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The Direct Activation of MIK, a Germinal Center Kinase (GCK)-like Kinase, by MARK, a Maize Atypical Receptor Kinase, Suggests a New Mechanism for Signaling through Kinase-dead Receptors*

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Blanca Llompart‡, Enric Castells, Adolfo Río§, Ramon Roca¶, Alejandro Ferrando‖, Virginia Stiefel, Pere Puigdomènech, and Josep M. Casacuberta**

From the Departament de Genètica Molecular, IBMB-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

Signaling by receptor protein kinases (RPKs) involves their dimerization and transphosphorylation. However, atypical RPKs with kinase-defective domains have been described recently. Some of them are essential for proper signaling in animal systems, although the precise mechanism involved is unknown in most cases. Here we describe the cloning and characterization of an atypical plant receptor kinase from maize, MARK, which does not phosphorylate *in vitro*. A yeast two-hybrid approach has allowed us to identify a new germinal center kinase (GCK)-related protein, MIK, that interacts with MARK. Interestingly, the interaction of the intracellular domain of MARK with the regulator domain of MIK strongly induces MIK kinase activity. As some GCK-related proteins connect cell-surface receptors to the intracellular MAPK cascades, the activation of MIK by direct interaction with MARK could illustrate a new mechanism for signaling through atypical RPKs.

Receptor protein kinases (RPKs)[1] are essential components of the cell regulation machinery that transmits extracellular signals to the inside of the cell. Although RPKs show high variability in their receptor domain, they share highly conserved cytoplasmic kinase domains, and they are assumed to function by a relatively well conserved general mechanism. After ligand binding and receptor oligomerization, the intracellular kinase domain becomes activated. This results in intermolecular auto-phosphorylation and conformational changes that allow the receptor to bind downstream signaling proteins (see Refs. 1 and 2). Nevertheless, atypical RPKs that transduce signals by phosphorylation-independent mechanisms have been described recently (3). They include phosphorylation-capable PRKs that can also signal through other mechanisms but also completely kinase-defective atypical RPKs, such as CCK-4 (4), H-Ryk (5, 6), ErbB-3 (7, 8), and DNT (9). These atypical RPKs have substitutions within the kinase-conserved motifs, especially in the aspartic acid in subdomain VIb and in the DFG activation loop motif of subdomain VII. Among them, ErbB3 and H-Ryk are probably the best known examples. It has been shown that mice knockouts for ErbB-3 or Ryk present severe mutant phenotypes suggesting that both proteins are essential for signaling (10, 11). ErbB3 forms heterodimers with other members of the family of epidermal growth family receptors and is phosphorylated by these kinase-active RPKs (12). Upon phosphorylation, ErbB3 serves as docking sites for multiple downstream signaling proteins. H-Ryk also forms heterodimers with other kinase-active RPKs, although in this case the interaction does not result in phosphorylation (13). A chimeric receptor approach has shown that the ligand stimulation of H-Ryk results in activation of the MAPK pathway (6), suggesting that activated H-Ryk can interact with and activate other downstream signaling proteins. Indeed, it has been proposed that signaling through atypical RPKs could involve regulated protein-protein interactions through their intracellular domains, which would explain the high conservation of the kinase-like structure of these domains through evolution (14). Nevertheless, the signal transduction mechanism for most atypical RPKs is still unknown.

MAP kinases play a key role on signal transduction in eukaryotes. MAP kinase modules are usually connected to the membrane-located receptors through G proteins, such as Ras or heterotrimeric complexes, or kinases that phosphorylate MAPKKK and are denoted as MAPKKKK (15–17).

Here we report the cloning and characterization of two maize genes coding for an atypical plant receptor kinase (PRK) and for a kinase of the GCK subfamily of MAPKKKK, which have been named MARK and MIK, respectively. We show that MARK and MIK interact in yeast, *in vitro*, and in mammalian cells. Moreover, phosphorylation experiments show that this interaction results in the induction of the kinase activity of MIK, suggesting a new mechanism for signaling through atypical receptor kinases.

EXPERIMENTAL PROCEDURES

Plant Material—Zea mays cv. W64A plants were grown at 26 °C on a 16-h light/8-h dark cycle.

Cloning of MARK and MIK cDNAs—A partial MARK cDNA was obtained from a differential screening of a 12-day after pollination (DAP) maize embryo cDNA library using 12-DAP embryo cDNA and leaf cDNA as probes, as described elsewhere (18). This clone was used

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‡ Present address: ERA Plantech, Parc Cientific de Barcelona, Joseph Samitier 1-5, 08028 Barcelona, Spain.

§ Present address: Hospital Universitari Germans Trias i Pujol, Carretera del Canyet, 08915 Badalona, Spain.

¶ Present address: Almirall Prodespharma, Parc Cientific de Barcelona, Joseph Samitier 1-5, 08028 Barcelona, Spain.

‖ Present address: Universitat de Barcelona, Plant Physiology Dept., Diagonal 643, 08028 Barcelona, Spain.

** To whom correspondence should be addressed. Tel.: 34-93-4006142; Fax: 34-93-2045904; E-mail: jcsgmp@cid.csic.es.

[1] The abbreviations used are: RPK, receptor protein kinases; PRK, plant receptor kinases; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase kinase; DAP, days after pollination; RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase; HA, hemagglutinin; MBP, myelin basic protein; ID, intracellular domain; GCK, germinal center kinase.

to screen maize cDNA and genomic libraries (18) to obtain the full-length cDNA and genomic sequence.

The full-length MIK sequence was obtained by RACE PCR on 15-DAP maize embryo cDNA with the primers MIKRACE1 (5′-CATCCATGCCTTTTGC-3′) and MIKRACE2 (5′-GATGCTTGAGAAGCTCCTTGG-3′) designed from the partial cDNA obtained from a yeast two-hybrid cDNA library (see below), using a RACE PCR kit (Invitrogen) according to manufacturer's instructions.

Northern Analysis—Total RNA from immature seeds and adult leaves was obtained as described (18). 10 μg of the different RNAs was subjected to denaturing electrophoresis, blotted to nylon membranes (Nytran; Schleicher & Schuell), and hybridized with radiolabeled MARK or MIK cDNA probes following standard procedures as reported previously (18).

Recombinant Proteins and Production of Antibodies—A partial cDNA corresponding to the last 325 amino acids of MARK protein, which coincide with its putative intracellular domain (ID-MARK), and the partial MIK cDNA corresponding to the last 143 amino acids of MIK (C-MIK) obtained from the yeast two-hybrid screening (see below) were cloned into a pET28 vector, and the recombinant His_6-tagged ID-MARK and His_6-tagged MIK were obtained as described (19). ID-MARK cDNA and C-MIK cDNA were also cloned into a pGEX-KG expression vector (Amersham Biosciences) in order to obtain the GST-ID-MARK and GST-C-MIK as described (19).

Antibodies against ID-MARK and C-MIK were obtained in rabbits inoculated with the His_6-tagged ID-MARK and His_6-tagged C-MIK recombinant proteins.

ID-MARK and MIK full-length cDNAs were cloned into pFLAG-CMV (Sigma) and pCMV5-HA vector (Clontech) in order to express both tagged proteins in mammalian COS-7 cells.

Immunofluorescence Assays—For immunolocalization assays, 15-DAP immature maize embryos were fixed in an ethanol/formaldehyde/glacial acetic acid (80:3.5:5) fixative solution for 1 h at room temperature and for 7 days at 4 °C. Embryos were dehydrated and embedded in paraffin. 8-μm sections were deparaffinized with xylol, dehydrated, and permeabilized with 0.5% Triton X-100. Sections were incubated with the corresponding antibodies (1:200) in phosphate-buffered saline buffer with 2% bovine serum albumin, 0.1% Triton X-100, for 1 h at room temperature plus 1 h at 4 °C. After extensive washing sections were incubated with an anti-rabbit Cy3-conjugated antibody (Jackson ImmunoResearch) (1:400) in phosphate-buffered saline with 0.1% Triton X-100, 2% bovine serum albumin for 1 h. After extensive washing, sections were mounted with Mowiol and stored at 4 °C. Fluorescence was analyzed by confocal laser scanning microscopy (Leica TCS SP).

Two-hybrid Analysis and GST Pull-down Assays—To construct the pAS2-1 bait plasmid, a partial cDNA corresponding to the last 325 amino acids of MARK was fused to the Gal4 DNA binding domain in plasmid pAS2-1 to generate pAS2-MARK (Clontech, Palo Alto, CA). This plasmid was used to screen a maize embryo (7 DAP) cDNA library cloned in the pACT2 plasmid kindly provided by Dr. Werr (Köln University). The screening was performed in strain Y190 of *Saccharomyces cerevisiae* (*MAT*α*, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, gal4*Δ*, gal80*Δ*, cyh*^{r}*2, LYS2::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS, MEL1 URA::GAL1*$_{UAS}$*-GAL1*$_{UAS}$*-lacZ*), and transformants were grown on selective medium lacking tryptophan, leucine, and histidine in the presence of 15 mM 3-amino-1′,2′,4′-triazole to select positive growing clones. The activity of the *lacZ* reporter gene was monitored visually by using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) filter assay. To test the specificity of MARK-MIK interaction, yeast cells were co-transformed with the pACT2-MIK1 clone obtained from the screening and either pAS2-MARK or the full-length cDNAs of lamin C, CDK2, and SNF1 cloned into the pAS2-1 plasmid that was used as control. The analysis of the possible interactions was performed as described (19).

GST pull-down experiments were performed as described (19) using the His_6-tagged ID-MARK as an input on different MIK GST fusion proteins bound to glutathione-Sepharose 4B beads.

Phylogenetic Analysis—Sequences were aligned using the ClustalW multiple alignment program (version 1.5) (20). DNAdist, Neighbor, Seqboot, and Consense programs in Felsestein's PHYLIP package (21) were used to generate a distance matrix based on the Jukes-Cantor algorithm and the corresponding neighbor-joining tree, respectively. Bootstrap analyses were performed using the Seqboot and Consense programs from Felsestein's PHYLIP package (21).

Gel Filtration—15 DAP maize embryos were ground in liquid nitrogen, and proteins were extracted with extraction buffer (10 mM NaCl, 100 mM Tris (pH 7.5), and 40 mM octyl glucoside). The homogenate was centrifuged twice at 3000 rpm, and the supernatant was ultracentrifuged at 100,000 × *g* for 90 min before loading onto a precalibrated Superose 6 column (Amersham Biosciences). Gel filtration was carried out in 100 mM Tris (pH 7.5), 10 mM NaCl, and 0.05% Triton buffer at 0.4 ml/min. 0.5-ml fractions were collected, and 15 μl of each was analyzed.

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1    MRSPPPPPWRAGRLHSPLPMLLLFVAALAARAGA                    SP

35   DDLASDARALLAFRDAVGRRLTWNASDVAGACSWTGVSCE

75   NGRVAVLRLPGATLSGAVPAGTLGN
100  LTALHTLSLRLNGLSGALPADLAS
124  AAALRNVFLNGNRLSGGFPQAILA                              LRR
148  LPALVRLSLGGNDLSGPIPAELGS
172  LTHLRVLLLENNRFSGEISDVK
194  LPPLQQFNVSFNQLNGSIPASLRS

218  QPRSAFLGTGLCGGPLGPCPGEVSPSPAPAGQTPSLTPVP
258  SSGGGNGSGSGSGGTIGGNGGESGHKNKKLSGGAIA


294  GIAIGSALGAGLLLFLLVCLC                                 TM

315  RRSGGTRTRSLEMPPPEAPAAAAAAGGRKPPEMTSGAAVA
355  PLTTIGHPNAPIGQSTSGKKLVFFGSAAAVAPFDLEDLLR
395  ASAEVLGKGAFGTTYKAVLESGATVAVKRLKDVTLSEAEF              ID
435  RERISEIGELQHEFIVPLRAYYYSKDEKLLVYDFMPMGSL
475  SAVLHGNVSSGRTPLNWDLRSSIALAAARGVEYIHSTTST
515  ASHGNIKSSNVLLGKSYQARVSENGLTTLVGPSSSSSRTT
555  GYRAPEVIDSRRVSQKADVYSFGVLLLELVTGKAPSQAAL
595  NDEGVDLPRWVQSVNRSEWGSLVFDMELMRHQTGEEPMAQ
635  LVLLAMDCTAQVPEARPSMAHVVMRIEEIKKSSVTPNIEQ
675  VDDQSSKAESEAAPTNPFAT
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FIG. 1. **Sequence and structure of MARK.** The subdomains of the protein are indicated as follows: *SP,* signal peptide; *LRR,* leucine-rich repeats; *TM,* transmembrane domain; and *ID*, intracellular domain. Conserved amino acids within leucine-rich repeats are shown in *boldface.* MARK sequence data will appear in EMBL, GenBank™, and DDBJ sequence data bases under the accession number AY188755.

Cell Culture, Transfection, Cell Lysis, Immunoprecipitation, Immune Complex Kinase Assay, and Western Blotting—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 10 μg/ml penicillin/streptomycin. Cells were transfected using LipofectAMINE transfection reagent (Invitrogen) according to the manufacturer's recommendation. Transfected cells were lysed 24 h after transfection in 0.5 ml of immunoprecipitation buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 μg/ml E-64, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 10,000 rpm for 10 min. 100 μl of supernatant was pre-cleared with 12 μl of a 25% slurry of protein G-Sepharose 4B fast flow (Sigma) and incubated with anti-HA antibody (Sigma) or anti-FLAG antibody (Sigma) for 2 h at 4 °C. 25 μl of a 25% slurry of protein G-Sepharose 4B fast flow (Sigma) was added to the mixture and incubated for 1 h at 4 °C. Immunocomplexes were washed twice in immunoprecipitation buffer.

Cell lysates or immunoprecipitates were separated by 10% SDS-PAGE before electrophoretic transfer onto polyvinylidene difluoride membrane. Proteins were detected with the respective antibodies.

Kinase Assays—ID-MARK was cloned into pGEX-KG expression vector and the GST_ID-MARK fusion was purified as mentioned before. MARK auto-phosphorylation and trans-phosphorylation on myelin basic protein (MBP, Sigma) was performed as described (22), using GST as negative control and GST-SERK (22) as a positive control. After phosphorylation, proteins were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to verify equal loading and then dried. The radioactivity was quantified with a PhosphorImager using the Quantity One program (Bio-Rad).

Kinase reactions on immunoprecipitated proteins were performed in 20 μl of phosphorylation buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 0.01% Triton, 1 mM dithiothreitol, 10 mM $MgCl_2$) containing 2 μg of MBP, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP 3000 Ci/mmol (Amersham Biosciences). The protein kinase reactions were performed at 30 °C for 15 min, and the reactions were stopped by adding 2× loading buffer. To verify equal loading of HA-MIK, samples were subjected to immunoblotting with anti-MIK.

RESULTS

MARK, a Maize Atypical PRK with a Kinase-dead Domain—As part of a project aimed to identify genes involved in

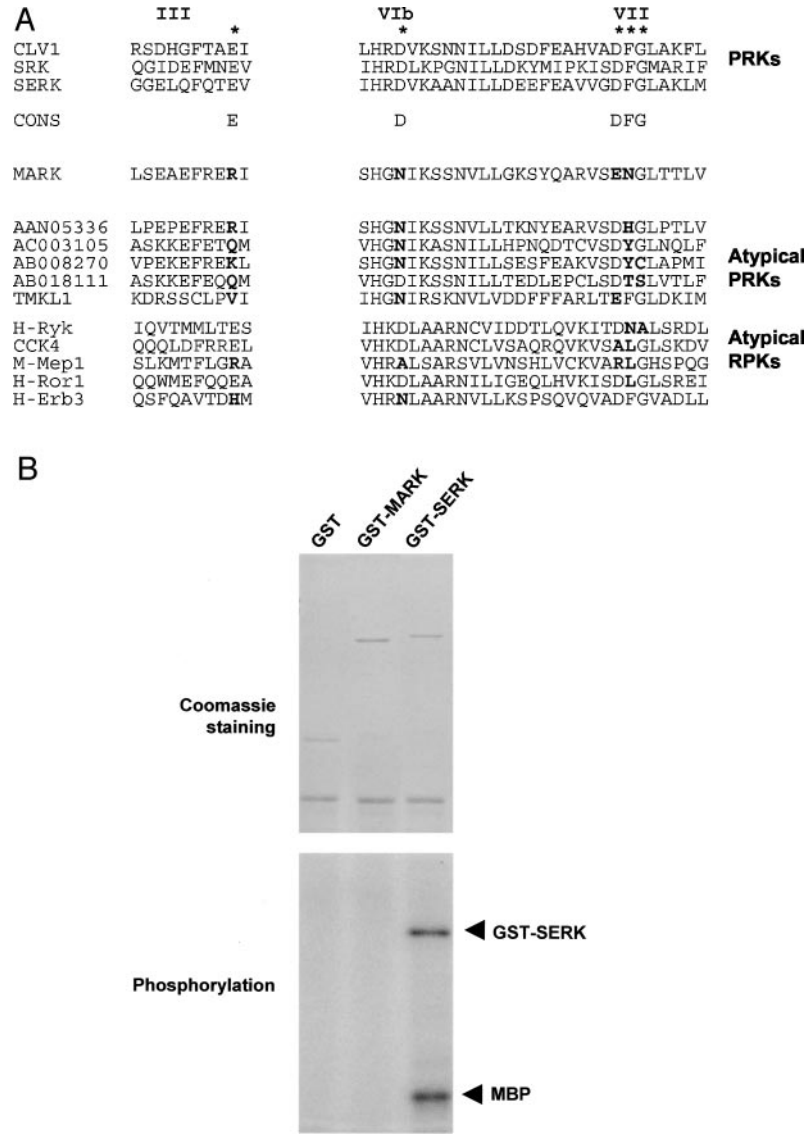

FIG. 2. **Analysis of MARK kinase activity.** *A*, sequence comparison of Ser/Thr kinases subdomains III, VIb, and VII. Comparison of ID-MARK sequence with typical and atypical PRKs and RPKs. The invariant and highly conserved amino acids among kinases are indicated with an *asterisk,* and the substituted amino acids are shown in *boldface.* Proteins shown in the figure are as follows: CLV1 (*Arabidopsis thaliana*) NP_177710; SRK (*B. rapa*) 2106157B; RLK5 (*A. thaliana*) CAB79651, AAN05336, AC003105, AB008270, AB018111; and TMKL1 (*A. thaliana*) CAA51385; H-RYK (*Homo sapiens*) NP_002949; CCK4 (PTK7) (*H. sapiens*) Q13308; M-Mepl (*Mus musculus*) AAM53410; H-Ror1 (*H. sapiens*) NP_005003; H-Erb3 (*H. sapiens*) P21860. *B*, MARK *in vitro* phosphorylation assay. The ability of GST_ID-MARK to auto- or trans-phosphorylate MBP was compared with GST-SERK (Shah *et al.* (22)) and GST alone as positive and negative controls, respectively. Reaction products were separated by SDS-PAGE and visualized by autoradiography (*lower panel*). Coomassie Blue staining (*upper panel*) was used to verify equal loading.

maize embryogenesis control, we performed a differential screening of an embryo cDNA library with embryo and adult leaf cDNA probes. One of the putative embryo-specific cDNA clones showed high sequence similarity with genes coding for Ser/Thr kinases. This clone was chosen for further study and used as a probe to screen embryo cDNA and genomic maize libraries. The complete cDNA and genomic sequences predicted an open reading frame of 694 amino acids with high sequence similarities with PRKs (Fig. 1). We named this protein MARK (from maize atypical receptor kinase). As deduced from the predicted protein sequence, MARK presents a signal peptide, an extracellular domain with 6 imperfect leucine-rich repeats, a single transmembrane domain, and an intracellular domain displaying high sequence similarity with Ser/Thr kinases.

Although the intracellular domain of MARK (ID-MARK) contains the 11 conserved subdomains of Ser/Thr kinases, some of the invariant and highly conserved amino acids within these subdomains are substituted (Fig. 2*A*). In particular, the aspartic acid present in the subdomain VIb, which is assumed to part of the kinase-active site (23), by an asparagine (residue 519), and the aspartic acid and phenylalanine within the DFG activation loop are replaced by a glutamic acid and an asparagine (residues 537 and 538) (see Fig. 2*A*). Mutation of the aspartic acid residue to glutamic acid, despite being relatively conservative, is sufficient to yield inactive the v-Fps kinase (24), and the critical role of the phenylalanine residue of the DFG triplet has been demonstrated in the case of H-Ryk (6). This suggests that the intracellular domain of MARK could be an atypical PRK with a kinase-dead domain. We thus expressed ID-MARK in *Escherichia coli* as a translational fusion with GST, and we checked the ability of the recombinant protein to auto- or trans-phosphorylate MBP *in vitro*. The results presented in Fig. 2*B* show that ID-MARK did not phosphorylate MBP nor was it auto-phosphorylated in the conditions in which the intracellular domain of SERK, an Arabidopsis PRK with a typical intracellular kinase domain (22), used here as a positive control, efficiently auto-phosphorylates and trans-phosphorylates MBP.

Cloning MIK, a New GCK-like Kinase—Atypical PRKs are understood to participate in signal transduction by means of regulated protein-protein interactions (3). We thus searched for proteins that could interact with the intracellular domain of MARK using a yeast two-hybrid approach.

The screening of 100,000 clones of a yeast two-hybrid immature embryo cDNA library using ID-MARK as bait gave a number of positive clones. Two of them contained overlapping sequences coding for a polypeptide with high sequence similarity with the C-terminal domain of two highly related MAP4K from *Brassica napus* (25). We named the corresponding gene MIK, from MARK interacting kinase. The analysis of MARK-MIK interaction by a yeast two-hybrid assay is shown in Fig.

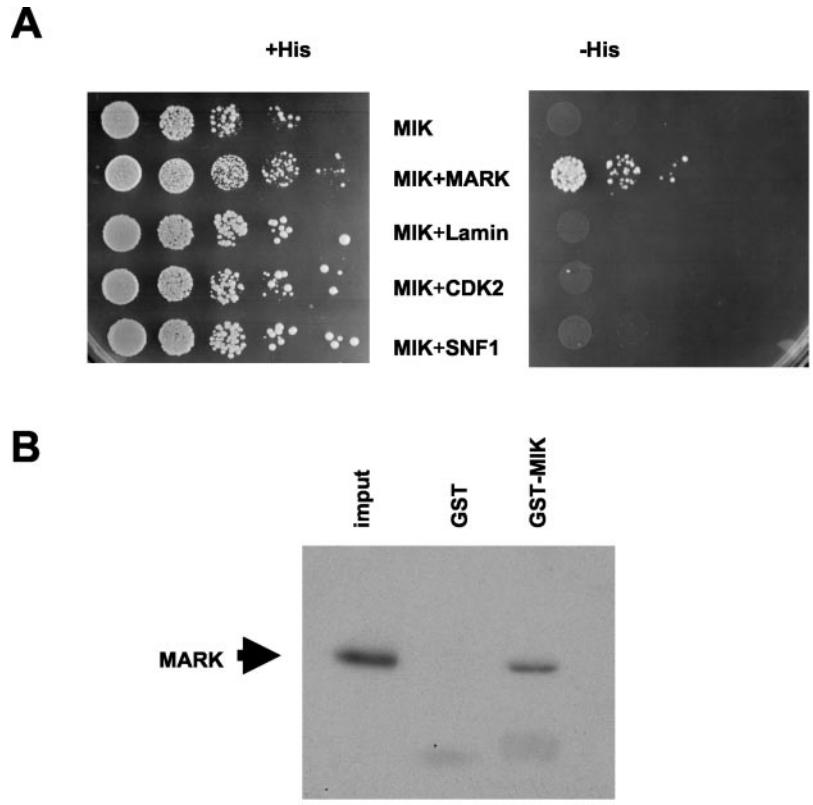

FIG. 3. **Analysis of MARK-MIK interaction.** *A*, yeast two-hybrid assay of the interaction of MARK with MIK. Yeast two-hybrid assay of the MARK-MIK interaction. Lamin C, CDK2, and SNF1 fused to Gal4 DNA binding domain were used as control proteins. Twenty microliters of the culture was dropped on His^+ (*left*) and His^- (*right*) plates at a cell density of 2×10^6 cells/ml (*1st lane*) and serial 10-fold dilutions (*2nd* to *5th lanes*). *B*, GST pull-down assay of the MARK-MIK interaction. GST pull-down assay of the interaction of recombinant His_6-tagged ID-MARK with GST and GST-MIK. The *1st lane* shows 1/10 of the input protein. Reaction mixtures were immunoblotted with Anti-MARK polyclonal antibody.

3*A*. Confluent growth was obtained when the culture transformed with the MIK partial cDNA, corresponding to the most C-terminal 143 amino acids of the protein. ID-MARK in the corresponding pACT2 and pAS2.1 plasmids was plated on selective medium at the highest density, and significant growth was observed when the culture was plated at a 10^2-fold dilution. On the contrary, cultures co-transformed with the MIK partial cDNA and different unrelated control proteins, such as lamin, CDK2, or SNF1 do not show significant growth even when plated at the highest density. The interaction between the C-terminal part of MIK and ID-MARK was confirmed using GST pull-down experiments. ID-MARK bound an immobilized GST-MIK polypeptide containing the most C-terminal 143 amino acids of the MIK protein, whereas it did not bind to an immobilized GST (Fig. 3*B*).

The partial MIK cDNA clones obtained from the yeast two-hybrid screen were used to screen maize embryo cDNA and genomic DNA libraries. The analysis of corresponding complete cDNA and genomic clones allowed us to deduce the complete MIK sequence (Fig. 4*A*). MIK contains an N-terminal kinase domain with high sequence similarity to Ser/Thr kinases of the GCK subfamily of Ste20 family of MAPK. Moreover, this domain contains the sequence VGTPFWMAPEV, which matches the signature motif of Ste20-like kinases, (v/i)GTPyWMAPEv (lowercase letters indicate a lower degree of conservation) (17). Fig. 4*B* shows a Neighbor-joining tree comparing the kinase domain of MIK (MIK-KD) with those of previously described GCK proteins. MIK-KD plots in a separate branch includes the Arabidopsis and *Brassica* GCK-like proteins described to date (25). This group of proteins resemble the GCK-III group of proteins, which include the human MASK, MST3, and SOK-1, as well as the *Dictyostelium* SEVERIN proteins. The C-terminal domain of GCKs is highly variable in sequence and is assumed to act as a regulatory domain. The C-terminal domain of MIK does not show significant sequence similarity with other GCK proteins except Arabidopsis and *B. napus* MAP4K (25).

To explore the possible biological significance of MARK-MIK interaction, we analyzed the expression of both genes. Northern blot hybridization analysis show that both *MARK* and *MIK* are expressed during early and mid-embryogenesis, from 2 until 30–40 DAP (days after pollination), with a peak at 15 DAP, in embryo and endosperm, whereas they are not expressed in adult leaves (Fig. 5). Preliminary analysis also showed that both genes are excluded from most adult tissues except the meristem (not shown), which are the structures that allow plants to continuously generate organs all along their life. Antibodies raised against the intracellular domain of MARK, α-MARK, and against the regulator domain of MIK, α-MIK, were used to perform immunolocalization studies. Fig. 6 shows that both MARK and MIK accumulate in 15-DAP immature embryos. MARK strongly accumulates in the provascular tissues of the coleoptile and in the pericycle of the radicle, which are the tissues that are most active in differentiation and proliferation in 15-DAP immature embryos. MARK also accumulates in the scutellum cells as a gradient from outside to inside, coinciding with the proliferation and differentiation activity of this organ at this stage of development. Although the differences in MIK accumulation are less pronounced, it also accumulates in the already mentioned proliferating and differentiating tissues of the immature embryos. Within the cell, MARK displays a membrane-associated pattern of accumulation, whereas most MIK seems to accumulate in the cytoplasm of the cells. *MARK* and *MIK* are thus expressed with a coincident pattern of expression during embryogenesis and are probably restricted to developing tissues.

MARK Interacts with MIK in Vivo and Induces Its Kinase Activity—To determine whether MARK and MIK interact *in vivo* and are components of high molecular weight complexes, we fractionated total protein extracts from 15-DAP maize immature embryos by gel filtration. MARK and MIK co-fractionate on a Superose 6 gel filtration column with identical peaks at ~600 kDa (fraction 18) suggesting that they form stable complexes *in vivo* (Fig. 7). We could not co-immunoprecipitate both

A

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1   MSDSASMAAAIEARFSSRDLIGCGSFGDVY
31  KGFDKELNKEVAIKVIDLEEAEDDIEDIQK
61  EISVLSQCRCPYITDYYGSYLHQTKLWIVM
91  EYMAGGSVADLLQAGPPLDEMSIACILRDL
121 LHAIEYLHSEGKIHRDIKAANILLTESGDV
151 KVADFGVSAQLTKTMSRRKTFVGTPFWMAP
181 EVIQNSEGYNEKADIWSLGITAIEMAKGEP
211 PLADIHPMRVLFIIPRENPPQLDEHFSKPM
241 KEFVSLCLKKNPAERPSAKELLKHRFI

268 KNARKTPKLLERIRERPKFAAKGMDATQNG
298 QTHDEEEDFGTGTIKVNRTKDTAPALSQGT
328 VRKSAVRDFSDRSEGTGTVRVVSRPPQIAS
358 TKDGRSDMPKSPKAPIRTADRENQWRSSGT
388 GSEESLSLRDTQSGRGRVESSTDDNDHSVS
418 GSGTVVLRSPRASQLYSTASNHSAKPPSRF
448 SSYEDMSNSGTVVQTQNEDPETPRSSRSRL
478 GIQEKASNAPLEDSAINLAEAKAALQAGLR
508 KGNARERPFINRREKGSHEHRSSGVNSQEV
538 QSEDVDTQKGHKLRQLPDGQSASRASSVAS
568 PAVSSLLLPSLKEATGDKFDRPAVHAFLDS
598 LMDLERDIPGSCEVLVGRLLHRLGSSKDSS
628 LQGLQETAMSIFTKEPEPPSEKAGDNKLAN
658 MPPLAAPTVSPLARFLLTRWQNQVSQDLNS
688 V
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KD

RD

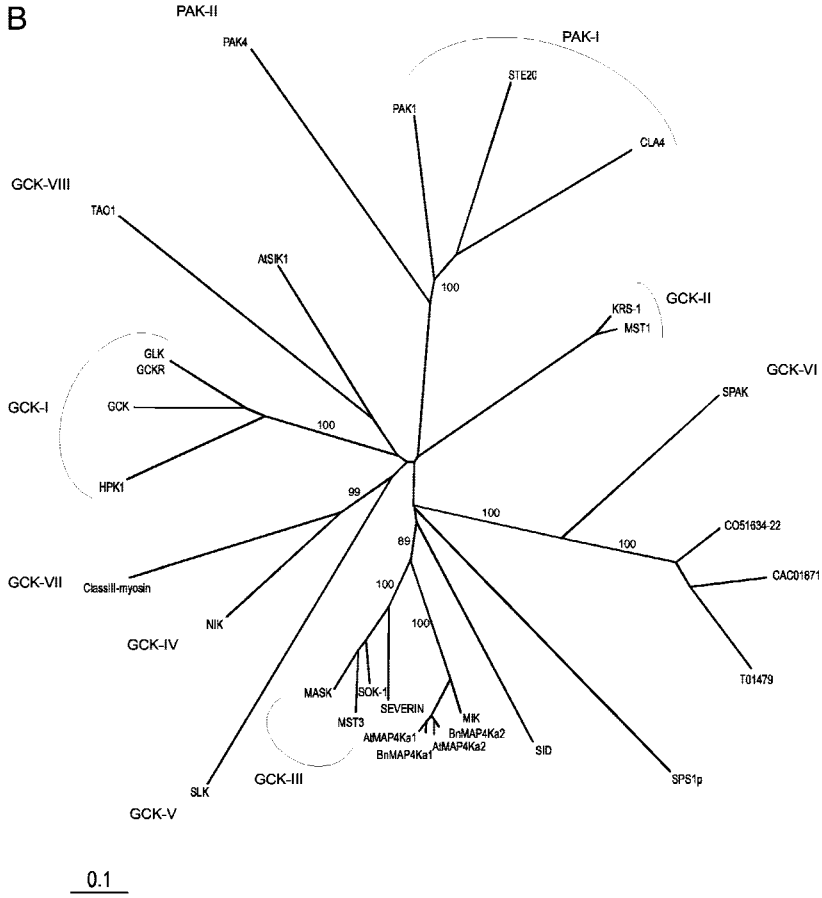

FIG. 4. **MIK sequence and structure.** *A*, sequence of MIK. The different subdomains of the protein are indicated as follows: *KD*, kinase domain; *RD*, regulator domain. The sequence that matches the consensus of Ste20 kinases is *underlined.* The peptide obtained from the two-hybrid screen is shown in *boldface.* MIK sequence data will appear in EMBL, GenBank™, and DDBJ sequence data bases under the accession number AY18875. *B*, phylogenetic analysis of GCK proteins. Neighbor-joining tree obtained comparing the kinase domains of proteins representative of the different GCK subfamilies. Proteins shown are as follows: SEVERIN (AAC24522); BnMAP4Ka1 (CAA08757); BnMAP4Ka2 (CAA08758); AtMAP4Ka1 (AC008007); AtMAP4Ka2 (AP000413); MASK (XP_029574); MST3 (Q9Y6E0); KRS-1 (NP_006272); MST-1 (CAB89421); SPS1p (NP_010811); GCK (NP_004570); GCKR (NP_003609); HPK1 (NP_003609); PAK1 (AAC24716); PAK4 (NP_005875); TAO (NP_057235); SLK (NP_055535); SPAK (XP_002444); SID (gi2370557); AtSIK1 (U96613.1). The following protein sequences were deduced from EST or genomic sequences: putative Ste20 from *Oryza sativa* (Co51634_22); putative Ste20 from *A. thaliana* (CAC01871 and T01479). Bootstrap values above 50% supporting major clusters are shown.

proteins from peak fractions probably because the antibodies used were raised against the interacting domains of both proteins and were competing with the proteins for interaction. We thus tested whether MARK and MIK interact *in vivo* in COS-7 cells transfected with an HA-ID-MARK construct and a FLAG-MIK construct. An antibody against HA efficiently immunoprecipitated ID-MARK, and MIK was also detected in co-transfected cells immunoprecipitated with this antibody (Fig. 8*A*), showing that MIK interacts with ID-MARK *in vivo*.

We have also used the COS-7 cell expression system to analyze MIK phosphorylation activity. Constructs containing MIK- and MARK-tagged proteins were transfected into COS-7 cells alone or in combination, and MIK-tagged or MARK-tagged proteins were immunoprecipitated and used for phosphorylation assays. FLAG-ID-MARK does not auto-phosphorylate or trans-phosphorylate MBP (Fig. 8*B*), confirming that ID-MARK is a kinase-dead domain. HA-MIK weakly phosphorylated MBP in the conditions tested (Fig. 8*B*). Interestingly, the co-expression of FLAG-ID-MARK induced a 7-fold increase of HA-MIK phosphorylation activity, whereas the co-expression of a control GFP construct had no effect (Fig. 8*B*).

DISCUSSION

We report the cloning and characterization of two new plant genes coding for an atypical PRK and a GCK-like MAP kinase

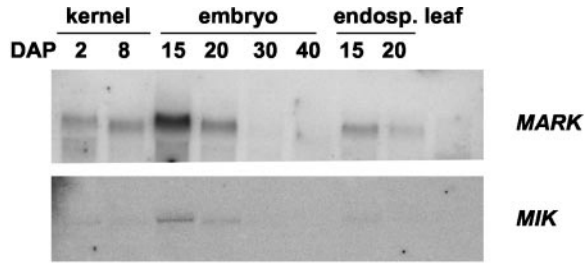

FIG. 5. **MARK and MIK expression patterns.** Northern blot analysis of *MARK* (*top*) and *MIK* (*bottom*) in developing seeds and adult leaves.

that could participate in signal transduction in maize. MARK is an atypical PRK with amino acid substitutions in its kinase-like domain that are sufficient to render inactive other kinases. Our results show that, indeed, the intracellular domain of MARK does not phosphorylate *in vitro*. We performed site-directed mutagenesis to replace the atypical residues present in subdomains III, VIb, and VII of MARK by the consensus residues, but the modified MARK protein did not auto- or trans-phosphorylate (not shown). This suggests that other modifications of amino acids important for kinase activity have occurred during MARK evolution. This is also the case of the

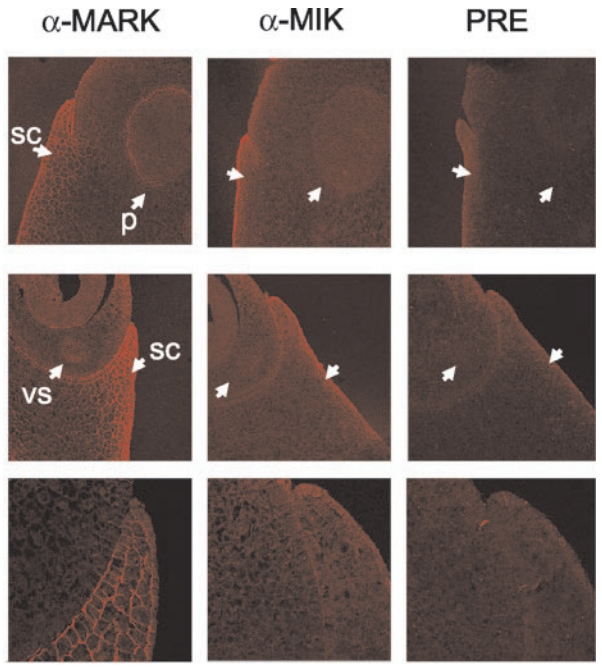

FIG. 6. **Immunolocalization of MARK and MIK.** Transversal sections of 15-DAP immature embryos at the level of the radicle (*top panels*) or the coleoptile (*middle* and *bottom panels*) incubated with α-MARK (*left*), α-MIK (*middle*), or preimmune (*right*) sera. The scutellum (*sc*), pericycle (*p*), and provascular (*vs*) tissues are shown by *white arrows*. *Bottom panels* show the coleoptile sections (*middle panel*) at higher magnification.

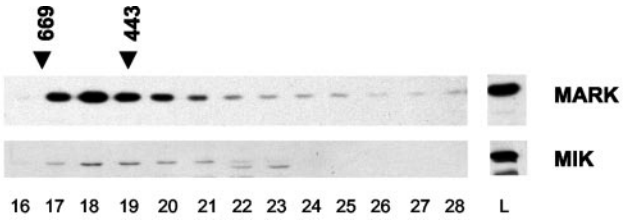

FIG. 7. **Participation of MARK and MIK in high molecular weight complexes.** Total protein extract from 15-DAP maize embryos were fractionated on a Superose 6 column. Fractions were analyzed by Western blot for the proteins indicated to the *right* of each panel. Fraction numbers are shown *below lanes*. *Arrows* indicate where size standards eluted from the column. *L* indicates 1/100 of the amount of protein loaded onto the column.

mammalian ErbB3 atypical RPK, which does not phosphorylate even after the recognizable atypical residues have been modified (26). Our results thus indicate that MARK is a kinase-dead PRK that signals by kinase-independent mechanisms. Although only one plant protein with the sequence characteristics of an atypical PRK has been described to date, *Arabidopsis* TMKL1 protein (27), the data bases contain a large number of *Arabidopsis* and rice sequences with these characteristics (see Fig. 3*B*), thus suggesting that phosphorylation-independent mechanisms, mediated by atypical PRKs, are important in signal transduction in plants, as they have been shown to be in animal systems.

In the last few years an increasing number of PRKs have been described in plants. Although they are assumed to transduce signals by mechanisms similar to their animal counterparts (28), only a handful of putative downstream signaling proteins interacting with them have been described (29–36), and the intracellular signaling cascade they regulate is unknown in most cases. In other cases, like flagellin receptor FLS2, for which a complete MAP kinase cascade acting downstream the receptor has been characterized (37), it is still

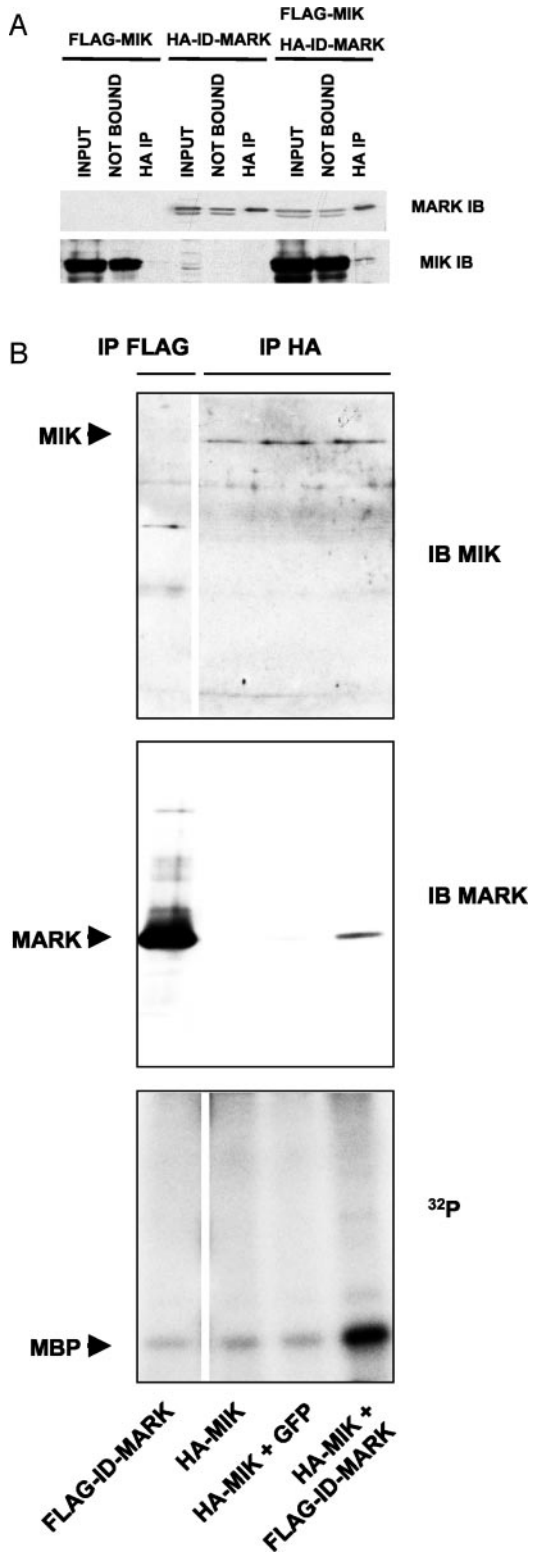

FIG. 8. **Activation of MIK activity by interaction MARK-MIK *in vivo*.** *A*, MARK and MIK interact in COS-7 cells. COS-7 cells were transfected with clones corresponding to the indicated proteins. 1/3 of the anti-HA immunoprecipitates, 1/10 of the extracts, and 1/10 of the not bound fraction were subjected to immunoblot with antibodies against MARK (*upper panel*) and MIK (*lower panel*). *B*, activation of MIK phosphorylation activity. COS-7 cells were transfected with clones corresponding to the indicated proteins. Anti-HA (*2nd* to *4th tracks*) or anti-FLAG (*1st track*) immunoprecipitates (*IP*) were assayed for phosphorylation activity on MBP. Reaction products were separated by SDS-PAGE and visualized by autoradiography (*lower panel*). The amount of MIK and MARK proteins in the immunocomplexes was determined by immunoblotting (*IB*) with anti-MARK (*middle panel*) and anti-MIK (*upper panel*) antibodies.

unknown whether the receptor interacts directly with the MAP kinase cassette or whether an unknown protein connects the two signaling modules.

MAPK cascades are usually connected to membrane receptors through G proteins such as Raf, heterotrimeric complexes, or MAP4K (15–17). Only one small G protein has been shown to interact with a membrane receptor in plants (32) although its capacity to activate MAP signaling cascades has not been demonstrated. On the other hand, although more than 10 genes in *Arabidopsis* probably code for MAPKKKK (38), and they are thus assumed to be relatively abundant in plants, nothing is known of their possible participation in signaling. For this reason, the interaction shown here between MARK and MIK, an atypical PRK and an MAP4K, resulting in the activation of the latter, is particularly relevant. It is interesting to note that MARK and MIK are expressed in developing tissues, and different components of the MAP kinase cassettes are expressed in proliferating tissues in plants (38). In particular, NPK1, a tobacco MAP3K, accumulates in growing tissues, cotyledons, vascular systems, and the meristems of the plant (39) where *MARK* and *MIK* are also expressed. Recently, it has been demonstrated that NPK1 controls the expansion of the cell plate during cytokinesis in tobacco (40), activating a complete set of MAP kinases (reviewed in Ref. 38). We are at present searching for proteins that could interact with MIK in order to identify its possible downstream signaling partners.

MAP kinases, and in particular GCK-related kinases, usually contain a regulatory domain that inhibits their kinase activity (41–43). Full activation of GCKs requires the release of this auto-inhibition, which can be accomplished by different mechanisms including the cleavage of the regulatory domain by caspases (44, 45), or by conformational changes induced by protein-protein interactions through the regulatory domain (43). Our results show that MIK has a low kinase activity when expressed in COS-7 cells and that the co-expression of ID-MARK greatly increases this activity. As ID-MARK specifically interacts with the C-terminal regulatory domain of MIK, we propose that MARK-MIK interaction results in the release of a possible autoinhibition of MIK kinase activity by this regulatory domain.

In summary, our results suggest a new mechanism by which atypical RPKs can transduce signals and could constitute the first step of a still unknown signaling cascade associated with developmental processes in plants.

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