MRK, a Mixed Lineage Kinase-related Molecule That Plays a Role in γ-Radiation-induced Cell Cycle Arrest*

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Mitogen-activated protein (MAP) kinase pathways are three-kinase modules that mediate diverse cellular processes and have been highly conserved among eukaryotes. By using a functional complementation screen in yeast, we have identified a human MAP kinase kinase kinase (MAPKKKK) that shares homology with members of the mixed lineage kinase (MLK) family and therefore was called MRK (MLK-related kinase). We report the structure of the MRK gene, from which are generated two splice forms of MRK, MRK-α and MRK-β, encoding for proteins of 800 and 456 amino acids, respectively. By using a combination of solid phase protein kinase assays, transient transfections in cells, and analysis of endogenous proteins in stably transfected Madin-Darby canine kidney cells, we found that MRK-β preferentially activates ERK6/p38γ via MKK3/MKK6 and JNK through MKK4/MKK7. We also show that expression of wild type MRK increases the cell population in the G2/M phase of the cell cycle, whereas dominant negative MRK attenuates the G2 arrest caused by γ-radiation. In addition, exposure of cells to γ-radiation induces MRK activity. These data suggest that MRK may mediate γ-radiation signaling leading to cell cycle arrest and that MRK activity is necessary for the cell cycle checkpoint regulation in cells.

In a wide range of organisms, from yeast to mammals, mitogen-activated protein kinase (MAPK) pathways mediate a variety of signals that regulate multiple physiological processes, including cell proliferation, cell differentiation, and cell death as well as stress-induced responses (1–3). These MAPK modules consist of distinct cascades of kinases, beginning with a serine/threonine kinase, MAPKKK, which phosphorylates and activates a dual specificity kinase, MAPKK or MEK, that in turn transfers phosphates onto threonine and tyrosine residues of a third enzyme, MAP kinase. The MAP kinase subsequently phosphorylates and activates various transcription factors, among other substrates. In mammals, the best characterized MAPK pathways are defined by the four main classes of MAPK they activate: extracellular signal-regulated protein kinases (ERK-1 and -2), Jun amino-terminal kinases (JNK-1, -2, and -3), p38 proteins (p38α, -β, -γ, and -δ), and ERK5 (4). The MAPKKK family consists of at least 14 members that include the MEKK group (MEKK1–4), the mixed lineage kinase group (MLK1–3, DLK, and Lzk), the ASK proteins (ASK1 and -2), TAK1, TAO, andTpl2/Cot. Although members within each group are highly homologous, with identity ranging between 50 and more than 90%, the homology between groups is significantly reduced and is restricted to the kinase domain. The large number of structurally diverse MAPKKKs may reflect tissue specificity or stimulus-specific signaling. Although substantial progress has been made in linking each of the known MAPKKK proteins to specific MAP kinase pathways, their precise contribution has not been clearly defined. For instance, MEKK1–3, DLK, MLK, and Tpl2 have been reported to activate preferentially JNK or ERK, rather than the p38 MAPK (5–10). Conversely, TAK1, MEKK4, TAO, and ASK1 more effectively activate the p38 pathway (11–14). The link between individual MAPKKKs and specific upstream control molecules has only been identified for some family members and remains to be firmly established for most. Despite our growing knowledge of the signaling elements involved in each cascade, no upstream MAPKKKs have yet been described for some MAP kinases, such as ERK3 (15) and ERK6/p38γ (16).

In Saccharomyces cerevisiae, there are five MAPK modules, one of which is the well characterized mating pheromone pathway (17). In this system, Ste11 is the MAPKKK that activates Ste7, the MEK counterpart, which in turn activates the MAPK, Fus3 (18). We and others (19, 20) have shown that loss of Ste11 by gene knock out can be functionally complemented in this system by an active mammalian Raf protein and its substrate MEK. In the present study we conducted a functional screen in this system to identify novel components of MAPK pathways, and we discovered a gene that encodes a serine/threonine kinase, designated MRK for MLK-related kinase. Here we describe the characterization of the structure of the MRK gene and the effect of the MRK protein on the known mammalian MAP kinase cascades. We found that MRK expression preferentially activated the ERK6/p38γ and JNK path-

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† The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAP kinase; MAPKKK, MAP kinase kinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; MDCK, Madin-Darby canine kidney; JNK, c-Jun NH2-terminal kinase; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; UTR, untranslated region; Gy, gray; MBP, myelin basic protein.
ways, both in transiently transfected and stable cell lines, whereas it had a marginal effect on p38a and no significant effect on ERK. The activation of these pathways is accompanied by stimulation of their respective MKKs, in particular MKK3/MKK6 and MKK4. We also report that expression of wild type MRK induces an increase in the G2/M cell population. Conversely, γ-ray-mediated G1 and G2 arrest are decreased in cells expressing the dominant negative allele of MRK. The effect of γ-radiation is accompanied by activation of endogenous MRK. These findings suggest a role for MRK in the regulation of cell cycle checkpoints.

**EXPERIMENTAL PROCEDURES**

**Plasmids, cDNA Library Constructions, and Genomic DNA Analysis**—The 2-μm based plasmid pAB233BX2, containing the URA3 selectable marker, was derived from pAB23-BX2 (21) by inserting a new polyclinker (SacI, SacII, AstII, and XhoI) between BstXI and NotI and used as the library vector. cDNA synthesis was performed on 5 μg of poly(A)+ RNA, isolated from 2 × 10⁷ Jurkat cells using the Invitrogen Fast track kit. The first strand was primed with a linker-primer from the ZAP-cDNA synthesis kit (Stratagene) that contains an XhoI site. Protection of this 3′-cloning site allowed for unidirectional cloning of the finished cDNA. This protection was provided by ligation of a BstXI adaptor. The cDNA library was size-fractionated to collect the portion containing inserts above 500 bp and ligated to the prepared vector. Transformation of *Escherichia coli* DH10B (Invitrogen) yielded approximately 1 × 10⁸ total transformants of which vector religation represented a 3% background.

Catalytically inactive MRK mutants were generated using the Quick-Change, site-directed mutagenesis kit from Stratagene. The following primer was used to convert Lys-45 to Ala: 5′-GGAGTGCCGCTGTCGCGACGAG-CTCCTCA-3′, generating MRK-β-K45A. Mutagenesis was conducted as suggested by the manufacturer. pBS-MRK-β-KT3 was constructed by inserting the KT3 tag after the last codon of MRK, using the following 3′ end oligo in a PCR: GGATCCACACCAACGAGAAGAG-ACCAGGCCGC.

The pCDV vector, containing the SV40 promoter, was obtained from George Martin (Onyx Pharmaceuticals) and was used to generate pCDV-MRK-β-WT and pCDV-MRK-β-K45A by subcloning *SalI* (filled in)–NotI fragments from the pBS constructs. pEXV-ERK2, GST-ERK2, and pEXV-JNK1 were obtained from John Lyons and Jerry Bertman (Onyx Pharmaceuticals). FLAG-p38 and FLAG-MKK6-glu were kindly provided by Roger Davis (University of Massachusetts). PCEFL-HA-ERK6 (p38) was a generous gift of Silvio Gutkind (National Institutes of Health). pTRE-MRK-β and pTRE-MRK-β-K45A were constructed by inserting a 1.4-kb HindIII (filled-in)–NotI fragment containing the MRK-β-KT3-tagged genes into the pUWII-NotI sites of the pTRE2 vector (CLONTECH).

A human whole blood *A. thaliana* genomic library (Stratagene) was screened with an MRK kinase domain probe, generated using the following primers: 5′-GGAGTGCCGCTGTCGCGACGAG-CTCCTCA-3′ and 5′-GGTGATCATATTCATCTTCCTC-3′. A High Density CITB Human BAC colony DNA membrane (Research Genetics) was screened with the same probe. The cDNA library was size fractionated to collect the portion containing inserts above 500 bp and ligated to the prepared vector. Transformation of *E. coli* DH10B (Invitrogen) yielded approximately 1 × 10⁸ total transformants of which vector religation represented a 3% background.

**RNA and Protein Analysis**—Poly(A)+ RNA from HT1080 cells was prepared with the Invitrogen Fast Track kit. Human Multiple Tissue Northern blots I and II were purchased from CLONTECH. Mononclonal (4-23) and polyclonal (40-5) anti-MRK antibodies were generated against recombinant MRK expressed in *E. coli*. These antibodies were typically used at the 1:1000 dilution. Mammalian cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂), supplemented prior to use with 1 mM sodium orthovanadate, 50 mM NaF, protease inhibitor mixture tablets (1 tablet/7 ml) (Roche Molecular Biochemicals). Cells were lysed on ice for 4°C for 20 min, and the debris was collected at 14,000 rpm at 30°C. Typically, 50 μg of proteins were immunoprecipitated with the appropriate antibodies, either coupled to protein A-Sepharose beads (KT3 antibodies) or together with protein A beads, for 2 h at 4°C. Immune complexes were washed three times with lysis buffer, and the proteins were eluted in Laemmli sample buffer heated for 5 min at 95°C. Samples were processed for Western blot analysis. Kinase reactions were performed on the immune complexes in the presence of 30 μM Tris-HCl, pH 8.0, 20 mM MgCl₂, 1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 10 μM cold ATP, 5 μCi of [γ-^32^P]ATP, and the appropriate substrate as described in the figure legends. MKK4, MKK7, and MKK6 recombinant proteins were purchased from Upstate Biotechnology, Inc., GST-Jun from Stratagene, and ATF-2 from Cell Signaling Technologies.

**Cell Culture and Transfections**—CO5-1 cells were transiently transfected by electroporation essentially as described in Porfirri and McCormick (24).

The parental MDCK T23 clone (25), which expresses the tetracycline-repressible transactivator (26), was used to generate stable lines expressing wild type (pTRE-MRK-β), catalytically inactive MRK-β-K45A, and MRK-β-K45A by subcloning *SacI*–(filled-in)–NotI fragment containing the hygromycin resistance gene. Cells were transfected with the Effectene method from Qiagen, following the manufacturer’s protocol. Clone selection was carried out in the presence of 200 μg/ml hygromycin B (Invitrogen) and 20 ng/ml doxycycline (Sigma). Drug-resistant clones were further tested for expression of the transgenes after removal of doxycycline to induce expression of the recombinant MRK-β gene. Growth arrest in the G1 phase of the cell cycle was monitored by FACS analysis with a FACSCalibur flow cytometer (BD Pharmingen). Data were analyzed using the Cell Quest (BD Pharmingen) and ModFit (Verity) analysis software.

**RESULTS**

**Cloning of the MRK Gene and Characterization of Its Genomic Structure**—To identify novel signal transduction molecules affecting MAP kinase pathways, we used a yeast system in which mammalian Raf and MEK proteins were expressed to complement the yeast Ste11 protein (19, 23). Strain SY1984, expressing Raf and MEK (SY1984 R-L M-T), responded to Raf activation by signaling through the yeast MAP kinase, Fus3. In this strain, activation of Fus3 induces transcription of the *HIS3* gene off the *FUSI* promoter, which leads to growth in the absence of exogenous histidine. Cells were transformed with a human cDNA library prepared from Jurkat cells, and colonies that grow in the absence of histidine were characterized. This screen could in principle identify activators of each member of the MAPK cascade regulating the expression of the *HIS* gene, as well as genes that could indirectly affect growth on selection media. Therefore, to identify the functional target of each cloned library gene, cDNAs from each colony were isolated and re-tested in four strains as follows: the original strain used in the primary screen (SY1984 R-L M-T), a strain
that lacked Raf (SY1984 M-T); one that lacked MEK (SY1984 R-L); and a Ste7 null (SY1943-L21). The screen yielded several genes that activated Raf, MEK, or the yeast MEK, Ste7. Different Ras and 14-3-3 clones were isolated as Raf activators in that they were capable of stimulating growth in the absence of histidine only when Raf was present. Among the MEK activators, MEKK1 and MEKK2 partial clones, encompassing the kinase domains of these genes, were obtained. In addition, two novel genes were isolated as activators of Ste7. They allowed growth on media lacking histidine in the wild type STE7 strain SY1984 but not in the ste7 null strain SY1493-L21. These clones, designated J42 and J207, encoded identical kinase domains but diverged in their 3’ sequences, suggesting that they might represent splice variants of the same gene. Northern blot analysis using a cDNA probe from the common kinase domain identified a predominant band of 7.5 kb and less abundant forms of 3.8 and 1.6 kb. A specific probe from the 3’ end of J42 identified the longest transcript as J42 mRNA, whereas a probe from the 3’ end of J207 recognized the 3.8-kb message (Fig. 1A). The identity of the 1.6-kb species remains to be elucidated. Analysis of mRNA distribution indicated that J42 and J207 are ubiquitously expressed in normal tissues but are most abundant in skeletal muscle and heart (Fig. 1B). Although the signal from brain and kidney tissues is very weak, protein analysis in cells derived from such tissues (see below and data not shown) indicated expression of these genes. As the original J42 cDNA clone was only 2.2 kb long, we screened several cDNA libraries and performed 5’ -RACE analysis on poly(A)+ RNA as well as on 5’-capped mRNA (which represents a population of full-length mRNAs), to identify the additional sequences corresponding to the 7.5-kb mRNA. These approaches identified the 5’ start site of the two major RNA forms located 195 bp upstream of the ATG start codon. Analysis of 2 genomic

<FIG. 1. Northern blot analysis of MRK messages. A, 10 μg of poly(A)+ RNA from HT1080 human cells were loaded in each lane and hybridized to 32P-labeled probes as follows. A PCR probe derived from the common kinase domain region (nucleotides 193–513) was used in lane 1, a J42-specific probe, generated from the PstI fragment (nucleotides 1322–2181) in the unique 3’ end sequences, was hybridized to RNA in lane 2, and a J207-specific probe (nucleotides 1291–2650) was used in lane 3. B, a blot containing poly(A)+ RNA isolated from various human tissues was hybridized with the same kinase probe used in A, lane 1. The 3rd panel represents a longer exposure of the indicated samples, to illustrate the low but detectable presence of J42 and J207 transcripts in these tissues. All tissue samples expressed similar levels of β-actin mRNA (data not shown).>
encodes a highly acidic domain. The carboxyl terminus of J207, on the other hand, has a sterile α-motif domain, which has been implicated in protein-protein interactions (33). The isolated J42 and J207 cDNAs have a coding potential for proteins of calculated molecular masses of 51.5 and 91.1 kDa, respectively. Interestingly, the long mRNA encodes for the shorter of the two proteins. We named the J207 and J42 genes (and their corresponding products) MRK-α and -β, respectively, for MLK Related Kinases. In this paper we focus primarily on the characterization of MRK-β.

Characterization of the MRK Proteins—To characterize the function of the MRK proteins, we tagged the MRK-α and -β cDNAs with FLAG and KT3 (34) tags, respectively. When expressed in COS-1 cells, the two cDNAs yielded proteins with SDS-PAGE mobility of about 98 and 55 kDa (Fig. 4A), in agreement with the coding potential of the two cDNA clones. To verify that these clones represented the full-length endogenous proteins, we generated a monoclonal antibody, 4-23, against the MRK common kinase domain. We used this reagent to identify endogenous MRK polypeptides in the human epithelial cell line, MCF-10A. Fig. 4 shows that the anti-MRK antibody recognizes two bands with SDS-PAGE mobility similar to that of the respective recombinant proteins. The slight electrophoretic retardation observed for recombinant MRK-β (see Fig. 4, A and B) when compared with the endogenous protein was attributed to the KT3 tag, as demonstrated by removal of the tag (Fig. 4C).

To verify that MRK-β possessed kinase activity, we tested recombinant MRK-β for kinase activity when expressed in COS-1 cells. Fig. 4D shows that recombinant MRK-β, in addition to autophosphorylation, exhibits kinase activity toward the generic substrate, MBP. Both auto- and exogenous substrate phosphorylation depend on the integrity of the MRK kinase domain, as a substitution of alanine for a critical lysine in the active site in the mutant MRK-β-K45A abolishes these activities (Fig. 4D, lane 3). Thus, the MRK-β protein is a functional kinase.

Effect of MRK-β on MAPK Pathways—The functional screen used to clone MRK, as well as the kinase domain sequence, suggested that the MRK proteins belong to the MAPKKK family. To test whether MRK affects the known MAP kinase pathways, we transiently co-expressed MRK-β with ERK, JNK, or p38α in COS-1 cells. The MAP kinases were immunoprecipitated and tested for kinase activity in vitro against their respective substrates. To assess the relative stimulation of the different MAP kinases by MRK-β, in each group we compared the effect of known activators and stimuli of each pathway with that of MRK-β. Fig. 5 shows that MRK-β stimulated ERK2 activity by about 2.5-fold over background, whereas Ras and serum activated ERK2 6- and 11-fold, respectively. A slightly greater stimulation of p38α was observed, although its extent was still about a third of that obtained with MKK6 or with osmolarity treatment. On the other hand, we found that MRK-β activated JNK 10-fold above background, and this level was comparable with that caused by UV treatment and MEKK1.

Because the activation of the p38α MAP kinase was weak but reproducible, we asked whether MRK-β affected a less well characterized member of the p38 subfamily, ERK6/p38γ. These members of the p38 subfamily have been reported to be differentially activated by several stimuli, such as hypoxia and γ-irradiation (35, 36), and respond differently to the inhibitory compound, SB203580 (37). In contrast to p38α, we observed a 17-fold activation of ERK6/p38γ by MRK-β, as reflected by the phosphorylation of ATF-2. This effect was comparable with that obtained with MKK6, the direct activator of ERK6/p38γ, and it depended on MRK kinase activity, as expression of the kinase inactive mutant, MRK-β-K45A, did not result in activation. This level of activation was also observed when the translation factor PHAS-1 (38, 39) was used as substrate in the in vitro kinase reaction (data not shown). These results indicate that MRK-β is upstream of the stress kinases JNK and ERK6/p38γ.

To assess whether MRK-β could activate endogenous MAP kinases, we generated stable Madin-Darby canine kidney (MDCK) (25) cell lines that expressed wild type or catalytically inactive MRK-β-K45A under the control of a tetracycline-repressible transactivator (26). In this system, removal of the tetracycline analog doxycycline induced expression of the recombinant proteins. This expression system offered the advantage of controlling the recombinant protein expression levels by titration of doxycycline. Vector-transfected clones were used as

![Genomic structure of the MRK locus](image)

**Fig. 2.** Genomic structure of the MRK locus. The BAC clones used to characterize the MRK locus are indicated. The genomic contigs represent partial sequences deposited in the Washington University Genome Sequencing Center Data base. Gaps between non-overlapping contigs are of unknown length; however, they encompass intron sequences. Exons are indicated by dark lines or boxes and are identified by the numbers above them. The correspondence between the domains in the MRK open reading frame and the different exons is indicated. LZ, leucine zipper; SAM, sterile α-motif.
control to monitor the effect of the induction treatment. Fig. 6 shows that, upon induction of the MRK-H9252 wild type protein, both endogenous JNK and ERK6/p38/H9253 are activated. Although the anti-phospho-ERK6/p38/H9253 antibodies cross-react with phospho-p38/H9251, a parallel set of samples blotted with the anti-phospho-p38/H9251-specific antibodies showed a very weak signal. These results confirmed our observations with transient transfection that JNK and ERK6/p38/H9253 are strongly activated by MRK-H9252, whereas no significant activation of p38/H9251 or ERK1 and -2 was detected. Activation of these pathways depended on the kinase activity of MRK-H9252, as stimulation was not observed after expressing similar levels of the recombinant catalytically inactive mutant, MRK-H9252-K45A (Fig. 6).

To confirm that MRK-H9252 activated JNK and ERK6/p38/H9253 via their respective MKK proteins, we tested the activation state of the endogenous MKK4 and MKK3/MKK6 proteins in the MDCK clones using antibodies specific for these phosphokinases. Fig. 6 shows that, upon induction of MRK-H9252 expression, these MKK proteins became phosphorylated at their active sites. Interestingly, both the MKK3/MKK6 and ERK6/p38/H9253 proteins are activated as early as 8 h after induction of MRK-H9252 expression, when the levels of the recombinant protein are relatively low. In contrast, the JNK pathway is activated at later time points. Therefore, in this system MRK-H9252 strongly stimulates the ERK6/p38/H9253 pathway.

MRK-H9252 Phosphorylates MKK4, -6, and -7 in Vitro—The sequence homology between MRK and members of the MAPKKK family suggests that it might phosphorylate and activate members of the MAPKK family of proteins. We therefore tested some of the known members of this family as substrates to be phosphorylated directly by MRK-H9252 in vitro. We transiently transfected COS-1 cells with MRK-H9252, MEKK1 as positive control for MEK activation, or the empty vector as negative control. The recombinant proteins were immunoprecipitated with

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Fig. 3. Sequence homology among the kinase domains of MRK, MLK2, MLK1, and TAK1. A, sequence alignment encompassing the kinase domain and the leucine zipper region of MRK. Alignment was determined by the ClustalW method. The underscored region corresponds to the putative leucine zipper domain. B, phylogenetic tree indicating the sequence distances between MRK, the MLK proteins, and TAK1.
the respective anti-tag antibodies and incubated with purified recombinant kinase-inactive MEK mutants (MEK-B), MKK4, MKK7, or MKK6. Fig. 7 shows that MRK-H9252 did not phospho-
rylate a kinase-inactive version of recombinant MEK, which, however, was a good substrate for MEKK1. This result is in agreement with the lack of substantial activation of the ERK pathway by MRK-β and suggests that the ERK activation observed in COS-1 cells is indirect and a possible result of production of autocrine growth factors. On the other hand, we found good incorporation of \(^{32}\)P-ATP in the other three MKK proteins.

These in vitro results, together with the activation observed in cells, suggest that MRK-β preferentially activates the JNK pathway by phosphorylating MKK4 and MKK7 and the ERK6/p38 pathway via MKK3/MKK6. A distinction between the latter two proteins is not possible because the anti-phospho-MKK3/6 antibodies cannot discriminate between these two kinases.

**Constitutive Activation of MRK Increases the G2/M Cell Population**—The activation of ERK6/p38 by MRK prompted us to investigate the role of MRK in the checkpoint regulation in response to DNA damage, a pathway that has been reported to be mediated by ERK6/p38 (35). We first analyzed the cell cycle profile of the MDCK cell line expressing wild type MRK. Upon
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FIG. 6. Activation of endogenous JNK and ERK8/p38 by MRK. MDCK cells stably transfected with vector plasmid (vector), wild type MRK-β or kinase-inactive mutant, MRK-β-K45A, were induced for the expression of the ectopic genes for the indicated times and analyzed by Western blot for the activation of endogenous proteins with corresponding phospho-specific antibodies as indicated. Anti-JNK antibodies were used for loading controls. The KT3 antibody identified the recombinant MRK proteins. The 40-5 antibodies recognize recombinant as well as endogenous MRK-β, which in these cells migrates as two closely running bands. Experiments were repeated at least twice.

The MRK-mediated Pathway Is Needed for Cell Cycle Arrest in Response to γ-Radiation—Because MRK activation increases the cell population in the G2/M phase of the cell cycle (28%) compared with the control cells (17%), whereas the other phases were not significantly affected. Because overexpression of MRK results in activation of its kinase activity, this observation suggested that activation of MRK might be involved in the regulation of the G2 checkpoint control.

The percentage of cells in the cell cycle phases was calculated using the side legend. Data are means ± S.E. of three independent observations.

MRK expression increases the G2/M cell population. MDCK cells stably transfected with wild type MRK or vector plasmid control were induced to express the recombinant genes for 48 h and then analyzed by FACS as described under “Experimental Procedures.” The percentage of cells in the cell cycle phases was calculated using the ModFit software. The cell cycle phases are represented by the different shades of the histograms as indicated by the side legend. Data are means ± S.E. of three independent observations.

The endogenous MRK pathway is involved in the cellular response to DNA damage, we asked whether its kinase activity is induced in response to γ-radiation. We used the MDCK vector control clones and subjected them to the same treatment as described above, 20 Gy of γ-radiation, and at different times after irradiation, we tested the kinase activity of endogenous MRK after immunoprecipitation with MRK-specific antibodies. Fig. 10 shows that MRK activity, as measured by autophosphorylation and by MBP phosphorylation, is increased 2-fold over the untreated population of control cells and MRK-K45A clones have a similar cell cycle profile. However, after irradiation the clones expressing dominant negative MRK had a reduced percentage of cells arrested in G2 compared with the control population (52% versus 67%, p < 0.02). In addition, whereas control cells had a very low percentage in S phase, indicating a G1 block, this fraction was more than doubled in the MRK-K45A population (13 and 32%, respectively). The presence of more cells in S phase indicates that the G1 arrest is also reduced and is consistent with the lower percentage of cells in G1 (16 versus 20%, p < 0.001). These data collectively indicate that expression of dominant negative MRK attenuated the γ-radiation-induced G1 and G2 arrest.

γ-Radiation Induces MRK Activation—To assess whether
background 15 min after irradiation, and it is still above background 4 h post-treatment. Thus, γ-irradiation stimulates a pathway that leads to MRK activation, which contributes to cell cycle arrest.

**DISCUSSION**

In this study, we describe the identification of a human serine/threonine kinase, MRK, discovered as an activator of *S. cerevisiae* Ste7, the yeast MEK homolog that mediates the mating pheromone response. We also characterize MRK-β as a member of the MAPKKK family and show that MRK-β preferentially activates the ERK6/p38γ and the JNK MAP kinase pathways. In addition, we provide evidence that the MRK-β-mediated pathway is activated by γ-radiation and is necessary for the G1 and G2 arrest induced by DNA damage.

We identified two splice variants of the *MRK* gene, as supported by the characterization of the genomic structure of the MRK locus. The gene is spread over more than 200 kb, a rather long stretch of genomic sequence. Interestingly, the MRK-β mRNA has an unusually long 5.6-kb 3′-UTR that could be involved in post-transcriptional regulation. Although rare, a long 3′-UTR has been reported for other mRNAs, such as one of the FGF-2 mRNA species (40) where it has been implicated in modulating translation (41). The role of this region in MRK mRNA stability or in protein expression remains to be investigated. It is also possible that the transcript length may control splicing, yielding a much less abundant mRNA encoding the...
alternative splice form, MRK-α. This form is, in fact, expressed at much lower levels than MRK-β in all tissues with the exception of liver, where it appears to be the major species.

The MRK proteins share significant homology in the kinase domain with proteins of the MAPKKK family. The most closely related members are those in the MLK subfamily. However, the homology is restricted to the kinase domain and remains in the 50% similarity range. There is a single leucine zipper domain in the MRK proteins, whereas this is found as a double domain in the MLK family members.

The functional identification and the primary sequence suggest that MRK-β is a member of the MAPKKK family. This was confirmed by its activation of specific MAP kinase pathways. Although activation of the three major MAP kinase pathways was observed when MRK was greatly overexpressed with the respective MAP kinases in cells, we found that the effects of relatively low levels of MRK on endogenous MAP kinases were more specific. Of the pathways tested, the ERK6/p38γ and JNK cascades were predominantly activated, whereas the ERK and the p38α pathways were marginally affected. In vitro phosphorylation studies demonstrated that the effect on ERK is indirect, as shown by the inability of MRK-β to phosphorylate MEK directly. Therefore, the activation of MEK, when co-transfected with MRK in cells, is likely to be secondary to new gene expression of autocrine factors. In line with this interpretation, we did not observe any effect on the activation of endogenous ERK1 and -2 in MRK-expressing MDCK cells (Fig. 6). In contrast, the MKK proteins upstream of JNK and p38, MKK4 and MKK3/MKK6, respectively, were found to be good substrates in vitro as well as in cells. Remarkably, the activation of endogenous MKK3/MKK6 and ERK6/p38γ proteins was already obvious at a time when recombinant MRK is expressed at relatively low levels, 8 h after induction, underlining the preferential stimulation of this pathway. The activation of these stress-activated kinases did not appear to be the result of cellular stress caused by overexpression of proteins in cells, because expression of similar levels of the catalytically inactive MRK kinase did not elicit any of the responses observed with the expression of the wild type protein. This observation, therefore, supports the specificity of the MRK-induced effects.

While this work was in progress, Gotoh et al. (42) reported the isolation of two mouse clones orthologous to the MRK genes, called MLTK. However, they reported indiscriminate activation of the ERK, JNK, p38, and ERK5 pathways. It is possible that the experimental approach used in their study, namely co-expression of the kinases with each of the potential substrates in cells, could explain the lack of discrimination observed among these signaling pathways. As discussed above, autocrine factors induced by the recombinant proteins may account for the observed effects on some of the pathways tested.

Members of the p38 pathway, such as MKK3, MKK6, and ERK6/p38γ, are preferentially expressed in heart or skeletal muscle (16, 43–45). Interestingly, the levels of MRK-β are particularly elevated in these tissues. It will be of interest to explore the possibility that MRK-β, via the ERK6/p38γ pathway, plays an important role in the physiology of these tissues.

This work also identifies a role for MRK in the cell cycle checkpoint regulation in response to DNA damage-inducing radiation. In the MDCK cell system, the effect on the cell cycle caused by wild type MRK suggests that this kinase mediates signals leading to G2 arrest. This hypothesis is supported by the finding that dominant negative MRK reduces the effects of γ-radiation on the G1 and G2 phases of the cell cycle. This observation is consistent with a physiological function of MRK in the regulation of cell cycle checkpoints. These data were supported by the finding that endogenous MRK is activated by γ-irradiation shortly after treatment. How does MRK affect checkpoint regulation? The ERK6/p38γ cascade has been implicated in γ-radiation-induced cell cycle arrest (35). Given the prominent activation of ERK6/p38γ by MRK, this member of the p38 family was expected to be a good candidate. Surprisingly, no activation of ERK6/p38γ was detected in response to γ-irradiation (data not shown). Therefore, at this time we cannot implicate ERK6/p38γ in the observed cell cycle effects of γ-radiation in this system. It is possible that cell type differences are responsible for this discrepancy.

In conclusion, we have shown that MRK, a member of the MAPKKK family, preferentially activates the ERK6/p38γ and JNK pathways and plays a role in the regulation of DNA damage-induced checkpoints. Future studies will address the identification of the elements that relay the signals initiated by γ-radiation to MRK and those that act downstream of MRK in response to DNA damage.

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