The Stress Kinase MRK Contributes to Regulation of DNA Damage Checkpoints through a p38γ-independent Pathway*

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DNA damage induced by ionizing radiation (IR) activates a complex cellular response that includes checkpoints leading to cell cycle arrest. The stress-activated mitogen-activated protein kinase (MAPK) p38γ has been implicated in the G2 phase checkpoint induced by IR. We recently discovered MRK as a member of the MAPK kinase family that activates p38γ. Here we investigated the role of MRK in the checkpoint response to IR. We identified autophosphorylation sites on MRK that are important for its kinase activity. A phosphospecific antibody that recognizes these sites showed that MRK is activated upon IR in a rapid and sustained manner. MRK depletion by RNA interference resulted in defective S and G2 checkpoints induced by IR that were accompanied by reduced Chk2 phosphorylation and delayed Cdc25A degradation. We also showed that Chk2 is a substrate for MRK in vitro and is phosphorylated at Thr68 by active MRK in cells. MRK depletion also increased sensitivity to the killing effects of IR. In addition, MRK depletion reduced IR-induced activation of p38γ but had no effect on p38α activation, indicating that MRK is a specific activator of p38γ after IR. Inhibition of p38γ by RNA interference, however, did not impair IR-induced checkpoints. Thus, in response to IR MRK controls two independent pathways: the Chk2-Cdc25A pathway leading to cell cycle arrest and the p38γ MAPK pathway.

DNA damage induced a complex cellular response that involves interacting networks controlling cell cycle arrest, DNA repair, and apoptosis (1). Defects in any of these functions may contribute to chromosome instability and predisposition to cancer. DNA damage-induced cell cycle arrest is thought to be important to allow time for DNA repair. This process is controlled by signaling events collectively referred to as checkpoints (1, 2). ATM (the ataxia-telangiectasia-mutated protein) is a crucial and most proximal transducer of the S and G2 phase checkpoints in response to DNA double-strand breaks, the most severe type of DNA damage caused by ionizing radiation (IR)†.

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† The abbreviations used are: IR, ionizing radiation; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase, and MAPK. The c-Jun N-terminal kinase and p38 MAPK pathways respond to cellular stress including DNA damage. In addition to participating in the apoptotic response that follows extensive DNA damage, the p38 pathway has been reported to be required for checkpoint regulation. In particular, p38α and p38β have been implicated in the UV radiation checkpoint response (29), whereas p38γ is thought to participate in the control of G2 arrest following γ-radiation (30). The pathway that leads to p38γ activation by IR is, however, not clear.

MRK is a MAPKKK protein that belongs to the mixed lineage kinase (MLK) protein family (31). We have characterized the most abundant of the two MRK splice forms, MRK-β (32), and throughout this study we refer to this form simply as MRK. Overexpression of MRK activates the c-Jun N-terminal kinase and p38 MAPK pathways respond to cellular stress including DNA damage. In addition to participating in the apoptotic response that follows extensive DNA damage, the p38 pathway has been reported to be required for checkpoint regulation. In particular, p38α and p38β have been implicated in the UV radiation checkpoint response (29), whereas p38γ is thought to participate in the control of G2 arrest following γ-radiation (30). The pathway that leads to p38γ activation by IR is, however, not clear.

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pndependent pathways: the Chk2-Cdc25A pathway and the stress-activated p38 MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Transfections, and in Vitro Kinase Assays**—The MRK mutants carrying alanine substitutions for threonine at positions 161, 162, and 165 and the double mutant MRK-T161A/S165A were generated by site-directed mutagenesis using the QuikChange kit from Stratagene. The following primers were used to convert the respective threonine to alanine: T161A, 5'-CTTCGCGCTTCCATACACGTACCGACACACATTGGCTTTGTT-3'; T162A, 5'-CGGTCGACAATACACATACGGCCACACATGGCCTTGGTTG-3'; S165A, 5'-AACATTCAACACAGCTGGCCTTGGTTGAACCTTCAAC-3'; and T161A/S165A, 5'-CGGTCGCAAATAGCGGTCTTGGTGGAACCTTCAAC-3'.

**Cell Lines, Cell Culture, and siRNA Treatment**—Human U2-OS, HCT116, and HEK293 cells were obtained from ATCC. The U2-OS and HCT116 cells were grown in McCoy's media (Invitrogen), and the HEK293 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker); all media were supplemented with 10% fetal calf serum. The U2-OS, HCT116, and HEK293 cells were transfected using the Effectene reagent (Qiagen) and the LipofectAMINE 2000 reagent (Invitrogen) under the control of the cytomegalovirus promoter. The catalytically inactive MRK-K45A plasmid has been previously described (32). Plasmid pCEP-FLAG ERRKp38 was obtained from Silvio Gutkind (National Institutes of Health). The active MRK construct was generated by subcloning a Xbal fragment containing wild type MRK into the XbaI site of a derivative of pc2-P1. IE, pc2-α, 522, the plasmid that is part of the ARGENT-regulated RRE-modification kit (ARIAD; www.ariad.com/regulationkits). The resulting MRK construct was found to be constitutively active even in the absence of the diminging drug.

**HeK293 and U2-OS cells were transfected using the Effectene reagent (Qiagen) and the LipofectAMINE 2000 reagent (Invitrogen), respectively, according to the manufacturers' suggestions. In vitro kinase assays were as previously described (32).**

**Results**

The MRK K45A mutant has been previously described (32). Plasmid pCEP-FLAG ERRKp38 was obtained from Silvio Gutkind (National Institutes of Health). The active MRK construct was generated by subcloning a Xbal fragment containing wild type MRK into the XbaI site of a derivative of pc2-P1. IE, pc2-α, 522, the plasmid that is part of the ARGENT-regulated RRE-modification kit (ARIAD; www.ariad.com/regulationkits). The resulting MRK construct was found to be constitutively active even in the absence of the diminging drug.

**Cell Lines, Cell Culture, and siRNA Treatment**—Human U2-OS, HCT116, and HEK293 cells were obtained from ATCC. The U2-OS and HCT116 cells were grown in McCoy's media (Invitrogen), and the HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Bio Whittaker); all media were supplemented with 10% fetal calf serum (HyClone), glutamine, and penicillin/streptomycin. The control GMH 162, H9251, H9253, and H11032 AT cell lines were purchased from the Coriell Cell Repository and grown in minimum essential Eagle Earle's basal salts solution with 2% concentration of essential and nonessential amino acids and vitamins, 15% fetal calf serum, glutamine, and penicillin/streptomycin.

**siRNAs**—siRNA transfections were performed using LipofectAMINE 2000 (Invitrogen) essentially following the manufacturer's recommendations, with 4 μM/well of lipid reagent in a six-well plate. All of the siRNA duplexes used in this study were designed following the rules suggested by Elbashir et al. (33). A BLAST search was conducted against GenBank™ to ensure that their sequence did not cross-hybridize to non-specific targets. In addition, these duplexes were used at the lowest concentration that gave at least 80% target inhibition. In some experiments the concentration was 150 nM, but subsequently it was reduced to 75 nM in order to reduce non-specific inhibition. The MRK expression was observed 2 days after transfection, and the effect was sustained up to 5 days after transfection (data not shown). All of the siRNA duplexes were supplied by Dharmaco Research. Specific oligonucleotide sequences for each target gene were as follows: 5'-GAAGUGUCGGAGGCUUATAATT-3' (M1), 5'-CUUGUGUUAUUCAGUCAGCGUUATTT-3' (M3-1), and 5'-CAUGCCAGGACCGGACAAATTT-3' (M13) targeting MRK; 5'-UGGAGAAGGUCUGUAAUCUT-3' (29), and 5'-GGCAUCAUCAGCACCTATTT-3' (33) targeting p38; 5'-GGCUACCCGGAUAACUUCGATTT-3' luciferase as control, and 5'-GAUAACUUCCUUCAUCATT-3' targeting CDC25A.

**Radio-resistant DNA Synthesis**—Forty-four hours after siRNA transfection, U2-OS cells were labeled for 18 h with 10 nCi/ml of [14C]thymidine, washed twice with phosphate-buffered saline, and incubated with regular media for 24 h. The cells were irradiated at different doses using a 137Cs source, incubated for 30 min, and labeled with [3H]thymidine (2.5 μCi/ml) for 15 min. The cells were then harvested and fixed in 70% methanol for 1 h, transferred to Whatman filters, and sequentially fixed with 70% and 95% methanol. The filters were air-dried, and the amount of radioactivity was assayed in a liquid scintillation counter.

**Cycloheximide Treatment, Protein Analysis, and Antibodies**—Forty-four hours after transfection, the cells were treated with cycloheximide (25 μg/ml) (Sigma), immediately exposed to 6 Gy of IR, and harvested at the indicated times.

**Cytotoxicity Treatments, Protein Analysis, and Antibodies**—Forty-four hours after transfection, the cells were treated with cycloheximide (25 μg/ml) (Sigma), immediately exposed to 6 Gy of IR, and harvested at the indicated times.

**Ionizing Radiation Induces MRK Autophosphorylation and Activation**—As a first approach to study the role of endogenous MRK in the DNA damage response, we generated a phospho-specific antibody that recognizes active MRK. Within the MRK kinase loop between the DFG and subdomain III and APE (sub-domain VIII) residues, there are three conserved threonine/serine residues (Thr161, Thr162, and Ser165) (Fig. 1A) that in other MKL family proteins are important for activation (31, 35). To test the role of these residues in MRK activation, we mutated them to alanine, and upon expression in COS cells, we tested the mutant proteins for their activity in vitro. Fig. 1B shows that alanine substitution at positions 161, 165, and, to a lesser extent 162, results in loss of MRK autophosphorylation as well as loss of phosphorylation of a substrate (MRP) in vitro. In order to confirm that these residues are autophosphorylation sites, we constructed GST fusions with peptides containing the wild type MRK kinase loop (GST-KL-WT) or its double alanine mutant counterpart (GST-KL-AA). We used these proteins as substrates in an in vitro kinase reaction with baculovirus-expressed wild type MRK, which has constitutive kinase activity. Fig. 1C shows that the GST-KL-WT protein is indeed phosphorylated by active MRK. These data demonstrate that these residues are phosphorylated by MRK and that autophosphorylation at these sites is required for MRK activity in vitro.

The MRK-T161A/S165A double mutant did not show lower activity than each single mutant. However, to increase specificity, we used a peptide that is phosphorylated at both sites to raise an antibody that detects active MRK. To characterize this antibody, we expressed wild type or mutant MRK proteins in human embryonic HEK293 cells and induced MRK activation by osmotic shock. Fig. 1D shows reactivity of the phospho-specific anti-MRK antibody with activated wild type MRK but not with mutant MRK-T161A/S165A (M-AA). In agreement with our published in vitro data (32), loss of MRK enzymatic activity, caused by alanine substitution for lysine in the active site of the MRK-K45A mutant (M-KA), also abolished phosphorylation at positions 161/165. These observations demonstrate that phosphorylation of the MRK kinase loop is the result of autophosphorylation in cells.

Using this reagent, we examined activation of endogenous MRK by IR in the human osteosarcoma U2-OS cells. We found that MRK is phosphorylated at these sites within 30 min after IR and that this signal persists for up to 12 h (Fig. 2A, top two panels). The phosphorylation signal is specific for MRK because it is lost in cells treated with a specific MRK siRNA (Fig. 2A, bottom panel), which is described in the following paragraph. These results indicate that MRK undergoes rapid autophosphorylation upon exposure to IR. Previously, we demonstrated p38γ activation by MRK (32). To confirm that IR-induced phos-
Forty-eight hours post-transfection, the cells were treated with 0.5M T161AS165A (M-AA) activation. The recombinant KT3-tagged MRK proteins were immunoprecipitated or MRK mutants (T161A, T162A, S165A, or T161A/S165A) as indicated.

COS cells were transfected with vector control (WT), wild type (M-WT), MRK-K45A (M-WT), or MRK-T161AS165A (M-AA) mutation. Forty-eight hours after transfection, the cells were exposed to 6 Gy of IR and harvested 1 h later, and processed for Western blot analysis with the P-MRK and tubulin antibodies. B, U2-OS cells were co-transfected with p38γ and control plasmid (Vector), wild type MRK (M-WT), or MRK-T161AS165A (M-AA) mutant. Forty-eight hours after transfection, the cells were exposed to 6 Gy of IR and harvested 1 h after IR. The p38γ proteins were immunoprecipitated and subjected to in vitro kinase assay with ATP-2 as substrate and [γ-32P]ATP. The top panel is an autoradiogram of the labeled ATF-2 proteins. The bottom two panels are Western blot analyses of the immunoprecipitated p38γ proteins and the recombinant MRK proteins. The data are representative of at least two independent experiments.

Therefore, we conclude that IR induces MRK autophosphorylation and activation in vivo.

MRK Depletion Causes Radio-resistant DNA Synthesis and Progression through Mitosis upon IR—To test the functional involvement of MRK in IR-induced cell cycle arrest, we down-regulated the expression of endogenous MRK in cells using RNA interference. A siRNA duplex M1, directed against MRK, efficiently depleted cells of endogenous MRK (Fig. 3A). Reduction of MRK expression did not change the growth properties of the cells during the time frame of the experiments performed in this study. To examine the role of MRK in S phase checkpoint regulation, we measured DNA synthesis in control and MRK siRNA-treated cells after IR. [3H]Thymidine incorporation into DNA was monitored after exposure of siRNA-transfected cells to various doses of IR (Fig. 3B). A decrease in [3H]thymidine incorporation into newly synthesized DNA in control cells indicated that these cells had an intact S phase checkpoint. In contrast, MRK-depleted cells continued to initiate DNA replication at all doses of IR as indicated by radio-resistant DNA synthesis. Thus, MRK is required for the S phase arrest induced by IR.

We monitored G2 arrest by measuring the number of cells that entered mitosis after IR. The cells were co-stained with propidium iodide to determine DNA content and with anti-phospho-histone H3 antibody to identify mitotic cells (34).
MRK-depleted cells had three times as many mitotic cells 1 h after IR compared with control cells (Fig. 3C). Reduction in G2 phase arrest by MRK siRNA was also observed in the human colorectal carcinoma HCT116 cells; after exposure to 6 Gy of IR, the fraction of mitotic cells in MRK-depleted cells was twice as large as that in control cells (0.8% versus 0.4%; data not shown). Although the impairment of G2 arrest is not as robust as that observed in S phase, collectively, these data indicate that MRK contributes to IR-induced cell cycle arrest in both S and G2.

MRK Expression Is Dependent on ATM—ATM is rapidly activated by IR and is one of the most upstream checkpoint signaling elements in the DNA damage response. To address the dependence of MRK activation on ATM, we tested the AT cell lines GM09607B and GM05849D and the control cells GM0637I for the MRK response to IR. In the course of these experiments, we found that MRK levels in the AT cells were reduced by at least 10-fold compared with control cells (Fig. 4). Although the low levels of MRK in the AT cells made it impossible to test the phosphorylation state of MRK after IR in these cells, our data indicate that expression of MRK is dependent on the expression of ATM.

MRK Inhibition Reduces IR-induced Chk2 Activation and Cdc25A Degradation—To begin dissecting the biochemical pathway controlled by MRK in checkpoint regulation, we determined the level of Chk2-Thr68 phosphorylation. ATM phosphorylates this site in response to IR, a step that is necessary for Chk2 activation (36–39). Fig. 5A shows that 1 h after IR, Chk2 was phosphorylated at Thr68 in control cells. In contrast, MRK-depleted cells showed a marked and significant decrease in Chk2 phosphorylation, as determined by quantification of the Thr68 phosphorylation levels in the respective samples (Fig.
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One of the Chk2 substrates that participate in checkpoint regulation is the Cdc25A phosphatase, which upon phosphorylation by Chk2 is degraded. Consistent with reduced Chk2 phosphorylation, we observed that Cdc25A degradation was delayed in MRK-deficient cells (Fig. 6). These data indicate that MRK operates upstream of Chk2 and Cdc25A in checkpoint regulation. 

**MRK Is Essential for IR-induced Activation of p38γ, but Its Role in S and G2 Cell Cycle Checkpoints Is Independent of p38γ**—Using dominant negative mutants of p38γ and its activator MKK6, Wang et al. (30) reported that p38γ is required for G2 arrest induced by IR and that it operates upstream of Chk2. We previously showed that overexpression of MRK in MDCK cells activates p38γ (32), and Fig. 2 shows that exposure to IR enhances p38γ activation in U2-OS cells expressing ectopic MRK. We therefore tested whether MRK mediates IR-induced checkpoint regulation via p38γ. We first measured p38γ activation by IR in cells depleted of MRK. After exposure to IR, p38γ was immunoprecipitated from cells transfected with control or MRK siRNA, and its activity was measured in vitro against ATF2 as substrate. Fig. 7A shows that the 2-fold activation of p38γ induced by IR was almost completely abolished in MRK-depleted cells. These data demonstrate that MRK is necessary for p38γ activation after IR. In contrast, reduced MRK expression did not decrease p38α activation in response to IR (Fig. 7B). Thus, MRK specifically activates p38γ upon IR.

We then tested whether p38γ mediates the effects of MRK on the S phase checkpoint, using two different p38γ siRNAs. Although the levels of p38γ were greatly reduced in cells treated with the siRNAs (Fig. 8A, inset), measurement of DNA synthesis upon IR showed no significant difference between the p38γ-depleted cells and control cells (Fig. 8A). This result indicated that p38γ is not required for the S checkpoint. More surprisingly, determination of mitotic cells by phospho-histone H3 staining revealed that p38γ is not involved in the G2 arrest (Fig. 8B). Consistent with this conclusion, Fig. 8C shows that the IR-induced levels of Chk2-Thr(P)68 remained unchanged in p38γ-depleted cells. This result was confirmed in a different
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To better probe the role of MRK in IR-induced checkpoint regulation, we asked whether MRK could directly phosphorylate Chk2. To this end, we first tested MRK’s ability to phosphorylate Chk2 in vitro. We used this protein in an in vitro kinase assay as described for Fig. 2B. The top panel is an autoradiogram of the labeled ATF-2 proteins. The numbers indicate the fold activation compared with the untreated control. The bottom three panels are Western blot analysis of the indicated endogenous proteins to demonstrate inhibition by the respective siRNAs.

Fig. 9. Residual p38γ activity in MRK and p38γ siRNA treated cells. Depletion of p38γ by siRNA results in minimal p38γ residual activity that is comparable with that caused by MRK depletion. U2-OS cells were transfected with control (L), MRK (M1), or p38γ (29+33) siRNAs and exposed to 0 (−) or 6 Gy (+) of IR and harvested 1 h after IR. Western blot analysis was performed with the indicated antibodies. The data are representative of two independent experiments.

One caveat of these experiments is that the residual p38γ activity in cells treated with the p38γ siRNAs may be sufficient to provide the required signal for checkpoint regulation. To rule out this possibility, we compared the IR-induced levels of p38γ activity in the p38γ siRNA-treated cells with those of cells transfected with the MRK siRNA. To further reduce p38γ levels, we transfected cells with a mixture of two p38γ siRNA duplexes and tested IR-induced p38γ activation as well as Chk2-Thr68 phosphorylation (Fig. 8C) to monitor cell cycle checkpoint functions. Fig. 8 shows that the residual p38γ activity in cells treated with the p38γ siRNA was comparable with that seen after MRK depletion. However, in contrast to MRK, p38γ knockdown does not result in checkpoint defects. Therefore, the residual p38γ activity following p38γ knockdown cannot account for lack of a checkpoint defect in these cells. We conclude that, although MRK mediates the IR-induced activation of p38γ, its role in checkpoint regulation is independent of p38γ. We therefore propose a model in which MRK stimulates two independent pathways in response to IR: one controlling Chk2 activation and Cdc25A degradation that leads to cell cycle arrest and the other leading to p38γ activation that could be involved in the stress response.

MRK Directly Phosphorylates Chk2 and Thus Contributes to Its Activation—To better probe the role of MRK in IR-induced checkpoint regulation, we asked whether MRK could directly participate in Chk2 activation. To this end, we first tested whether purified MRK can phosphorylate Chk2 in vitro. Wild type MRK expressed in the baculovirus system is constitutively active. We used this protein in an in vitro kinase assay with GST-Chk2(D368N) as substrate. The kinase inactive form of Chk2 prevented autophosphorylation from interfering with the assay. Fig. 10A shows a dose-dependent phosphorylation of Chk2 by MRK. Thus, Chk2 is a substrate for MRK in vitro. We then asked whether this phosphorylation occurred in cells. We transfected U2-OS cells with wild type or an active form of MRK, generated as a fusion between wild type MRK and the drug-binding domain of an FKBP12 derivative (see “Experimental Procedures”). Fig. 10B shows that expression of active MRK in cells induces phosphorylation of endogenous Chk2 at Thr388. Thus, MRK phosphorylates Chk2 in vitro and in vivo.

Role of MRK in Modulating Radiation Sensitivity—Failure to arrest upon DNA damage may lead to cell cycle progression with damaged DNA, which can induce apoptosis in subsequent cell cycles. We asked whether MRK knockdown, by interfering...
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Figure 10. In vitro and in vivo phosphorylation of Chk2 by MRK. A, in vitro kinase assay. Baculovirus-produced MRK (Bv-MRK) was incubated with GST-Chk2(D368N) in the presence of $[^{32}P]ATP$. The phosphorylated proteins were resolved on SDS-PAGE gels, dried onto paper, and exposed to film for autoradiography. This experiment was repeated three times with similar results. B, in vivo phosphorylation of Chk2 in MRK expressing cells. U2-OS cells were transfected with the indicated plasmids. Twenty-four hours later, the Chk2 proteins were immunoprecipitated and processed for Western blot analysis with the Chk2-Thr(P)68-specific antibody. The bottom panel shows equal amounts of immunoprecipitated Chk2. The data are representative of two independent experiments.

Discussion

In this study, we established that MRK contributes to the DNA damage response by directly phosphorylating Chk2 and thus participating in its activation. We showed that MRK gene silencing by RNAi inhibits IR-induced checkpoint functions, impairing the S phase and to some extent also the G$_2$ phase cell cycle arrest. We also showed that although p38$\gamma$ is activated by IR in an MRK-dependent fashion, p38$\gamma$ does not mediate MRK regulation of checkpoints. Thus, MRK controls two independent signaling cascades upon activation by IR: the Chk2-Cdc25A pathway, leading to cell cycle arrest, and the stress-activated p38$\gamma$ MAPK pathway (Fig. 12).

Activation of MRK by IR—Autophosphorylation plays an important role in MRK activation, as demonstrated by in vitro studies with mutated MRK proteins and the use of a phosphospecific anti-MRK antibody (Figs. 1 and 2). The autophosphorylation step in the activation of MRK is similar to that found for other members of the MLK family proteins (31). In addition, autophosphorylation plays an essential role in the activation of checkpoint regulators such ATM and Chk2 (3, 41–43). Autophosphorylation of MRK occurs within 30 min of irradiation, which is consistent with detectable Chk2 phosphorylation after exposure to IR (21, 36, 43, 44). However, in contrast to Chk2-Thr$^{68}$ phosphorylation, which appears to decrease by 4 h (Ref. 15 and Fig. 5A), possibly as a consequence of IR-induced Chk2 degradation (43), MRK remains active for up to 12 h. This extended activation suggests that MRK may have additional functions beyond contributing to Chk2 activation. One interesting question raised by these data is the identity of the trigger event for MRK autophosphorylation after IR. Interestingly, although autophosphorylation is important for MRK activation, the residual kinase activity of the MRK-AA mutant (Fig. 2B) suggests that other mechanisms contribute to MRK activation. In the case of MLK3, binding of an activating protein induces homodimerization, which leads to autophosphorylation and activation (35, 45, 46). Chk2 also forms homodimers following phosphorylation by ATM, which is proposed to induce autophosphorylation and activation (37, 38, 42). Whether MRK forms dimers and the nature of the autophosphorylation trigger remain to be investigated.

A possible element upstream of MRK is ATM. Our attempts to test this dependence were impaired by the finding that MRK expression is very much reduced in AT cells, linking MRK expression to that of ATM. How ATM regulates MRK expression is not known at this time. However, in the case of the insulin-like growth factor–1 receptor, ATM regulates its expression at the level of transcription (47). Interestingly, the activation of p38$\gamma$ by IR has been shown to be dependent on ATM (30). Here we have shown that p38$\gamma$ activation by IR depends on MRK. Thus, it is likely that MRK depends on ATM for its IR-induced activation.

MRK Contributes to Chk2 Activation and Cdc25A Degradation upon IR—We have shown that MRK depletion reduces the extent of Chk2 phosphorylation and function, as demonstrated...
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by delayed Cdc25A degradation after IR. Threonine 68 in Chk2 is targeted by ATM (36–38) and can also occur in cells that lack ATM (43), suggesting that other kinases may phosphorylate this site. PLK1 in fact was found to phosphorylate Chk2 on Thr68 (48). In this study we have shown that MRK directly phosphorylates Chk2 in vitro and leads to Thr68 phosphorylation in cells (Fig. 10). It is possible that in addition to Thr68, MRK phosphorylates other sites in Chk2. Future studies will determine whether this is the case.

The fact that Chk2 phosphorylation at Thr68 is reduced but not completely abolished by MRK depletion is consistent with the notion that Chk2 activation is a complex process (43). Therefore, following IR, Chk2 may integrate several input stimuli that include MRK, as well as other elements of the checkpoint pathway. Impaired Chk2-Thr68 phosphorylation after IR was also observed in cells depleted of MDC1 and 53BP1 by RNA interference (11, 15) and in cells null for Nbs1 (18). More recently these proteins were found to be required upstream of ATM, which provides an explanation for their involvement in Chk2 activation (16, 17).

Consistent with reduced Chk2 activation, we find that MRK depletion is accompanied by a delay in Cdc25A degradation following IR. This phosphatase has been shown to be a substrate of Chk2. Cdc25A is also a substrate of Chk1, another checkpoint protein that recently has been shown to contribute to checkpoint regulation induced by IR (49–51). Attempts to monitor the IR-induced activation and phosphorylation of Chk1 in cells depleted of MRK did not show significant differences from control cells (data not shown). However, we cannot exclude the possibility that MRK controls other aspects of Chk1 function, such as localization (52) or interaction with partners like Claspin (53).

Role of p38α—Overexpression of MRK in cells activates both p38α (54) and p38γ (32). In this study, however, we show that in cells that are challenged by IR, MRK depletion specifically interferes with the activation of p38γ and not p38α (Fig. 7). This observation is somewhat surprising, because the p38 family members share the immediate upstream activators MKK6 and MKK3. Thus, our results using RNA interference strongly suggest the existence of distinct signaling complexes that regulate p38γ and p38α activation, only one of which is regulated by MRK upon IR treatment. A likely mechanism underlying this specificity is the interaction of scaffold proteins with components of the MAPK cascades (55). Furthermore, our results suggest that upon overexpression of individual members of the MAPK cascades, signaling specificity may be lost.

The requirement of MRK for IR-induced p38γ activation suggests that MRK regulates some functions via p38γ. However, checkpoint functions are not impaired by loss of p38γ under conditions where the residual p38γ activity is comparable to that achieved in MRK-depleted cells. These results are in contrast to the reported role of p38γ in the IR-induced G2 checkpoint regulation (30). A possible explanation for the different outcome of that study is the use of high levels of dominant negative mutant MKK6 and p38γ proteins that may result in nonspecific effects. The role of p38γ in the response to IR remains unclear. p38γ depletion also did not alter cell survival after IR (data not shown). Although this protein is known to be activated by a variety of stress stimuli, including osmotic shock, protein synthesis inhibitors (56), hypoxia (57), and IR (Ref. 30 and this study), the events downstream of p38γ are not well characterized (58).

The lack of reduction of p38α activity in MRK-depleted cells also indicates that p38α is not involved in checkpoint regulation in response to IR. This conclusion is concordant with previously reported data, indicating that p38α is not required for the G2/M checkpoint after IR (29). Thus, we have shown that MRK, a MAPKKK, controls the Chk2-Cdc25A pathway independently of a MAPK cascade (Fig. 12). Although this signal bifurcation is rather unusual for a MAPKKK, another example is that of TAK1, a MAPKKK protein that activates both the p38 pathway as well as the IKK-NF-κB pathway (59, 60).

Protective Role of MRK in the DNA Damage Response—MRK depletion sensitizes cells to the toxic effects of IR. This observation is consistent with a protective role of MRK in the checkpoint response and with the radiosensitive phenotype observed in cells defective for other checkpoint regulators, including ATM and Nbs1 (26–28). Interestingly, this response is different from that observed in cells lacking Chk2. Cells derived from Chk2-deficient mice are in fact impaired in p53-dependent apoptosis after IR (61, 62). One explanation for the different outcome of cells depleted of MRK and Chk2-deficient cells is that, in addition to regulating Chk2 activity, MRK might control other pathways that are important for cell survival.

Collectively, our data demonstrate that MRK participates in the regulation of cell cycle arrest in response to IR by directly contributing to Chk2 phosphorylation and activation. This role of MRK in checkpoint regulation is independent of p38γ activation. The protective property of MRK in IR-treated cells suggests that this kinase, like other regulators of DNA damage responses, including ATM and Nbs1 (7), could be a potential drug target for sensitizing tumors to ionizing radiation.

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