PKCε-mediated ERK1/2 activation involved in radiation-induced cell death in NIH3T3 cells

Yoon-Jin Lee[a,b], Jae-Won Soh[c], Doo-Il Jeoung[d], Chul-Koo Cho[a], Gil Ja Jhon[b], Su-Jae Lee[a], Yun-Sil Lee[a,*]

[a]*Laboratory of Radiation Effect, Korea Cancer Center Hospital, 215-4 Gongneung-Dong, Nowon-Ku, Seoul 139-706, South Korea*
[b]*Division of Molecular Life Sciences, Ewha Womans University, Seoul 120-750, South Korea*
[c]*Department of Biochemistry and Molecular Biophysics and Herbert Comprehensive Cancer Center, Columbia University, New York, NY 10032, USA*
[d]*Division of Functional Genomics, In2Gen Company, 28, Yongon-dong, Chongno-gu, Seoul 110-799, Seoul, South Korea*

Abstract

Protein kinase C (PKC) isoforms play distinct roles in cellular functions. We have previously shown that ionizing radiation activates PKC isoforms (α, δ, ε, and ζ), however, isoform-specific sensitivities to radiation and its exact mechanisms in radiation mediated signal transduction are not fully understood. In this study, we showed that overexpression of PKC isoforms (α, δ, ε, and ζ) increased radiation-induced cell death in NIH3T3 cells and PKCε overexpression was predominantly responsible. In addition, PKCε overexpression increased ERK1/2 activation without altering other MAP-kinases such as p38 MAPK or JNK. Co-transfection of dominant negative PKCε (PKCε-KR) blocked both PKCε-mediated ERK1/2 activation and radiation-induced cell death, while catalytically active PKCε construction augmented these phenomena. When the PKCε overexpressed cells were pretreated with PD98059, MEK inhibitor, radiation-induced cell death was inhibited. Co-transfection of the cells with a mutant of ERK1 or -2 (ERK1-KR or ERK2-KR) also blocked these phenomena, and co-transfection with dominant negative Ras or Raf cDNA revealed that PKCε-mediated ERK1/2 activation was Ras–Raf-dependent. In conclusion, PKCε-mediated ERK1/2 activation was responsible for the radiation-induced cell death.
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1. Introduction

ERK1/2 pathway is known to be critical in the control of cellular growth and cell survival responses to mitogenic signals in many different cell systems. It is generally accepted that activation of the ERK1/2 pathway delivers a survival signal that counteracts proapoptotic effects associated with JNK and p38 activations [1,2]. On the other hand, a requirement for ERK1/2 in mediating cisplatin-induced apoptosis of human cervical carcinoma and ovarian cell lines [3,4] has also been demonstrated. Moreover, persistent activation of ERK1/2 also contributes to glutamate-induced oxidative toxicity [5].

The pathways involved in the activation of these MAPK cascades have been well established for ERK1/2 and are activated by phosphorylation of both threonine and tyrosine residues which are catalysed by MAPK kinase, namely MEK1 and MEK2 [6]. MEKs are in turn regulated by serine phosphorylation by several MAPK kinase kinases (MKKKs), including Raf-1. However, upstream elements of the cascade are not well characterized. ERK pathway triggered by GPCRs is shown to be sensitive to genestein, suggesting the involvement of tyrosine kinases [7–9]. More precisely, several recent studies implicate Src kinases in GPCR-mediated activation of the ERK1/2 pathway [10]. Protein kinase C (PKC)-dependent and independent pathways have been suggested for the upstream elements. Concerning PKC-dependent mechanisms, it is well established that phorbol esters lead to a rapid and massive activation of ERK1/2 in most cell types [11]. While both conventional PKCs (cPKCs like α, $\beta1$, $\beta2$, and γ) and novel PKCs (nPKC such as δ, ε, θ and η) are activated by phorbol-12-myristate 13-acetate (PMA), only cPKCs are Ca^{2+}-dependent. Atypical PKCs (aPKCs such as ζ and ι/λ) are neither activated by Ca^{2+} nor phorbol esters, although

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* Corresponding author. Tel.: +82-2-970-1325; fax: +82-2-977-0381.
E-mail address: yslee@kcchsun.kcch.re.kr (Y.-S. Lee).

they can bind diacylglycerol (DAG). The existence of a family of PKC isoforms suggests that individual PKCs may exert specific role in signal transduction.

In vitro, glycated serum albumin-induced vascular smooth muscle cell proliferation is dependent on ERK1/2 pathway which is regulated by PKCδ [10]. PKCα and -ε have been shown to activate Raf-1 [12] and to regulate transcription factors including AP-1 and NF-κB [13]. In addition, respiratory syncytial virus infection results in activation of PKCβ1, δ, ε, and μ, leading to activation of ERK1/2 [14]. Recent studies show that translocation of PKCε and -δ to membrane is required for UV-induced activation of MAPK and apoptosis [15]. Moreover, PKCε modulates NF-κB and AP-1 via MAPK [16] and is required for mechano-sensitive activation of ERK1/2 [17]. PKCε-mediated induction of Ras-ERK pathway by α-phenyl-N-$tert$-butylnitron treatment [18] and thrombin-induced ERK activation via PKCε-dependent pathway [19] have also been reported. The above descriptions clearly indicate that PKC-dependent ERK1/2 activation is isoform-specific for cell type and treatment as well.

The activation of specific PKC isoforms occurs in response to a variety of apoptotic stimuli, suggesting that this family of protein kinase may contribute to regulation of the apoptotic pathway. The role of PKC in apoptosis, however, is controversial with data supporting either pro- [20,21] or anti-apoptotic functions [22–24]. Studies have shown that PKC can be activated transiently by variety of DNA-damaging agents including ionizing radiation and PKC is involved in radiosensitivity [25,26]. Our previous study also demonstrated that ionizing radiation activated PKCα, δ, ε and ζ [25], however, isoform-specific sensitivities and its exact mechanisms are not fully elucidated.

In this study, since it is very difficult to examine the involvement of endogenous PKC isoforms in radiation-induced cell death, we overexpressed PKC isoforms (PKCα, δ, ε, and ζ) in NIH3T3 cells and found that PKCε overexpressed cells had increased radiation-induced cell death, which correlated with Ras–Raf-dependent ERK1/2 activation.

2. Materials and methods

2.1. Materials

PD98059 was purchased from Calbiochem (La Jolla, CA, USA) and dissolved in DMSO, and control dishes were treated with an equal amount of DMSO. Bisbenzimide trihydrochloride (Heochst No. 33258) was from Sigma Chemical Co. (St. Louis, MO, USA). Anti-PKCα, δ, ε, and ζ antibodies, anti-phopho Akt, anti-Akt, anti-HA, anti-His and anti-Flag antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MAPK, anti-phospho MAPK (P202/Y204), anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, anti-phospho-JNK, anti-MEK1/2, and anti-phospho-MEK1/2 (Ser-217/221) polyclonal antibodies were from New England BioLabs (Beverly, MA, USA).

2.2. Plasmid construction

pHANE is a mammalian expression vector that contains a CMV promoter, Kozak translation initiation sequence, ATG start codon, N-terminal HA epitope tag, EcoRI cloning site, and stop codon. It was generated into pcDNA3 (Invitrogen)
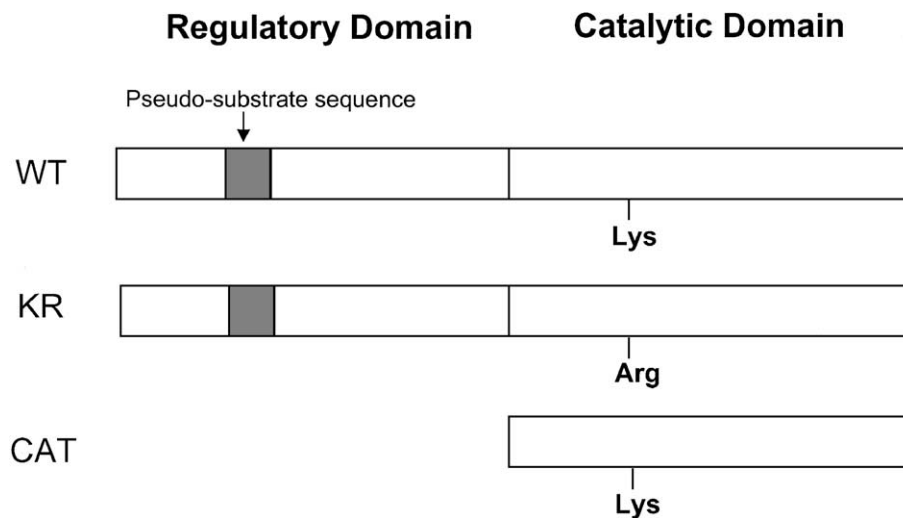

Fig. 1. Construction of dominant negative and constitutively active mutant of PKCα, δ, ε and ζ. Structures of PKC mutants. PKC-WT constructs contain a full-length PKC open reading frame. Also shown are the psuedosubstrate sequences in the N-terminal regulatory domain and the essential lysine residue in the ATP binding region of the catalytic domain. PKC-KR constructs encode a full-length PKC with a point mutation that abolishes the ATP binding ability. PKC-CAT constructs encode a truncated protein in which the catalytic domain (CAT) of PKC is preserved, but the regulatory N-terminal domain is deleted.

after digestion with *Bam*H1 and *Eco*RI [27], and was used to generate PKC mutants with an N-terminal HA tag. pHACE-PKC-WT expression plasmids were generated by ligating full-length open reading frames of different PKC isoforms at the ATP binding into pHACE digested with *Eco*RI. PHACE-PKC-KR expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a K → R point mutation at the ATP binding site into pHACE digested with *Eco*RI. All the cDNA fragments of PKC mutants were generated by PCR and were analyzed to confirm their sequences with an automated DNA sequencer. pcDNA3-Raf-K375M (Raf-KR) was constructed by subcloning the *Bam*H1 fragment of c-Raf-1 cDNA with a K375 → M point mutation [27]. The pM2NRasN17 plasmid containing the dominant-negative Ras (RasN17) under the control of a metallothionein promoter as well as the empty vector were used [28]. The eukaryotic expression vectors encoding His-tagged rat ERK2-KR and human ERK1-KR under the control of the cytomegalovirus promoter were produced by cloning the inserts from the respective NpT7-5 clones [29] into pCMV5 [30]. Plasmid pEGFP (Clontech, Palo Alto, CA, USA) was used for co-transfection with PKCε-WT to detect apoptosis.

2.3. Transfection

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine and 10% calf serum (DMEM). The cells were transiently transfected with either empty vector as a control (pcDNA3) or various expression vectors using lipofectamine (GIBCO BRL, Gaithersburg, MD) by following the procedure recommended by the manufacturer. At 6 h after transfection, the cells were fed with fresh medium and incubated overnight. Four micrograms of each plasmid per 10-cm dish was transfected.

2.4. Irradiation

Cells were plated in 3.5-, 6-, or 10-cm dishes and incubated at 37 °C under humidified 5% CO_2−95% air in culture medium until 70−80% confluent. Cells were then exposed to γ-rays with ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Canada) with dose rate of 3.81 Gy/min.

2.5. Detection of cell death

Two kinds of cell death detection methods were used. (1) Cells were plated on glass slides and irradiated. After indicated hours, cells were fixed in 70% ethanol, washed with PBS, and were incubated with 1 μg/ml bisbenzimide trihydrochloride in PBS (Heochst no. 33258) for 30 min in the dark. Specimens were viewed by fluorescence microscopy using Olympus BX-40 microscope. At least 200 cells for each determination were scored. (2) The extent of

apoptosis was determined by flow cytometry, using either PI (Sigma) staining of hypodiploid DNA or Annexin V (PharMingen) double staining. The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis.

2.6. Immunofluorescence microscopy

Cells were washed twice in PBS, fixed for 30 min in 2% formaldehyde in phosphate buffer and washed in PBS.
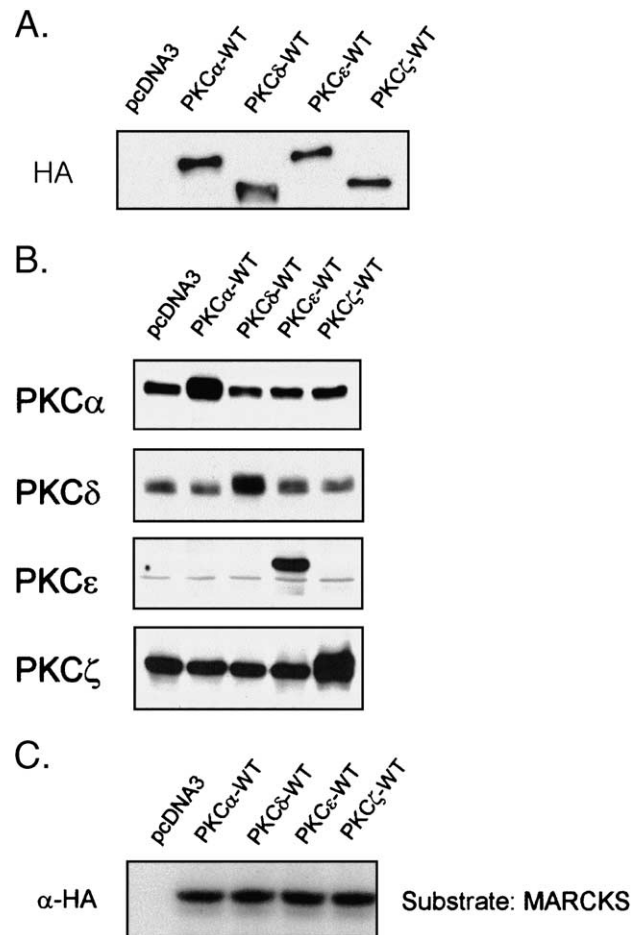
Fig. 2. Transient overexpression of PKC wild-type. Western blot analysis of transiently expressed PKC wild-type. An empty control vector (pcDNA3) or expression vectors containing PKC wild-type or mutant sequences were transiently transfected into NIH3T3 cells, and total cell lysates were subjected to Western blot analysis with anti-HA (A) or-PKC antibodies (B). NIH3T3 cells were transfected with the PKC wild-type expression vectors and control vector pcDNA3, and cellular proteins were extracted by lysis with PKC extraction buffer. HA-tagged PKC proteins were immunoprecipitated from 300 μg of cell extracts by using 3 μg of an anti-HA antibody and 30 μl of protein G-Sepharose at 4 °C after 3-h incubation. Immune complex kinase reactions were performed at 30 °C for 30 min in the presence of 10 μg of the GST-MARCKS substrate and 5 μCi of [γ-^{32}P]ATP. The reaction products were then analyzed by SDS-PAGE and autoradiography. The apparent molecular mass of the recombinant GST-MARCS protein was about 50 kDa.

Specimens were viewed by fluorescence microscopy using Olympus BX-40 microscope.

2.7. Polyacrylamide gel electrophoresis and Western blot

Cells were solubilized with lysis buffer [120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40] and boiled for 5 min, and an equal amount of protein (40 μg/well) was analyzed on 7.5%–10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. When antibodies against phospho-specific peptides were used, blots were stripped by washing three times with TBS-T [10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 (0.1%)] for 5 min each at room temperature (RT), 30 min at 55 °C with stripping buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol], and finally three times with TBS-T at RT for 5 min each. The stripped blots were then reprobed with corresponding non-phospho-specific antibod-

ies to ensure equal protein loading. For visualization of antibody binding, the Amersham ECL detection system was used.

2.8. PKC assay

NIH3T3 cells were transfected with the indicated expression vectors or the control vector pcDNA3, and cellular proteins were extracted by lysis in PKC extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 2.5 mM EGTA, and 10% glycerol] that contained protease inhibitors (10 μg aprotinin/ml, 10 μg leupeptin/ml, and 0.1 mM phenylmethylsufonyl fluoride) and phosphatase inhibitors [1 mM NaF, 0.1 mM Na$_3$VO$_4$, and 10 mM beta-glycerophosphate]. HA-tagged PKC proteins were immunoprecipitated from 300 μg of cell extracts with 3 μg of the anti-HA antibody and 30 μl of protein G-Sepharose at 4 °C after 3-h incubation. The immunoprecipitates were washed twice with PKC
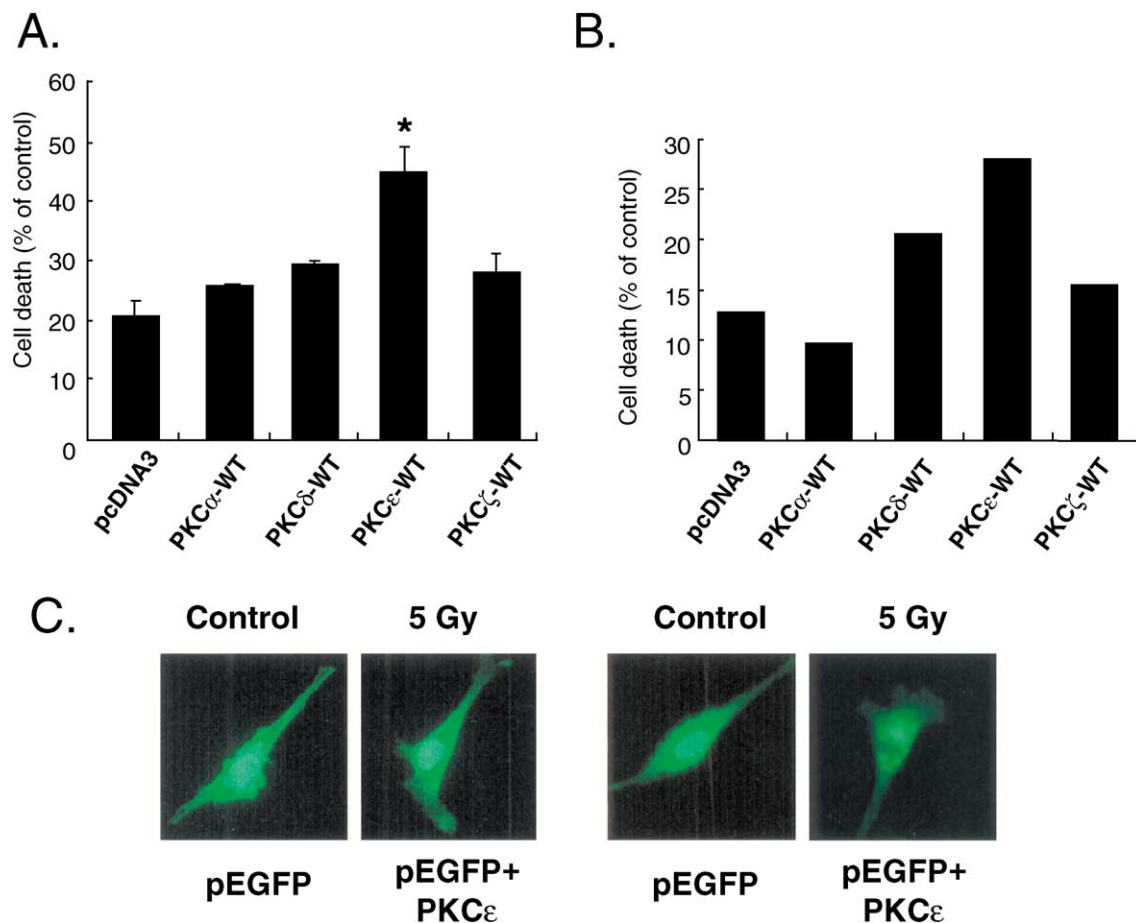

Fig. 3. Radiation-induced cell death in wild-type PKC overexpressing cells. NIH3T3 cells were transfected with the PKC wild-type expression vectors and control vector pcDNA3, and DNA fragmentation was measured by Hoechst 33258 staining 48 h after 5 Gy radiation as described in Materials and methods. Error bar indicates mean ± S.D. from three independent experiments. At least 200 cells for each determination were scored. Apoptosis was characterized by chromatin condensation and fragmentation (A) or assessed by PI staining as well as Annexin V–FITC double staining. The results represent one of three independent experiments (B). Plasmid pEGFP was used for contrasfection with PKCε-WT to detect DNA fragmentation. Cells were washed twice in PBS, fixed for 30 min in 2% formaldehyde in phosphate buffer and washed in PBS. Cells were viewed by fluorescence microscopy (C).

extraction buffer and then twice with PKC reaction buffer [50 mM HEPES (pH 7.5), 10 mM MgCl$_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na$_3$VO$_4$, and 10 mM beta-glycerophosphate], and resuspended in 20 μl of PKC reaction buffer. The kinase assay was initiated by adding 40 μl of PKC reaction buffer containing 10 μg of glutathione-S-transferase (GST)-myristoylated alanine-rich C kinase substrate (MARCKS) and 5 μCi of [γ-^{32}P] ATP. The reactions were carried out at 30 °C for 30 min, terminated by adding SDS sample buffer, and the mixtures were boiled for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography. Recombinant GST-MARCKS proteins were expressed in *Escherichia coli* BL21 (DE3)/LysS and purified to homogeneity with glutathione-S-Sepharose beads (Pharmacia) [27].

2.9. In vitro ERK1 and -2 kinase assays

Cells were washed twice with ice-cold PBS, were lysed with 1% Triton-based lysis buffer (TLB) [containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 40 mM β-glycerophosphate, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml), leupeptin (1 μg/ml), and 20 mM *p*-nitropheyl phosphate], and the lysates were incubated on ice for 30 min. The cell debris was removed by centrifugation and protein concentrations were determined with Bio-Rad protein assay kit (Hercules, CA). Monoclonal antibody against HA epitope was coupled to protein G-Sepharose beads by adding 20 μg of antibody to 1 ml of 50:50 slurry of protein G-Sepharose for 30 min at 4 °C; 40 μl of the antibody–protein G-Sepharose complex was added to 300 μg of cellular lysate protein and the mixture was incubated for 2 h at 4 °C. Immune complexes were then washed three times with TLB and twice in kinase buffer [1 × kinase buffer contained 25 mM HEPES (pH 7.4), 10 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM dithiothreitol, and 0.2 mM sodium vanadate]. The final pellet resuspended in 30 μl of kinase buffer was incubated with 50 μM ATP, 5 μCi of [γ-^{32}P]-ATP, and 5 μg of myelin basic protein (MBP) for 20 min at 30 °C. Reactions were stopped by the addition of 10 μl of 6 × concentrated sample buffer and boiling for 5 min. Beads were pelleted by centrifugation and supernatants were loaded onto 10% acrylamide separating gel. Proteins were transferred to nitrocellulose and subjected to autoradiography.

3. Results

3.1. Generation of cells overexpressing specific PKC isoforms (α, δ, ε, and ζ)

Since it is extremely difficult to examine the involvement of endogenous PKC isoforms in radiation-induced cell death, we overexpressed each PKC isoform (α, δ, ε, and

ζ) in NIH3T3 cells whose signal transduction has been well characterized, and Western blot analysis revealed that the NIH3T3 cells expressed at least four PKC isoforms, namely, PKCα, -δ, -ε, and -ζ (data not shown). Therefore, the respective cDNAs were inserted into the mammalian expression vector pHACE, and PKC-WT constructs contained the full-length open reading frames of PKCα, -δ,-ε, or -ζ (Fig. 1). Subsequently, the expression vectors for PKC-WT were transfected into NIH3T3 cells to verify that they expressed the predicted protein (HA), and the constructs were found to express the corresponding proteins with expected sizes at comparable levels (Fig. 2A). Whenever expressions of PKC isoform protein were detected by Western blot, increased level of each PKC isoform was detected (Fig. 2B). In addition, increased PKC kinase activity with GST-MARCKS as a substrate was also found in each PKC isoform of overexpressed cells (Fig. 2C), suggesting that activation levels of each PKC isoform were almost the same.

3.2. PKCε overexpression increased radiation-induced cell death

To further elucidate closely the inter relationship between each PKC isoform and radiation sensitivity, cell death was examined using Hoechst 33258 staining. As shown in Fig.
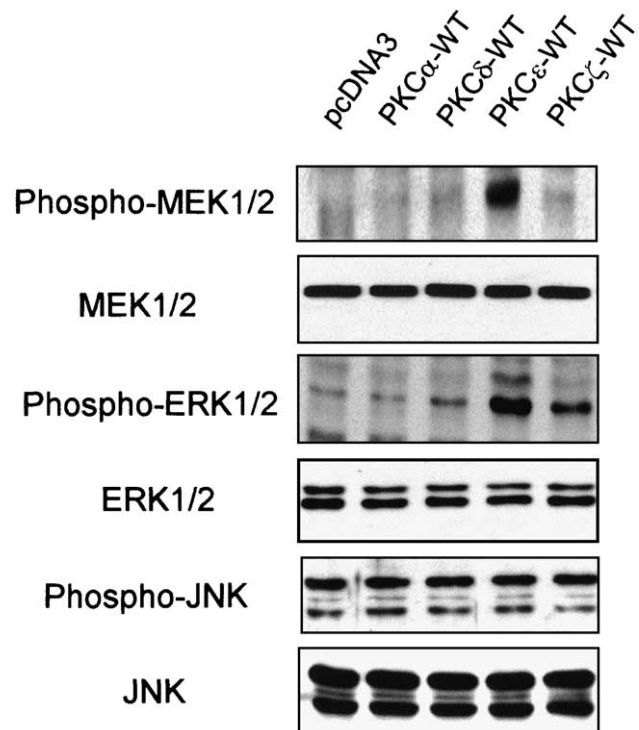

Fig. 4. MAP kinase phosphorylation of wild-type PKC overexpressing cells. NIH3T3 cells were transfected with the PKC wild-type expression vectors and control vector pcDNA3, and protein extracts were prepared, separated by SDS-PAGE, and analyzed by Western blot analysis.

3A, even though overexpression of any of all the PKC isoforms increased the radiation-induced cell death, PKCε-WT transfectant cells showed the most dramatic increase (21.9% increase from the control cells) among the isoforms. When we detected apoptosis using Annexin V–FITC kit, similar results were obtained (Fig. 3B). When we co-transfected with pEGFP and PKCε-WT plasmids, PKCε-WT transfected cells showed increased apoptosis in confocal microscopy, when compared to pEGFP plasmid alone transfected cells (Fig. 3C). From the results, PKCε was mostly responsible for the cell death.

3.3. PKCε overexpression increased ERK1/2 activation without altering activation of other MAPkinases such as p38 MAPK and JNK

To elucidate the signaling mechanisms of PKCε-mediated apoptosis, activation of MAP kinases, ERK1/2, p38 MAPK and JNK, was examined. Among the isoforms, only PKCε-WT overexpressed cells were found to have increased phosphorylation of ERK1/2 proteins, while p38 MAPK or JNK were not activated (Fig. 4). Expression of phospho-MEK1/2, an upstream molecule of ERK1/2, was
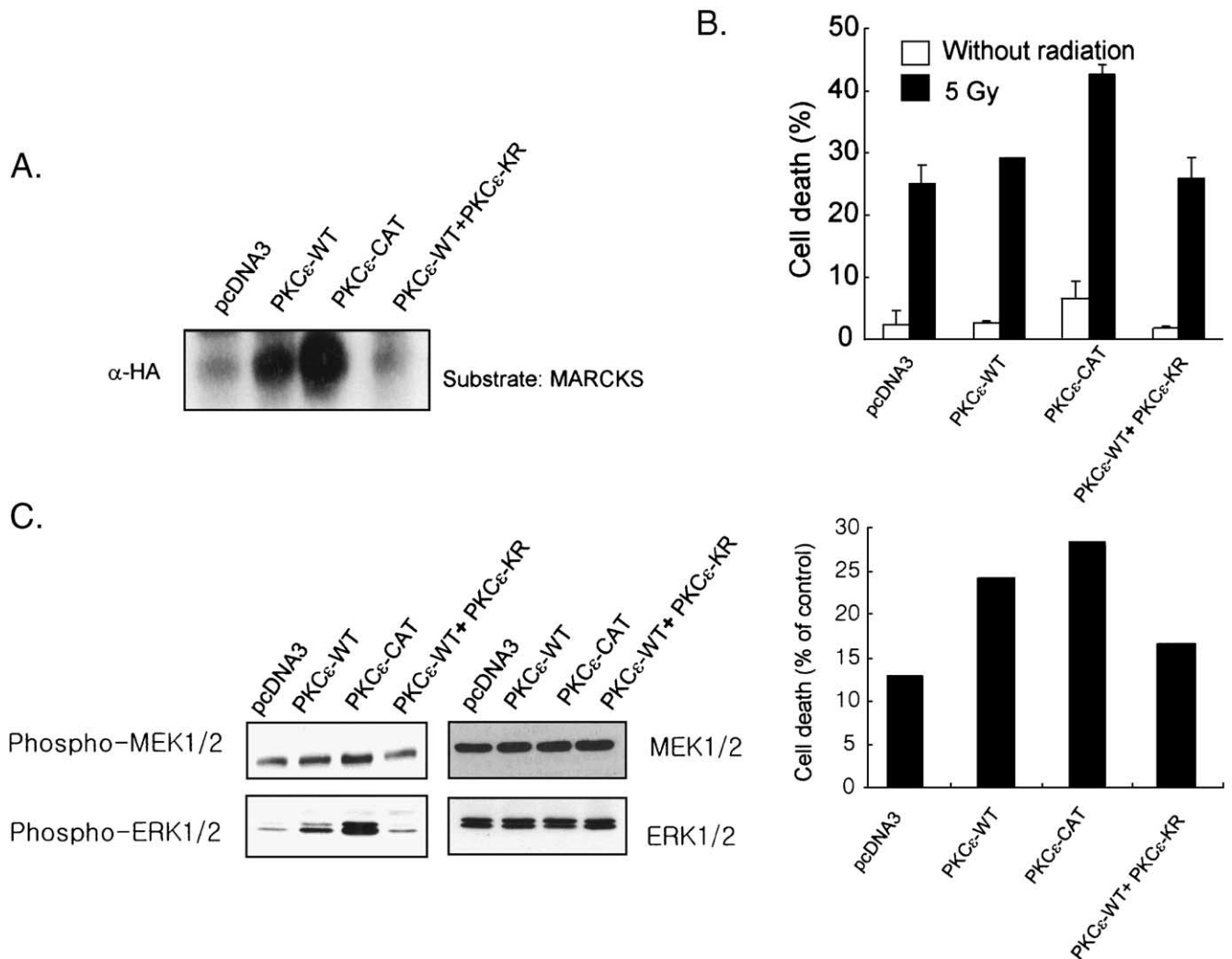

Fig. 5. PKCε overexpression increased radiation-induced cell death through ERK1/2 activation. (A) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, PKCε-CAT, or PKCε-KR) vectors and control vector pcDNA3, and cellular proteins were extracted by cell lysis in PKC extraction buffer. HA-tagged PKC proteins were immunoprecipitated from 300 μg of cell extracts by using 3 μg of an anti-HA antibody and 30 μl of protein G-Sepharose at 4 °C, after a 3-h incubation. Immune complex kinase reactions were performed at 30 °C for 30 min in the presence of 10 μg of the GST-MARCKS substrate and 5 μCi of [γ-^{32}P]-ATP. The reaction products were then analyzed by SDS-PAGE and autoradiography. The apparent molecular mass of the recombinant GST-MARCS protein was about 50 kDa. (B) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, PKCε-CAT, or PKCε-KR) vectors and control vector pcDNA3, and DNA fragmentation was measured by Hoechst 33258 staining 48 h after 5 Gy radiation, as described in Materials and methods. Error bar indicates mean ± S.D. from three independent experiments (upper) or assessed by PI staining as well as Annexin V–FITC double staining. The results represent one of three independent experiments (lower). (C) After 5-Gy radiation, protein extracts (60 μg) of growing vector control and various PKCε sequences (PKCε-WT, PKCε-CAT, or PKCε-KR) overexpressing cells were prepared, separated by SDS-PAGE, and analyzed by Western blot.

also upregulated by PKCε-WT overexpression, suggesting that ERK1/2 might be a major signal target of overexpressed PKCε.

3.4. Transfection of dominant negative PKCε (PKCε-KR) inhibited PKCε-mediated ERK1/2 activation and radiation-induced cell death

Whether PKCε-mediated ERK1/2 activation was associated with the radiation-induced cell death, PKCε-KR whose constructs contained full-length open reading frame with a K → R point mutation in the ATP binding site and PKCε-CAT whose constructs contained only the catalytic domains with the inhibitory N-terminal domains deleted (Fig. 1) were transfected to NIH3T3 cells (Fig. 5A). As shown in Fig. 5B, PKCε-CAT transfection showed more increased induction of cell death than that of PKCε-WT transfection, when detected by morphology and flow cytometry using Annexin V staining. In addition, when dominant negative PKCε, PKCε-KR, was co-transfected with PKCε-WT, induction ratio of cell death decreased to the control level (Fig. 5B), suggesting that PKCε was responsible for ERK1/2 activation as well as for the radiation-induced cell death. In addition, ERK1/2 activation was increased when PKCε-CAT was transfected, whereas co-transfection with PKCε-KR disappeared these phenomena (Fig. 5C).

3.5. Treatment of PD98059, MEK inhibitor, reduced radiation-induced cell death in the PKCε overexpressed cells

Whether PKCε-mediated ERK1/2 activation was responsible for the radiation-induced cell death, radiation-induced cell death in the PKCε-WT and PKCε-CAT overexpressed cells was examined using PD98059, blocker of ERK1/2 phosphorylation. As shown in Fig. 6A and B, ERK1/2 phosphorylation and increased induction of radiation-induced cell death by PKCε-WT and PKCε-CAT overexpression were inhibited by pretreatment with PD98059 for 30 min, suggesting that PKCε-mediated ERK1/2 activation was the major factor in PKCε-mediated radiation-induced cell death.

3.6. Cotransfection of dominant negative ERK1/2 in the PKCε overexpressed cells blocked radiation-induced cell death

To elucidate the relationship between the level of ERK1/2 and PKCε-mediated cell death, interfering mutants of the MAP kinase ERK1 (ERK1-KR) or ERK2 (ERK2-KR), in which a lysine residue in the ATP binding site was mutated to arginine [31], were coexpressed in PKCε-WT or PKCε-CAT (Fig. 7A), and kinase activity of respective ERK1 or ERK2 was found to be also inhibited (data not shown). Moreover, radiation-induced cell death was repressed by
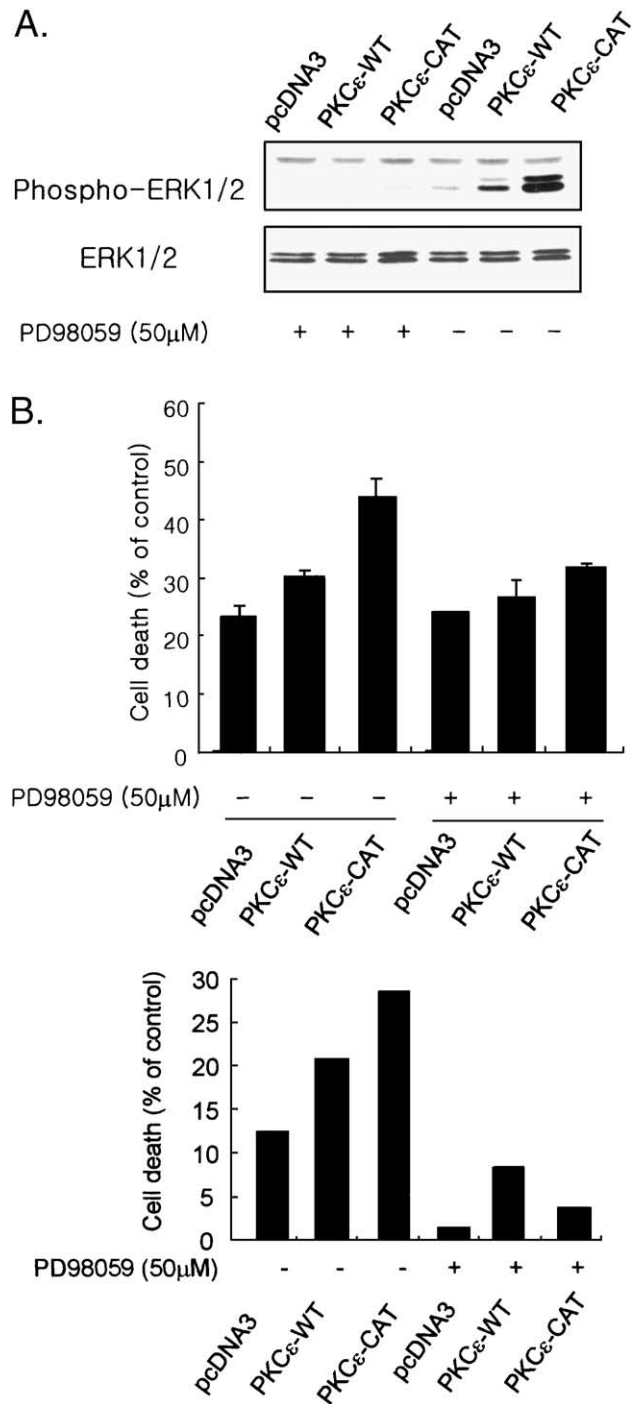

Fig. 6. PD98059 pretreatment inhibited PKCε-mediated radiation-induced cell death and ERK1/2 activation. (A) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, or PKCε-CAT) vectors and control vector pcDNA3 with or without pretreatment with 50 μM PD98059, and proteins were prepared, separated by SDS-PAGE, and analyzed by Western blot. (B) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, PKCε-CAT, or PKCε-KR) vectors and control vector pcDNA3, and DNA fragmentations were measured by Hoechst 33258 staining 48 h after 5-Gy radiation with or without pretreatment with 50 μM PD98059, as described in Materials and methods. Error bar indicates mean ± S.D. from three independent experiments (upper) or assessed by PI staining as well as Annexin V–FITC double staining. The results represent one of three independent experiments (lower).
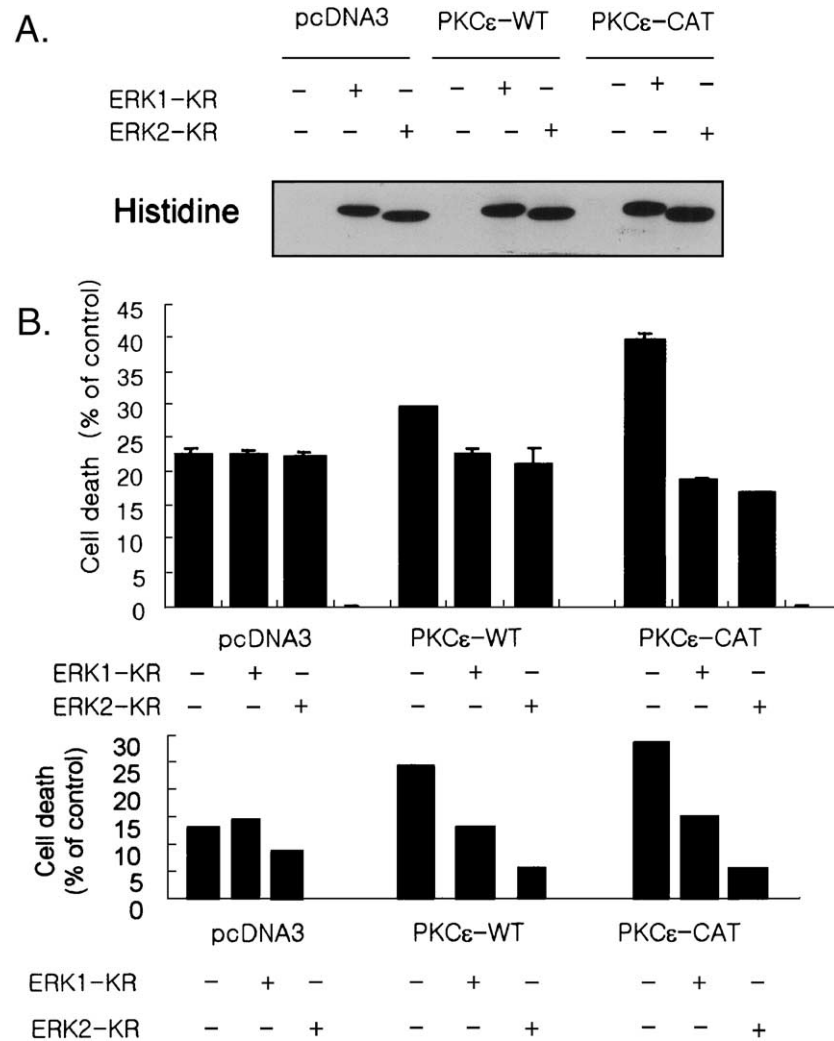
Fig. 7. ERK1/2 dominant-negative mutant blocked PKCε-mediated radiation-induced cell death. (A) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, or PKCε-CAT) vectors and control vector pcDNA3 with or without pCMV-ERK1-KR or pCMV-ERK2-KR plasmid expressing dominant negative kinases, and proteins were prepared, separated by SDS-PAGE, and analyzed by Western blot with anti-histidine antibody. (B) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, or PKCε-CAT) vectors with or without pCMV-ERK1-KR or pCMV-ERK2-KR plasmid and control vector pcDNA3, and DNA fragmentations were measured by Hoechst 33258 staining 48 h after 5-Gy radiation, as described in Materials and methods. Error bar indicates mean ± S.D. from three independent experiments (upper) or assessed by PI staining as well as Annexin V–FITC double staining. The results represent one of three independent experiments (lower).

both ERK1-KR and ERK2-KR co-transfection (Fig. 7B), suggesting that ERK1/2 pathway was essential for PKCε-mediated radiation-induced cell death.

3.7. PKCε-mediated ERK1/2 activation was Ras- and Raf-dependent

To elucidate upstream molecules of PKCε, Ras- or Raf-dominant negative mutants were used. When co-transfected PKCε-WT with Raf-dominant and negative mutants (Raf-KR), PKCε-mediated radiation-induced cell death and ERK1/2 phosphorylation were abolished (Fig. 8A). In addition, when RasN17, Ras-dominant negative mutant, was co-transfected to the PKCε-WT overexpressed cells, PKCε-mediated radiation-induced cell death and ERK1/2 and MEK1/2 phosphorylations were also abolished (Fig.

8B), thus demonstrating that PKCε-mediated ERK1/2 activation and radiation-induced cell death were dependent on Ras and Raf pathways.

4. Discussion

As described earlier in Introduction, remarkable progress has recently been made in elucidating the details of the signal transduction pathways. There are numerous indirect evidences to indicate that PKC plays a role in this pathway; however, it is not yet known with certainty which isoforms of PKC are involved. Indeed, studies on the specific cellular effects of individual isoforms have in general been hampered by several factors, including the facts that individual cells often express several isoforms of PKC, that the PKC

activator TPA can activate all of the PKC isoforms except PKCζ and PKCι, and that PKC isoform specific inhibitors are not yet available.

Recently, accumulating evidence indicates that activation of ERK requires selective activation of a specific PKC isoform, and both Ras-dependent and Ras-independent mechanism of activation have been described [1]. PKCδ activated ERK pathway by treatment of growth factors [31]. Similarly, PKCζ cooperates with PI-3 kinase to mediate Ras-independent ERK activation [32] and mediates platelet-derived growth factor-induced ERK activation by a Raf-1 and phospholipase C-dependent cascade [33]. In addition, PKCζ has been implicated in the activation of ERK and a dominant-negative mutant of PKCζ severely impairs activation of ERK kinase (MEK) [34].

The major finding in our present study was that when overexpressed in NIH3T3 cells, PKCε was the major component of a radiation-sensitive signal transduction pathway that led to the activation of ERK1/2. Furthermore, PKCε was highly specific for this pathway, since PKCα, δ, and ζ were not required for ERK1/2 activation (Fig. 4); PKCε-CAT activated ERK1/2 more than PKC-WT, while co-transfection of mutant PKCε, PKCε-KR, with PKCε-WT did less, suggesting that PKCε regulated ERK1/2 activation without altering activation of other MAPKs such as p38-MAPK and JNK. When the upstream molecule of ERK1/2, MEK1/2, was examined, a similar pattern was also observed.

Co-transfection of dominant negative construction of Ras or Raf, which are the upstream molecules of ERK1/2, revealed that PKCε-mediated ERK1/2 activation was Ras–Raf-dependent. Evidence indicates that PKCε can interact directly with Raf-1 to activate ERK1/2 [35], and that expression of active PKCε results in increased Raf-1 activity even in the context of dominant negative Ras [12], suggesting PKCε being able to directly activate Raf in vitro.

As discussed above, PKCε-mediated ERK1/2 activation was responsible for the radiation-induced cell death (Fig. 3); PKCε-CAT induced more cell death, while mutant PKCε, PKCε-KR, abolished this phenomena, indicating that PKCε was the major component of radiation-induced cell death. Moreover, treatment of the PKCε-WT and PKCε-CAT overexpressed cells with ERK1/2 inhibitor, PD98059, and co-transfection of ERK1 or ERK2 dominant negative mutants (ERK1-KR or ERK2-KR) revealed that ERK1/2 pathways were critical in radiation-induced cell death (Fig. 6). These observations were somewhat surprising, since ERK pathway is known to be critical in the control of cellular growth and cell survival responses to mitogenic signals in many different cell systems, i.e. signals including those received by tyrosine kinase, G protein-coupled and cytokine receptors [30]. Many studies support a general view that activation of the ERK pathway delivers a survival signal that counteracts proapoptotic effects associated with JNK and p38 activation [4]. However, requirement for ERK in mediating cisplatin-induced apoptosis of human cervical carcinoma HeLa cells and ovarian cell lines [2,3] has also been demonstrated.

Moreover, persistent activation of ERK1/2 contributes to glutamate-induced oxidative toxicity [5]. In agreement with the above observations, our data also suggested that PKCε-mediated ERK1/2 activation was a positive regulator of radiation-induced cell death.
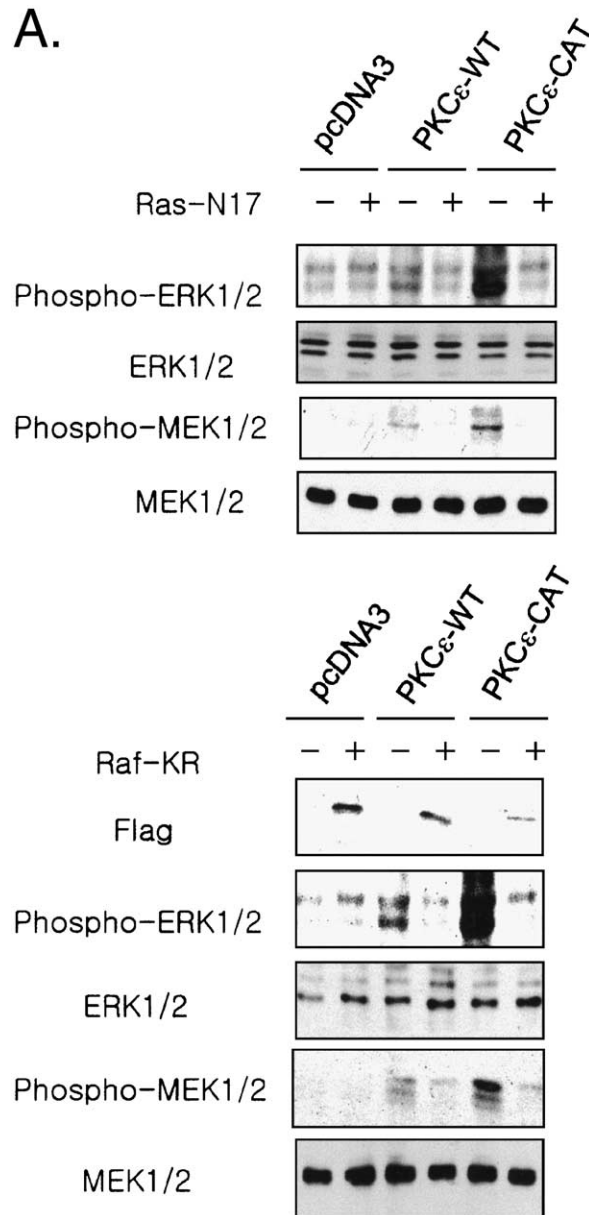

Fig. 8. PKCε-mediated radiation-induced cell death and ERK1/2 activation was Ras- and Raf-dependent. (A) NIH3T3 cells were transfected with PKCε sequences (PKCε-WT, or PKCε-CAT) vectors and control vector pcDNA3 with or without Ras-N17 or Raf-KR plasmid expressing dominant negative kinases, and proteins were prepared, separated by SDS-PAGE, and analyzed by Western blot. (B) NIH3T3 cells were transfected with PKCε sequence (PKCε-WT or PKCε-CAT) vectors and control vector pcDNA3 with or without Ras-N17 or Raf-KR plasmid expressing dominant negative kinases, and DNA fragmentations were measured by Hoechst 33258 staining 48 h after 5-Gy radiation, as described in Materials and methods. Error bar indicates mean ± S.D. from three independent experiments (left) or assessed by PI staining as well as Annexin V–FITC double staining. The results represent one of three independent experiments (right).
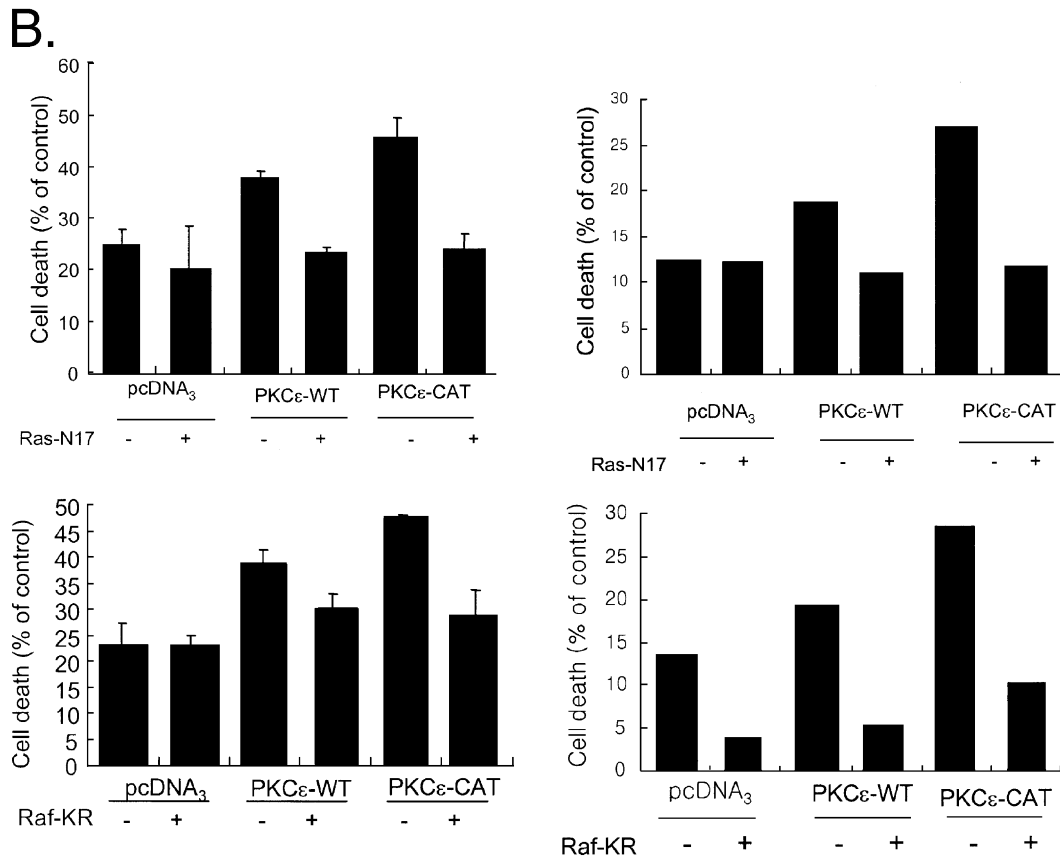
B.

Fig. 8 (*continued*).

In the present study, we generated mammalian expression vectors that could be used to transiently overexpress either wild-type, constitutively active mutant, or dominant negative mutants of PKC isoforms and these constructs were used for transient transfection assays to discern PKC isoforms specific in radiation-induced cell death. We found that PKCε was the major component of radiation-induced cell death when overexpressed to the same extent as other PKC isoforms. Compared with other isoforms, the amount of PKCε in NIH3T3 cells is, however, generally small. Although depending on the stimuli and cell types, overexpression of PKCε displays oncogenic transformation [36,37] and PKCε transgenic mice induce highly malignant/metastasis squamous cell carcinoma of skin cancer [38]. Therefore, our data suggest that cancer cells with activated or overexpressed PKCε are radiosensitive, and we are in the process of extending our study to other cell systems such as human tumor cell lines.

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