Protein kinase C δ is responsible for constitutive and DNA damage-induced phosphorylation of Rad9

Kiyotsugu Yoshida^{1,2}, Hong-Gang Wang³,
Yoshio Miki² and Donald Kufe^{1,4}

¹Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, ²Drug Discovery Program, H.Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, FL, USA and ³Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

⁴Corresponding author
e-mail: donald_kufe@dfci.harvard.edu

The mammalian homolog of the *Schizosaccharomyces pombe* Rad9 is involved in checkpoint signaling and the induction of apoptosis. While the mechanisms responsible for the regulation of human Rad9 (hRad9) are not known, hRad9 is subject to hyperphosphorylation in the response of cells to DNA damage. The present results demonstrate that protein kinase C δ (PKC δ) associates with Rad9 and that DNA damage induces this interaction. PKC δ phosphorylates hRad9 *in vitro* and in cells exposed to genotoxic agents. The functional significance of the interaction between hRad9 and PKC δ is supported by the finding that activation of PKC δ is necessary for formation of the Rad9–Hus1–Rad1 complex. We also show that PKC δ is required for binding of hRad9 to Bcl-2. In concert with these results, inhibition of PKC δ attenuates Rad9-mediated apoptosis. These findings demonstrate that PKC δ is responsible for the regulation of Rad9 in the Hus1–Rad1 complex and in the apoptotic response to DNA damage.

Keywords: apoptosis/DNA damage/PKC δ /Rad9/RNAi

Introduction

The cellular response to genotoxic stress includes cell cycle arrest, activation of DNA repair and, in the event of irreparable damage, induction of apoptosis. The signaling mechanisms responsible for regulation of the DNA damage response are largely unknown. Certain insights have been derived from the finding that isoforms of the protein kinase C (PKC) family are activated in response to DNA damage. The calcium-independent PKC δ isoform is cleaved to a 40 kDa catalytically active fragment by caspase-3 in cells treated with DNA-damaging agents (Emoto *et al.*, 1995, 1996). The finding that overexpression of the PKC δ catalytic fragment (PKC δ CF) induces chromatin condensation and DNA fragmentation has supported a role for PKC δ cleavage in the induction of apoptosis (Ghayur *et al.*, 1996). Interaction of PKC δ CF with the nuclear DNA-dependent protein kinase catalytic subunit (DNA-PKcs) inhibits the function of DNA-PKcs to form complexes with DNA and to phosphorylate its

downstream target, p53 (Bharti *et al.*, 1998). In addition, cells deficient in DNA-PK are resistant to apoptosis induced by overexpressing PKC δ CF (Bharti *et al.*, 1998). Other studies have shown that PKC δ interacts with the c-Abl tyrosine kinase in cells treated with DNA-damaging agents (Yuan *et al.*, 1998). c-Abl-mediated phosphorylation activates PKC δ and induces translocation of PKC δ to the nucleus (Yuan *et al.*, 1998). In concert with these findings, tyrosine phosphorylation of PKC δ is necessary for its nuclear translocation and subsequent caspase-dependent cleavage in the apoptotic response to DNA damage (Blass *et al.*, 2002).

In *Schizosaccharomyces pombe*, cell cycle arrest in response to incomplete DNA replication or DNA damage is dependent on expression of spRad9, spHus1 and spRad1 (al-Khodairy and Carr, 1992; al-Khodairy *et al.*, 1994). Studies in human cells have demonstrated that this checkpoint pathway is conserved through expression of hRad9, hHus1 and hRad1 (Freire *et al.*, 1998; Kostrub *et al.*, 1998; Parker *et al.*, 1998; Udell *et al.*, 1998). As found in yeast, hRad9 forms a complex with hHus1 and hRad1 (St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Nuclear hRad9 is expressed constitutively as multiple phosphorylated forms and is phosphorylated in response to DNA damage (St Onge *et al.*, 1999, 2001; Volkmer and Karnitz, 1999; Chen, X. *et al.*, 2001). The product of the gene mutated in ataxia telangiectasia (ATM) has been shown to phosphorylate Rad9 in response to DNA damage (Chen, M.J. *et al.*, 2001). Other studies have shown that hRad9 localizes to extraction-resistant nuclear complexes in response to DNA lesions (Burtelow *et al.*, 2000). The finding that hRad9, hHus1 and hRad1 share regions of similarity with the proliferating cell nuclear antigen (PCNA) has suggested a potential role for their involvement in the DNA replication machinery (Thelen *et al.*, 1999). In this context, hRad17, a clamp loader protein, associates with the hRad9–hHus1–hRad1 complex, and DNA damage induces dissociation of these nuclear complexes (Rauen *et al.*, 2000; Lindsey-Boltz *et al.*, 2001). Another nuclear function for hRad9 may be related to the demonstration that recombinant hRad9 exhibits 3'–5' exonuclease activity (Bessho and Sancar, 2000). Furthermore, the recent finding that hRad9 interacts with anti-apoptotic Bcl-2 family proteins through a BH3-like region has supported a role for hRad9 in the apoptotic response to DNA damage (Komatsu *et al.*, 2000a,b).

The present studies demonstrate that PKC δ interacts with hRad9. We show that phosphorylation of hRad9 by PKC δ is necessary for formation of the hRad9–hHus1–hRad1 complex. The results also show that PKC δ regulates the interaction of hRad9 with Bcl-2 and the hRad9-mediated apoptotic response to DNA damage.

Results

PKC δ associates with hRad9

To determine whether PKC δ associates with hRad9, lysates from human U-937 cells were subjected to immunoprecipitation with anti-PKC δ . Analysis of the immunoprecipitates with anti-Rad9 demonstrated binding of PKC δ and Rad9 (Figure 1A). Moreover, treatment with the genotoxic agent, 1- β -D-arabinofuranosylcytosine (ara-C), was associated with increased formation of PKC δ -Rad9 complexes (Figure 1A). In the reciprocal experiment, immunoblot analysis of anti-Rad9 immunoprecipitates with anti-PKC δ demonstrated a low level of binding between PKC δ and Rad9 in control cells that was increased by ara-C treatment (Figure 1B). To confirm the association of PKC δ and Rad9, nuclear extracts from control and ara-C-treated U-937 cells were analyzed for co-sedimentation in glycerol gradients. Immunoblot analysis of the gradient fractions demonstrated co-sedimentation of PKC δ and Rad9 in the nuclear lysates from ara-C-treated cells (Figure 1C). In contrast, there was little if any PKC δ in the nuclear lysates from control U-937 cells (data not shown). In concert with these results, treatment with ara-C was associated with targeting of PKC δ to the nucleus (Figure 1D). Purity of the nuclear fraction was confirmed by immunoblotting with antibodies against the cytosolic Ikb α protein and the membrane-associated platelet-derived growth factor (PDGF) receptor (Figure 1D). Based on these findings, nuclear lysates were subjected to immunoprecipitation with anti-Rad9. Immunoblot analysis of the precipitates with anti-PKC δ demonstrated that PKC δ associates with Rad9 in cells treated with ionizing radiation (IR) or ara-C, but not in control cells (Figure 1E). These findings indicate that PKC δ translocates to the nucleus and associates with nuclear Rad9 in the response of cells to genotoxic stress.

PKC δ phosphorylates hRad9

To determine whether PKC δ phosphorylates hRad9 *in vitro*, kinase-active recombinant PKC δ was incubated with His-tagged hRad9 and [γ - 32 P]ATP. Analysis of the reaction products demonstrated that, as shown previously (Yoshida and Kufe, 2001), the recombinant kinase-active PKC δ undergoes autophosphorylation and that hRad9 functions as a substrate for PKC δ (Figure 2A). To assess the effects of PKC δ -mediated phosphorylation on the electrophoretic mobility of hRad9, recombinant PKC δ was incubated with His-hRad9 in the absence and presence of ATP. Immunoblot analysis of the reaction products with anti-His showed that phosphorylation of hRad9 by PKC δ is associated with a decrease in electrophoretic mobility (Figure 2B). To extend these findings, 293T cells were transfected to express green fluorescent protein (GFP) or GFP-PKC δ CF. Immunoblot analysis with anti-hRad9 demonstrated that PKC δ CF also induces a decrease in the electrophoretic mobility of hRad9 (Figure 2C). These findings demonstrate that PKC δ phosphorylates hRad9 *in vitro* and that PKC δ can induce Rad9 phosphorylation above that found constitutively in cells.

To determine whether treatment of cells with ara-C is associated with a shift in electrophoretic mobility of hRad9, U-937 cells were exposed to different ara-C concentrations for 2 h and then analyzed by immunoblot-

ting with anti-hRad9. The results demonstrate that treatment with 1 μ M ara-C causes a decrease in electrophoretic mobility of hRad9 (Figure 3A). Moreover, maximal decreases in electrophoretic mobility were observed following treatment with 10 μ M ara-C (Figure 3A). The effects of 10 μ M ara-C were detectable at 1 h and persisted through 8 h of treatment (data not shown). Similar results were obtained when DNA damage was induced with IR or cisplatin (CDDP) (Figure 3B). To assess whether PKC δ contributes to the phosphorylation of hRad9 in response to genotoxic stress, cells were exposed to the PKC δ inhibitor, rottlerin (Gschwendt *et al.*, 1994; Yoshida and Kufe, 2001). Immunoblot analysis with anti-hRad9 demonstrated that rottlerin increases the electrophoretic mobility of hRad9 in control and ara-C-treated cells (Figure 3C, left). In contrast, treatment with the PKC α , β and γ inhibitor, Gö 6976, had no apparent effect on electrophoretic mobility of hRad9 in either control or ara-C-treated cells (Figure 3C, middle). To confirm that the electrophoretic mobility shift induced by DNA damage is a consequence of phosphorylation, anti-hRad9 immunoprecipitates were prepared from lysates of ara-C-treated cells. Incubation of the immunoprecipitates with calf intestinal phosphatase (CIP) alone resulted in an increase in electrophoretic mobility (Figure 3C, right). In contrast, incubation with CIP and the phosphatase inhibitor, β -glycerophosphate, attenuated this effect (Figure 3C, right). Whereas rottlerin inhibits other kinases (Davies *et al.*, 2000; Soltoff, 2001), 293T cells were treated with small interfering RNA (siRNA) duplexes that target PKC δ . The results demonstrate that PKC δ siRNA1 and PKC δ siRNA2 downregulate PKC δ expression (Figure 3D, left). As a control, treatment with GFPsiRNA had little effect (Figure 3D, left). Significantly, treatment with PKC δ siRNA1, and not GFPsiRNA, increased the electrophoretic mobility of Rad9 (Figure 3D, left). Moreover, PKC δ siRNA1, and not GFPsiRNA, blocked DNA damage-induced decreases in electrophoretic mobility of Rad9 (Figure 3D, right). These findings indicate that PKC δ contributes to both constitutive and DNA damage-induced phosphorylation of hRad9.

Activation of PKC δ is necessary for targeting nuclear hRad9

To determine whether activation of PKC δ is necessary for the interaction with nuclear hRad9, anti-PKC δ immunoprecipitates from nuclear lysates of ara-C-treated cells were first analyzed for PKC δ activity. Using histone H1 as substrate (Konishi *et al.*, 1997), the results demonstrate that nuclear PKC δ activity is increased in response to ara-C (Figure 4A). In concert with these findings, nuclear levels of full-length PKC δ (PKC δ FL) were increased at 1 and 2 h of ara-C treatment (Figure 4A). Longer periods of ara-C exposure were associated with proteolytic cleavage of PKC δ FL to the catalytic fragment (PKC δ CF) (Figure 4A) and shuttling of PKC δ CF to the cytosol (data not shown). Similar findings were obtained in IR- and CDDP-treated cells (Figure 4A; data not shown). Inhibition of PKC δ with rottlerin attenuated nuclear targeting of PKC δ and increased the electrophoretic mobility of nuclear hRad9 in control and ara-C-treated cells (Figure 4B). In concert with these results, rottlerin attenuated ara-C-induced binding of PKC δ to nuclear

Fig. 1. Association between PKC δ and Rad9. (A and B) U-937 cells were left untreated or treated with 10 μ M ara-C for 2 h. Cell lysates were subjected to immunoprecipitation with pre-immune rabbit serum (PIRS), anti-Rad9 (A) or anti-PKC δ (B). Cell lysates and immunoprecipitates were subjected to immunoblot analysis (IB) with anti-PKC δ and anti-Rad9. (C) Nuclear extracts from U-937 cells treated with 10 μ M ara-C for 2 h were layered onto a 10–30% glycerol gradient. The indicated fractions were subjected to SDS-PAGE and immunoblotting with anti-PKC δ (upper panel) or anti-Rad9 (lower panel). (D) U-937 cells were treated with 10 μ M ara-C for the indicated times. Lysates from the nuclear, cytosolic and membrane fractions were subjected to immunoblot analysis with anti-PKC δ (top panel), anti-laminB (second panel), anti-I κ B α (third panel) or anti-PDGFR (bottom panel). (E) U-937 cells were treated with 20 Gy of IR and harvested at 2 h or treated with 10 μ M ara-C for 2 h. Anti-Rad9 immunoprecipitates (IP) from nuclear lysates were analyzed by immunoblotting with anti-PKC δ (upper panel) and anti-Rad9 (lower panel).

hRad9 (Figure 4B). To confirm these findings, immunofluorescence microscopy was performed to assess the intracellular distribution of PKC δ . The reactivity of anti-PKC δ was predominantly extranuclear in control cells and

nuclear in ara-C-treated cells (Figure 4C). Moreover, treatment with rottlerin blocked nuclear targeting of PKC δ in the response to ara-C (Figure 4C). In contrast, localization of hRad9 was predominantly nuclear in

Fig. 2. Phosphorylation of Rad9 by PKC δ . (A) Recombinant PKC δ was incubated with purified His-Rad9 and [γ - 32 P]ATP for 15 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography. (B) Recombinant PKC δ was incubated with His-Rad9 in the presence or absence of ATP. The protein complexes were subjected to immunoblot analysis with anti-His (upper panel) or anti-PKC δ (lower panel). (C) 293T cells were transfected with GFP vector or GFP-PKC δ CF. Cell lysates were analyzed by immunoblotting with anti-Rad9 (upper panel) or anti-GFP (lower panel).

control cells, and treatment with ara-C and/or rottlerin had no apparent effect (data not shown). These findings demonstrate that activation of PKC δ is necessary for its nuclear targeting and interaction with hRad9.

Phosphorylation of hRad9 by PKC δ is required for formation of the hRad9–hHus1–hRad1 complex

Previous work has demonstrated that hRad9 forms a complex with hHus1 and hRad1 in the response to DNA damage (St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). To define the functional significance of the interaction between PKC δ and hRad9, cells were treated with ara-C and rottlerin or Gö 6976. Anti-Rad9 immunoprecipitates from cell lysates were subjected to immunoblot analysis with anti-Rad1 or anti-Hus1. The results demonstrate that ara-C treatment is associated with binding of hRad9 to hHus1 and hRad1 (Figure 5A). In concert with involvement of PKC δ , treatment with rottlerin blocked ara-C-induced binding of hRad9 to hHus1 and hRad1 (Figure 5A). In contrast, treatment with Gö 6976 had little effect (Figure 5A). To confirm these findings, nuclear lysates from cells treated with ara-C alone, ara-C + Gö 6976 or ara-C + rottlerin were subjected to sedimentation in glycerol gradients. Immunoblot analysis of the fractions demonstrated cosedimentation of hRad9 with hHus1 and hRad1 in cells treated with ara-C alone or ara-C + Gö 6976 (Figure 5B; data not shown). In contrast, rottlerin attenuated the formation of hRad9–hHus1–hRad1 complexes (Figure 5C). Rottlerin also attenuated binding of hRad9 to hHus1–hRad1 in cells treated with IR or CDDP (data not shown).

ATM functions upstream of PKC δ activation

Previous studies have demonstrated that ATM phosphorylates Rad9 after exposure to IR (Chen, M.J. *et al.*, 2001). To assess the involvement of ATM in PKC δ -mediated regulation of Rad9, we transfected 293T cells with siRNAs that target ATM. The results show that ATMsiRNA1 and ATMsiRNA2 decrease ATM expression (Figure 6A). In contrast, GFPsiRNA had no apparent

effect (Figure 6A). To determine whether ATM regulates PKC δ activation in response to DNA damage, 293T cells transfected with ATMsiRNA1 were treated with IR or ara-C. Analysis of PKC δ immunoprecipitates from nuclear lysates demonstrated that ATM is required in part for PKC δ activation (Figure 6B). In this regard, PKC δ activation is required for the nuclear targeting of PKC δ (Figure 4; Yuan *et al.*, 1998). Consistent with these findings, cells deficient in ATM exhibited less PKC δ in the nucleus (Figure 6B). Downregulation of ATM expression was also associated with attenuation of DNA damage-induced decreases in the electrophoretic mobility of Rad9 (Figure 6C). ATM functions upstream of c-Abl in the DNA damage response (Baskaran *et al.*, 1997; Shafman *et al.*, 1997) and c-Abl interacts with Rad9 (Yoshida *et al.*, 2002). Moreover, c-Abl and PKC δ interact in response to DNA damage (Yuan *et al.*, 1998). To assess the respective roles of PKC δ and c-Abl in the regulation of Rad9, studies were performed on wild-type and c-Abl $^{-/-}$ mouse embryo fibroblasts (MEFs). As shown previously (Yoshida *et al.*, 2002), ara-C-induced binding of Rad9 to Bcl-2 was dependent on c-Abl expression (Figure 6D). The findings also show that the formation of Rad9–Bcl-2 complexes is inhibited by rottlerin (Figure 6D). These results indicate that Rad9 is regulated by both PKC δ and c-Abl in the interaction between Rad9 and Bcl-2.

PKC δ regulates hRad9-mediated cell death

To assess further the functional role of PKC δ in the regulation of hRad9, U-2 OS cells were stably transfected to express kinase-inactive PKC δ CF(K-R) under the control of the metallothionein-II promoter. Expression of PKC δ CF(K-R) was absent in control cells and induced in the presence of cadmium sulfate (Figure 7A). Exposure to cadmium sulfate for 24 h was associated with attenuation of constitutive and ara-C-induced PKC δ activity (Figure 7B). Induction of PKC δ CF(K-R) expression was also associated with an increase in electrophoretic mobility of hRad9 in control and ara-C-treated cells (Figure 7B).

To determine whether PKC δ regulates the interaction between hRad9 and Bcl-2, U-2 OS cells were studied in

Fig. 3. Phosphorylation of Rad9 in response to DNA damage. (A) U-937 cells were treated with the indicated concentrations of ara-C for 2 h. Cell lysates were subjected to immunoblot analysis with anti-Rad9 (upper panel) or anti-tubulin (lower panel). (B) U-937 cells were treated with 15 Gy of IR or 50 μ M CDDP for 2 h. Cell lysates were subjected to immunoblot analysis with anti-Rad9 (upper panel) or anti-tubulin (lower panel). (C) U-937 cells were left untreated or treated with 5 μ M rottlerin (left panel) or 50 nM Gö 6976 (middle panel) for 30 min followed by treatment with 10 μ M ara-C for 2 h. Cell lysates were analyzed by immunoblotting with anti-Rad9 or anti-tubulin. U-937 cells were treated with 10 μ M ara-C for 2 h (right panel). Anti-Rad9 immunoprecipitates from cell lysates were left untreated or treated with CIP in the presence or absence of 25 mM β -glycerophosphate (β -GP). Immune complexes were subjected to immunoblotting with anti-Rad9. (D) 293T cells were transfected with GFPsiRNA, PKC δ siRNA1 or PKC δ siRNA2. Cell lysates were analyzed by immunoblotting with anti-PKC δ , anti-Rad9 or anti-tubulin (left panel). 293T cells transfected with GFPsiRNA or PKC δ siRNA1 were treated with 20 Gy of IR and harvested at 2 h. Lysates were analyzed by immunoblotting with anti-Rad9, anti-PKC δ or anti-tubulin (right panel).

Fig. 4. Nuclear translocation of PKC δ in response to DNA damage. (A) U-937 cells were treated with 10 μ M ara-C or 15 Gy of IR for the indicated times. Nuclear lysates were subjected to immunoprecipitation with anti-PKC δ . The immunoprecipitates were incubated with histone H1 and [γ -³²P]ATP for 15 min at 30°C. Reaction products were resolved by SDS-PAGE and analyzed by autoradiography (upper panel). Nuclear lysates were also subjected to immunoblot analysis with anti-PKC δ (lower panel). (B) U-937 cells were left untreated or treated with 5 μ M rottlerin for 30 min followed by the treatment with 10 μ M ara-C for 2 h. Nuclear lysates were analyzed by immunoblotting with anti-PKC δ (top panel), anti-Rad9 (second panel) or anti-laminB (third panel). Nuclear lysates were also subjected to immunoprecipitation with anti-Rad9. The immunoprecipitates were analyzed by immunoblotting with anti-PKC δ (bottom panel). (C) U-937 cells were left untreated or treated with 5 μ M rottlerin for 30 min followed by the treatment with 10 μ M ara-C for 2 h. After fixation and blocking, cells were incubated with anti-PKC δ or anti-Rad9 (data not shown) and counterstained with DAPI. PIRS was used as a negative control (data not shown).

the absence and presence of PKC δ CF(K-R) expression. Immunoblot analysis of anti-hRad9 immunoprecipitates with anti-Bcl-2 demonstrated that constitutive binding of hRad9 to Bcl-2 is increased in response to ara-C treatment (Figure 7B). Expression of PKC δ CF(K-R) abrogated the formation of hRad9-Bcl-2 complexes in both control and ara-C-treated cells (Figure 7B). To determine whether phosphorylation of hRad9 by PKC δ is associated with induction of apoptosis, U-2 OS cells were transfected with a GFP or GFP-hRad9 vector. At 24 h after transfection, cells were treated in the absence and presence of cadmium sulfate for 24 h. Analysis of GFP-positive cells for sub-G₁ DNA demonstrated that, in

the absence of cadmium sulfate, expression of hRad9 induces apoptosis (Figure 7C). In contrast, expression of PKC δ CF(K-R) attenuated hRad9-induced apoptosis (Figure 7C). Treatment with ara-C potentiated the induction of apoptosis in hRad9-expressing cells (Figure 7C). Moreover, the apoptotic response to treatment of hRad9-expressing cells with ara-C was substantially attenuated by PKC δ CF(K-R) (Figure 7C). Similar results obtained in multiple experiments confirm that PKC δ CF(K-R) expression attenuates hRad9-induced apoptosis (Figure 7D). These findings provide support for involvement of hRad9 phosphorylation by PKC δ in the apoptotic response to DNA damage.

Fig. 5. Dissociation of the hRad9-hHus1-hRad1 complex by inhibition of PKC δ . (A) U-937 cells were left untreated or treated with 5 μ M rottlerin or 50 nM Gö 6976 for 30 min followed by treatment with 10 μ M ara-C for 2 h. Cell lysates were subjected to immunoprecipitation with PIRS or anti-Rad9. The immunoprecipitates were analyzed by immunoblotting with anti-Rad1 (upper panel), anti-Hus1 (middle panel) or anti-Rad9 (lower panel). (B and C) U-937 cells were treated with 50 nM Gö 6976 (B) or 5 μ M rottlerin (C) for 30 min, followed by the treatment with 10 μ M ara-C for 2 h. Nuclear extracts were layered onto a 10–40% glycerol gradient. After fractionation, the indicated fractions were subjected to SDS-PAGE and immunoblotting with anti-Rad9 (upper panel), anti-Hus1 (middle panel) or anti-Rad1 (lower panel).

Discussion

Rad9 is a nuclear target of PKC δ

Previous work has shown that PKC δ is expressed predominantly in the cytoplasm and to a lesser extent in the nucleus (Bharti *et al.*, 1998; Yuan *et al.*, 1998). While nuclear PKC δ associates constitutively with DNA-PKcs (Bharti *et al.*, 1998), the nuclear targets of PKC δ otherwise are largely unknown. The present findings demonstrate that nuclear PKC δ also associates constitutively at low levels with hRad9. Targeting of PKC δ to the nucleus in response to DNA damage was associated with increases in the formation of PKC δ -hRad9 complexes. Nuclear hRad9 forms a ternary complex with hHus1 and hRad1 (St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Moreover, DNA damage increases the formation of hRad9-hHus1-hRad1 complexes (Volkmer and Karnitz, 1999; Burtelow *et al.*, 2000). In concert with the involvement of PKC δ , treatment with rottlerin to inhibit PKC δ activation (Yoshida and Kufe, 2001) blocked DNA damage-induced formation of hRad9-hHus1-hRad1 complexes. In contrast, inhibition of the cPKCs (α , β and γ) had no effect on nuclear hRad9 complexes in the DNA damage response. Inducible expression of PKC δ CF(K-R) as another approach to inhibit PKC δ activation also blocked the effects of DNA damage on hRad9-hHus1-hRad1 complex formation (data not shown). Cleavage of PKC δ to PKC δ CF could also contribute to the regulation of nuclear hRad9. The generation of PKC δ CF by caspase-3 is, however, a later event (4–6 h), while nuclear targeting of PKC δ and the

regulation of hRad9-hHus1-hRad1 complexes occurs within 2 h of treatment with ara-C and other genotoxic agents. These findings thus support a model in which activation of PKC δ and thereby nuclear targeting of PKC δ in the DNA damage response is necessary for induction of the interaction of hRad9 with hHus1 and hRad1.

PKC δ phosphorylates hRad9

Nuclear hRad9 is constitutively detectable as multiple phosphorylated forms (St Onge *et al.*, 1999). Moreover, hRad9 is subject to additional phosphorylation in the response to DNA damage (Volkmer and Karnitz, 1999). Whereas recent findings have indicated that ATM contributes to the phosphorylation of hRad9 (Chen, M.J. *et al.*, 2001), other work has shown that c-Abl binds directly to the C-terminal region of hRad9 and phosphorylates the hRad9 BH3 domain on Y28 (Yoshida *et al.*, 2002). The present results demonstrate that PKC δ phosphorylates hRad9 *in vitro* and that such modification is associated with a decrease in electrophoretic mobility. hRad9 contains nine potential consensus PKC δ phosphorylation sites (S/TXXR/K). *In vitro* studies using PKC δ fragments indicate that most if not all of these sites are subject to PKC δ phosphorylation. The results of our studies in cells provide further support for PKC δ -mediated phosphorylation of hRad9. Significantly, inhibition of PKC δ with rottlerin or siRNA resulted in a substantial increase in electrophoretic mobility. The finding that CIP has no apparent effect on mobility of hRad9 from rottlerin-treated cells indicates that constitutive phosphorylation of hRad9 is mediated predominantly by a PKC δ -dependent

Fig. 6. Involvement of ATM in PKC δ -mediated regulation of Rad9. (A) 293T cells were transfected with GFPsiRNA, ATMsiRNA1 and ATMsiRNA2. Cell lysates were analyzed by immunoblotting with anti-ATM (upper panel) or anti-tubulin (lower panel). (B) 293T cells transfected with GFPsiRNA or ATMsiRNA1 were treated with 20 Gy of IR or 10 μ M ara-C and harvested at 2 h. Anti-PKC δ immunoprecipitates from nuclear lysates were analyzed for phosphorylation of histone H1 as a substrate (top panel). Nuclear lysates were also subjected to immunoblot analysis with anti-PKC δ (second panel), anti-ATM (third panel) or anti-laminB (bottom panel). (C) 293T cells transfected with GFPsiRNA or ATMsiRNA1 were treated with 20 Gy of IR or 10 μ M ara-C and harvested at 2 h. Lysates were analyzed by immunoblotting with anti-Rad9 (upper panel), anti-ATM (middle panel) or anti-tubulin (lower panel). (D) Wild-type and *c-abl*^{-/-} MEFs were left untreated or treated with 5 μ M rottlerin for 30 min, followed by exposure to 10 μ M ara-C for 2 h. Anti-Rad9 immunoprecipitates were subjected to immunoblot analysis with anti-Bcl-2 (upper panel). Lysates were also analyzed by immunoblotting with anti-Bcl-2 (lower panel).

mechanism (data not shown). The demonstration that rottlerin or siRNA also blocks DNA damage-induced decreases in hRad9 mobility provides further support for involvement of PKC δ in phosphorylation of hRad9 in response to DNA damage. In concert with these observations, similar results were obtained with knock-down of PKC δ expression with siRNA and with inducible expression of PKC δ CF(K-R) to inhibit PKC δ activity. These findings indicate that PKC δ regulates both constitutive and DNA damage-induced phosphorylation of hRad9.

PKC δ regulates the interaction between hRad9 and Bcl-2

The hRad9 BH3 domain binds to the pro-apoptotic Bcl-2 and Bcl-x_L proteins (Komatsu *et al.*, 2000b). Phosphorylation of the hRad9 BH3 domain by c-Abl induces the interaction of hRad9 and Bcl-x_L (Yoshida *et al.*, 2002). In contrast, c-Abl-mediated phosphorylation of hRad9 had no effect on binding of hRad9 to hHus1 or hRad1 (Yoshida *et al.*, 2002). The present results demonstrate that, in addition to regulating formation of hRad9–

Fig. 7. Attenuation of Rad9-mediated apoptosis by inhibition of PKC δ . (A) U-2 OS/PKC δ CF(K-R) cells were treated with 2 μ M cadmium sulfate (CdSO₄) for the indicated times. Cell lysates were analyzed by immunoblotting with anti-PKC δ . Lysates from U-2 OS cells were used as a control. (B) U-2 OS/PKC δ (K-R) cells were left untreated or treated with 2 μ M CdSO₄ for 24 h, followed by the treatment with 10 μ M ara-C for 2 h. Cell lysates were subjected to immunoprecipitation with anti-PKC δ (top panel). Anti-PKC δ immunoprecipitates were incubated with histone H1 and [γ -³²P]ATP and analyzed by SDS-PAGE and autoradiography (top panel). Cell lysates were analyzed by immunoblotting with anti-Rad9 (second panel) or anti-Bcl-2 (bottom panel). Anti-Rad9 immunoprecipitates were also subjected to immunoblot analysis with anti-Bcl-2 (third panel). (C and D) U-2 OS/PKC δ CF(K-R) cells were transfected with GFP vector or GFP-Rad9. At 24 h post-transfection, cells were left untreated or treated with 10 μ M ara-C for 24 h in the presence or absence of 2 μ M CdSO₄. DNA content in cells positive for green fluorescence was analyzed by FACscan. The flow cytometry patterns represent DNA content from GFP-Rad9-transfected cells (C). The results (mean $\pm$ SD of three independent experiments) are presented as the percentage of apoptotic cells with sub-G₁ DNA (D). Control cells (open bars); ara-C-treated cells (closed bars).

hHus1-hRad1 complexes, PKC δ -mediated phosphorylation of hRad9 is involved in binding of hRad9 to Bcl-2. Inhibition of PKC δ activity with rottlerin or PKC δ CF(K-R) expression blocked constitutive binding of hRad9 to Bcl-2. The results also demonstrate that inhibition of PKC δ activation blocks binding of hRad9 to Bcl-2 in response to DNA damage. These results and the demonstration that c-Abl is necessary for DNA damage-induced binding of hRad9 to Bcl-x_L (Yoshida *et al.*, 2002) indicate that both PKC δ and c-Abl may be required for interactions of hRad9 and Bcl-2/Bcl-x_L. In addition to the c-Abl phosphorylation site at Y28, the hRad9 BH3 domain contains two potential consensus PKC δ phosphorylation sites. The findings with ATMsiRNA-mediated interference indicate that DNA damage-induced PKC δ activation is in part dependent on

ATM. Whereas ATM activates c-Abl (Baskaran *et al.*, 1997; Shafman *et al.*, 1997), and c-Abl activates PKC δ (Yuan *et al.*, 1998), a potential explanation is that DNA damage induces a ATM $\rightarrow$ c-Abl $\rightarrow$ PKC δ pathway. Alternatively, ATM may directly activate PKC δ in the DNA damage response. Our data also show that inhibition of PKC δ activity with rottlerin or siRNA is associated with decreases in both constitutive and DNA damage-induced phosphorylation of Rad9.

PKC δ potentiates hRad9-mediated apoptosis

Overexpression of hRad9 induces apoptosis by a mechanism that is blocked by Bcl-2 or Bcl-x_L (Komatsu *et al.*, 2000b). Moreover, the demonstration that downregulation of hRad9 expression by an antisense vector suppresses cell

death by the genotoxic agent methyl methanesulfonate has supported a role for hRad9 in the apoptotic response to DNA damage (Komatsu *et al.*, 2000b). Our results demonstrate that hRad9-mediated apoptosis is attenuated by inhibition of PKC δ activity. The results also show that hRad9 potentiates DNA damage-induced apoptosis, and these effects of hRad9 are attenuated by inducible expression of PKC δ CF(K-R). Our other studies have demonstrated that the interaction between c-Abl and hRad9 also potentiates hRad9-mediated apoptosis (Yoshida *et al.*, 2002). Therefore, in concert with the involvement of both c-Abl and PKC δ in regulating the interaction of hRad9 with Bcl-2/Bcl-x_L, these kinases may exhibit independent or cooperative effects on signaling of hRad9 in the induction of apoptosis. Recent work has shown that knock-down of hRad9 with siRNA partially (~50%) abrogates the G₂ checkpoint in response to genotoxic stress (Hirai and Wang, 2002). Thus, PKC δ -mediated regulation of hRad9 could contribute, at least in part, to control of the G₂ checkpoint. Defining the role of PKC δ in regulation of the hRad9 checkpoint function may, however, be complicated by interactions between PKC δ and nuclear effectors of cell cycle progression, such as c-Abl and p73 (Yuan *et al.*, 1998; Ren *et al.*, 2002). In this regard, further studies are needed to define the nature of the interaction, if any, between c-Abl and PKC δ in regulating both hRad9-mediated apoptosis and G₂ arrest in the response to DNA damage. Based on the present findings, activation of PKC δ by genotoxic stress contributes to binding of hRad9 to Bcl-2 and thereby potentiation of hRad9-induced apoptosis.

Materials and methods

Cell culture

Human U-937 myeloid leukemia cells and human U-2 OS osteogenic sarcoma cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. 293T embryonal kidney cells, MEFs and *c-abl*^{-/-} MEFs (Tybulewicz *et al.*, 1991) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics. Cells were treated with ara-C (Sigma-Aldrich), CDDP (Sigma-Aldrich), rottlerin (Calbiochem) or Gö 6976 (Calbiochem). Irradiation was performed at room temperature with a Gammacell 1000 (Atomic Energy of Canada) and a ¹³⁷Cs source emitting at a fixed rate of 0.21 Gy/min.

Cell transfections

PKC δ CF(K-R) cDNA (Yoshida and Kufe, 2001) was cloned into a pMEP4 vector (Invitrogen) which has an expression cassette downstream from a metallothionein-II promoter. The pMEP4-PKC δ CF(K-R) vector was stably expressed in U-2 OS cells by electroporation (Gene Pulser; Bio-Rad) and selection in hygromycin B (Roche Molecular Biochemicals). For induction of PKC δ CF(K-R) expression, transfected cells were treated with cadmium sulfate (Sigma-Aldrich) at a final concentration of 2 μ M. 293T cells were transiently transfected by the calcium phosphate method.

Immunoprecipitation and immunoblot analysis

Cell lysates were prepared as described (Yoshida *et al.*, 2000) and cleared by centrifugation at 12 000 g for 15 min. Soluble proteins were incubated with anti-Rad9 [sc-8324; Santa-Cruz Biotechnology (SCBT)] or anti-PKC δ (sc-937; SCBT) antibodies for 2–6 h at 4°C followed by a 1 h incubation with protein A/G-Sepharose beads (SCBT). The immune complexes were washed three times with lysis buffer. Cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with anti-Rad9 (Oncogene), anti-PKC δ , anti-ATM (sc-7230; SCBT), anti-tubulin (Sigma-Aldrich), anti-Bcl-2 (Zymed), anti-His (Novagen) or anti-GFP

(Roche Molecular Biochemicals) for 1–4 h at room temperature. After washing, the filters were incubated with anti-rabbit or anti-mouse IgG-peroxidase conjugate (SCBT). The antigen-antibody complexes were visualized by chemiluminescence (NEN Life Science Products). In certain experiments, immune complexes were treated with 25 U of CIP (New England Biolabs) for 30 min at 30°C in the presence or absence of 25 mM β -glycerophosphate before immunoblot analysis.

Glycerol gradient sedimentation analysis

Nuclear extracts were prepared as described (Dignam *et al.*, 1983). A 200 μ l aliquot of nuclear extracts was layered on top of a glycerol gradient (5 ml) formed in 50 mM Tris-HCl pH 7.3, 0.1 M KCl, 0.2 mM EDTA, 10 mM β -mercaptoethanol and 0.1% NP-40. After centrifugation in a Beckman SW55Ti rotor at 250 000 g for 12 h at 4°C, fractions (150 μ l) were collected from the bottom of the tube. Equal volumes (20 μ l) of each sample were analyzed by SDS-PAGE and immunoblotting with anti-PKC δ , anti-Rad9, anti-Hus1 (sc-8323; SCBT) or anti-Rad1 (NeoMarkers, Inc).

siRNA transfections

siRNA duplexes (siRNAs) were synthesized and purified by Japan Bio Service (Saitama, Japan). The siRNA sequences for targeting PKC δ were PKC δ siRNA1 (5'-GAUGAAGGAGGCGCUCAGTT-3') and PKC δ siRNA2 (5'-GGCUGAGUUCUGGCUGGACTT-3'). The siRNA sequences for targeting ATM were ATMsiRNA1 (5'-GCGCCUGAUUCGAGAUCCUTT-3') and ATMsiRNA2 (5'-UGGUGCUAUUUACGAGCUTT-3'). GFPsiRNA was used as a negative control (Hirai and Wang, 2002). Transfection of siRNAs was performed as described (Elbashir *et al.*, 2002).

Immunofluorescence assays

Cells were harvested by centrifugation at 1000 g for 5 min, washed once with phosphate-buffered saline (PBS) and spun onto glass slides. Cells were fixed in 3.2% paraformaldehyde and permeabilized in 0.5% Triton X-100 in PBS for 10 min. Fixed cells were washed twice in PBS and blocked with 2% bovine serum albumin in PBS-T for 1 h. After washing with PBS-T three times, cells were incubated with anti-Rad9 or anti-PKC δ for 3 h. Immune complexes were then stained with fluorescein-conjugated anti-rabbit antibodies (SCBT). Stained cells were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and analyzed with a Nikon Eclipse TE2000-U microscope.

In vitro kinase assays

In vitro kinase assays were performed as described (Yoshida and Kufe, 2001) using His-Rad9 as a substrate. For analysis of PKC δ activity in the anti-PKC δ immunoprecipitates, the immune complexes kinase assays were performed using histone H1 as a substrate (Yoshida and Kufe, 2001).

Subcellular fractionation

Subcellular fractionation was performed as described (Kharbanda *et al.*, 1996). Purity of the fractions was monitored by immunoblot analysis with anti-laminB (Oncogene), anti-I κ B α (sc-37; SCBT) and anti-PDGFR receptor (Oncogene).

Assessment of apoptosis

DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton Dickinson). The numbers of cells positive for green fluorescence with sub-G₁ DNA content were determined with a CELLQuest program (Becton Dickinson).

Acknowledgements

The authors acknowledge Kamal Chauhan and Tomoko Yamaguchi for excellent technical support. This work was supported by grants CA55241 and CA29431 (D.K.) and CA90315 (H.-G.W.) awarded by the National Cancer Institute.

References

- al-Khodayry, F. and Carr, A.M. (1992) DNA repair mutants defining G₂ checkpoint pathways in *Schizosaccharomyces pombe*. *EMBO J.*, **11**, 1343–1350.
- al-Khodayry, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., Lehmann, A.R.

- and Carr, A.M. (1994) Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. *Mol. Biol. Cell*, **5**, 147–160.
- Baskaran, R. *et al.* (1997) Ataxia telangiectasia mutant protein activates c-abl tyrosine kinase in response to ionizing radiation. *Nature*, **387**, 516–519.
- Bessho, T. and Sancar, A. (2000) Human DNA damage checkpoint protein hRAD9 is a 3' to 5' exonuclease. *J. Biol. Chem.*, **275**, 7451–7454.
- Bharti, A. *et al.* (1998) Inactivation of DNA-dependent protein kinase by protein kinase C δ : implications for apoptosis. *Mol. Cell. Biol.*, **18**, 6719–6728.
- Blass, M., Kronfeld, I., Kazimirsky, G., Blumberg, P. and Brodie, C. (2002) Tyrosine phosphorylation of protein kinase C δ is essential for its apoptotic effect in response to etoposide. *Mol. Cell. Biol.*, **22**, 182–195.
- Burtelow, M.A., Kaufmann, S.H. and Karnitz, L.M. (2000) Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. *J. Biol. Chem.*, **275**, 26343–26348.
- Chen, M.J., Lin, Y.T., Lieberman, H.B., Chen, G. and Lee, E.Y. (2001) ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation. *J. Biol. Chem.*, **276**, 16580–16586.
- Chen, X., Zheng, Y., Zhu, J., Jiang, J. and Wang, J. (2001) p73 is transcriptionally regulated by DNA damage, p53 and p73. *Oncogene*, **20**, 769–774.
- Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.*, **351**, 95–105.
- Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
- Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods*, **26**, 199–213.
- Emoto, Y. *et al.* (1995) Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J.*, **14**, 6148–6156.
- Emoto, Y., Kisaki, H., Manome, Y., Kharbanda, S. and Kufe, D. (1996) Activation of protein kinase C δ in human myeloid leukemia cells treated with 1- β -D-arabinofuranosylcytosine. *Blood*, **87**, 1990–1996.
- Freire, R., Murguia, J.R., Tarsounas, M., Lowndes, N.F., Moens, P.B. and Jackson, S.P. (1998) Human and mouse homologs of *Schizosaccharomyces pombe* rad1(+) and *Saccharomyces cerevisiae* RAD17: linkage to checkpoint control and mammalian meiosis. *Genes Dev.*, **12**, 2560–2573.
- Ghayur, T. *et al.* (1996) Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med.*, **184**, 2399–2404.
- Gschwendt, M., Muller, H., Keilbassa, K., Zang, R., Kittstein, W., Rinke, G. and Marks, F. (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.*, **199**, 93–98.
- Hirai, I. and Wang, H.G. (2002) A role of the C-terminal region of human Rad9 (hRad9) in nuclear transport of the hRad9 checkpoint complex. *J. Biol. Chem.*, **277**, 25722–25727.
- Kharbanda, S., Saleem, A., Yuan, Z.-M., Kraeft, S., Weichselbaum, R., Chen, L.B. and Kufe, D. (1996) Nuclear signaling induced by ionizing radiation involves colocalization of the activated p56/p53^{lyn} tyrosine kinase with p34^{cdc2}. *Cancer Res.*, **56**, 3617–3621.
- Komatsu, K., Hopkins, K.M., Lieberman, H.B. and Wang, H. (2000a) *Schizosaccharomyces pombe* Rad9 contains a BH3-like region and interacts with the anti-apoptotic protein Bcl-2. *FEBS Lett.*, **481**, 122–126.
- Komatsu, K., Miyashita, T., Hang, H., Hopkins, K.M., Zheng, W., Cuddeback, S., Yamada, M., Lieberman, H.B. and Wang, H.G. (2000b) Human homologue of *S.pombe* Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. *Nat. Cell Biol.*, **2**, 1–6.
- Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U. and Nishizuka, Y. (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H₂O₂. *Proc. Natl Acad. Sci. USA*, **94**, 11233–11237.
- Kostrub, C.F., Knudsen, K., Subramani, S. and Enoch, T. (1998) Hus1p, a conserved fission yeast checkpoint protein, interacts with Rad1p and is phosphorylated in response to DNA damage. *EMBO J.*, **17**, 2055–2066.
- Lindsey-Boltz, L.A., Bermudez, V.P., Hurwitz, J. and Sancar, A. (2001) Purification and characterization of human DNA damage checkpoint Rad complexes. *Proc. Natl Acad. Sci. USA*, **98**, 11236–11241.
- Parker, A.E., Van de Weyer, I., Laus, M.C., Oostveen, I., Yon, J., Verhasselt, P. and Luyten, W.H. (1998) A human homologue of the *Schizosaccharomyces pombe* rad1⁺ checkpoint gene encodes an exonuclease. *J. Biol. Chem.*, **273**, 18332–18339.
- Rauen, M., Burtelow, M.A., Dufault, V.M. and Karnitz, L.M. (2000) The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1 and hRad9. *J. Biol. Chem.*, **275**, 29767–29771.
- Ren, J., Datta, R., Shioya, H., Li, Y., Oki, E., Biedermann, V., Bharti, A. and Kufe, D. (2002) p73 β is regulated by protein kinase C δ catalytic fragment generated in the apoptotic response to DNA damage. *J. Biol. Chem.*, **277**, 33758–33765.
- Shafman, T. *et al.* (1997) Interaction between ATM protein and c-Abl in response to DNA damage. *Nature*, **387**, 520–523.
- Soltoff, S.P. (2001) Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase C δ tyrosine phosphorylation. *J. Biol. Chem.*, **276**, 37986–37992.
- St Onge, R.P., Udell, C.M., Casselman, R. and Davey, S. (1999) The human G₂ checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1. *Mol. Biol. Cell*, **10**, 1985–1995.
- St Onge, R.P., Bestley, B.D., Park, M.Y., Casselman, R. and Davey, S. (2001) DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein. *J. Biol. Chem.*, **276**, 41898–41905.
- Thelen, M.P., Venclovas, C. and Fidelis, K. (1999) A sliding clamp model for the Rad1 family of cell cycle checkpoint proteins. *Cell*, **96**, 769–770.
- Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.K., Bronson, R.T. and Mulligan, R.C. (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. *Cell*, **65**, 1153–1163.
- Udell, C.M., Lee, S.K. and Davey, S. (1998) RAD1 and MRAD1 encode mammalian homologues of the fission yeast rad1⁺ cell cycle checkpoint control gene. *Nucleic Acids Res.*, **26**, 3971–3976.
- Volkmer, E. and Karnitz, L.M. (1999) Human homologs of *Schizosaccharomyces pombe* rad1, hus1 and rad9 form a DNA damage-responsive protein complex. *J. Biol. Chem.*, **274**, 567–570.
- Yoshida, K. and Kufe, D. (2001) Negative regulation of the SHPTP1 protein tyrosine phosphatase by protein kinase C δ in response to DNA damage. *Mol. Pharmacol.*, **60**, 1431–1438.
- Yoshida, K., Weichselbaum, R., Kharbanda, S. and Kufe, D. (2000) Role for Lyn tyrosine kinase as a regulator of stress-activated protein kinase activity in response to DNA damage. *Mol. Cell. Biol.*, **20**, 5370–5380.
- Yoshida, K., Komatsu, K., Wang, H.-G. and Kufe, D. (2002) c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol. Cell. Biol.*, **22**, 3292–3300.
- Yuan, Z.-M., Utsugisawa, T., Ishiko, T., Nakada, S., Huang, Y., Kharbanda, S., Weichselbaum, R. and Kufe, D. (1998) Activation of protein kinase C δ by the c-Abl tyrosine kinase in response to ionizing radiation. *Oncogene*, **16**, 1643–1648.

Received October 29, 2002; revised January 10, 2003;
accepted January 27, 2003