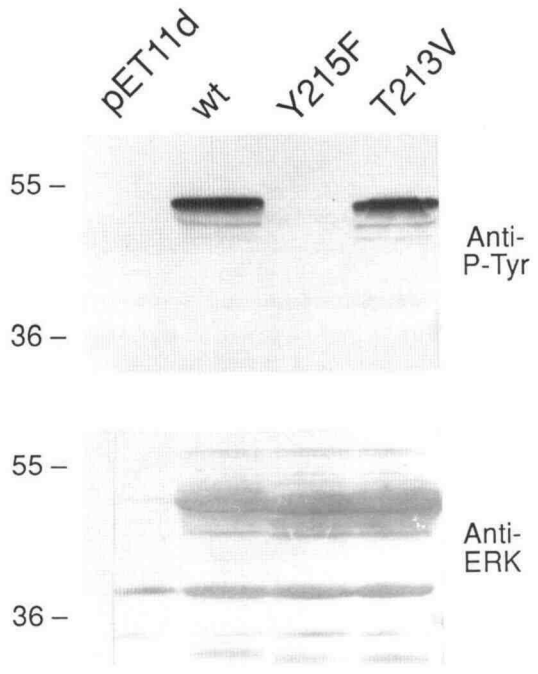

Figure 7. Anti-Phosphotyrosine–Probed Immunoblot of Site-Directed Mutants of MsERK1 Overexpressed in *E. coli.*

Lane designations are as follows: pET11d, *E. coli* carrying an empty expression vector; wt, wild-type rMsERK1; Y215F, rMsERK1 carrying Tyr→Phe mutation at position 215; T213V, rMsERK1 carrying Thr→Val mutation at position 213. The gel in the upper panel was probed with anti-phosphotyrosine antibody 4G10; the gel in the lower panel was probed with anti–rat ERK peptide antibody 691. Molecular mass standards are given at left in kilodaltons.

components of the cell division regulatory network described in yeast and animal systems are present in higher plants (Jacobs, 1992). On the basis of primary sequence comparisons alone, MsERK1 is arguably a member of the MAP kinase family. However, it should be noted that there are lengthy stretches of amino acid sequence identity among rat ERK1, rat ERK2, and Xenopus p42, which MsERK1 does not share (Figure 2). MsERK1's divergence in these otherwise conserved domains suggests that its cellular role, regulation, and/or substrates may differ from those of the heretofore identified ERKs.

ERK genes occur in small families in animal cells (Boulton et al., 1991; Gonzalez et al., 1991). The polymorphic pattern revealed in genomic DNA gel blot analysis of MsERK1 in alfalfa cultivar Iroquois (Figure 3) suggests that this plant MAP kinase gene may be a member of a small family as well. However, alfalfa is an autotetraploid with a tendency to outcross. Commercial varieties of tetraploid alfalfa such as cultivar Iroquois are not inbred, but are, in fact, mixtures of genotypes, selected for their relative phenotypic homogeneity. Thus, a single plant can carry as many as four alleles at a given locus, and a population of plants, such as those pooled in our DNA gel blot analysis, could carry as many alleles as are extant within the population. Further analyses of individual Iroquois alfalfa plants and additional alfalfa cDNA library screens should contribute to a more complete understanding of the genomic organization of alfalfa MAP kinases.

MAP kinase from insulin-stimulated Swiss 3T3 cells is phosphorylated on the first and third residues of the conserved TEYVxTR motif (corresponding to residues 213 to 219 in MsERK1) (Payne et al., 1991). rMsERK1 is recognized by anti-phosphotyrosine antibodies, but the Y215F mutant is not (Figure 6). However, a threonine at position 213 is not essential

DISCUSSION

In animal systems, the proliferative or differentiation response to a particular extracellular signal is frequently mediated via an intracellular phosphorylation signaling cascade, initiated by a ligand's binding to its receptor. MAP kinases (ERKs) have been identified as downstream effectors in such cascades in a variety of ligand/receptor systems (Sturgill and Wu, 1991). These pathways include responses of mammalian cells to insulin and mitogenic phorbol esters (Tobe et al., 1991), cytokines (Bird et al., 1991), and both mitogenic and differentiation-inducing growth factors (Rossomando et al., 1989; Schanen-King et al., 1991) as well as yeast cells' response to mating pheromone (Courchesne et al., 1989; Elion et al., 1990, 1991).

We have cloned an alfalfa gene whose translation product compares favorably in primary sequence, size, antigenicity, and enzyme activity with animal cell MAP kinases. The finding of a MAP kinase homolog in alfalfa is in keeping with mounting evidence that more than just the $p34^{cdc2}$ and cyclin

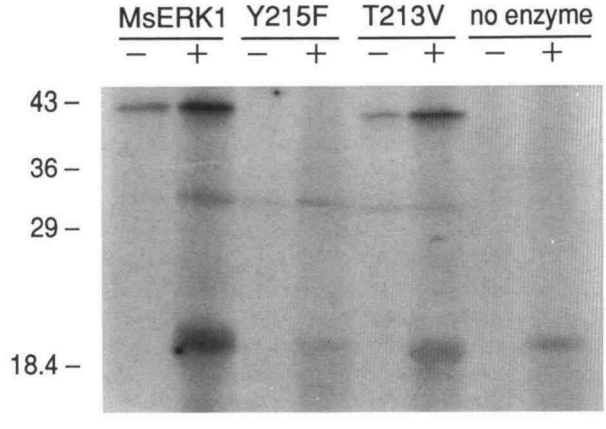

Figure 8. Phosphorylation Products of Wild-Type (MsERK1) and Mutant (Y215F and T213V) Proteins.

The lanes labeled "–" contain products of autophosphorylation assays, whereas those labeled "+" contain products of MBP kinase assays. Molecular mass standards are given at left in kilodaltons.

for tyrosine autophosphorylation in *E. coli* (Figure 7). This is consistent with the Xenopus system, in which in vivo phosphorylation of Xp42 at a residue analogous to MsERK1's Tyr-215 is independent of the presence of a threonine two residues upstream (Posada and Cooper, 1992). We have yet to determine whether Thr-213 of MsERK1 is phosphorylated in *E. coli* or in alfalfa roots. Recent results from Xenopus and mammalian systems suggest that autophosphorylation plays a role secondary to that played by distinct upstream protein kinases in the MAP kinase activation pathway (Adams and Parker, 1992; Crews et al., 1992a; Crews and Erickson, 1992; Ettehadieh et al., 1992; L'Allemain et al., 1992; Posada and Cooper, 1992).

Regulatory protein tyrosine phosphorylation has yet to be unequivocally detected in plant proteins, although members of the p34^{cdc2} family have now been reported in plants (John et al., 1989; Feiler and Jacobs, 1990; Colasanti et al., 1991) and that protein is regulated by tyrosine phosphorylation/dephosphorylation in yeast and animal cells. The failure of researchers in the past to detect protein tyrosine phosphorylation in higher plants may have been largely the result of inadequate analytical tools and a lack of distinctly identified candidate proteins. Extrapolating from the MsERK1 autophosphorylation data, it is not unreasonable to propose that this alfalfa protein is regulated in the plant by a post-translational phosphorylation mechanism not unlike that reported in Xenopus, mouse, and rat cells (Ray and Sturgill, 1988; Posada et al., 1991). Further application of the immunological tools developed in this work should resolve this issue, at least with respect to MsERK1.

There is little overlap between the pharmacopoeias of effective mitotic inducers for plant versus animal and yeast cells. Mitotic responses in plants are generally elicited by low molecular weight signal molecules and intrinsic developmental cues, but not generally by peptides or phorbol esters, factors which induce MAP kinase activation in animal cells. Therefore, it is inevitable that the upstream elements of any plant mitotic signal transduction cascade containing MsERK1 must diverge from those in analogous animal systems. Substrate quantities of alfalfa MAP kinase will provide valuable tools for characterizing upstream steps in the pathway (Gomez and Cohen, 1991). Additional upstream elements in the animal cell MAP kinase activation pathway include MEK, a MAP kinase kinase from murine cells (Crews et al., 1992a), and the *raf-1* protooncogene product, an enzyme with inducible protein kinase activity against the MAP kinase kinase (Kyriakis et al., 1992).

The MsERK1 cDNA was cloned from a library representing mRNAs expressed in the infectible zone of *Rhizobium*-inoculated alfalfa roots. This plant–bacteria interaction is a developmentally appropriate system in which to begin the study of higher plant MAP kinases because, as part of its infection process, *Rhizobium* induces unscheduled mitoses in the differentiating cortex of the host root. It will be interesting to determine whether MsERK1 is a component of the signal transduction pathway linking *Rhizobium*-produced signals (Roche et al., 1991) and the mitotic response machinery of the host plant.

METHODS

cDNA Library Construction and Screening

A cDNA library was constructed in the plasmid-primer vector system pCGN1703 (Alexander, 1987) from mRNA purified (de Vries et al., 1988) from 4 cm of the infectible zone of alfalfa (*Medicago sativa* cv Iroquois; Allied Seed, Napa, ID) seedling roots that had been inoculated with *Rhizobium meliloti* 1021, 4 days prior to harvest. Approximately 50,000 colonies were screened with a ^{32}P-labeled probe made from a partial *cdc2* clone from pea (*Pisum sativum*) (Feiler and Jacobs, 1990). Plasmid DNA was prepared from positive colonies and rescreened by DNA gel blot hybridization. The largest of three positive clones that passed the second screen was subcloned into pTZ18R (Mead et al., 1986) (this subclone was named pTZMSERK1), and the complete DNA sequences of both strands were determined by the dideoxy chain termination method with ^{35}S-labeled nucleotides. Plasmid miniprep DNA was used as sequencing template (Kraft et al., 1988) throughout and, in addition to T7 and universal sequencing primers, custom oligonucleotides were obtained as needed from the Genetic Engineering Facility of the University of Illinois Biotechnology Center (Urbana). DNA Strider and the SeqMan module of DNAStar software were used for sequence data management and analysis.

Alfalfa Genomic DNA Preparation and Gel Blot Analysis

Seeds of alfalfa cultivar Iroquois (Allied Seed, Napa, ID) were surface sterilized and grown to 4 inches tall in vermiculite in a lighted growth chamber. Genomic DNA was prepared (Dellaporta et al., 1983) from 6 g of leaves removed from ~100 17-day-old seedlings. DNA was CsCl-gradient purified, digested (10 g per lane) for gel blot analysis with restriction enzymes as indicated in Figure 3, separated on 1% agarose/Tris/EDTA/acetic acid gels, and transferred to Magnagraph membranes (Micron Separations, Inc., Westboro, MA) using the manufacturer's protocol. Membranes were baked under vacuum at 80°C for 30 min and UV-cross-linked at 0.02 J/cm^2 in a Stratalinker (Stratagene) on automatic setting. The full-length MsERK1 cDNA clone was gel-purified (Qian and Wilkinson, 1991) and ^{32}P-labeled by the random primer method (Feinberg and Vogelstein, 1984) to a specific activity of 4.8 × 10^8 cpm/μg. Filters were hybridized in 2.5 × 10^6 cpm/mL hybridization solution overnight at 42°C and were washed in 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.5% SDS at room temperature, 42°C, and 65°C for 20 min each. A final wash was performed with 0.2 × SSPE, 0.2% SDS for 20 min at room temperature. Filters were exposed overnight to XAR film (Kodak) with two intensifying screens.

Expression of MsERK1 in *Escherichia coli*

Custom oligonucleotide primers, 5′-CGCCATGGAAGGAGGAGGAGCTCC-3′ and 5′-GCGGATCCACATAACAAATTCCAAATCC-3′, were designed to amplify the coding region of MsERK1 by polymerase chain reaction (PCR) and subclone the product into the NcoI-BamHI sites of the T7 polymerase-driven *E. coli* overexpression plasmid pET11d (Novagen, Inc., Madison, WI). A subclone verified by restriction enzyme analysis was transformed into the T7 polymerase-expressing *E. coli* host strain BL21(DE3) (Studier and Moffatt, 1986) and induced for *lac* promoter-driven T7 polymerase expression with 0.4 mM isopropyl-β-

thiogalactopyranoside. After 3 hr, cells were harvested, resuspended in SDS–polyacrylamide gel sample buffer, and frozen at −20°C.

Antibody Production

A peptide corresponding to the carboxy-terminal 16 amino acids of MsERK1 (CELIYREALAFNPEYQQ) was synthesized by the University of Illinois Biotechnology Center Genetic Engineering Laboratory. The amino-terminal cysteine was included to facilitate coupling to a carrier. The peptide was coupled to keyhole limpet hemacyanin by glutaraldehyde chemistry (Harlow and Lane, 1988), and the purified conjugate was injected subcutaneously into New Zealand White rabbits. The animals were boosted with freshly prepared peptide conjugate 3 weeks following the primary injections. Preimmune serum was collected prior to the first injections, and immune serum was collected 2 and 5 weeks following the primary injections. The peptide conjugate was emulsified in Freund's Complete and Incomplete Adjuvant for the primary and booster injections, respectively. For probing electroblotted alfalfa proteins (Figure 6), antiserum from the week 5 collection was affinity purified by binding to and elution from a Sulfo-Link Coupling Gel column (Pierce, Rockford, IL) to which the peptide had been coupled according to the manufacturer's instructions.

Immunoblots

Bacterial proteins, prepared by boiling the frozen extracts described above, were separated on 12% SDS–polyacrylamide gels and transferred to nitrocellulose by a semidry electroblotting method (Multi-phor; Pharmacia LKB Biotechnology Inc.). Dilution of the various primary anti-MAP (mitogen-activated protein) kinase antibodies was antibody specific, but antibody 691, used for all studies following the initial antibody survey, was diluted 1:8000. Anti-phosphotyrosine monoclonal antibody 4G10 was diluted 1:4000. Goat anti–mouse or anti–rabbit alkaline phosphatase–conjugated secondary antibodies and alkaline phosphatase substrates were obtained from Promega and used according to the supplier's recommendations.

Monoclonal antiserum 4G10 was preincubated for 48 hr at 4°C with either phosphotyrosine, phosphoserine, phosphothreonine, each at 1 mM and pH 8.0, or buffer alone, prior to incubation with protein blots (Figure 5).

Site-Directed Mutagenesis

Mutant MsERK1 clones were generated by a "megaprimer" PCR procedure. PCR synthesis was first performed with pTZMSERK1 as template, and as primers, a mutagenic antisense oligonucleotide (5′-AACAACATATTCAACCATAAAATCGGTT-3′ for Thr-213→Val-213 and 5′-TCTAGTAACAACAAATTCAGTCATAAA-3′ for Tyr-215→Phe-215) and a sense oligonucleotide complementary to vector sequences flanking the 5′ end of the clone in pTZ18R. The entire amplification product of this synthesis was purified by isolation from a 4% Nuseive gel (FMC BioProducts, Rockland, ME) and used as the sense primer in a second PCR synthesis with pTZMSERK1 as template and an antisense oligonucleotide complementary to vector sequences flanking the 3′ end of the clone in pTZ18R. The final PCR product was digested with appropriate enzymes and ligated into pTZ18R. The authenticity of each mutant clone was confirmed by complete DNA sequence analysis.

Partial Purification of MsERK1

Wild-type and mutant recombinant MsERK1 (rMsERK1) proteins were semipurified by a modification of published procedures (Boulton et al., 1991). One liter cultures of *E. coli* overexpressing MsERK1 were grown and induced as described above. Cells were harvested by centrifugation, frozen, thawed, resuspended in 3 mL lysis buffer (containing 0.5 mg lysozyme and 32 U DNase per mL) per gram of cell paste, and lysed by gentle agitation overnight. Lysis buffer consisted of 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 20 mM para-nitrophenylphosphate, 50 mM sodium fluoride, 50 mM sodium vanadate, 60 mM β-glycerolphosphate, 2 mM phenylmethylsulfonylfluoride, 5 mM benzamidine, 3 μg/mL leupeptin, 0.5 μg/mL aprotinin, 5 mM dithiothreitol, and 10% glycerol. The lysate was clarified by centrifugation, diluted 10-fold with Buffer B (20 mM Hepes, pH 7.5, 0.1 mM EDTA, 20 mM NaCl) and loaded onto a 1.5 × 24 cm Affigel Blue (Bio-Rad, Richmond, CA) column equilibrated with Buffer B. The column was washed with Buffer B until the A_{280} of the eluate dropped below 0.05. Bound proteins were then eluted with a gradient of 0 to 2 M NaCl in Buffer B. Eluate fractions were assayed by SDS–polyacrylamide gel electrophoresis. rMsERK1 eluted between 1.8 and 2.0 M NaCl. Selected fractions were dialyzed against Buffer B containing 10% glycerol, assayed for total protein content (Bradford, 1976), aliquoted, and frozen at −80°C. The Y215F mutant protein bound considerably less tightly to the Affigel Blue column than did the wild-type or T213V mutant proteins. All protein purification steps were carried out at 4°C.

Protein Kinase Assays

Autophosphorylation reaction mixtures consisted of 1 μg semipurified MsERK1 protein, 20 mM Hepes, pH 7.5, 2 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EGTA, 100 μM rATP, 10 μCi γ-^{32}P-rATP (3000 Ci/mmole), 2 mM phenymethylsulfonylfluoride, 2 mM sodium vanadate, 10 mM β-glycerolphosphate, 10% glycerol, 0.01% Triton X-100, 10 μg/mL pepstatin, 3 μg/mL leupeptin, and 0.5 μg/mL aprotinin in a total reaction volume of 15 μL. For myelin basic protein (MBP) kinase assays, reaction mixtures were supplemented with 1 μg/μL MBP. Reactions were carried out at 30°C for 1 hr (autophosphorylation) or 20 min (MBP kinase) and were terminated by adding 10 μL of SDS–polyacrylamide gel sample buffer and boiling for 3 min. Products were immediately separated on SDS–polyacrylamide gels and visualized by autoradiographic exposure to XAR film (Kodak) with two intensifying screens.

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