The Death Domain Kinase RIP Has an Important Role in DNA Damage-induced, p53-independent Cell Death*

Gang Min Hur†, You-Sun Kim‡, Minho Won§, Swati Choksi‡, and Zheng-gang Liu††

From the †Cell and Cancer Biology Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the ‡Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon 301-131, Korea

Tumor suppressor p53 plays a critical role in cellular responses, such as cell cycle arrest and apoptosis following DNA damage. DNA damage-induced cell death can be mediated by a p53-dependent or p53-independent pathway. Although p53-mediated apoptosis has been well documented, little is known about the signaling components of p53-independent cell death. Here we report that the death domain kinase, RIP (receptor-interacting protein), is important for DNA damage-induced, p53-independent cell death. DNA damage induces cell death in both wild-type and p53−/− mouse embryonic fibroblast cells. We found that RIP−/− mouse embryonic fibroblast cells, which have a mutant form of the p53 protein, are resistant to DNA damage-induced cell death. The reconstitution of RIP protein expression in RIP−/− cells restored the sensitivity of cells to DNA damage-induced cell death. We also found that RIP mediates this process through activating mitogen-activated protein kinase, JNK1. Furthermore, knocking down the expression of RIP blocked DNA damage-induced cell death in the human colon cancer cell line, p53 null HCT 116. Taken together, our study demonstrates that RIP is one of the critical components involved in mediating DNA damage-induced, p53-independent cell death.

The cellular responses to DNA damage include cell cycle arrest, DNA repair, and apoptosis. It is known that the tumor suppressor gene p53 plays a critical role in these processes (1, 2). In response to genotoxic stress, such as ionizing radiation, UV radiation, and a variety of DNA-alkylating agents, the p53 protein is stabilized and activated. Induction of p53 leads to cell cycle arrest, apoptosis, or senescence, which results in limited cell proliferation (3–6). p53-mediated inhibition of cell proliferation is largely achieved through transcriptionally activating p53 target genes, although it has been reported that p53 could play a role in apoptosis independent of its transcriptional activity (1, 2). However, a recent study (7) demonstrated that DNA-damaging agents are able to induce necrotic cell death in p53-deficient cells, which suggests that DNA damage could also induce cell death through a p53-independent pathway. Although the molecular mechanism of p53-mediated apoptosis has been well documented, little is known about the signaling components of the p53-independent necrotic cell death in response to DNA damage.

When cells undergo necrotic cell death induced by death factors, such as TNF2 and FasL, or oxidative stress, one of the key mediators is the death domain kinase, receptor-interacting protein (RIP). RIP is a key effector molecule of TNF-induced activation of the transcription factor NF-κB and the mitogen-activated protein kinase JNK (8, 9). Although RIP is not required for TNF/FasL-induced apoptosis, RIP has been found to be essential for TNF/FasL or reactive oxygen species-induced necrotic cell death (10–12). One of the signaling pathways engaged by genotoxic stress is the c-Jun N-terminal kinase/stress-activated protein kinase pathway (13, 14). JNK/ stress-activated protein kinase is the critical regulator of the activity of the transcriptional factor AP-1 through phosphorylating its target proteins such as c-Jun and ATF2 (13, 14). It has been suggested that JNK activation may play a role in TNF or reactive oxygen species-induced necrotic cell death (12, 15, 16). Although JNK is activated by a variety of genotoxic stress, the molecular mechanism that mediates DNA damage-induced JNK activation remains elusive.

Our previous studies found that RIP plays an essential role in DNA damage-induced NF-κB activation and in oxidative stress-induced cell death (12, 17). Therefore, we investigated the involvement of RIP in DNA damage-induced cell death with RIP null MEF cells. We report here that RIP is essential for DNA damage-induced, p53-independent cell death. We also demonstrated that RIP mediates this process through activating JNK.

EXPERIMENTAL PROCEDURES

Reagents—Anti-JNK-1 and anti-p53 antibodies were purchased from Pharmingen; anti-RIP antibody was from Calbiochem. Anti-actin antibody, Ara-C, and CHX were purchased from Sigma; Adr, Cpt, and SP600125 were from Calbiochem. Anti-TNF-R1 and recombinant mTNF-α were purchased from R & D Systems. Protein A-Sepharose was purchased from R&D Systems. Protein A-Sepharose was purchased from Calbiochem. Anti-actin antibody, Ara-C, and CHX were purchased from Sigma; Adr, Cpt, and SP600125 were from Calbiochem.
DNA Damage-induced, p53-independent Cell Death Mediated by RIP

purchased from Amersham Biosciences. RIP-specific and control double-stranded small interfering RNA oligonucleotides were purchased from Santa Cruz Biotechnology.

Cell Culture and Treatment—All MEF and HCT 116 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The RIP-reconstituted cells, RIP−/−, were cultured in the same medium, including 300 μg/ml hygromycin (17). The RIP-reconstituted cell line was established by ectopically expressing Myc-tagged RIP protein in RIP−/− MEFs. To do so, RIP−/− cells were co-transfected with a Myc-RIP plasmid and a hygromycin selection plasmid. Then RIP expression in RIP−/− cells was confirmed by Western blotting.

Western Blot Analysis—After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris, pH 7.5, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin). Fifty micrograms of the cell lysates was fractionated by SDS-PAGE and Western-blotted. The proteins were visualized by enhanced chemiluminescence, according to the manufacturer’s instruction (Amersham Biosciences).

Verification of p53 Status—Identification of mutated p53 protein in cells was achieved by immunoprecipitation of mutant p53 with the monoclonal antibody pAb240, which specifically recognizes mutant forms of p53 under nondenaturing conditions. Immunoprecipitants were examined by Western blotting with an anti-p53 antibody, pAb122, which recognizes both wild-type and mutant p53 under denaturing conditions. The cDNA of p53 gene was cloned from the total RNAs purified with RIP−/− cells using RNAzol B reagent (Tel-Test, Friendswood, TX). Reverse transcription-PCR was carried out with sense (5’-gcacctagcttcaggc-3’) and antisense (5’- catacaagcagcaagc-3’) primers according to the protocol provided, and the PCR products were purified and ligated into pgEM-Teasy vector (Promega). The sequence of the cloned cDNA was obtained by autosequencing and compared with the one of wild-type p53 gene (cited from the NCBI data base, accession number AAH05448).

Small Interfering RNA Knockdown of RIP—p53−/− HCT 116 cells were plated in 12-well plates, and cells were transfected with either a RIP-specific double-stranded small interfering RNA oligonucleotide (RIP-siRNA, 150 μmol/well) or a control small interfering RNA (150 μmol/well) with no known homology to mammalian genes using Lipofectamine Plus reagent (Invitrogen). After 48 h of incubation, the transfected cells were lysed, and the efficiency of RIP knockdown was analyzed by Western blotting.

JNK Kinase Assay—Cells were collected and lysed in M2 buffer. JNK1 was immunoprecipitated with the anti-JNK1 antibody and protein A-Sepharose beads by incubation at 4 °C for 4 h to overnight. The beads were washed with lysis buffer, and the kinase assay was then performed in complete kinase assay buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerol phosphate, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM p-nitrophenyl phosphate, 50 μM sodium vanadate, 20 μM ATP) with the addition of [γ-32P]ATP and 1 μg of GST-c-Jun-(1–79), as substrate. After 20 min at 30 °C, sample buffer was added, and proteins were resolved in 4–20% SDS-polyacrylamide gels, and phosphorylated substrates were visualized by autoradiography.

Detection of Cell Death—Cell death of various MEF cells was quantified using a cytotoxicity detection kit, which measures the percentage of lactate dehydrogenase leakage, according to the manufacturer’s instructions (Roche Applied Bioscience). Briefly, 25 μl of culture medium was used for each assay, and 25 μl of reaction mixture was added to each sample. After 10–20 min of incubation, each sample was measured by an enzyme-linked immunosorbent assay reader.

RESULTS

RIP Has a Critical Role in p53-independent Cell Death Induced by DNA Damage—DNA damage can induce cell death through p53-dependent or independent pathways (6, 7, 20). To test whether p53 is required for cell death induced by different DNA-damaging agents other than MNNG (7), several commonly used DNA-damaging agents such as adriamycin (Adr), camptothecin (Cpt), and 1-β-D-arabinofuranosylcytosine (Ara-C) were employed in our experiments. These agents cause DNA damage through different mechanisms of action. Wild-type (WT) and p53 null MEF were used to examine the role of p53 in cell death induced by these DNA-damaging agents. As shown in Fig. 1A, all of these DNA-damaging agents, Adr, Cpt, and Ara-C, induced cell death in WT and p53 null MEFs very efficiently, even though the extent of cell death in p53 null cells is slightly less than wild-type cells. These results indicate that these agents can induce cell death by a p53-independent pathway in MEF cells. Consistent with the previous study (7), we found that caspase activation is not required for cell death induced by these DNA-damaging agents in p53 null cells because all of the caspase inhibitors we tested, including benzoyloxy carbonyl-γ-1-Val-Asp-D, and the poxvirus protein Crm A, failed to block the cell death (data not shown).

In our earlier studies, we found that RIP plays an essential role in DNA damage-induced NF-κB activation and reactive oxygen species-induced caspase-independent cell death (12, 17). Hence, we addressed whether RIP is involved in DNA damage-induced cell death by using RIP null (RIP−/−) MEF cells. Because p53 is often mutated during establishing MEF cell lines, we first examined the status of p53 in the RIP−/− MEF cells. As shown in Fig. 1B, whereas the basal level of p53 is undetectable in nontreated wild-type MEF cells, the induction of p53 protein is first detected at 1 h after treatment with Adr, and the level of p53 protein continued to accumulate as examined at different time points following treatment. In contrast, the basal level of p53 protein in RIP−/− cells was significantly increased, and there was no detectable induction of p53 protein following Adr treatment. Similar results were observed when cells were treated with other DNA-damaging agents such as Cpt (Fig. 1B, bottom panel) and Ara-C (data not shown). The protein levels of RIP and actin were checked as controls (Fig. 1B). Because it is known that p53 protein is stabilized when mutated and the protein level of the mutated p53 does not increase in response to DNA damage (21), our results in Fig. 1B imply that the p53 protein is a mutant form in RIP−/− MEF
DNA damage-induced, p53-independent cell death mediated by RIP

To further confirm this conclusion, we performed immunoprecipitation experiments with a monoclonal antibody, which only recognizes mutant p53 protein. Immunoprecipitants from wild-type and RIP/H11002/H11002 cells following Adr treatment were separated by SDS-PAGE and analyzed by Western blotting with the p53 antibody used in Fig. 1B, which recognizes both wild-type and mutant p53 proteins. As shown in Fig. 1C, the same amount of p53 protein was immunoprecipitated from different samples of RIP/H11002/H11002 cells regardless of treatment, indicating that p53 was mutated and not induced in response to DNA damage in these cells. However, as expected, no detectable level of p53 protein was immunoprecipitated from any sample of wild-type cells even though high levels of p53 protein were induced after Adr treatment (Fig. 1C, bottom panel). Hence, the p53 protein in RIP/H11002/H11002 MEF cells is a mutant one.

FIGURE 1. RIP−/− cells are resistant to DNA damage-induced cell death. A, DNA-damaging agents induce cell death by a p53-independent pathway in MEF cells. MEF cells from wild-type and p53−/− mice were treated with Adr (10 μg/ml), Cpt (120 μM), and Ara-C (100 μM). 24 h after treatment, the percentage of cell death was determined by lactate dehydrogenase release assay as described under “Experimental Procedures.” The results represent the mean values of at least three repeated experiments. B, impaired induction of p53 in response to DNA damage in RIP−/− cells. Wild-type and RIP−/− cells were treated with Adr and Cpt for various times as indicated. Cell extracts were applied to SDS-PAGE for Western blotting with anti-p53, anti-RIP, and anti-actin antibodies. C, immunoprecipitation with anti-p53 antibody, pAb240. Immunoprecipitants were analyzed by Western blotting with anti-p53, pAb122, anti-RIP, and anti-actin antibodies. One percent of extract from each sample was used as a control of protein input. D, RIP−/− cells are resistant to Adr-, Cpt-, or Ara-C-induced cell death. Wild-type, p53−/−, and RIP−/− MEF cells were treated with Adr, Cpt, and Ara-C. At 24 h after treatment, the percentage of cell death was determined as in A.

FIGURE 2. Reconstitution of RIP expression in RIP−/− cells restored the sensitivity of cells to DNA damage-induced cell death. A, the protein level of RIP in WT, RIP−/−, and RIP−/− (RIP) cells. B, ectopic expression of RIP restored the sensitivity of RIP−/− cells to DNA damage-induced cell death. RIP−/− and RIP-reconstituted cells, RIP−/− (RIP), were treated with Adr (10 μg/ml) or Cpt (120 μM) for 24 h. The percentage of cell death was determined as in Fig. 1A. C, impaired induction of p53 in response to DNA damage in RIP−/− (RIP) cells. RIP−/− and RIP−/− (RIP) cells were treated with Adr and Cpt for various times as indicated. Cell extracts were applied to SDS-PAGE for Western blotting with anti-p53, anti-RIP, and anti-actin antibodies.
DNA Damage-induced, p53-independent Cell Death Mediated by RIP

A

**FIGURE 3.** Knocking down the expression of RIP protein results in the decrease of the sensitivity of p53 

We further confirmed this conclusion by sequencing the cDNA sequence of p53 gene cloned from RIP 

Because the p53 protein is mutated in RIP 

To rule out the possibility that the presence of mutant p53 protein interferes with DNA damage-induced cell death in RIP 

To test whether this finding can be extended to other types of cells, particularly some human cancer cells, we examined the sensitivity of the p53 null human tumor cell line, HCT 116 (p53 

The Requirement of RIP in DNA Damage-induced, p53-independent Cell Death Is Not Linked to TNFRI Signaling—Previous studies have suggested that DNA damage could induce the expression of death receptors such as Fas or the release of TNF (23, 24). Because it is known that RIP is required for Fas or TNF-mediated necrotic cell death (10, 11), it is important to explore the possibility that the requirement of RIP in DNA damage-induced cell death in the p53 null human carcinoma HCT 116 cells.

The Requirement of RIP in DNA Damage-induced, p53-independent Cell Death Is Not Linked to TNFRI Signaling—Previous studies have suggested that DNA damage could induce the expression of death receptors such as Fas or the release of TNF (23, 24). Because it is known that RIP is required for Fas or TNF-mediated necrotic cell death (10, 11), it is important to explore the possibility that the requirement of RIP in DNA damage-induced cell death in the p53 null human carcinoma HCT 116 cells.

with RIP-siRNA and control siRNA were exposed to DNA-damaging agents, including Adr, Cpt, or Ara-C for an additional 24 h, and the percentage of cell death was determined as in Fig. 1A.
DNA Damage-induced, p53-independent Cell Death Mediated by RIP

DNA damage-induced cell death is because of the increased presence of these death mediators. First we tested whether the blockage of de novo protein synthesis using the protein synthesis inhibitor CHX has any effect on DNA damage-induced cell death in p53−/− cells. As shown in Fig. 4A, the presence of CHX did not affect the cell death induced by Adr treatment in p53−/− cells, although CHX does effectively inhibit protein synthesis as indicated by the protein level of the short half-life protein c-Myc (data not shown). Therefore, it is unlikely that a de novo synthesized death receptor or factor plays a major role in this process. To rule out the possibility that TNF signaling could be engaged by the release of pre-existing TNF in response to DNA damage (24), we examined DNA damage-induced cell death in TNFR1−/− MEF cells. As shown in Fig. 4B, the treatment of Adr, Cpt, or Ara-C resulted in a similar susceptibility to cell death in WT and TNFR1−/− cells, whereas TNF plus CHX failed to initiate TNF-induced cell death in TNFR1−/− cells (data not shown). These results suggest that the RIP-mediated cell death induced by DNA damage was not achieved through TNF signaling.

We also examined whether DNA damage-induced cell death requires FADD protein, which is a key effector of Fas and TNF-induced apoptosis. We found that FADD protein is not essential for DNA damage-induced cell death and JNK activation by using FADD−/− MEFs (Fig. 5A and B). In addition, RIP and FADD protein do not interact following DNA damage (Fig. 5C). RIP cleavage and ubiquitination during TNF-induced apoptosis has been reported by us and others. However, we did not observe any RIP degradation or ubiquitination following DNA damage (Fig. 1, B and C, and Fig. 5C). These observations further indicate that the role of RIP in DNA damage-induced cell death is distinct from its role in TNF-induced apoptosis.

RIP Mediates DNA Damage-induced Cell Death through Activating JNK—It has been reported that JNK activation is critical for TNF-induced necrotic cell death (15, 16). Therefore, it is important to investigate whether JNK activation plays any role in DNA damage-induced cell death. Because JNK is only activated by certain classes of DNA-damaging agents (13, 14), we examined JNK activation in response to Adr and Cpt in wild-type MEF cells and the possible role of RIP in JNK activation with RIP−/− cells. As shown in Fig. 6A, left panels, both Adr and Cpt activate JNK potently in wild-type MEF cells. However, RIP activation by Adr or Cpt is severely impaired in RIP−/− cells (Fig. 6A, right panels), indicating that RIP is needed for DNA damage-induced JNK activation. This conclusion is further supported by the observation that reconstitution of RIP expression in RIP−/− cells significantly restores JNK activation in response to Adr or Cpt (Fig. 6B). DNA damage-induced JNK activation is independent of p53 because JNK is activated normally in p53−/− cells (data not shown). Next, we sought

FIGURE 4. The requirement of RIP in DNA damage-induced cell death is not linked to TNF signaling pathway. A, DNA damage-induced cell death does not require de novo protein synthesis. p53−/− cells were treated with 10 μg/ml Adr for 24 h with or without 10 μg/ml CHX. Then cell death was determined as described in Fig. 1A. B, DNA damage-induced cell death is independent of TNFR1 receptor signaling. Wild-type and TNFR1−/− cells were treated with various DNA-damaging stimuli, including Adr, Cpt, or Ara-C for 24 h, and the percentage of cell death was determined as in Fig. 1A.

FIGURE 5. FADD is not involved in adriamycin-induced cell death and JNK activation. A, wild-type and FADD−/− cells were treated with various DNA-damaging stimuli, including Adr, Cpt, and Ara-C, and the percentage of cell death was determined as in Fig. 1A. B, wild-type and FADD−/− cells were treated with Adr (10 μg/ml) for various times as indicated. Cell extracts were applied to SDS-PAGE for Western blotting with anti-p-JNK and anti-JNK1 antibodies. C, wild-type cells were treated with Adr for various times as indicated, and cell extracts from each sample were immunoprecipitated with anti-RIP antibody. Immunoprecipitants were analyzed by Western blotting with anti-FADD and anti-RIP antibodies. One percent of cell extracts from each sample was used as a control of protein input.
DNA Damage-induced, p53-independent Cell Death Mediated by RIP

FIGURE 6. RIP is required for DNA damage-induced JNK activation. A, impaired JNK activation in RIP−/− cells in response to DNA damage. Wild-type and RIP−/− cells were treated with Adr (10 μg/ml) or Cpt (120 μM) for various times as indicated. Cell extracts were subjected to immunoprecipitation with anti-JNK1 antibody, and its activity was determined by immune complex kinase assay with [γ-32P] ATP and GST-c-Jun-(1–79) as a substrate. GST-c-Jun phosphorylation was assessed by SDS-PAGE and autoradiography. JNK protein content was analyzed by Western blotting with anti-JNK1 antibody. B, restoration of JNK activation in response to Adr or Cpt treatment in RIP−/− (RIP) cells. RIP−/− and RIP−/− (RIP) cells were left untreated (−) or treated with Adr and Cpt. JNK activity was measured as described in A. C, JNK, but not p38, null cells are resistant to DNA damage-induced cell death. Wild-type, JNK−/−, and p38−/− cells were treated with Adr, Cpt, or Ara-C as indicated, and cell death was determined by lactate dehydrogenase release assay as described in Fig. 1A.

to determine whether DNA damage-induced, RIP-mediated JNK activation contributes to cell death in response to DNA damage by using JNK1−/− cells. As shown in Fig. 6C, when cells were treated with Adr, Cpt, or Ara-C, JNK1−/− cells were found to be much more resistant to cell death as compared with wild-type or p38−/− cells. These results suggested that JNK is involved in DNA damage-induced cell death. This observation was further confirmed by experiments using the JNK-specific inhibitor SP600125. The pretreatment of SP600125 led to a significant suppression of cell death induced by Adr or Cpt in WT and p53−/− cells (data not shown). Therefore, these results suggest that RIP-mediated JNK activation is likely the pathway that is responsible for DNA damage-induced cell death.

DISCUSSION

p53 mutation is one of the most common genetic abnormalities in cancer, and loss of p53 function is associated with pronounced resistance of cancer to chemotherapeutic agents (1, 25). Improving the efficiency of chemotherapeutic agents in cancer cells with mutant p53 protein will have a significant impact on cancer treatment. The findings by Zong et al. (7) indicated that alkylating DNA-damaging agents induce p53-independent necrosis-like cell death, suggesting the possibility for using DNA-damaging agents to kill cancer cells through a p53-independent pathway. Therefore, it is critical to understand the molecular basis of this p53-independent pathway. In this study, we identified RIP-mediated JNK activation as the signaling pathway to regulate DNA damage-induced cell death independently of p53, and we provided a potential mechanism for this process.

RIP is a critical effector molecule of TNF signaling and is essential for TNF-induced JNK and NF-κB activation (8, 26). In a previous study, we showed that RIP also plays a pivotal role in DNA damage-induced NF-κB activation (17). In this study, we found that RIP mediates p53-independent, DNA damage-induced cell death. The involvement of RIP in DNA damage-induced cell death does not require TNFR1 signaling. RIP fulfills its function in DNA damage-induced cell death through mediating JNK activation. Recently, Janssens et al. (27) reported that the death domain protein PIDD functions upstream of RIP in DNA damage-induced NF-κB activation. It will be interesting to see whether PIDD has a similar function in DNA damage-induced cell death.

DNA damage can induce cells to undergo apoptosis or necrosis. However, alkylating DNA damage causes necrotic cell death, and this process is p53- and caspase-independent (7). In our study, we found that DNA damage-induced cell death in RIP−/− cells does not require caspase activation and has a necrotic morphology (data not shown). Because p53 is mutated in RIP−/− cells, the cell death we observed in those cells is p53-independent, necrosis-like cell death. Although it is still not understood how RIP senses the death signal of DNA damage, our study clearly establishes RIP as a key regulator of cell death in response to DNA damage and raises the possibility that RIP could function as a tumor suppressor. Especially, because many types of cancer cells are resistant to chemotherapies despite there being two distinct death pathways in response to DNA damage, it is of great importance to examine the status of the RIP/JNK pathway in addition to p53 protein in cancer cells.

Acknowledgments—We thank Dr. L. Neckers for p53−/− MEF cells, Dr. M. Karin for JNK1−/− and p38−/− MEF cells, and Dr. M. Keliiher for RIP−/− MEF cells.

REFERENCES

DNA Damage-induced, p53-independent Cell Death Mediated by RIP

The Death Domain Kinase RIP Has an Important Role in DNA Damage-induced, p53-independent Cell Death
Gang Min Hur, You-Sun Kim, Minho Won, Swati Choksi and Zheng-gang Liu

doi: 10.1074/jbc.M605577200 originally published online July 6, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605577200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 27 references, 9 of which can be accessed free at
http://www.jbc.org/content/281/35/25011.full.html#ref-list-1