The EMBO Journal vol.14 no.24 pp.6148–6156, 1995

Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells

Y.Emoto, Y.Manome, G.Meinhardt, H.Kisaki, S.Kharbanda, M.Robertson[1], T.Ghayur[2], W.W.Wong[2], R.Kamen[2], R.Weichselbaum[3] and D.Kufe[4]

Divisions of Cancer Pharmacology and [1]Hematologic Malignancies, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, [2]BASF Bioresearch Corporation, Worcester, MA 01605 and [3]Department of Radiation and Cellular Oncology, University of Chicago, Chicago, IL 60637, USA

[4]Corresponding author

These studies demonstrate that treatment of human U-937 cells with ionizing radiation (IR) is associated with activation of a cytoplasmic myelin basic protein (MBP) kinase. Characterization of the kinase by gel filtration and in-gel kinase assays support activation of a 40 kDa protein. Substrate and inhibitor studies further support the induction of protein kinase C (PKC)-like activity. The results of N-terminal amino acid sequencing of the purified protein demonstrate identity of the kinase with an internal region of PKCδ. Immunoblot analysis was used to confirm proteolytic cleavage of intact 78 kDa PKCδ in control cells to the 40 kDa C-terminal fragment after IR exposure. The finding that both IR-induced proteolytic activation of PKCδ and endonucleolytic DNA fragmentation are blocked by Bcl-2 and Bcl-x_L supports an association with physiological cell death (PCD). Moreover, cleavage of PKCδ occurs adjacent to aspartic acid at a site (QDN) similar to that involved in proteolytic activation of interleukin-1β converting enzyme (ICE). The specific tetrapeptide ICE inhibitor (YVAD) blocked both proteolytic activation of PKCδ and internucleosomal DNA fragmentation in IR-treated cells. These findings demonstrate that PCD is associated with proteolytic activation of PKCδ by an ICE-like protease.

Keywords: physiological cell death/protein kinase C δ/ proteolytic cleavage

Introduction

The response of eukaryotic cells to ionizing radiation (IR) includes cell cycle arrest, activation of DNA repair and apoptosis or physiological cell death (PCD). The available evidence indicates that IR induces these effects by direct interaction with DNA or through the formation of reactive oxygen intermediates (ROIs) which damage DNA and cell membranes (Hall, 1988). IR predominantly induces the production of hydroxyl radicals and superoxides (Limoli and Ward, 1993). Studies in bacteria have demonstrated different adaptive responses to superoxides and peroxides (Demple and Amabile-Cuevas, 1991). The soxRS gene products induce transcription of genes involved in the response to superoxides, while the oxyR gene product functions in the activation of genes which constitute the response to peroxides (Demple and Amabile-Cuevas, 1991). The signals that control the response of mammalian cells to IR are less well studied than those activated by genotoxic stress in both bacteria and yeast. However, the finding that IR induces transcription of the jun/fos and EGR-1 gene families (Sherman *et al.*, 1990; Hallahan *et al.*, 1991; Datta *et al.*, 1992) supports the involvement of nuclear signaling cascades. IR also induces expression and DNA binding of nuclear factor κB (NF-κB) (Brach *et al.*, 1991). Moreover, levels of the tumor suppressor p53 increase during the arrest in G_1 phase that occurs following IR exposure (Kastan *et al.*, 1991). IR-induced activation of transcription factors conceivably represents the transduction of early nuclear signals to longer term changes in gene expression that reflect responses to this agent. In this regard, certain other genes induced by IR include tumor necrosis factor (TNF), platelet-derived growth factor, fibroblast growth factor and interleukin 1 (Hallahan *et al.*, 1989; Witte *et al.*, 1989; Woloschak *et al.*, 1990).

Few insights are available regarding the activation of protein kinases which may transduce IR-induced signals to the nucleus. Recent studies have demonstrated that IR treatment is associated with selective activation of the Src-like p56/p53^{lyn} tyrosine kinase (Kharbanda *et al.*, 1994a). Activated p56/p53^{lyn} binds to p34^{cdc2} in irradiated cells. Since p34^{cdc2} controls entry of cells into mitosis, binding of p56/p53^{lyn} to p34^{cdc2} may contribute to the regulation of IR-induced G_2 arrest. Other work has supported the involvement of serine/threonine protein kinases in the radiation response. c-*jun* and EGR-1 are activated by both IR treatment and mitogenic signals. Consequently, protein kinases involved in growth control and induction of early response genes, such as mitogen activated protein (MAP) kinase and pp90^{rsk}, have been studied in irradiated cells. The finding that stimulation of MAP kinase phosphorylation and activity by IR is temporally related to pp90^{rsk} activation has supported the induction of a MAP kinase kinase/MAP kinase/pp90^{rsk} signaling cascade (Kharbanda *et al.*, 1994b). A role for protein kinase C (PKC) in the radiation response has also been proposed as a result of the finding that non-specific PKC inhibitors and prolonged phorbol ester exposure block IR-induced expression of c-*jun* and EGR-1 (Hallahan *et al.*, 1991). Moreover, since treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces c-*jun* and EGR-1, as well as genes associated with genotoxic stress, PKC has been implicated in signaling pathways induced by DNA-damaging agents (Herrlich *et al.*, 1992). However, cells resistant to TPA-induced c-*jun* and EGR-1 expression respond to IR with activation of these genes (Datta *et al.*, 1992, 1993). These findings have suggested that PKC

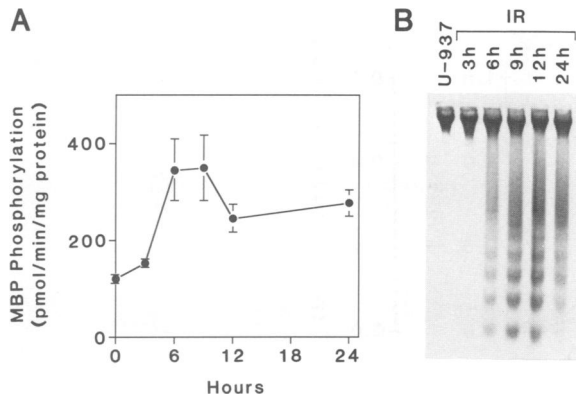

Fig. 1. IR induction of MBP kinase activity. U-937 cells were irradiated with 20 Gy and harvested at the indicated times. (**A**) Cytoplasmic extracts were applied to a Q-Sepharose column. Proteins eluting with 0.35 M NaCl were assayed for protein kinase activity using MBP as substrate. The results are expressed as the mean ± SE of three experiments. (**B**) DNA fragmentation was monitored by electrophoresis in 2% agarose gels.

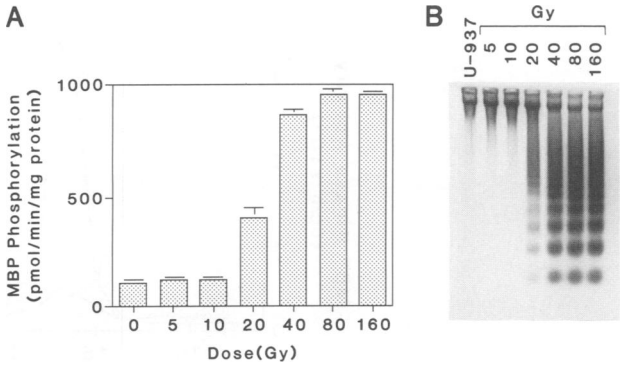

Fig. 2. IR dose-dependent effect on MBP kinase activity and internucleosomal DNA fragmentation. U-937 cells were exposed to the indicated IR doses and harvested at 6 h. (**A**) Cytoplasmic proteins were applied to Q-Sepharose, eluted with 0.35 M NaCl and assayed for phosphorylation of MBP. The results are expressed as the mean ± SE of two experiments. (**B**) DNA fragmentation was monitored by electrophoresis in 2% agarose gels.

isoforms not downregulated by phorbol esters may contribute to IR-induced signaling or that genotoxic stress stimulates PKC-independent pathways which activate early response genes.

The present studies have addressed the role of PKC in the cellular response to IR. The results demonstrate proteolytic cleavage of PKCδ to an activated form in irradiated cells. IR-induced activation of PKCδ is temporally associated with both induction of c-*jun* expression and PCD. The results also demonstrate that PKCδ activation is inhibited in cells which overexpress genes (bcl-2, bcl-x) involved in the control of PCD. The finding that PKCδ is cleaved at a site adjacent to aspartic acid further supports cleavage by a protease associated with apoptosis.

Results

Treatment of human U-937 myeloid leukemia cells with IR is associated with increased phosphorylation of myelin basic protein (MBP) (Kharbanda *et al.*, 1994b). In order to define further MBP kinase activities following IR exposure, U-937 cells were exposed to 20 Gy and then incubated for varying intervals. Soluble extracts were partially purified by Q-Sepharose chromatography. Proteins eluting at 0.35 M NaCl demonstrated an increase in MBP kinase activity at 6 and 9 h after irradiation (Figure 1A). Longer incubations were associated with partial declines in this activity (Figure 1A). Other studies have shown that treatment of U-937 cells with IR results in induction of internucleosomal DNA fragmentation (Manome *et al.*, 1993). The kinetics of endonucleolytic DNA cleavage were similar to induction of MBP kinase activity with the appearance of DNA fragments at multiples of ~200 bp at 6 and 9 h, while longer intervals after IR exposure were associated with a more diffuse pattern of degradation (Figure 1B). Induction of the MBP kinase activity and DNA fragmentation were also similar at different IR doses. While treatment with 10 Gy had little effect on MBP phosphorylation, maximal induction of the kinase activity occurred at 40 Gy and higher (Figure 2A). A similar pattern was observed for internucleosomal DNA fragmentation (Figure 2B).

In order to characterize the MBP kinase activity further, irradiated cells were harvested at 6 h and the fraction eluted from Q-Sepharose was applied to Mono Q anion-exchange beads. The column was eluted with a linear 0.1–0.6 M NaCl gradient and the fractions assayed for MBP phosphorylation (Figure 3A). Two peaks of MBP kinase activity were detectable in irradiated cells. The first peak eluted at 0.28 M NaCl, while the second peak corresponded to elution with 0.45 M NaCl. In contrast, similar preparations from control cells exhibited comparable levels of MBP phosphorylation in the second peak, but little activity eluting at 0.28 M NaCl (Figure 3A). The kinase activity of the Q-Sepharose fraction was also examined in gels containing MBP as substrate (Figure 3B). While several radioactive bands were apparent when using extracts from control and irradiated cells, a kinase of ~40 kDa was detectable only after IR exposure (Figure 3B). Mono Q column fractions 16 and 17 from irradiated cells (Figure 3A) were pooled, concentrated and subjected to Superose 12 gel filtration. Under these conditions, MBP kinase activity was detectable as a single peak that corresponded to an apparent M_r of 40 kDa (Figure 3C). Taken together, these findings supported the activation of a 40 kDa MBP kinase in irradiated cells.

Several other proteins and synthetic peptides were assayed for their ability to act as substrates for the 40 kDa kinase activity. Using reciprocal plots, K_m values were calculated from coefficients of the linear regression fits. The K_m for MBP was 2.3 μM (Table I). A synthetic peptide derived from amino acids 4–14 of MBP also served as a substrate with a K_m of 7.0 μM (Table I). Since MBP(4–14) has been shown to be phosphorylated on serine by PKC (Kishimoto *et al.*, 1985; Yasuda *et al.*, 1990), we assayed other known substrates of the PKC family. Replacement of alanine25 with serine in the pseudosubstrate region (amino acids 19–31) of PKC has been identified as a PKC phosphorylation site (House and Kemp, 1987). This peptide, [Ser25]PKC(19–31), also served as a phosphate acceptor for the IR-induced protein kinase (Table I). Phosphorylation of the 80 kDa MARCKS protein, another PKC substrate (Graff *et al.*, 1991) was similarly detectable with the purified kinase from radiation-

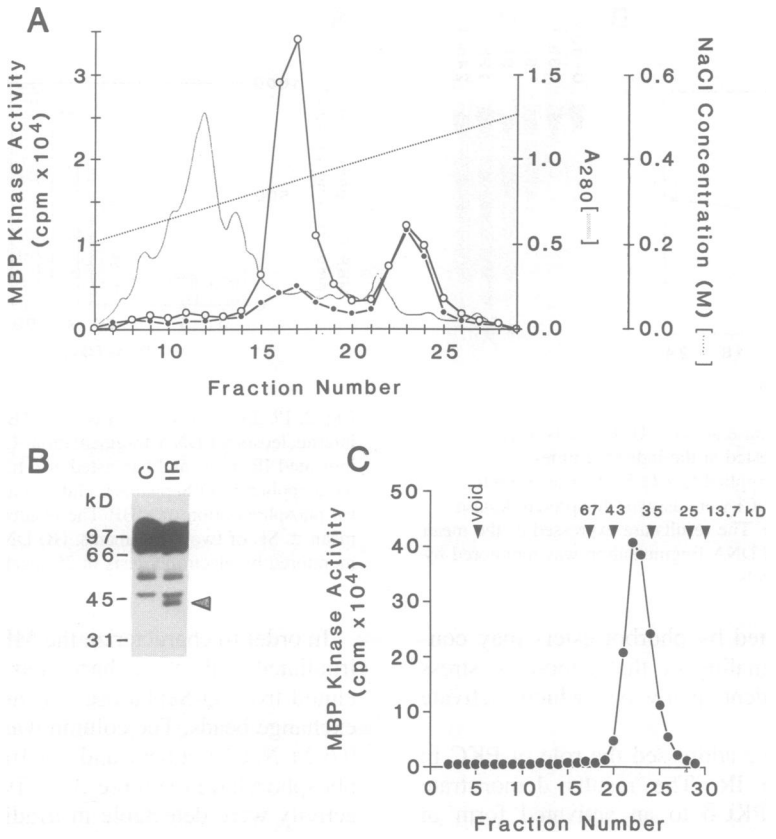

Fig. 3. Separation of IR-induced MBP kinase activity. (**A**) Cytoplasmic extracts from control (●) and IR-treated cells harvested at 6 h (○) were separated on Q-Sepharose columns. Proteins eluting at 0.35 M NaCl were then applied to Mono Q columns. The columns were washed with 0.1 M NaCl and proteins eluted with a linear 0.1–0.6 M NaCl gradient. Fractions were monitored for MBP phosphorylation and absorbance at 280 nm. (**B**) Proteins eluting at 0.35 M NaCl from the Q-Sepharose column were separated in SDS–polyacrylamide gels polymerized with MBP. The gels were subjected to renaturation and *in vitro* phosphorylation. (**C**) Proteins eluting at 0.35 M NaCl from the Mono Q column (fractions 16 and 17) were concentrated and separated by Superose 12 gel filtration. Fractions were assayed for MBP phosphorylation.

treated cells (Table I). Moreover, a synthetic peptide derived from the C terminus (amino acids 231–239, RRRLSSLRA) of the ribosomal S6 protein that includes a PKC phosphorylation site (House and Kemp, 1987) served as a substrate for the radiation-induced activity (Table I). In contrast, peptides derived from the X (amino acids 70–77, LASPELER) and Y (amino acids 56–69, NSDLLTSPDVGLLK) regions of the c-Jun protein that serve as substrates for MAP kinase (Pulverer *et al.*, 1991), JNK (Hibi *et al.*, 1993) and SAPK (Kyriakis *et al.*, 1994) were not phosphorylated by the IR-induced activity (Table I). These findings indicated that the IR-induced activity has a substrate specificity similar to that of PKC isozymes.

Previous work has demonstrated that the regulatory domain of PKC (amino acids 19–36) contains a pseudo-substrate region and that the corresponding synthetic peptide acts as a substrate antagonist (House and Kemp, 1987). Using MBP(4–14) as a substrate, PKC(19–36) inhibited the activity of the IR-induced protein kinase (Table II). This activity was also blocked by the indole carbazole staurosporine, an inhibitor of PKC and other serine/threonine protein kinases (Tamaoki *et al.*, 1986; Vegesna *et al.*, 1988) (Table II). The isoquinolinesulfonamide derivatives, H7 and H8 (Hidaka *et al.*, 1984), and bisindolylmaleimide (Toullec *et al.*, 1991) similarly blocked the IR-induced kinase, while herbimycin A, an inhibitor of tyrosine kinases (Uehara *et al.*, 1986), and PKI(6–22) amide, an inhibitor of protein kinase A, had no detectable effect (Table II). These results further supported the isolation of a PKC activity. The PKC isozymes have been grouped according to their dependence on Ca^{2+}, phospholipid and diacylglycerol or phorbol ester (Nishizuka, 1992). Consequently, we asked whether the IR-induced protein kinase is stimulated by the presence of Ca^{2+}. The addition of 1 mM Ca^{2+} had little effect, while 10 mM Ca^{2+} partially inhibited the activity (Table II). These results supported the isolation of a Ca^{2+}-independent PKC activity.

The IR-induced PKC-like kinase was also purified by pooling active Mono Q fractions (Figure 3) and subjecting

Table I. Substrate specificity of the purified IR-induced protein kinase

Substrate	K_m (μM)
MBP	2.3
MBP(4–14)	7.0
MARCKS	0.15
[Ser^{25}]PKC(19–31)	1.6
S6 (231–239)	6.4
X-peptide: c-Jun (70–77)	ND
Y-peptide: c-Jun (56–69)	ND

ND, no phosphorylation was detectable.

the concentrated protein to PKC(19–36) pseudosubstrate affinity chromatography. Stepwise elution with NaCl revealed a single peak of kinase activity (data not shown). The active fractions were pooled and applied to a phenyl–Superose column. Elution with $(NH_4)_2SO_4$ (Figure 4A) and sizing by Superose 12 filtration revealed a single peak of activity at ~40 kDa (Figure 4B). Substrate and inhibition specificities of this activity were the same as those listed in Tables I and II. Analysis of the fractions by SDS–PAGE and silver staining demonstrated two 40–46 kDa proteins that corresponded to elution of the kinase activity (Figure 5A and B). Consequently, after transfer to a PVDF membrane we subjected these proteins to amino acid sequencing. The results demonstrated the same N-terminal sequence of NSGTYGKIxEGS. A search in Swissprot revealed identity with an internal region of PKCδ (amino acids 331–342, NSGTYGKIWEGS). The detection of 40–46 kDa PKCδ fragments was further supported by the reactivity of these proteins with an antibody prepared against a peptide derived from the C-terminal region of PKCδ (Figure 5C). The calculated molecular mass of PKCδ from amino acids 331–676 is 40 kDa. Taken together, these findings supported the detection of a proteolytic fragment of PKCδ.

Table II. Effects of protein kinase inhibitors on IR-induced activity

Phosphorylation inhibitor	Concentration	MBP(4–14) phosphorylation (% control)
PKC(19–36)	0.2 μM	57
	2.0 μM	14
	20.0 μM	2
Staurosporine	0.1 nM	75
	1.0 nM	57
	10.0 nM	5
H7	0.5 μM	55
	5.0 μM	21
	50.0 μM	3
H8	0.5 μM	44
	5.0 μM	11
	50.0 μM	2
Bisindolylmaleimide	1.0 nM	86
	10.0 nM	53
	100.0 nM	4
Herbimycin A	0.1 μM	91
	1.0 μM	102
	10.0 μM	81
PKI(6–22) amide	1.0 μM	112
	10.0 μM	94
Ca^{2+}	1.0 mM	78
	10.0 mM	3

In order to confirm proteolytic cleavage of PKCδ in irradiated cells, Q-Sepharose eluates from control and irradiated cells were subjected to immunoblot analysis with anti-PKCδ. In control cells, PKCδ was detectable as a 78 kDa band, while this reactivity shifted to a 40 kDa fragment following IR exposure (Figure 6). Incubation of anti-PKCδ with the peptide used to prepare the antibody completely blocked reactivity with the 78 and 40 kDa proteins (data not shown). PKC consists of at least 10 subspecies which can be categorized with respect to Ca^{2+} and phospholipid dependence (Nishizuka, 1992). To determine whether cleavage of PKCδ following IR exposure also occurs in multiple members of the PKC family, we subjected the same Q-Sepharose eluate to immunoblot analysis with other anti-PKC antibodies. In contrast to the findings for PKCδ, there was no detectable effect of IR treatment on PKCα or PKCβ (Figure 6). Similar patterns of PKCε and PKCζ expression were also obtained before and after irradiation (Figure 6). These results supported selective cleavage of PKCδ.

The relationship between proteolytic activation of PKCδ and PCD was further explored in cells which overexpress products that inhibit this process. For example, Bcl-2 blocks PCD in most cells deprived of growth factors (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Nunez *et al.*, 1990) or exposed to radiation (Sentman *et al.*, 1991; Strasser *et al.*, 1991). U-937 cells transfected with bcl-2 (U-937/bcl-2) are resistant to IR-induced PCD, while transfectants with control vector (U-937/neo) exhibit internucleosomal DNA fragmentation following irradiation (Manome *et al.*, 1993). IR treatment of U-937/neo was associated with activation of PKCδ, while there was no detectable stimulation of PKCδ activity in irradiated U-937/bcl-2 cells (Figure 7A). Similar findings were obtained with U-937 cells transfected with a bcl-2-related gene, designated bcl-x, which functions as a bcl-2-independent regulator of PCD (Boise *et al.*, 1993) (Figure 7A). The U-937/bcl-2 and U-937/bcl-x transfectants were resistant to IR-induced internucleosomal DNA fragmentation (Figure 7B). Moreover, while proteolytic cleavage

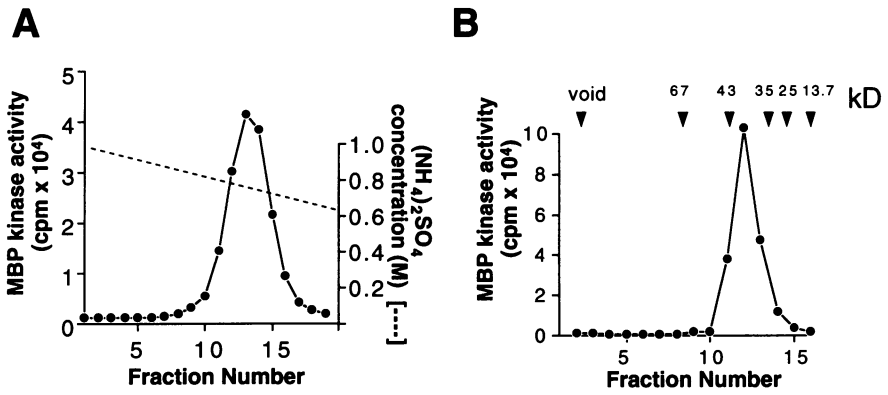

Fig. 4. Partial purification of IR-induced MBP kinase activity. Peak activity fractions obtained by Mono Q chromatography were applied to a PKC pseudosubstrate affinity column. Proteins were collected by stepwise elution. (**A**) Proteins eluted at 0.4 M NaCl from the PKC pseudosubstrate were applied to a phenyl–Superose column. The column was washed with 1.2 M ammonium sulfate and proteins eluted with a linear 1.2–0 M ammonium sulfate gradient. (**B**) Peak fractions (nos 13 and 14) from the phenyl–Superose column were pooled and subjected to Superose 12 gel filtration.

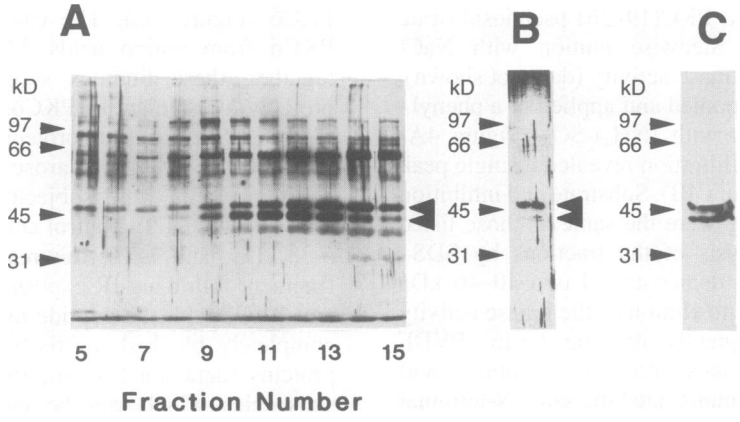

Fig. 5. SDS–PAGE and immunoblot analysis of the partially purified MBP kinase activity. (**A**) The indicated fractions obtained from phenyl–Superose chromatography (Figure 4A) were subjected to SDS–PAGE and silver staining. The proteins were transferred to a PVDF membrane and the p40 and p45 proteins were analyzed by amino acid sequencing. (**B**) The peak fraction (No. 12) from Superose 12 gel filtration (Figure 4B) was analyzed by SDS–PAGE and silver staining. (**C**) Immunoblot analysis was performed with the anti-PKCδ antibody.

of PKCδ was detectable in irradiated U-937/neo cells, overexpression of Bcl-2 or Bcl-x blocked appearance of the 40 kDa PKCδ fragment (Figure 7C).

Treatment of U-937 cells with TNF or anti-Fas antibody is associated with induction of internucleosomal DNA fragmentation (Trauth *et al.*, 1989; Yonehara *et al.*, 1989; Elias and Berry, 1991; Obeid *et al.*, 1993). Consequently, we asked whether TNF or anti-Fas treatment is also associated with proteolytic activation of PKCδ. Analysis of extracts from TNF- or anti-Fas-treated U-937 cells demonstrated induction of an MBP kinase activity which by further characterization was identical to the findings in irradiated cells (data not shown). Similar results were obtained with TNF- or anti-Fas-treated U-937/neo cells, while TNF or anti-Fas had no detectable effect on this activity in the U-937/bcl-2 or U-937/bcl-x transfectants (Figure 8A). These findings corresponded with the effects of TNF and anti-Fas on induction of internucleosomal DNA fragmentation in these cells (Figure 8B). TNF or anti-Fas treatment was also associated with proteolytic cleavage of PKCδ in U-937 and U-937/neo, but not in U-937/bcl-2 and U-937/bcl-x cells (Figure 8C).

Studies have demonstrated that proteases of the interleukin-1β converting enzyme (ICE) family are involved in the induction of PCD (Miura *et al.*, 1993; Yuan *et al.*, 1993; Gagliardini *et al.*, 1994). These proteases and granzyme B are unique in their ability to cleave adjacent to aspartic acid. The finding that proteolytic cleavage of PKCδ occurs between aspartic acid and asparagine at a site similar to one cleaved in proICE (Howard *et al.*, 1991) raised the possible involvement of ICE or an ICE-like protease. To address this issue, we examined the effects of a specific ICE inhibitor YVAD-chloromethyl ketone (Thornberry *et al.*, 1992) on IR-induced proteolytic activation of PKCδ and PCD. The results demonstrate that YVAD inhibits both activation (Figure 9A) and proteolytic cleavage (Figure 9B) of PKCδ in IR-treated cells. YVAD also blocked the effects of IR on internucleosomal DNA fragmentation (Figure 9C). In contrast, the calpain inhibitor leupeptin had no detectable effect on PKCδ cleavage or PCD (Figure 9A–C). These findings suggested that PKCδ cleavage involves ICE or an ICE-like protease. In order to determine the potential involvement of

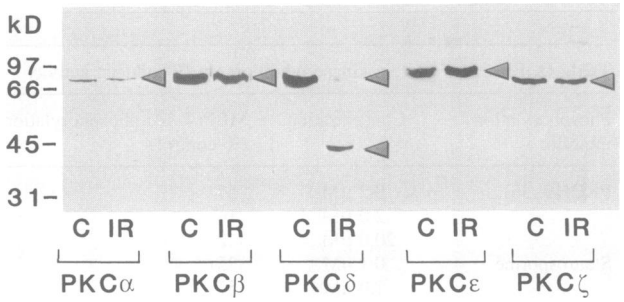

Fig. 6. Immunoblot analysis of PKC isoforms. Cytoplasmic extracts from control (C) and irradiated (IR) cells were subjected to Q-Sepharose chromatography and SDS–PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblotting with antibodies against the indicated PKC isoforms.

ICE, pro-IL-1β and PKCδ were labeled with [^{35}S]-methionine in a rabbit reticulocyte lysate system and then incubated with recombinant human ICE. While complete cleavage of pro-IL-1β was observed, there was no detectable cleavage of PKCδ to the 40 kDa fragment (Figure 9D). These results suggest that an ICE-like protease is involved in the proteolytic activation of PKCδ.

Discussion

Eukaryotic cells respond to IR with cell cycle arrest and activation of DNA repair mechanisms. The associated DNA damage can result in mutagenesis, transformation and, under certain circumstances, PCD. The cellular response to IR also includes transcriptional activation of early response genes. Temporal co-induction of c-*jun* expression and internucleosomal DNA fragmentation has been shown in irradiated cells (Manome *et al.*, 1993). The sensitivity of both IR-induced events to *N*-acetyl-L-cysteine (NAC), an antioxidant, supported the involvement of ROIs. However, the finding that H_2O_2 also induces c-*jun* expression by a NAC-sensitive mechanism, but has no effect on PCD, indicated that activation of c-*jun* and endonucleolytic DNA cleavage are regulated by different mechanisms. The present studies demonstrate that the response of cells to IR treatment includes activation of PKCδ. While this effect is temporally associated with

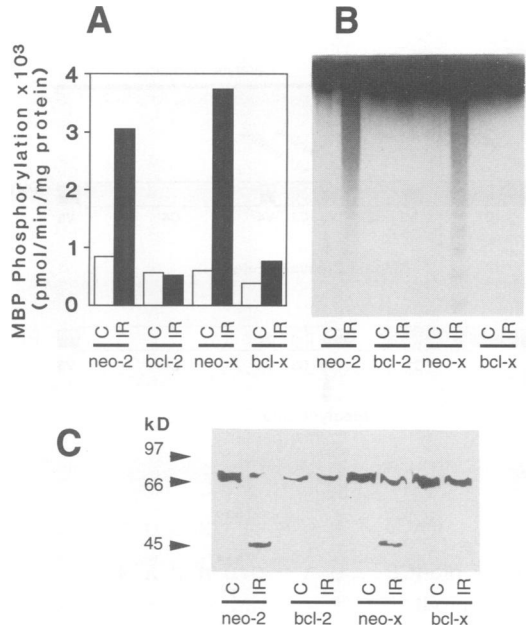

Fig. 7. Effects of Bcl-2 and Bcl-x_L on the proteolytic activation of PKCδ. U-937 cells overexpressing Bcl-2 or Bcl-x_L and corresponding neomycin-resistant clones (Neo-2, Neo-x) were treated with IR and harvested at 6 h. (**A**) Cytoplasmic extracts were partially purified by Q-Sepharose and Mono Q chromatography. Mono Q column fractions were assayed for MBP phosphorylation. The results represent MBP kinase activity for the peak fraction. (**B**) DNA fragmentation monitored by electrophoresis in 2% agarose gels. (**C**) Immunoblot analysis of Q-Sepharose eluates was performed with the anti-PKCδ antibody.

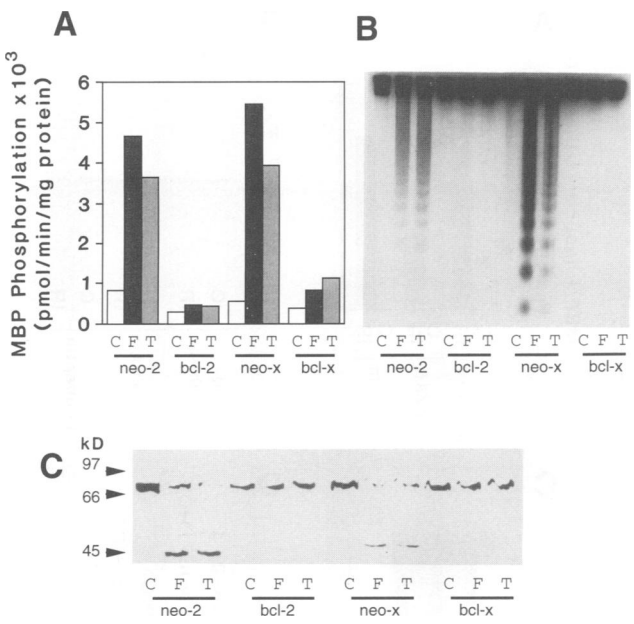

Fig. 8. Proteolytic activation of PKCδ by TNF and anti-Fas. U-937 cell clones (Bcl-2, Bcl-x, Neo-2 and Neo-x) were treated with 100 U TNF (T) or 5 μg/ml anti-Fas mAb 7C11 (F) for 6 h. (**A**) Cytoplasmic extracts were partially purified by Q-Sepharose and Mono Q chromatography. The results represent MBP kinase activity for the peak Mono Q column fraction. (**B**) DNA fragmentation was determined by electrophoresis in 2% agarose gels. (**C**) Immunoblot analysis of the Q-Sepharose eluates was performed with the anti-PKCδ antibody.

induction of c-*jun* and internucleosomal DNA fragmentation, the following results support involvement in PCD and not early response gene expression: (i) on exposure to IR, cells which overexpress anti-apoptosis genes respond with induction of c-*jun*, but not activation of PKCδ or PCD; (ii) treatment of cells with H_2O_2 is associated with c-*jun* expression, but no detectable effect on PKCδ or PCD; (iii) treatment of cells with an anti-Fas antibody activates PKCδ and PCD, but has no effect on induction of the c-*jun* gene. Other findings that lend support to the involvement of PKCδ activation in PCD include similar IR dose responses and the demonstration that TNF activates PKCδ in association with internucleosomal DNA fragmentation.

PKC consists of a number of subspecies, including α, β_I, β_{II}, γ, δ, ε, ζ and η (Nishizuka, 1992). While PKCα, β and γ (Group A) require Ca^{2+} for activity, the other isoforms (Groups B and C) are active in the absence of this cation. The Group A PKCs are cleaved by Ca^{2+}-dependent neutral proteases I and II (calpains I and II) to catalytically active fragments of 45–49 kDa (Kishimoto, 1990). Calpain I and II are sensitive to leupeptin and cleave at one or two specific sites in the third variable region of the Group A PKCs (Figure 10A). The present finding that PKCα and PKCβ remain intact suggests that the calpains are not activated in cells undergoing PCD. While less is known about proteolytic cleavage of the Group B and C isoforms, the present results demonstrate IR-induced cleavage of PKCδ to an active fragment. Analysis of the N-terminal sequence of the catalytic fragment demonstrated cleavage in the V3 region (Figure 10A). This cleavage site predicts the formation of a catalytic domain of 40 kDa and thus corresponds physically to the IR-induced kinase activity as determined by in-gel assays and by gel filtration. In addition to amino acid sequencing, confirmation of identity with PKCδ was obtained by immunoblotting with an anti-PKCδ antibody prepared against a C-terminal peptide. Cleavage of the 78 kDa form to a 40 kDa active PKCδ fragment was apparent in cell extracts and in highly purified preparations. The absence of detectable proteolytic cleavage of other Group B isoforms (PKCε and PKCζ) further supported the selectivity of IR-induced PKCδ cleavage. Studies with purified PKCδ have demonstrated 78 and 76 kDa species (Ogita *et al.*, 1992). Treatment of the 78 kDa protein with protein phosphatase 2A has provided evidence for phosphorylation of the 76 kDa form (Ogita *et al.*, 1992). In the present studies, the detection of two proteolytic PKCδ fragments with identical N-terminal sequences also supports some post-translational modification. Other studies have demonstrated that PKCδ undergoes tyrosine phosphorylation of the regulatory domain during activation of the full-length form (Li *et al.*, 1994). With regard to potential functions of PKCδ, CHO lines that overexpress this isoform, but not PKCα, PKCβ or the PKCζ subspecies, accumulate in G_2/M phase in response to TPA (Watanabe *et al.*, 1992). Similar findings have been obtained in NIH 3T3 cells which overexpress PKCδ (Mischak *et al.*, 1993). Neither of these studies, however, identified a proteolytic fragment of PKCδ or an association with PCD.

Treatment of human tumor cells with IR and cytotoxic drugs is associated with PCD (Kaufmann, 1989; Gunji *et al.*, 1991; Rubin *et al.*, 1991; Sentman *et al.*, 1991;

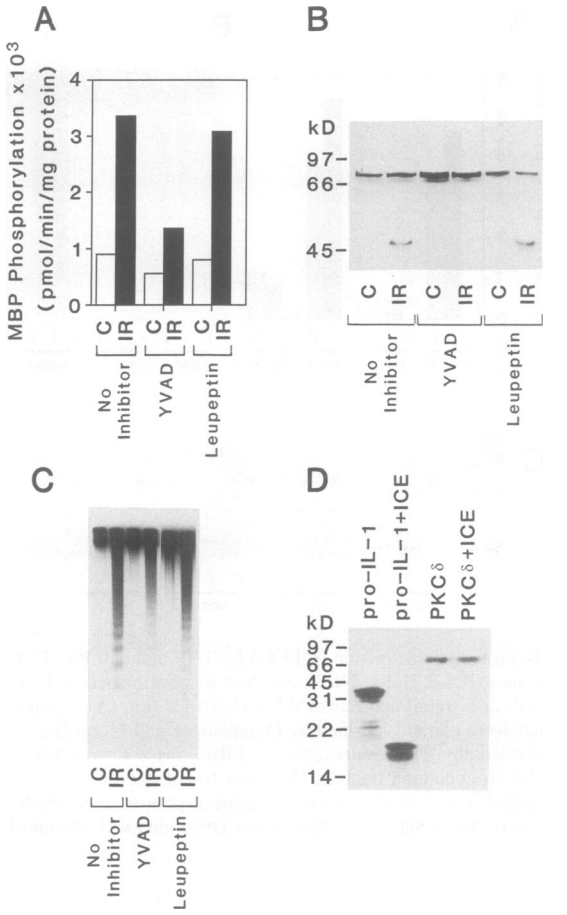

Fig. 9. U-937 cells were pre-incubated with 10 μM YVAD-chloromethylketone or 200 μM leupeptin for 30 min. The cells were then treated with IR and harvested at 6 h. (**A**) Cytoplasmic extracts were partially purified by Q-Sepharose and Mono Q chromatography. The results represent MBP kinase activity for the peak Mono Q column fraction. (**B**) Immmunoblot analysis of the Q-Sepharose eluates was performed with the anti-PKCδ antibody. (**C**) DNA fragmentation was monitored by electrophoresis in 2% agarose cells. (**D**) Pro-IL-1β and PKCδ were labeled with [^{35}S]methionine and incubated with recombinant human ICE. The reaction products were analyzed by SDS–PAGE and autoradiography.

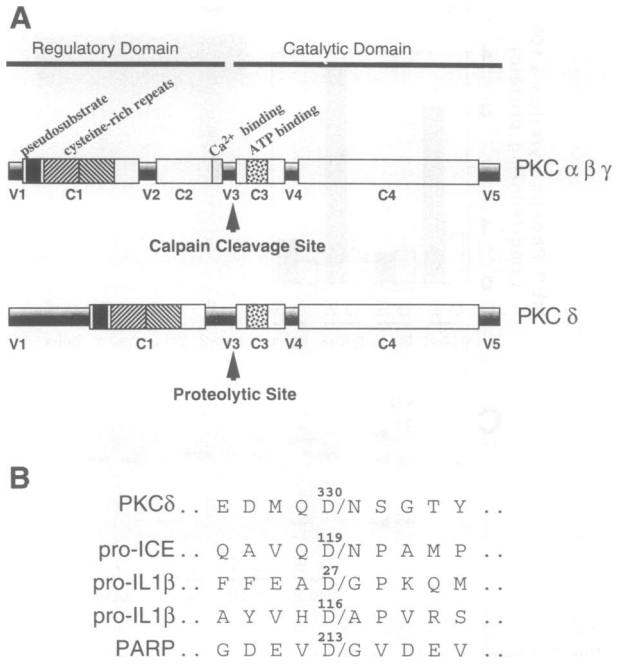

Fig. 10. Proteolytic cleavage sites in PKCδ and other proteins. (**A**) Schematic representation of PKC isoforms that undergo proteolytic cleavage. PKCα, β and γ are cleaved in the V3 region by calpains I and II. (**B**) Comparison of amino acid sequences at cleavage sites adjacent to aspartic acid.

Miyashita and Reed, 1992). The sensitivity of cells to undergo PCD is regulated in part by the bcl-2 gene (Sentman *et al.*, 1991; Miyashita and Reed, 1992). Bcl-2 blocks PCD in certain cells deprived of growth factors *in vitro* and during development *in vivo* (Hockenbery *et al.*, 1990; Sentman *et al.*, 1991; Strasser *et al.*, 1991; Garcia *et al.*, 1992; Siegel *et al.*, 1992; Allsopp *et al.*, 1993). The recent identification of a bcl-2-related gene, designated bcl-x, which functions as a bcl-2-independent regulator of PCD, has supported the existence of multiple regulatory mechanisms (Boise *et al.*, 1993; Gottschalk *et al.*, 1994). As a result of alternate mRNA splicing, expression of Bcl-x_L (long) inhibits PCD during growth factor withdrawal, while Bcl-x_s (short) inhibits the anti-apoptotic effects of Bcl-2 (Boise *et al.*, 1993). Both Bcl-2 and Bcl-x_L inhibit IR-induced PCD (Sentman *et al.*, 1991; Strasser *et al.*, 1991; Datta *et al.*, 1995). The present results indicate that Bcl-2 and Bcl-x_L also inhibit activation of PKCδ. Transfectants which overexpress bcl-2 or bcl-x fail to respond to IR with proteolytic cleavage of PKCδ to the 40 kDa active fragment. Similar findings were obtained in TNF-treated and anti-Fas-treated cells. While these results suggest that Bcl-2 and Bcl-x_L may function as inhibitors of proteolysis, the available evidence indicates that Bcl-2 regulates antioxidant pathways that block free radical formation (Hockenbery *et al.*, 1993). Thus, as one explanation for the present findings, Bcl-2 may inhibit the production of ROIs in IR- and TNF-treated cells, and thereby the activation of proteases. Sphingomyelin hydrolysis and ceramide production have been identified in TNF-treated cells (Hannun, 1994; Kolesnick and Golde, 1994). This pathway is also activated in cells exposed to IR and certain cytotoxic drugs, such as ara-C (Haimovitz-Friedman *et al.*, 1994; Strum *et al.*, 1994). Since ceramide has been shown to be a mediator of PCD (Obeid *et al.*, 1993), sphingomyelin hydrolysis appears to contribute to the induction of apoptosis by diverse agents. The finding that our bcl-2 and bcl-x transfectants are also resistant to ceramide supports a block in downstream signals which induce cleavage of PKCδ and internucleosomal DNA fragmentation (data not shown).

Genetic studies in *Caenorhabditis elegans* have demonstrated that mutations in the ced-3 gene block PCD during hermaphrodite development (Yuan and Horvitz, 1990). The CED-3 protein is similar to human ICE, a cysteine protease which cleaves the precursor of IL-1β to the active cytokine (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Yuan *et al.*, 1993). These findings have suggested that CED-3 may be a cysteine protease and that ICE may play a role in PCD. Other studies have demonstrated that overexpression of ICE in transient transfection assays induces cells to undergo PCD (Miura *et al.*, 1993). ICE is one of only two eukaryotic proteases that are known to cleave next to aspartic acid. The other is the serine protease granzyme B which induces cytotoxic lymphocyte-induced

PCD (Odake *et al.*, 1991; Shi *et al.*, 1992; Heusel *et al.*, 1994). Ich-1 is the third member of the ICE/CED-3 family which induces PCD, but has not as yet been shown to cleave at aspartic acid (Wang *et al.*, 1994). Proteolytic cleavage of PKCδ during PCD is also adjacent to aspartic acid, at the site QDN, which is similar to that cleaved in proICE (Figure 10B). Other work has shown that poly(ADP-ribose) polymerase (PARP) is cleaved during PCD at a peptide sequence similar to one of two ICE sites in human pro-IL-1β (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994) (Figure 10B). These results have suggested that a protease resembling ICE (prICE) is responsible for PARP cleavage (Lazebnik *et al.*, 1994). The site cleaved in PKCδ differs from those cleaved by ICE and prICE (Figure 10B). Furthermore, while ICE has no detectable activity against PKCδ *in vitro*, the specific ICE inhibitor YVAD blocked both proteolytic cleavage of PKCδ and PCD in irradiated cells. Taken together, these findings suggest that PKCδ is proteolytically activated by an ICE-like enzyme and that this event is sensitive to overexpression of genes (bcl-2, bcl-x) involved in the control of PCD.

Materials and methods

Cell culture and irradiation

Human U-937 myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Irradiation was performed with a gamma-ray source (cesium 137, Gamma Cell 1000, Atomic Energy of Canada, Ltd, Ontario) at a fixed dose rate of 13 Gy/min. Cells were also treated with TNF (BASF Bioresearch Corp., Worcester, MA) or anti-Fas mAb (Robertson *et al.*, 1995).

Protein kinase assays

Protein kinase activity was assayed in 50 μl reactions containing 20 mM Tris–HCl (pH 7.4), 0.25 mg/ml (Sigma), 20 μM [γ-^{32}P]ATP, 20 mM $MgCl_2$ and 0.4 mM dithiothreitol (DTT). After incubation for 5 min at 30°C, 25 μl of the reaction mixture were loaded onto phosphocellulose discs (Gibco-BRL, Grand Island, NY). The discs were washed three times with 1% phosphoric acid and then with water before scintillation counting.

Protein kinase purification

Cells (1×10^{10}) were washed three times with phosphate-buffered saline (PBS) and suspended in 120 ml of hypotonic solution (10 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 μg/ml aprotinin and 10 μg/ml leupeptin). After incubation on ice for 30 min to allow swelling, the cells were disrupted in a Dounce homogenizer (20–30 strokes). The homogenate was centrifuged at 190 000 *g* for 60 min and the resulting supernatant used as the cytoplasmic fraction. The cytoplasmic fraction was applied to a Q-Sepharose Fast Flow column (20 ml; Pharmacia, Piscataway, NJ). After washing with 0.1 M NaCl/buffer A [20 mM Tris–HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol], protein was eluted with 100 ml of 0.35 M NaCl/buffer A, adjusted to 0.1 M NaCl with buffer A and applied to a Mono Q column (HR 10/10; Pharmacia). The column was washed with 0.1 M NaCl/buffer A. Protein was eluted with a linear 0.1–0.6 M NaCl/buffer A gradient. Fractions (2.0 ml) were assayed for protein kinase activity using MBP as substrate. Appropriate regions were pooled and concentrated with Ultrafree-MC 10 000 MW filter units (Millipore, Bedford, MA). The protein concentrate was then separated on a Superose 12 column (HR 10/30; Pharmacia) with 0.1 M NaCl/buffer A or applied to a column containing Sepharose beads coupled to the pseudosubstrate peptide, PKC(19–36). The PKC(19–36) pseudosubstrate affinity column was washed with 0.2 M NaCl/buffer A and protein was eluted with 0.4 M NaCl/buffer A. The eluate was adjusted to 1.2 M $(NH_4)_2SO_4$/buffer A and applied to a phenyl Superose column (HR 5×5; Pharmacia). After washing with 1.2 M $(NH_4)_2SO_4$/buffer A, the column was eluted with a linear 1.2–0 M $(NH_4)_2SO_4$/buffer A gradient. Active fractions were pooled, concentrated and then separated on a Superose 12 column with 0.1 M NaCl/buffer A.

Analysis of DNA fragmentation

Cells (1×10^6) were washed with PBS and incubated in 20 μl of 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% SDS and 0.5 μg/ml proteinase K (Sigma) at 50°C for 30 min. Ten microliters of 0.5 μg/ml RNase A were added and incubated for an additional 1 h. The digested samples were incubated with 10 μl of 10 mM EDTA (pH 8.0) containing 2% (w/v) low-melting-point agarose, 0.25% bromophenol blue and 40% sucrose at 70°C. The DNA was separated in gels containing 2% agarose/TAE [40 mM Tris–acetate and 1.0 mM EDTA (pH 8.0)] buffer at 23 V for 16 h. The DNA was visualized by UV illumination after ethidium bromide staining

In-gel assays of protein kinase activity

Q-Sepharose eluates were subjected to electrophoresis in a SDS–4% polyacrylamide stacking gel and a SDS–10% polyacrylamide separation gel. MBP or bovine serum albumin (BSA) (0.2 mg/ml gel) was added to the separation gel just prior to polymerization. Following electrophoresis, SDS was removed from the gels by washing with 20% 2-propanol in 50 mM Tris–HCl (pH 7.5) for 1 h and then 50 mM Tris–HCl (pH 7.5) containing 5 mM β-mercaptoethanol (buffer B) for 1 h. Proteins were denatured with 6 M guanidine HCl (100 ml ×2) for 1 h and renatured with buffer B containing 0.04% Tween 20 (250 ml ×5) at 4°C for 16 h. The gel was subsequently incubated in 20 mM Tris–HCl (pH 7.5) containing 0.1 mM EGTA, 0.1 mM EDTA, 10 mM $MgCl_2$ and 0.4 mM DTT (buffer C) for 30 min at 30°C, and then with buffer C containing 50 μM ATP and 30 μCi [γ-^{32}P]ATP for 1 h. After the reaction, the gels were washed with 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate to remove labeled ATP. The gels were dried and analyzed by autoradiography.

Analysis of substrate specificity and inhibitors

The myristoylated alanine-rich C kinase substrate (MARCKS) was purified from rat brain as described previously (Knopf *et al.*, 1986). Peptides were synthesized by the stepwise solid-phase method using an automated synthesizer (Applied Biosystems). Further purification was achieved by reverse-phase HPLC in 0.1% trifluoroacetic acid–water–acetonitrile. As determined by the HPLC profile, the purity of the peptides was >95%. The amino acid composition of the purified peptide was confirmed by automated amino acid analyzer (Applied Biosystems). PKC(19–36), staurosporine, herbimycin A and PKI(6–22) amide were purchased from Gibco-BRL (Gaithersburg, MD). H-7 and H-8 were purchased from Seikagaku America, Inc. (St Petersburg, FL). Phosphorylation reactions (50 μl) containing 20 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 0.4 mM DTT, 5 μl of purified protein kinase and various concentrations of each substrate were performed at 30°C. The K_m was determined in the linear range at 5 min. Transfer of [^{32}P]phosphate was quantitated by the phosphocellulose paper binding method.

Immunoblot analysis

Proteins were subjected to electrophoresis in an SDS–4% polyacrylamide stacking gel and an SDS–10% polyacrylamide separation gel, and then transferred to nitrocellulose membranes (Costar, Cambridge, MA). The transfer buffer contained 25 mM Tris–HCl, 192 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol. The membranes were blocked with 5% dried milk, 0.1% Tween 20 and PBS. After washing three times with PBS/Tween, the membranes were incubated with anti-PKCβ (kindly provided by Dr Hiroyoshi Hidaka, Department of Pharmacology, Nagoya University School of Medicine, Nagoya, Japan), anti-PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PKCε or anti-PKCζ (Gibco-BRL). After washing three times with PBS/Tween, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (Gibco-BRL) for anti-PKCβ or anti-rabbit IgG (Amersham) for anti-PKCα, δ, ε and ζ.

Protease assays

An expression vector was generated by cloning the human full-length PKCδ (provided by Dr James D.Chang, Beth Israel Hospital, Boston) into the *Bam*HI and *Eco*RI sites of pcDNA3 (Invitrogen). PKCδ and recombinant human pro-IL-1β were labeled with [^{35}S]methionine by rabbit reticulocyte lysate translation (Promega, Madison, WI). Refolded recombinant ICE was prepared as described previously (Walker *et al.*, 1994). Labeled PKCδ or pro-IL-1β were incubated with 600 U/μl ICE in a buffer containing 50 mM HEPES (pH 7.5), 10% glycerol, 2.5 mM

DTT and 0.25 mM EDTA. After incubation at 37°C for 30 min, the reaction products were analyzed by SDS–PAGE and autoradiography.

Acknowledgements

This investigation was supported by PHS grant CA55241 awarded by the National Cancer Institute, DHHS.

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Received on July 24, 1995; revised on September 29, 1995