Rad9 Protects Cells from Topoisomerase Poison-induced Cell Death*

David Loegering‡, Sonnet J. H. Arlander§§, Jennifer Hackbarth¶¶, Benjamin T. Vroman¶¶, Pia Roos-Mattjus***, Kevin M. Hopkins‡‡, Howard B. Lieberman‡‡, Larry M. Karnitz‡§ §§, and Scott H. Kaufmann‡‡ §§

From the 3Division of Oncology Research, Mayo Clinic, and Departments of §Molecular Pharmacology and ||Biochemistry/Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, and ¶¶Center for Radiological Research, Columbia University, New York, New York 10032

Previous studies have suggested two possible roles for Rad9 in mammalian cells subjected to replication stress or DNA damage. One model suggests that a Rad9-containing clamp is loaded onto damaged DNA, where it participates in Chk1 activation and subsequent events that contribute to cell survival. The other model suggests that Rad9 translocates to mitochondria, where it triggers apoptosis by binding to and inhibiting Bcl-2 and Bcl-xL. To further study the role of Rad9, parental and Rad9−/− murine embryonic stem (ES) cells were treated with camptothecin, etoposide, or cytarabine, all prototypic examples of three classes of widely used anticancer agents. All three agents induced Rad9 chromatin binding. Each of these agents also triggered S-phase checkpoint activation in parental ES cells, as indicated by a caffeine-inhibitable decrease in [3H]thymidine incorporation into DNA and Cdc25A down-regulation. Interestingly, the ability of cytarabine to activate the S-phase checkpoint was severely compromised in Rad9−/− cells, whereas activation of this checkpoint by camptothecin and etoposide was unaltered, suggesting that the action of cytarabine is readily distinguishable from that of classical topoisomerase poisons. Nonetheless, Rad9 deletion sensitized ES cells to the cytotoxic effects of all three agents, as evidenced by enhanced apoptosis and diminished colony formation. Collectively, these results suggest that the predominant role of Rad9 in ES cells is to promote survival after replicative stress and topoisomerase-mediated DNA damage.

Recent studies have suggested two possible roles for Rad9 after DNA damage. The first involves participation of Rad9 in a heterotrimeric clamp that is assembled on chromatin following replication stress or other types of DNA damage. When replication forks stall, the single-stranded DNA-binding protein replication protein A binds areas of single-stranded DNA (1–4). Bound protein replication protein A facilitates the binding of the kinase ATR2 and its binding partner ATR-interacting protein to chromatin (4). At the same time, a preassembled complex of Rad9, Hus1, and Rad1 (5, 6), known as the 9-1-1 complex, is loaded onto damaged chromatin by a clamp loader consisting of Rad17 and the four replication factor C small subunits (7, 8). In a manner that remains poorly understood, the chromatin-bound 9-1-1 complex facilitates ATR-mediated phosphorylation and activation of Chk1 (reviewed in Ref. 9). Chk1 then phosphorylates (10–13) and causes proteolytic destruction of (14) Cdc25A, thereby abrogating the ability of this phosphatase to activate Cdk2/cyclin E complexes and drive S-phase progression (reviewed in Ref. 15). Chk1-mediated phosphorylation of Cdc25A and Cdc25C also prevents the activating dephosphorylation of Cdc2 cyclin B complexes and progression through G2 phase (reviewed in Ref. 16). Based on these data, along with the observation that disruption of Chk1 function sensitizes cells to a wide range of genotoxic stresses (17–22), one would predict that cells lacking Rad9 would be highly sensitive to replication stress and certain types of DNA damage.

Additional studies have suggested a potential second function for Rad9. Based on the observation that a nine-amino acid sequence near the N terminus of Rad9 is similar to the BH3 domain of proapoptotic Bcl-2 family members, it has been proposed that Rad9, like other BH3-only proteins (23), might traffic to mitochondria, bind to antiapoptotic Bcl-2 family members, and facilitate apoptosis (24). Consistent with this possibility, exogenous epitope-tagged Rad9 has been observed to translocate to mitochondria after DNA damage (24, 25), interact with Bcl-2 (24) and Bcl-xL (25), and induce apoptosis (24, 25). In agreement with this proposed role, Rad9 antisense oligonucleotides also reportedly diminish methyl methanesulfonate-induced apoptosis (24). Collectively, these results suggest that Rad9 plays a critical role in the induction of apoptosis after replicative stress or genotoxic damage and lead to the prediction that Rad9 deletion might result in resistance to apoptosis.

Among the agents that activate Chk1 are drugs that target DNA topoisomerases. Etoposide, a prototypic topoisomerase II poison (26), inhibits the religation steps in the topoisomerase II catalytic cycle, thereby stabilizing covalent DNA-topoisomerase II complexes that interact with helicases to produce frank DNA double-strand breaks (27). Camptothecin, the founding member of a large class of topoisomerase I poisons (26, 28), inhibits the religation step of topoisomerase I (29) to produce

---

* This work was supported in part by National Institutes of Health Grants CA73709 (to S. H. K.), CA84321 (to J. H. L.), and CA98816 (to H. B. L.), and predoctoral fellowships from the Mayo Foundation (to S. J. H. A., J. H., and P. R.-M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors contributed equally to this work.

‡‡ Present address: Turku Center for Biotechnology, FIN-20520 Turku, Finland.

§§ Both authors contributed equally to this work. To whom correspondence should be addressed: Div. of Oncology Research, Guggenheim 1301, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905; Tel.: 507-284-8950. Fax: 507-284-3906. E-mail: Kaufmann.Scott@mayo.edu or karntiz.larry@mayo.edu.

1 The abbreviations used are: ATR, mutated in ataxia telangiectasia (ATM)-Rad-3-related kinase; ES, embryonic stem; CPT, camptothecin; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; UV, ultraviolet.
covalent topoisomerase I-DNA complexes (30, 31) that likewise yield DNA double-strand breaks (32–34) after interaction with advancing replication forks (33, 35). More recently, it has been reported that cytarabine, a nucleoside analogue that is widely used to treat acute leukemias and lymphomas (36), might also act by trapping topoisomerase I-DNA covalent complexes after incorporation of the analogue into DNA (37, 38). Direct or indirect evidence has indicated that each of these prototypic anticancer agents also causes activation of Chk1 (19–21, 39), which ordinarily occurs in an ATR-dependent manner (39, 40). Because the ATR/Chk1 pathway is usually implicated in replicative stress, and whereas topoisomerase poisons induce DNA strand breaks, the exact roles of various DNA damage-activated signal transduction components in response to these agents remain to be more fully defined (41, 42).

To further evaluate the role of Rad9 in the response to topoisomerase poisons and replicative stress, we have now examined the effects of camptothecin, etoposide, and cytarabine in Rad9+/− ES cells and their wild-type counterparts. Results of this analysis failed to demonstrate any apoptotic defect in Rad9-deficient cells. To the contrary, deletion of this critical component of the checkpoint machinery sensitized cells to all three of the agents.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from the following suppliers: camptothecin, cytarabine, pacthaxel, and 2-mercaptoethanol from Sigma; etoposide from Biomol (Plymouth Meeting, PA); knock-out Dulbecco’s modified Eagle’s medium and l-glutamine from Invitrogen; and ES-GRO leukemia inhibitory factor from Chemicon (Temecula, CA). Anti-phospho-Ser345-Chk1 and procaspase-3 from Cell Signaling Technology (Beverly, MA); and murine anti-Chk1, goat anti-actin, and mouse anti-Cdc25A from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were obtained as described previously (43, 44).

Cell Lines and Tissue Culture—Rad9+/− ES cells, derived by targeted interruption of both murine Rad9 alleles, were generated and characterized by Kevin Hopkins, Wojtek Auerbach, Xiang Yuan Wang, M. Prakash Hande, Haiying Hang, Debra J. Wolgemuth, Alexandra L. Joyner, and Howard B. Lieberman, as described previously (44). Stable Rad9+/− ES cell clones expressing human wild-type Rad9 or a Rad9 mutant in which eight phosphorylation sites were converted to alanine (Rad9-8A) were derived by transfection and selection as described (44). ES cells were propagated on gelatinized tissue culture plates in knock-out Dulbecco’s modified Eagle’s medium containing 15% ES cell conditioned medium. Rad9−/− ES cells and their wild-type counterparts (Rad9+8A) were derived by transfection and selection as described (44). ES cells were propagated on gelatinized tissue culture plates in knock-out Dulbecco’s modified Eagle’s medium containing 15% ES cell conditioned medium. Rad9 and Topoisomerase Poisons

RESULTS

Drug-induced Rad9 Chromatin Binding—In light of previous studies showing that the ATR/Chk1 pathway plays a critical role in survival of cells after many genotoxic stresses, including topoisomerase poisons (reviewed in Refs. 22, 39, 40; see also Refs. 21, 51, 52), we assessed the role of the Chk1 regulator Rad9 in the response to camptothecin, etoposide, cytarabine, and, as a control, UV light. To evaluate the role of Rad9 in the response to camptothecin, we assessed the role of the Chk1 regulator Rad9 in the response to camptothecin, etoposide, cytarabine, and, as a control, UV light. To evaluate the role of Rad9 in the response to camptothecin, we assessed the role of the Chk1 regulator Rad9 (44) in K562 human leukemia cells, which were extensively utilized in previous studies of 9-1-1 chromatin binding (45), and murine ES cells. As shown in Fig. 1, submicromolar concentrations of each agent induced in-
increased binding of Rad9 to chromatin. It is worth noting that Rad9 chromatin binding occurs within 6 h of treatment with drug concentrations that kill less than 1 log of cells (see below). Moreover, these submicromolar drug concentrations are readily achievable in the clinical setting (28, 36, 53). Drug-induced phosphorylation of Chk1 and Chk2 in parental and Rad9−/− ES cells—Rad9 is required for Chk1 activation induced by ionizing radiation, UV light, and the ribonucleotide reductase inhibitor hydroxyurea (44). To determine whether Rad9 also participates in Chk1 activation induced by the anticancer drugs used in Fig. 1, we assessed Chk1 phosphorylation on Ser345, a modification that is catalyzed by ATR (40, 54). These studies examined parental ES cells, which express Rad9, and Rad9−/− ES cells expressing wild-type human Rad9 (hRad9), and the Rad9-8A phosphorylation-site mutant (hRad9-8A) were subjected to immunoprecipitation and immunoblottting with anti-Rad9 antibodies.

Drug-induced Phosphorylation of Chk1 and Chk2 in Parental and Rad9−/− ES Cells—Rad9 is required for Chk1 activation induced by ionizing radiation, UV light, and the ribonucleotide reductase inhibitor hydroxyurea (44). To determine whether Rad9 also participates in Chk1 activation induced by the anticancer drugs used in Fig. 1, we assessed Chk1 phosphorylation on Ser345, a modification that is catalyzed by ATR (40, 54). These studies examined parental ES cells, which express Rad9, and Rad9−/− ES cells expressing wild-type human Rad9 (hRad9), and the Rad9-8A phosphorylation-site mutant (hRad9-8A) were subjected to immunoprecipitation and immunoblottting with anti-Rad9 antibodies.

Parental ES and Rad9−/− ES cells were treated with 200 nM cytarabine, 500 nM CPT, 500 nM etoposide, or 15 J/m2 UV light. Cell lysates were prepared, separated by SDS-PAGE, and sequentially immunoblotted for phospho-Ser345-Chk1 and total Chk1. The same samples were also separated by SDS-PAGE and immunoblotted for total Chk2.

To evaluate whether Rad9 is also required for activation of Chk2 by these agents, we examined Chk2 mobility shifts in untreated and treated parental and Rad9−/− ES cells. In the absence of drugs, a biphasic Chk2 mobility shift is observed in parental ES cells (Fig. 2A, lane 5), reflecting the slow accumulations of Chk2 protein bands. Treatment with cytarabine, etoposide, or camptothecin is required for Chk1 activation (40, 54). These studies examined parental ES cells, which express Rad9, and Rad9−/− ES cells expressing wild-type human Rad9 (hRad9), and the Rad9-8A phosphorylation-site mutant (hRad9-8A) were subjected to immunoprecipitation and immunoblottting with anti-Rad9 antibodies.

Parental ES and Rad9−/− ES cells were treated with 200 nM cytarabine, 500 nM CPT, 500 nM etoposide, or 15 J/m2 UV light. Cell lysates were prepared, separated by SDS-PAGE, and sequentially immunoblotted for phospho-Ser345-Chk1 and total Chk1. The same samples were also separated by SDS-PAGE and immunoblotted for total Chk2.

Fig. 2. Drug-induced phosphorylation of Chk1 and Chk2 in parental and Rad9−/− ES cells. A, Rad9 expression in cell lines utilized in these studies. Lysates from parental ES cells (expressing mouse Rad9 [mRad9]), Rad9−/− ES cells, Rad9−/− ES cells expressing wild-type human Rad9 (hRad9), and the Rad9-8A phosphorylation-site mutant (hRad9-8A) were subjected to immunoprecipitation and immunoblottting with anti-Rad9 antibodies. B, parental and Rad9−/− ES cells were treated with 200 nM cytarabine, 500 nM CPT, 500 nM etoposide, or 15 J/m2 UV light. Cell lysates were prepared, separated by SDS-PAGE, and sequentially immunoblotted for phospho-Ser345-Chk1 and total Chk1. The same samples were also separated by SDS-PAGE and immunoblotted for total Chk2.

The activation of the S-phase checkpoint is readily achievable in the clinical setting (28, 36, 53). Drug concentrations in the following ranges were observed during high dose infusions (36); drug-induced phosphorylation of Chk1 and Chk2 in parental and Rad9−/− ES cells. A, Rad9 expression in cell lines utilized in these studies. Lysates from parental ES cells (expressing mouse Rad9 [mRad9]), Rad9−/− ES cells, Rad9−/− ES cells expressing wild-type human Rad9 (hRad9), and the Rad9-8A phosphorylation-site mutant (hRad9-8A) were subjected to immunoprecipitation and immunoblottting with anti-Rad9 antibodies. B, parental and Rad9−/− ES cells were treated with 200 nM cytarabine, 500 nM CPT, 500 nM etoposide, or 15 J/m2 UV light. Cell lysates were prepared, separated by SDS-PAGE, and sequentially immunoblotted for phospho-Ser345-Chk1 and total Chk1. The same samples were also separated by SDS-PAGE and immunoblotted for total Chk2.

Drug concentrations in the following ranges were observed during clinical trials: camptothecin, 3–40 μM 54 h after a single dose (70); cytarabine, up to 5 μM for 3 days during high dose infusions (36); etoposide, peak levels averaging 85 μM for 3 consecutive days in the bone marrow transplant setting (71).

Fig. 3. S-phase checkpoint activation. A and B, parental and Rad9−/− ES cells were pretreated with nothing (Untreated) or 3 mM caffeine for 30 min. The cells were then exposed to vehicle (Con), 50 nM cytarabine (Cyt), 500 nM CPT, or 500 nM etoposide (Etop) for 1 h and pulsed with [3H]thymidine for 20 min. Incorporation of radiolabeled thymidine into DNA is plotted as percent of incorporation relative to untreated cells. Values represent the means ± S.E. of the mean from four independent experiments. C, parental and Rad9−/− ES cells were treated with diluent (Con), 200 nM cytarabine, 500 nM camptothecin, or 500 nM etoposide for 3 h. Alternatively, cells were treated with 15 J/m2 UV irradiation and harvested 3 h later. At the completion of the incubation, Cdc25A was immunoprecipitated from cell lysates and analyzed by immunoblotting. The immunoglobulin heavy chain band on the same blot confirms the addition of equal amounts of antibody to each cell lysate and the uniform transfer of the samples.
Rad9 in toxic stress (22), we predicted that the Chk1 activation defect in the Chk1 signaling pathway decreases survival after genotoxic poisons and cytarabine treatment (circled arabine) and apoptotic cells after camptothecin or cytotoxic S-phase checkpoint activated by the topoisomerase inhibitors, which induced apoptosis in 43% of the parental cells (Fig. 4). Collectively, these results demonstrate that apoptosis occurs earlier in parental and Rad9−/− cells than in parental and Rad9−/− cells treated with camptothecin or etoposide (Fig. 4). To distinguish between these alternatives, parental and Rad9−/− cells were treated with camptothecin, etoposide, or cytarabine and analyzed for apoptotic morphological changes at various time points. Results of this analysis demonstrated that chromatin condensation and nuclear fragmentation occurs after drug treatment in the absence of Rad9 (Fig. 4A), ruling out the possibility that this polypeptide plays an essential role in the induction of apoptosis by these agents. To the contrary, quantitation of the morphological apoptotic changes (Fig. 4, B and C) demonstrated that apoptosis occurred earlier (Fig. 4B) and at lower camptothecin concentrations (Fig. 4C) in Rad9−/− cells. For example, in four separate experiments, 70 ± 12% (mean ± S.D.) of the Rad9−/− cells but only 31 ± 13% of the parental cells were apoptotic after treatment with 200 nM camptothecin for 48 h (p = 0.012 by paired t test). Similar results were obtained with etoposide (Fig. 4D), which induced apoptosis in 50 ± 2% of the Rad9−/− cells but only 24 ± 11% of the parental cells during a 48-h exposure to 125 nM drug (n = 4, p = 0.026), and cytarabine (Fig. 4E), which induced apoptosis in 43 ± 11%
of the Rad9−/− cells but in only 20 ± 11% of the parental cells during a 48-h exposure to 500 nM drug (n = 4, p < 0.05).

To provide independent support for the hypothesis that apoptosis can be triggered in the absence of Rad9, whole cell lysates prepared from Rad9−/− cells treated with varying concentrations of camptothecin (Fig. 4F) or etoposide3 were examined by immunoblotting. In both instances, caspase activation was readily detected, as indicated by the disappearance of procaspase-3 (Fig. 4F, top) and cleavage of multiple caspase-3 substrates to their signature fragments (Fig. 4F, arrowheads). Taken together, these results demonstrate that Rad9 is not essential for anticancer drug-induced apoptosis. Instead, this polypeptide, likely by means of activating the Chk1 signaling pathway, seems to play a role in protecting cells from these apoptotic stimuli.

**Effect of Rad9 on Clonogenic Survival**—To rule out the possibility that the increased apoptosis in Rad9−/− cells merely reflected enhanced kinetics without altering long-term survival, we assessed the ability of individual cells to proliferate using colony-forming assays. As illustrated in Fig. 5A, the IC50 for camptothecin was 2.9 ± 0.7-fold lower in Rad9−/− cells than in parental cells (n = 7, p = 0.001). Likewise, Rad9−/− cells displayed diminished colony-forming ability after treatment with SN-38, the active metabolite of the camptothecin analog irinotecan (Fig. 5B). Taken together, these results demonstrate that Rad9 enhances the survival of cells treated with these agents.

To rule out the possibility that these results reflected an alteration other than Rad9 gene deletion, Rad9−/− cells expressing wild-type human Rad9 or the human Rad9 mutant (Δ) with eight phosphorylation sites converted to alanines (Rad9-8A) (44) were treated with the indicated concentrations of camptothecin for 24 h, washed, and incubated for 6 additional days to allow colonies to form. Bars, mean ± 1 S.D. of triplicate samples.

**DISCUSSION**

The results of the present study provide new insight into the potential roles of Rad9 after treatment with agents that cause

---

3J. Hackbarth and S. H. Kaufmann, unpublished observations.
Topoisomerase I is slowed. Recent studies have suggested that cytarabine might act like a topoisomerase poison. After its incorporation into DNA (66), cells are hypersensitive to other topoisomerase poisons. These results have potentially important implications for the current understanding of the mechanisms of action of the agents studied as well as the functions of Rad9.

Previous studies using Rad9- and Hus1-deficient cells demonstrated that these cell lines had defects in the S-phase checkpoint activated by UV light and benzo(a)pyrene dihydrodiol epoxide, two agents that introduce bulky DNA lesions capable of stalling replication forks (44, 59). In contrast, neither Rad9 nor Hus1 was required for the S-phase checkpoint activated by ionizing radiation, which produces double-stranded breaks (44, 59). These results suggest that the lesions produced by UV light and ionizing radiation initially activate different signaling pathways that then lead to S-phase arrest.

Analysis of the chemotherapeutic agents used in this study revealed a similar dichotomy. As was observed previously with UV light (44), the ability of cytarabine to trigger S-phase checkpoint activation and Cdc25A degradation required Rad9 (Fig. 3). In contrast, topoisomerase poisons (Fig. 3), like ionizing radiation (44), activated these checkpoint events even in the absence of Rad9. These results indicate that the cellular responses produced by cytarabine and UV light are similar to each other and are somewhat different from the responses produced by ionizing radiation and the topoisomerase poisons.

The lesions that activate the S-phase checkpoint in a 9-1-1-dependent manner have in common the fact that they introduce replication stress (44, 59). The fact that cytarabine also requires Rad9 to induce S-phase arrest is consistent with earlier reports showing that cytarabine induces replication stress after its incorporation into DNA (66–69). On the other hand, recent studies have suggested that cytarabine might act like a topoisomerase I poison (37, 38). Evidence to support this claim has included (i) the demonstration that the religation step of topoisomerase I is slowed ~5-fold when an oligonucleotide containing cytidine arabinoside in place of cytidine at a topoisomerase I cleavage site is exposed to purified enzyme in vitro; (ii) the observation that covalent topoisomerase I-DNA complexes are increased in neoplastic cell lines several hours after the addition of cytarabine to cultures; and (iii) the demonstration that camptothecin-selected P388/camptothecin (CPT) murine lymphoma cells, which lack detectable topoisomerase I and are 4000-fold more resistant to CPT, are 5-fold resistant to cytarabine. In contrast, our observations indicate that the cell cycle response of ES cells to cytarabine is quite different from the response to the topoisomerase I poison CPT. We cannot at present rule out the possibility that our results reflect differences between ES cells and lymphoma cells. Likewise, we cannot exclude the possibility that cytarabine causes the accumulation of topoisomerase I-DNA complexes over time, possibly as a consequence of biochemical changes that contribute to apoptosis. Nonetheless, the present study suggests that the cell cycle response to cytarabine is most consistent with the action of a classical antimetabolite, as originally envisioned by earlier authors (36).

In contrast to the results seen with cytarabine, Rad9 is not required for activation of the S-phase checkpoint by topoisomerase I or topoisomerase II poisons (Fig. 3). This observation is similar to results seen previously with ionizing radiation. Because topoisomerase poisons and ionizing radiation all introduce double-stranded DNA breaks, it is likely that this lesion is the trigger for the Rad9-independent S-phase checkpoint described in Fig. 3 and our previous study (44). Additional work has also indicated that the topoisomerase I poison topotecan induces activation of the S-phase checkpoint in SV40-transformed human fibroblasts (39). Importantly, expression of a kinase dead ATR allele in these cells diminishes topotecan-induced S-phase slowing (39), suggesting a critical role for ATR in S-phase checkpoint activation. Consistent with this observation, the ATR substrate Chk1 is also required for activation of S-phase checkpoint and Cdc25A degradation after treatment with ionizing radiation in other cell types (11, 19). In contrast, Rad9 deficiency does not affect that S-phase checkpoint in ES cells treated with the topoisomerase poisons (Fig. 3) or ionizing radiation (44). Although the reasons for this discrepancy are not currently known, Chk2 is hyperactivated when Rad9−/− ES cells are treated with the topoisomerase poisons (Fig. 2B). Because both Chk1 and Chk2 phosphorylate overlapping sites on Cdc25A that participate in degradation of this phosphatase (13), one possible explanation for our findings is that the hyperactivation of the Chk2 signaling pathway contributes to topoisomerase poison-induced S-phase checkpoint activation in the Rad9−/− ES cells.

Even though Rad9−/− cells have no reproducible defects in their ability to activate the S-phase checkpoint after treatment with topoisomerase poisons, Rad9 deletion nonetheless sensitizes ES cells to camptothecin and etoposide (Figs. 4 and 5). These observations confirm and extend recent data showing that Rad9−/− ES cells are also hypersensitive to ionizing radiation in the absence of an S-phase checkpoint defect (44). In the present study, these differences in drug sensitivity were observed when apoptosis was quantified and when long-term antiproliferative effects of the various agents were assessed using colony forming assays. These results raise the possibility that Rad9 plays critical roles in other aspects of the response to DNA damage, in addition to its role in checkpoint activation. Further studies are required to identify these additional roles that contribute to survival of cells with wild-type Rad9 function.

Finally, recent studies have raised the possibility that Rad9 might also contribute to the DNA damage response by acting as a BH3-only polypeptide and trafficking to mitochondria, where it facilitates apoptosis by binding to and inhibiting the antiapoptotic polypeptides Bcl-2 and Bcl-xL. A previous study from our laboratory (45), however, failed to detect endogenous Rad9 in mitochondria by immunoblotting or immunofluorescence after ionizing radiation but could not rule out the possibility that the amount of mitochondrial Rad9 was below the limit of detection with the reagents utilized. Consistent with these earlier results, Rad9−/− cells displayed readily detectable apoptosis after a variety of DNA damaging treatments (Fig. 4), indicating that trafficking of Rad9 to mitochondria cannot be required for induction of apoptosis in ES cells. To the contrary, Rad9−/− cells were hypersensitive to camptothecin and etoposide as assessed by apoptotic and clonogenic assays (Figs. 4 and 5). We cannot at present rule out that mitochondrial trafficking of Rad9 makes a minor and nonessential contribution to induction of apoptosis in ES cells, nor can we eliminate the possibility that mitochondrial trafficking of Rad9 plays a more important role in other cell lines. Nonetheless, the present results suggest that the predominant role of Rad9 in ES cells relates to protection from agents that induce DNA double-strand breaks or replication stress.

Acknowledgments—We thank Junjie Chen and Guy Poirier for kind gifts of antibodies utilized in this study and Deb Strauss for editorial assistance.
Rad9 Protects Cells from Topoisomerase Poison-induced Cell Death
David Loegering, Sonnet J. H. Arlander, Jennifer Hackbarth, Benjamin T. Vroman, Pia Roos-Mattjus, Kevin M. Hopkins, Howard B. Lieberman, Larry M. Karnitz and Scott H. Kaufmann

J. Biol. Chem. 2004, 279:18641-18647.
doi: 10.1074/jbc.M313536200 originally published online February 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313536200

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 70 references, 52 of which can be accessed free at
http://www.jbc.org/content/279/18/18641.full.html#ref-list-1