

Tyrosine phosphorylation is a mandatory proximal step in radiation-induced activation of the protein kinase C signaling pathway in human B-lymphocyte precursors

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Communicated by George J. Todaro, September 14, 1992 (received for review April 12, 1992)

ABSTRACT Ionizing radiation triggers a signal in human B-lymphocyte precursors that is intimately linked to an active protein-tyrosine kinase regulatory pathway. We show that in B-lymphocyte precursors, irradiation with γ -rays leads to (i) stimulation of phosphatidylinositol turnover; (ii) downstream activation by covalent modification of multiple serine-specific protein kinases, including protein kinase C; and (iii) activation of nuclear factor κ B. All of the radiation-induced signals were effectively prevented by the protein-tyrosine kinase inhibitors genistein and herbimycin A. Thus, tyrosine phosphorylation is an important and perhaps mandatory proximal step in the activation of the protein kinase C signaling cascade in human B-lymphocyte precursors. Our report expands current knowledge of the radiation-induced signaling cascade by clarifying the chronological sequence of biochemical events that follow irradiation.

Weichselbaum and colleagues (1, 2) have proposed that irradiation initiates a cascade of cytoplasmic signaling events in mammalian cells. In particular, ionizing radiation stimulates the transcription of immediate early response genes that encode transcriptional factors, by activation of a protein kinase C (PKC)-dependent cytoplasmic signaling pathway (1, 2). However, radiation-induced signaling events proximal to PKC activation require further evaluation.

Protein-tyrosine kinases (PTKs) are key participants in the initiation of signal cascades that affect proliferation and survival of human B-lymphocyte precursors (3, 4). We have recently shown that ionizing radiation stimulates several PTKs in human B-lymphocyte precursors, including the Src family tyrosine kinases p59^{lyn} and p55^{blk}, leading to enhanced tyrosine phosphorylation of multiple substrates and triggering apoptosis (5). Since PTKs play myriad roles in the regulation of cell function and proliferation (6, 7), the activation of a PTK cascade and tyrosine phosphorylation may explain the pleiotropic effects of ionizing radiation on cellular functions.

The present study expands earlier work by specifically examining the role of tyrosine phosphorylation in radiation-induced activation of the PKC pathway in human B-lymphocyte precursors at discrete developmental stages of B-cell ontogeny. Notably, the tyrosine kinase inhibitors herbimycin and genistein effectively inhibited radiation-induced activation of PKC (PK76) and PKC-dependent serine kinases PK55 and PK50. Furthermore, the activation of nuclear factor κ B (NF- κ B) in irradiated B-lymphocyte precursors was abrogated by pretreatment with herbimycin or genistein. Our results indicate that radiation-induced activation of the PKC

signaling cascade leads to downstream activation of multiple serine-specific protein kinases, and activation of NF- κ B. The evidence provided reveals that these events are triggered by radiation-induced stimulation of tyrosine-specific protein kinases.

MATERIALS AND METHODS

Patient Material and Cell Lines. We used the CD19⁺CD10⁺C μ ⁻sIgM⁻ pre-pre-B cell line REH, the CD19⁺CD10⁺C μ ⁺sIgM⁻ pre-B cell line NALM-6, and the CD19⁺CD10⁻C μ ⁻sIgM⁺ early B/Burkitt lymphoma cell lines Daudi and Ramos. The immunophenotypic and genotypic features and the radiation sensitivity of these human B-lymphocyte precursor cell lines were detailed in a previous report (8). All of these cell lines undergo apoptosis within 24 hr after exposure to 2–4 Gy of γ -rays (5, 8). We also used primary bone marrow blasts from a pre-pre-B acute lymphoblastic leukemia patient (UPN 10), following the guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research for secondary use of pathologic or surgical tissue.

Irradiation of Cells and the Use of Kinase Inhibitors. Cells (5×10^5 per ml in plastic tissue culture flasks) were irradiated during a logarithmic growth phase and under aerobic conditions with a ¹³⁷Cs irradiator (J. L. Shephard and Assoc., Glendale, CA, model mark I), as previously described (9). For the inositol 1,4,5-trisphosphate (Ins-1,4,5- P_3) assays and the serine kinase renaturation assays, cells were irradiated with 100–400 cGy at a dose rate of 99 cGy/min. In analyses of radiation-induced activation of NF- κ B, cells were irradiated with 1000–4000 cGy at a dose rate of 326 cGy/min. Prior to irradiation, cells were incubated for 1 hr at 37°C with (a) phosphate-buffered saline, (b) the PTK inhibitor genistein (100 μ g/ml = 370 μ M; ICN), or (c) the PKC inhibitor 1-(5-isoquinolylsulfonyl)-2-methylpiperazine (H7, 11 μ g/ml = 30 μ M; GIBCO/BRL), or (d) for 24 hr at 37°C with the benzoquinoid ansamycin antibiotic herbimycin A (7 μ g/ml = 12 μ M; Sigma), which is a potent PTK inhibitor, according to published protocols (3, 4). At the applied concentrations, these compounds effectively inhibit their target enzymes (3, 4). Genistein competes with the ATP binding site of PTKs and inhibits their activation. In contrast, herbimycin is not a classic competitive inhibitor of PTKs but rather targets PTKs for degradation and leads to actual loss of these proteins. In general, herbimycin is a more potent tyrosine kinase inhibitor in B-lineage lymphoid cells. Whereas genistein usually attenuates tyrosine kinase-linked signals, herbimycin abolishes them. In some experiments,

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Abbreviations: Ins-1,4,5- P_3 , inositol 1,4,5-trisphosphate; NF- κ B, nuclear factor κ B; PAP, pokeweed antiviral protein; PKC, protein kinase C; PLC, phospholipase C; PtdIns, phosphatidylinositol; PTK, protein-tyrosine kinase.

cells were pretreated with B43 (anti-CD19)–pokeweed antiviral protein (PAP) immunotoxin (10) or mafosfamid prior to radiation exposure. Cells were treated with B43–PAP immunotoxin for 16 hr at 37°C. Under these conditions, B43–PAP kills >99.9% of human B-cell precursors by an irreversible inhibition of ribosomal protein synthesis (10). Mafosfamid (ASTA Werke, Bielefeld, Germany) is an *in vitro*-active congener of the oxazaphosphorine cyclophosphamide. Cells were incubated with mafosfamid (50 µg/ml) for 30 min at 37°C as described (10). Under these conditions, mafosfamid kills >99.9% of human B-cell precursors by DNA alkylation (10).

Measurement of Ins-1,4,5- P_3 Levels. After irradiation, Ins-1,4,5- P_3 levels in human B-lymphocyte precursors were measured with a highly specific D-*myo*-[^3H]Ins-1,4,5- P_3 assay system (Amersham), as described (3, 4). This assay is based on the competition between unlabeled Ins-1,4,5- P_3 and a fixed quantity of a high-specific-activity [^3H]Ins-1,4,5- P_3 tracer for a limited number of binding sites on a specific and sensitive bovine adrenal binding-protein preparation.

Renaturation Assay for Analysis of Protein-Serine Kinase Activation. We used the kinase renaturation method of Ferrell and Martin (11) to examine the effects of ionizing radiation on the activities of renaturable serine/threonine-specific protein kinases in human B-lymphocyte precursor cells, as previously described (4). In some experiments, kinase renaturation assays were performed with immunoprecipitated PKC as follows. Irradiated cells were lysed in 1 ml of immunoprecipitation buffer [150 mM NaCl/50 mM Tris Cl, pH 7.5/1% (vol/vol) Nonidet P-40/1 mM Na₃VO₄/1 mM sodium molybdate] containing phenylmethanesulfonyl fluoride (1 mM), leupeptin (10 µg/ml), and aprotinin (10 µg/ml). Nuclei were removed by centrifugation and the supernatants were used for immunoprecipitation of PKC with a rabbit polyclonal anti-pan PKC antibody (Upstate Biotechnology, Lake Placid, NY), which was generated by using a synthetic peptide corresponding to C-terminal sequences of rabbit protein kinase type βII as immunogen. Cell lysates were incubated with anti-PKC (final concentration, 5 µg/ml) for 1.5 hr at 0°C. Protein A-Sepharose (Sigma) was added to the lysates and incubated for 1 hr at 4°C to recover the immune complexes. The immunoprecipitates were washed six times in immunoprecipitation buffer and then boiled for 5 min in 2× SDS sample buffer. Samples were run in SDS/polyacrylamide gels and subsequently immunoblotted with anti-PKC (1 µg/ml). ^{125}I -labeled protein A was used to detect PKC. Kinase renaturation assays on the anti-PKC immunoprecipitates were performed as described above for whole cell lysates.

Nuclear Extraction and Electrophoretic Mobility-Shift Assays. After irradiation of cells, nuclear proteins were extracted according to published procedures (12, 13). Gel shift assays were performed as described (13, 14). Aliquots (50 ng) of a double-stranded oligonucleotide containing a tandem repeat of the 11-base-pair consensus sequence for the NF- κB DNA binding site (GGGGACTTCC), which was obtained in kit form from GIBCO/BRL, were end-labeled by using [γ - ^{32}P]ATP and T4 polynucleotide kinase according to the recommendations of the manufacturer. One nanogram of the radiolabeled oligonucleotide (400,000 cpm) was incubated with 10 µg of nuclear protein for 20 min at room temperature in 12 µl of 25 mM Tris Cl, pH 7.6/5 mM MgCl₂/0.5 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol containing 1 µg of poly(dI-dC) (Boehringer Mannheim). Competition studies were performed by preincubating the nuclear protein for 15 min on ice with a 500-fold excess of unlabeled NF- κB oligonucleotide prior to the addition of the ^{32}P -end-labeled NF- κB probe. Controls used 500-fold excess of unlabeled AP-1 and NF-1 oligonucleotide probes for competition. DNA–protein complexes in the reaction mixture were ana-

lyzed by polyacrylamide gel electrophoresis using a 4.5% running gel under nondenaturing conditions in 0.25× TBE buffer (25 mM Tris base/22.5 mM boric acid/0.25 mM EDTA, pH 8.2). The gels were pre-run at 150 V for 2 hr at 4°C before the samples were loaded and electrophoresed for an additional 3 hr at 150 V. Gels were dried overnight and exposed at –70°C to Kodak XAR-5 x-ray film with intensifying screens.

RESULTS AND DISCUSSION

Role of Tyrosine Phosphorylation in Radiation-Induced Stimulation of Phosphatidylinositol (PtdIns) Turnover in Human B-Lymphocyte Precursors. Previous studies by Weichselbaum and colleagues (1, 2) provided elegant, albeit circumstantial, evidence that ionizing radiation triggers PKC activation. PKC is an enzyme that plays a pivotal role in the transduction of growth-regulatory signals. Its activation under physiologic conditions is mediated by stimulation of PtdIns-specific phospholipase C- γ (PLC- γ) (15). Ionizing radiation causes radiolysis of bound and solvent water in the cell, producing hydroxyl radicals, superoxide radicals, and hydrogen peroxide (16, 17). Oxidants such as hydrogen peroxide and superoxide can induce activation of PLC- γ , leading to Ca²⁺ mobilization and PKC activation (18, 19). The catalytic activity of PLC- γ is regulated through tyrosine phosphorylation by receptor- and non-receptor-type PTKs (4, 20, 21), and biochemical signals that trigger tyrosine-specific protein phosphorylation have been shown to precede the activation of PLC- γ and stimulation of PtdIns turnover in many experimental systems (3, 4, 20, 21). These findings prompted us to examine the effects of ionizing radiation on inositolphospholipid turnover in human lymphocyte precursors. For this, we used a highly specific and quantitative D-*myo*-[^3H]Ins-1,4,5- P_3 assay system (3, 4). Ionizing radiation stimulated an increase in the production of Ins-1,4,5- P_3 by FL112 pro-B, REH pre-pre-B, NALM-6 pre-B, and Daudi early B cells in a dose-dependent fashion (data not shown). By comparison, treatment of these human B-lymphocyte precursor cell lines with B43 (anti-CD19)–PAP immunotoxin, which kills target cells by an irreversible inhibition of ribosomal protein synthesis (10), or with the *in vitro*-active cyclophosphamide congener mafosfamid, which kills target cells by DNA alkylation (10), did not stimulate PtdIns turnover. We next examined the kinetics of radiation-induced PtdIns turnover. Irradiation of REH pre-pre-B cells and Daudi early B cells with 200 cGy of γ -rays stimulated a rapid and monophasic increase in the production of Ins-1,4,5- P_3 leading to markedly elevated Ins-1,4,5- P_3 levels between 30 sec and 3 min after completion of radiation exposure (i.e., 2.5 min and 5 min after the start of radiation exposure at a dose rate of 99 cGy/min). Thereafter, the level of Ins-1,4,5- P_3 rapidly declined but at 5 min it was still higher than the baseline (Fig. 1). Taken together, these experiments provide conclusive evidence that the biochemical signal triggered in human B-lymphocyte precursors by ionizing radiation is intimately linked to signal-transduction pathways that stimulate inositolphospholipid turnover, producing Ins-1,4,5- P_3 as a second messenger. Notably, the PTK inhibitors genistein and herbimycin effectively prevented the radiation-triggered Ins-1,4,5- P_3 signals, whereas H7, a potent inhibitor of PKC and PKC-dependent serine/threonine kinases, did not. These observations provide strong evidence that tyrosine phosphorylation is a requisite step in the radiation-triggered stimulation of PtdIns turnover in human B-lymphocyte precursors.

Role of Tyrosine Phosphorylation in Radiation-Induced Activation of PKC and PKC-Dependent Renaturable Serine Kinases in Human B-Lymphocyte Precursors. Using the kinase renaturation method of Ferrell and Martin (11), we previously

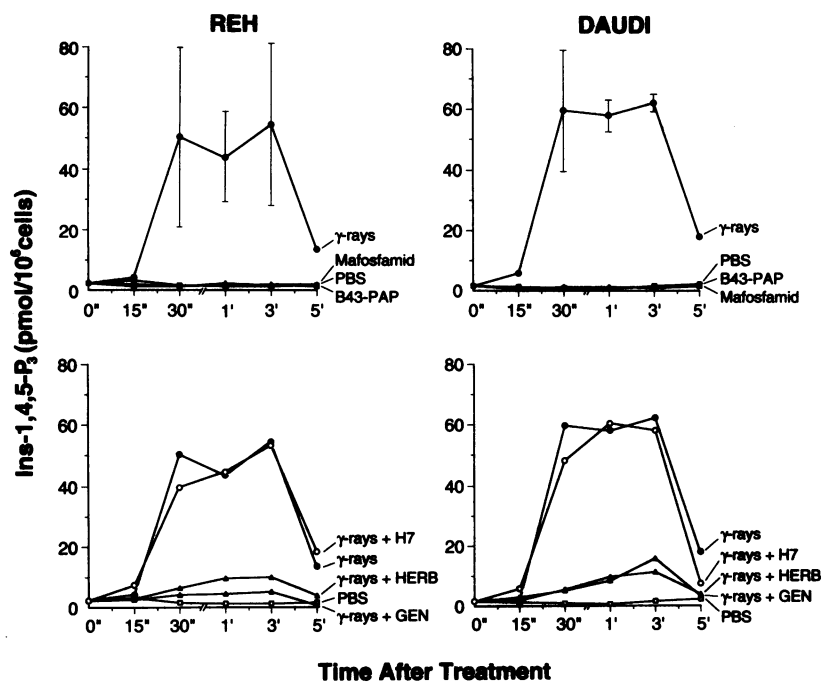


FIG. 1. Magnitude and kinetics of radiation-stimulated PtdIns turnover in human B-lymphocyte precursors. REH pre-pre-B and Daudi early B cells were irradiated in the presence or absence of the PTK inhibitors genistein (GEN) or herbimycin A (HERB) or the PKC inhibitor H7. Subsequently, the Ins-1,4,5- P_3 levels were determined at the indicated times [15 sec (15') to 5 min (5')] by using a highly specific D-myo-[3H]Ins-1,4,5- P_3 assay system (3, 4). The Ins-1,4,5- P_3 responses were analyzed in three independent experiments, each performed in quadruplicate. Results are expressed as the mean pmol amounts of Ins-1,4,5- P_3 per 10^6 cells. Control samples were treated with phosphate-buffered saline (PBS), mafosfamid, or B43-PAP immunotoxin.

found that the activation of the PLC/PKC cascade in human B-cell precursors by phorbol ester plus ionomycin or antibodies directed against sIgM, major histocompatibility complex class II, and CD40 antigens enhanced the activity of multiple serine/threonine kinases, as measured by the renaturable kinase activity toward the blocking protein substrate albumin (4). Activation of the PLC/PKC cascade in irradiated B-lymphocyte precursors prompted us to use this kinase renaturation method to examine the effects of ionizing radiation on the activities of serine-specific protein kinases in target cell populations. Irradiation of Ramos early B cells and REH pre-pre-B cells enhanced within 15 min the *in vitro* activity of three renaturable serine/threonine-specific protein kinases of apparent molecular masses of 76 kDa (PK76), 55 kDa (PK55), and 50 kDa (PK50) (Fig. 2 A and D). Phospho amino acid analysis of the kinase bands by two-dimensional thin-layer electrophoresis demonstrated that these renaturable kinases phosphorylate only serine residues (Fig. 2B). In Daudi early B cells, ionizing radiation stimulated PK76 and PK55, while in UPN10 pre-pre-B cells, only PK76 showed enhanced activity after radiation exposure (Fig. 2D). We have previously shown that PK55 and PK50 are PKC-dependent serine kinases (4). Since the apparent molecular mass of the renaturable serine/threonine kinase PK76 was very similar to that of PKC (77 kDa), we postulated that PK76 may indeed be PKC. To test this hypothesis, we immunoprecipitated PKC from whole cell lysates of unirradiated and irradiated Ramos cells with an anti-PKC antibody and examined its activity by the kinase renaturation assay. Equal amounts of PKC were immunoprecipitated from unirradiated as well as irradiated cells, as determined by Western blot analysis of the PKC immunoprecipitates with the same anti-PKC antibody that was used for immunoprecipitation. PKC that was immunoprecipitated from the lysates of irradiated cells showed enhanced renaturable kinase activity (Fig. 2C). These results corroborate and extend the findings of Weichselbaum and colleagues (1, 2) and provide direct

evidence that ionizing radiation stimulates PKC. The increased renaturable kinase activity of PKC most likely results from covalent modification, since any stimulatory factors (such as diacylglycerol) would be separated from the PKC protein by denaturation in SDS, subsequent gel electrophoresis, and blotting. Notably, the PTK inhibitors genistein and herbimycin effectively prevented the radiation-induced stimulation of PK76, PK55, and PK50 (Fig. 2) at concentrations that inhibited radiation-induced tyrosine phosphorylation. Renaturation assays of the kinase activity in anti-PKC immunoprecipitates from unirradiated and irradiated Ramos cells showed that herbimycin could prevent the stimulation of PKC after ionizing radiation more effectively than the potent PKC inhibitor H7 (Fig. 2). Taken together, these findings provide evidence that tyrosine phosphorylation has a fundamental role in the generation of the biochemical signal in irradiated human B-lymphocyte precursors that leads to stimulation of multiple serine-specific protein kinases, including PKC.

Role of Tyrosine Phosphorylation in Radiation-Induced Activation of NF- κ B in Human B-Lymphocyte Precursors. NF- κ B is an inducible transcription factor that is involved in regulation of a series of target genes (22). Brach *et al.* (23) have shown that ionizing radiation induces expression and binding activity of NF- κ B in KG-1 myeloid leukemia cells by a PKC-dependent mechanism. Furthermore, Schreck *et al.* (24) reported that diverse agents that can activate NF- κ B act through a common mechanism involving the synthesis of reactive oxygen intermediates. Therefore, we utilized electrophoretic mobility-shift assays to examine the effects of ionizing radiation on NF- κ B in B-lymphocyte precursors. Notably, irradiation with 10–40 Gy of γ -rays stimulated a NF- κ B-specific DNA-binding activity, as reflected by a marked increase in intensity of a shifted band that was observed when the NF- κ B probe was incubated with nuclear extracts from Ramos cells (Fig. 3). This retarded band was eliminated by competition with a 500-fold molar excess of

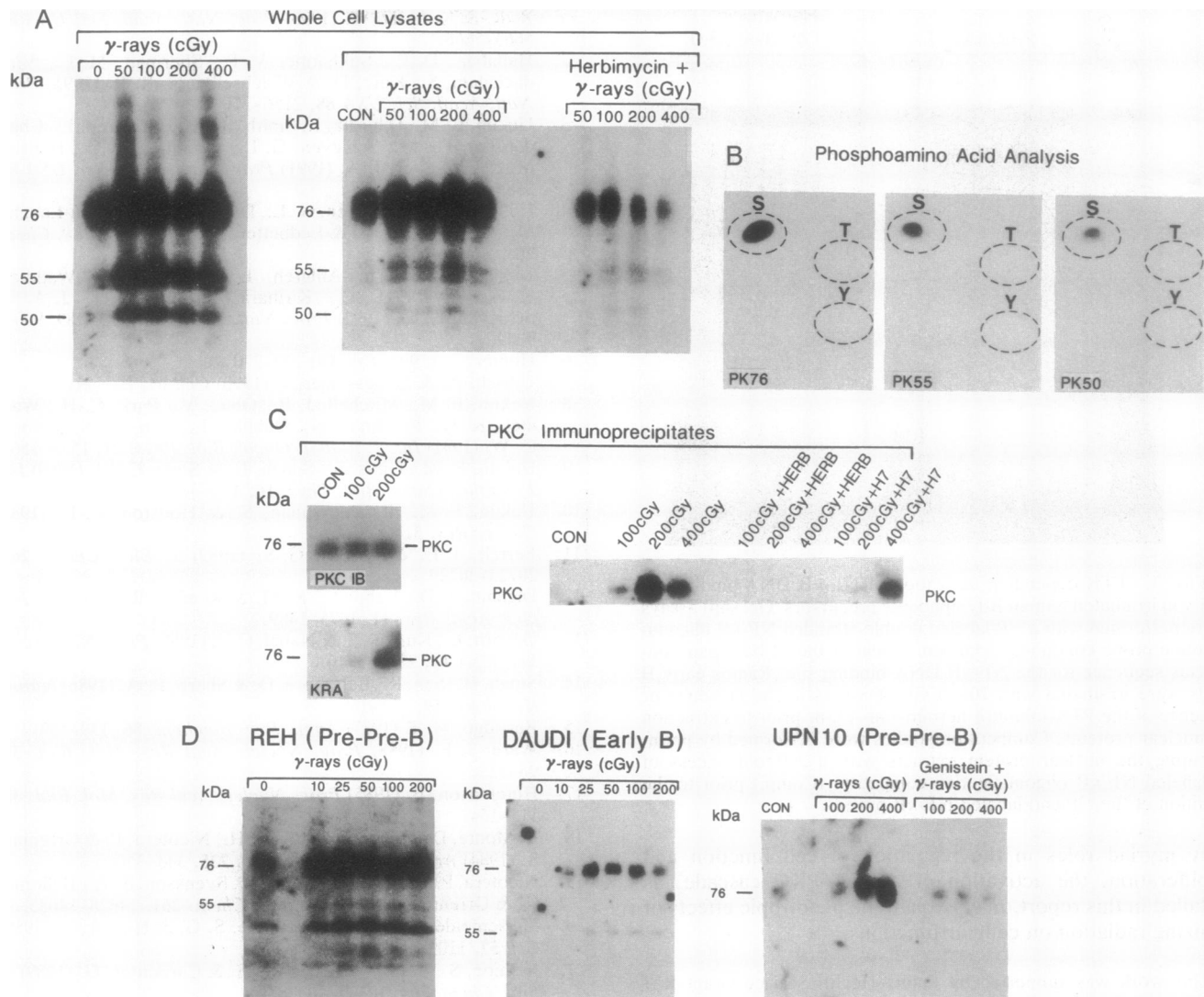


FIG. 2. PTK-dependent activation of PKC and PKC-dependent renaturable serine kinases in irradiated human B-lymphocyte precursors. (A and C) Ramos early B cells were irradiated in the presence or absence of the PTK inhibitor herbimycin A (HERB) or the PKC inhibitor H7. Cells were lysed 15 min after irradiation, and kinase renaturation assays were performed (10) with whole cell lysates (A) or immunoprecipitated PKC (C). Each band represents ³²P covalently bound to protein and corresponds to a distinct protein kinase. A shows a representative experiment in which ionizing radiation was found to activate multiple serine-specific protein kinases (Left) and an experiment demonstrating the inhibitory effects of herbimycin on radiation-induced activation of serine kinases (Right). CON, control. (B) Phospho amino acid analysis of the kinase bands was performed as described (4). Positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are indicated. (D) Kinase renaturation assays were performed using whole cell lysates of irradiated REH pre-pre-B, UPN pre-pre-B, and Daudi early B cells.

unlabeled NF-κB oligonucleotide, confirming the specificity of the DNA-protein interactions (Fig. 3). By comparison, a 500-fold excess of unlabeled NF-1 control oligonucleotide did not compete with the NF-κB probe for binding to the retarded complex (data not shown). These results extend the findings of Brach *et al.* (23) on myeloid leukemia cells and provide unambiguous evidence that ionizing radiation activates NF-κB in human B-lymphocyte precursors. We next examined the role of tyrosine phosphorylation in radiation-induced NF-κB activation. To this end, Ramos cells were treated overnight with the potent PTK inhibitor herbimycin A prior to irradiation. Herbimycin pretreatment substantially inhibited the formation of NF-κB protein-DNA complexes in extracts from irradiated Ramos cells (Fig. 3). This observation does not exclude the possibility that ionizing radiation can activate NF-κB by several different mechanisms, but it indicates that tyrosine phosphorylation is an important step in radiation-induced activation of NF-κB in human B-lymphocyte precursors.

In summary, we examined the biochemical nature of signaling events in irradiated human B-lymphocyte precursors. This report clarifies the chronological sequence of biochemical events that follow exposure to ionizing radiation. In human B-lymphocyte precursors, ionizing radiation induces tyrosine phosphorylation by stimulation of as yet unidentified PTKs that are probably sensitive to reactive oxygen intermediates (5). The mechanism by which ionizing radiation triggers PTKs is unknown. Irradiation subsequently leads to (i) stimulation of PtdIns turnover; (ii) downstream activation by covalent modification of multiple serine-specific protein kinases, including PKC; and (iii) activation of NF-κB. Our study of radiation-induced signaling events proximal to PKC activation has linked the downstream events of the PKC cascade to PTK-regulated proximal events. Further, this report confirms previous reports by Weichselbaum and colleagues (1, 2) that ionizing radiation can activate a PKC-dependent signaling pathway in human cells. Since both PTK and PKC or PKC-dependent kinases

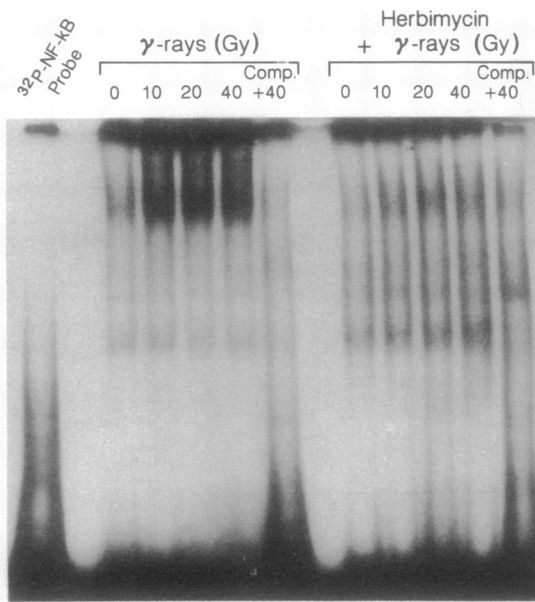


FIG. 3. PTK-dependent activation of NF- κ B DNA-binding activity in irradiated human B-lymphocyte precursors. Gel shift assays were performed with a 32 P-labeled double-stranded NF- κ B oligonucleotide probe containing a tandem repeat of the 11-base-pair consensus sequence for the NF- κ B DNA binding site. Ramos early B cells were irradiated with 10–40 Gy of γ -rays in the presence or absence of the PTK inhibitor herbimycin A 2 hr prior to extraction of nuclear proteins. Competition studies were performed by preincubating the nuclear protein extracts with a 500-fold excess of unlabeled NF- κ B oligonucleotide competitor (Comp.) prior to the addition of the 32 P-end-labeled NF- κ B probe.

play myriad roles in the regulation of cell function and proliferation, the activation of a PTK–PKC cascade, as detailed in this report, may explain the pleiotropic effects of ionizing radiation on cellular functions.

This work was supported by Public Health Service Grant R29 CA42111 from the National Cancer Institute. F.M.U. is a Scholar of the Leukemia Society of America. This is publication no. 95 from the Tumor Immunology Laboratory, University of Minnesota.

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