Involvement of Reactive Oxygen Intermediates in the Induction of c-jun Gene Transcription by Ionizing Radiation†

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ABSTRACT: Previous work has demonstrated that the cellular response to ionizing radiation includes transcriptional activation of the c-jun gene. The signaling events responsible for this response, however, remain unclear. The present studies have examined the effects of ionizing radiation on c-jun expression in a variant of HL-60 cells, designated HL-525, which is deficient in protein kinase C (PKC)-mediated signal transduction. The results demonstrate that these cells express low levels of PKCα and PKCβ transcripts and exhibit an attenuated induction of c-jun expression following treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In contrast, HL-525 cells respond to ionizing radiation with an increase in c-jun mRNA which is more pronounced than that in wild-type HL-60 cells. These cells similarly respond to ionizing radiation with increased expression of the jun-B, jun-D, c-fos, and fos-B genes. Nuclear run-on assays demonstrate that X-ray-induced c-jun expression in HL-525 cells is regulated by increases in the rate of c-jun gene transcription. Moreover, mRNA stability studies in irradiated HL-525 cells demonstrate that the half-life of c-jun transcripts is prolonged compared to that in wild-type cells. Studies with *N*-acetyl-L-cysteine (NAC), an antioxidant, suggest that X-ray-induced transcriptional activation of the c-jun gene is mediated at least in part through the formation of reactive oxygen intermediates (ROIs). In this context, H_2O_2 also induced c-jun expression in HL-525 cells, and this effect was inhibited by NAC. We further demonstrate that the induction of c-jun expression by X-rays, as well as H_2O_2, is inhibited (1) by prolonged exposure to TPA or bryostatin and (2) by H7, a nonspecific inhibitor of PKC-like protein kinases, but not HA1004, a more selective inhibitor of cyclic nucleotide-dependent protein kinase activity. Taken together, these results indicate that ionizing radiation induces c-jun gene transcription through the formation of ROIs and that a protein kinase, perhaps a PKC isoform distinct from PKCα and PKCβ, is also involved in this signaling pathway.

The response of eukaryotic cells to ionizing radiation includes activation of DNA repair mechanisms, cell cycle arrest, mutagenesis, and lethality. However, the events responsible for the control of these responses remain unclear. Ionizing radiation has been postulated to induce such effects either by direct interaction with DNA or through the formation of reactive oxygen intermediates (ROIs) which damage DNA (Hall, 1988). Recent studies have further suggested a role for the activation of immediate-early genes in the response to ionizing radiation. For example, exposure of cells to X-rays is associated with activation of the c-jun/c-fos and EGR-1 gene families which code for transcription factors (Sherman et al., 1990a; Hallahan et al., 1991a). Other studies have demonstrated that ionizing radiation induces expression and DNA binding activity of the nuclear factor κB (NF-κB) (Brach et al., 1991). The activation of transcription factors may represent a critical control point in transducing early nuclear signals to longer term changes in gene expression that reflect the response to X-ray-induced damage. Indeed, other studies have demonstrated that irradiation of cells is associated with increased expression of certain cytokines, including tumor nucrosis factor (TNF), platelet-derived growth factor, fibroblast growth factor, and interleukin-1 (Hallahan et al., 1989; Witte et al., 1989; Woloschak et al., 1990). The increase in TNF expression following exposure to ionizing radiation is regulated by transcriptional mechanisms (Sherman et al., 1991), although it is not known which DNA binding proteins confer this inducibility.

The c-jun gene codes for the major form of the 40–44-kDa AP-1 transcription factor (Mitchell & Tjian, 1989). As observed in irradiated cells (Sherman et al., 1990a; Hallahan et al., 1991a), this gene is induced as an immediate-early event in response to phorbol esters and certain growth factors (Quantin & Breathnach, 1988; Brenner et al., 1989; Wu et al., 1989). The Jun/AP-1 complex binds to the heptameric DNA consensus sequence $TGA^G/_CTCA$ (Mitchell & Tjian, 1989). The DNA binding domain of c-Jun is shared by a family of transcription factors, including Jun-B, Jun-D, and c-fos. Moreover, the affinity of c-Jun binding to DNA is related to the formation of homodimers or heterodimers with products of the fos gene family (Zerial et al., 1989; Nakabeppu et al., 1988; Halazonetis et al., 1988). Jun-B also forms dimers and binds to the AP-1 element (Ryder et al., 1988; Chiu et al., 1989), although the trans-acting properties of Jun-B differ from those of c-Jun (Chiu et al., 1989; Schutte et al., 1989). While the product of the Jun-D gene also interacts with c-Fos and has similar binding properties to that of c-Jun (Hirai et

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al., 1989), the function of Jun-D is unknown. Certain insights are available regarding the signals which contribute to the regulation of these genes. For example, the finding that phorbol esters activate c-jun transcription in diverse cell types has implicated the involvement of a protein kinase C (PKC)-dependent mechanism (Brenner et al., 1989; Angel et al., 1988a; Hallahan et al., 1991a). A similar pathway appears to play a role, at least in part, in the induction of c-jun expression by ionizing radiation. In this regard, prolonged treatment with phorbol esters to down-regulate PKC is associated with decreases in activation of c-jun by X-rays (Hallahan et al., 1991a). Furthermore, nonspecific inhibitors of PKC, such as the isoquinolinesulfonamide derivative H7, block X-ray-induced c-jun expression (Hallahan et al., 1991a). Taken together with the demonstration that ionizing radiation induces an activity with characteristics of PKC (Hallahan et al., 1991b), these findings have suggested that PKC or a related kinase transduces signals which confer X-ray inducibility of the c-jun gene.

The present studies have examined the effects of ionizing radiation on c-jun expression in an HL-60 cell variant, designated HL-525, which is deficient in PKC-mediated signal transduction (Homma et al., 1986). This variant is resistant to both phorbol ester-induced differentiation and X-ray-induced TNF gene expression (Hallahan et al., 1991b; Homma et al., 1986). The present results demonstrate that HL-525 cells are resistant to the induction of c-jun expression by phorbol esters while treatment of these cells with ionizing radiation is associated with superinduction of c-jun mRNA levels compared to phorbol ester-responsive HL-60 cells. The findings indicate that this effect of ionizing radiation is related at least in part to the formation of reactive oxygen intermediates and induction of a protein kinase activity.

MATERIALS AND METHODS

Cell Culture. Clone HL-205 was isolated from the human HL-60 myeloid leukemia cell line (Homma et al., 1986). The phorbol ester-resistant variant of HL-60 cells, designated HL-525, was isolated by exposing wild-type cells to low concentrations of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 0.5–3 nM) for 102 passages (Homma et al., 1986). These cells were maintained in RPMI 1640 medium containing 20% fetal bovine serum (FBS) with 1 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Irradiation was performed at room temperature using a Gammacell 1000 (Atomic Energy of Canada Ltd., Ontario) with a ^{137}Cs source emitting at a fixed dose rate of 13.3 Gray (Gy)/min as determined by dosimetry. The cells were also treated with 32 nM TPA (Sigma Chemical Co., St. Louis, MO), 50 μM H_2O_2 (Sigma), 10 nM bryostatin (kindly provided by Dr. George Pettit), 30 mM *N*-acetyl-L-cysteine (Sigma), 50 μM H7 (Seikagaku America, St. Petersburg, FL) and 50 μM HA1004 (Seikagaku).

Isolation and Analysis of RNA. Total cellular RNA was purified by the guanidine isothiocyanate–cesium chloride technique (Chirgwin et al., 1979). The RNA was analyzed by electrophoresis through 1% agarose–formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the following ^{32}P-labeled DNA probes: (1) the 1.8-kb *Bam*HI/*Eco*RI insert of a human c-jun gene purified from a pBluescript SK(+) plasmid (Angel et al., 1988b); (2) the 1.5-kb *Eco*RI fragment of the murine jun-B cDNA from the p465.20 plasmid (Ryder et al., 1988); (3) the 1.7-kb *Eco*RI fragment of a murine jun-D cDNA purified from the XHJ-12.4 plasmid (Ryder et al., 1989); (4) the 0.9-kb *Sca*I/*Nco*I insert of a human c-fos gene purified from the pc-fos-1 plasmid (Curran et al., 1983); (5) the 2.0-kb *Pst*I insert of a chicken β-actin gene purified from the pA1 plasmid (Cleveland et al., 1980); (6) a 1.3-kb *Eco*RI PKCα insert of a phPKC-α7 plasmid; and (7) a 1.7-kb *Eco*RI PKCβ insert of a phPKC-β1-15-Eco plasmid (PKC probes obtained from the American Type Culture Collection, Rockville, MD). Hybridizations were performed at 42 °C for 24 h in 50% (v/v) formamide, 2× SSC, 1× Denhardt's solution, 0.1% SDS, and 200 μg/mL salmon sperm DNA. The filters were washed twice in 2× SSC–0.1% SDS at room temperature and then in 0.1× SSC–0.1% SDS at 60 °C for 1 h. Autoradiographic bands were scanned using a LKB Produkter (Bromma, Sweden) Ultroscan XL laser densitometer and analyzed with the Gelscan XL software package (version 1.21). Signal intensity was determined in a linear range and normalized to that for the actin control.

Nuclear Run-On Assays. Nuclei were isolated from 10^8 cells and suspended in 100 μL of glycerol buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). An equal volume of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 5 mM dithiothreitol) was added to the nuclei in suspension and incubated at 26 °C for 45 min with 250 μCi of [α-^{32}P]UTP (3000 Ci/mmol; Dupont, Boston, MA). The nuclear RNA was isolated as described (Sherman et al., 1990b) and hybridized to the following DNAs: (1) a *Pst*I digest of the pA1 plasmid containing a fragment of the chicken β-actin gene (positive control) (Cleveland et al., 1980); (2) a 1.1-kb *Bam*HI insert of a human β-globin gene (negative control) (Wilson et al., 1978); and (3) a *Bam*HI/*Eco*RI digest of the pBluescript SK(+) plasmid containing a fragment of the human c-jun gene (Angel et al., 1988b). The digested DNAs were run in 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were performed with 10^7 cpm of ^{32}P-labeled RNA/mL in 10 mM Tris-HCl, pH 7.5, 4× SSC, 1 mM EDTA, 0.1% SDS, 2× Denhardt's solution, 40% formamide, and 100 μg/mL yeast tRNA for 72 h at 42 °C. The filters were washed in (a) 2× SSC–0.1% SDS at 37 °C for 30 min, (b) 200 ng/mL RNase A in 2× SSC at room temperature for 5 min, and (c) 0.1× SSC–0.1% SDS at 42 °C for 30 min. Signal intensity was determined by laser densitometry as described above.

Protein Kinase Activity (Hallahan et al., 1991b). Cells were extracted by the addition of 0.4 mL of ice-cold TEM lysis buffer (20 μM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM 2-mercaptoethanol) containing 0.5% Triton X-100, 25 μg/mL leupeptin, and 25 μg/mL aprotinin. After Dounce homogenization and incubation on ice for 30 min, the cell extract was spun in a microcentrifuge at 15000*g* for 2 min. The supernatants were applied to DEAE-cellulose ion-exchange columns (Whatman DE52; 0.25 g/column) and eluted at 4 °C with 2 mL of TEM buffer containing 0.2 M NaCl. The DEAE eluate was used directly or after dilution in TEM containing 0.2 M NaCl to obtain a range of enzyme concentrations. PKC activity was determined as described (protein kinase C assay system, GIBCO BRL, Grand Island, NY). Briefly, the partially purified protein extract in TEM containing 0.2 M NaCl was incubated for 5 min at 30 °C in phospholipid (0.28 mg/mL phosphatidylserine and 1 mM phorbol 12-myristate 13-acetate in 0.3% Triton X-100 mixed micelles), [γ-^{32}P]ATP (20–25 μCi/mL; NEN, Boston, MA), and 50 μM PKC synthetic peptide [Gln-Lys-Arg-Pro-Ser-(8)-Gln-Arg-Ser-Lys-Tyr-Leu] substrate derived from myelin basic protein. All assays were performed in both the presence and the absence of a PKC pseudosubstrate inhibitor

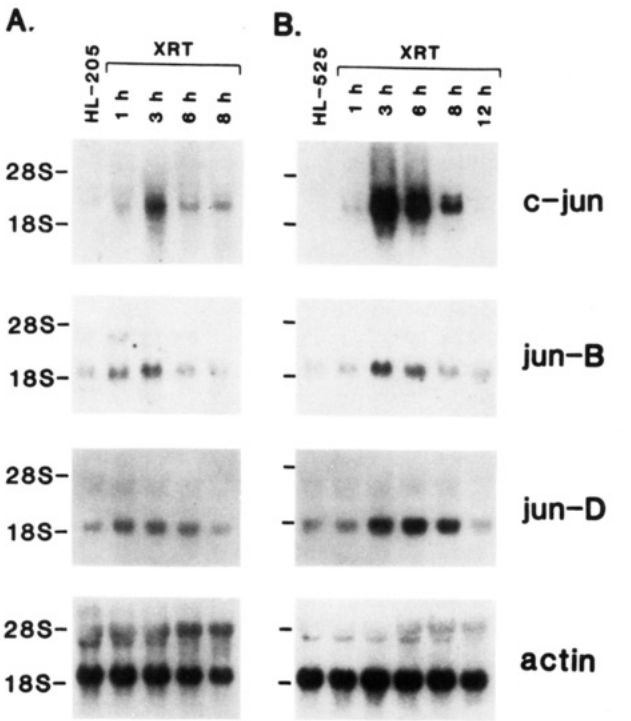

FIGURE 1: Induction of jun gene expression in HL-205 and HL-525 cells by ionizing radiation. HL-205 (A) and HL-525 (B) cells were exposed to 20-Gy ionizing radiation (XRT). Total cellular RNA (20 μg) was isolated at the indicated times and hybridized to ^{32}P-labeled c-jun, jun-B, jun-D, or actin probes.

peptide (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn) (GIBCO BRL). The samples were dried on phosphocellulose and washed in 1% H_3PO_4, and the amount of phosphorylated peptide was quantified by scintillation counting.

RESULTS

Ionizing Radiation Induces Early Response Gene Expression in TPA-Resistant HL-525 Cells. Previous studies have demonstrated that treatment of HL-205 cells with TPA is associated with translocation of PKC activity from the cytosolic fraction to the cell membrane while no cellular redistribution of PKC is detectable during similar exposures of HL-525 cells (Homma et al., 1986). Since previous work has also suggested that ionizing radiation induces early response gene expression by a PKC-dependent mechanism (Hallahan et al., 1991a), we sought to determine the effects of X-rays on a cell, such as HL-525, which is deficient in PKC-mediated signal transduction. A low level of c-jun transcripts was detectable in untreated HL-205 cells, while treatment with ionizing radiation was associated with a transient increase which was maximal at 3 h (Figure 1A). The kinetics and intensity of this response were identical to those reported for the parent HL-60 cells (Sherman et al., 1990a). Expression of the c-jun gene was also low in untreated HL-525 cells (Figure 1B). However, exposure of these cells to ionizing radiation resulted in c-jun mRNA levels which at 3 h were approximately 20-fold higher than that obtained in HL-205 cells (Figure 1B). Higher levels of c-jun expression were similarly detected in HL-525 cells at 6 and 8 h after X-ray exposure (Figure 1B). Expression of the jun-B and jun-D genes was also transiently increased following X-irradiation of the HL-205 line (Figure 1A). Similar findings were obtained with HL-525 cells, although mRNA levels for jun-B and jun-D at 3 h were 2.3- and 2.5-fold higher in this variant as compared to that in HL-205 cells (Figure 1).

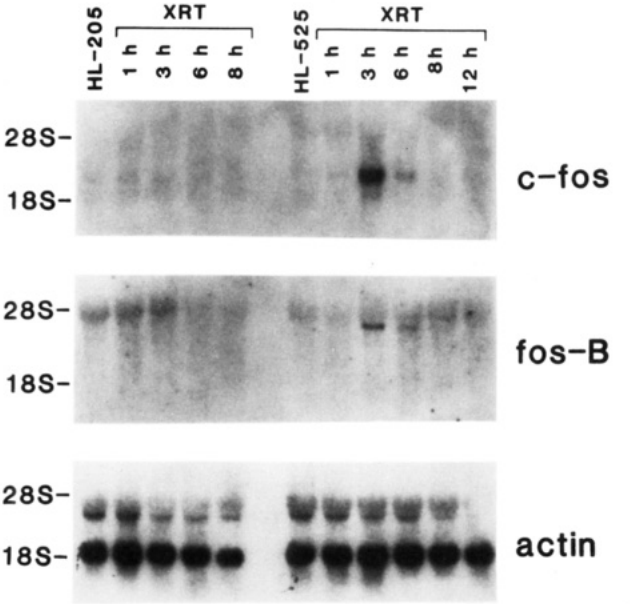

FIGURE 2: Ionizing radiation increases c-fos and fos-B mRNA levels. HL-205 and HL-525 cells were exposed to 20-Gy ionizing radiation (XRT). Total cellular RNA (20 Gy) was isolated at the indicated times and hybridized to ^{32}P-labeled c-fos, fos-B, or actin probes.

Proteins encoded by members of the jun gene family can form heterodimers with fos gene products (Zerial et al., 1989; Nakabeppu et al., 1988; Halazonetis et al., 1988; Hirai et al., 1989). Consequently, we also studied the effects of X-rays on c-fos and fos-B expression. c-fos transcripts were present at low levels in HL-205 cells, and there was little if any effect of ionizing radiation on expression of this gene (Figure 2). Similar findings were obtained for fos-B (Figure 2). In contrast, while expression of c-fos and fos-B was also low in HL-525 cells, X-ray exposure was associated with transient increases in transcripts for both of these genes (Figure 2). The kinetics of these increases in fos gene expression were similar to those obtained for members of the jun gene family. Thus, activation of multiple jun and fos genes could contribute to diverse nuclear signals in the response of cells to X-rays.

Treatment of HL-60 cells and other myeloid leukemia cells with TPA is associated with induction of the c-jun gene (Sherman et al., 1990b; Szabo et al., 1991). Similar effects were obtained in TPA-treated HL-205 cells (Figure 3A). The response of these cells to TPA was associated with increases in c-jun expression that were detectable at 6 h and reached maximal levels by 24 h (Figure 3A). In contrast, exposure of HL-525 cells to TPA resulted in an increase in c-jun expression which was transient at 12 h and attenuated compared to that in the HL-205 line (Figure 3A). Similar results were obtained for the c-fos gene (Figure 3A). These findings indicated that HL-525 cells are resistant at least in part to the effects of TPA on Jun/AP-1-mediated signaling events. Since TPA activates PKC (Nishizuka, 1984) and translocation of this enzyme is undetectable in HL-525 cells (Homma et al., 1986), we compared expression of PKC in the HL-205 and HL-525 lines. HL-60 cells have been shown to express the PKCα and PKCβ isozymes (Makowske et al., 1988; Hocevar & Fields, 1991). Indeed, PKCα and PKCβ transcripts were detectable in HL-205 cells (Figure 3B). However, constitutive expression of these genes was decreased by approximately 90% in HL-525 cells (Figure 3B). These results suggested that the relative resistance of HL-525 cells to TPA-induced c-jun transcription could be attributable to low levels of PKC expression. In this regard, we have previously demonstrated that exposure of HL-60 cells to

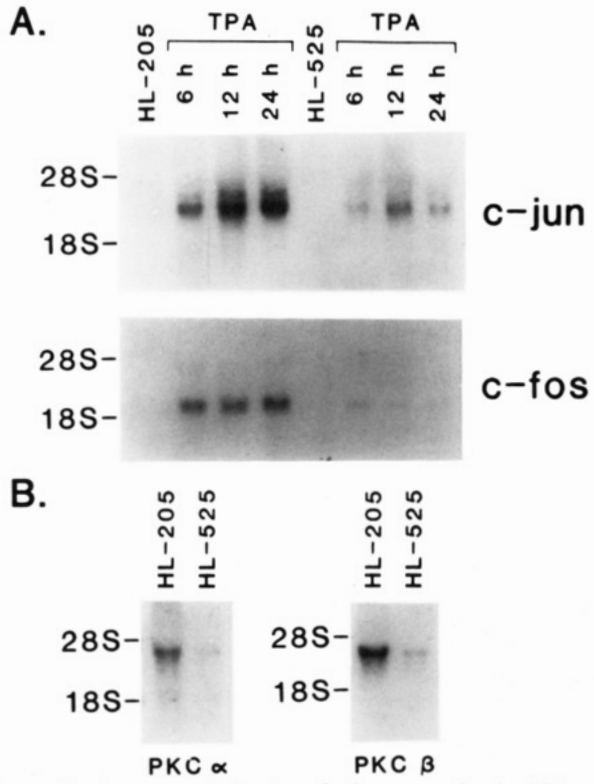

FIGURE 3: Attenuated induction of c-jun expression by TPA and decreased levels of PKC transcripts in HL-525 cells. (A) HL-205 and HL-525 cells were exposed to 32 nM TPA. Total cellular RNA (20 μg) was isolated at the indicated times and hybridized to the c-jun and c-fos probes. (B) Total cellular RNA (20 μg) from untreated HL-205 and HL-525 cells was hybridized to the PKCα and PKCβ probes. Actin hybridization demonstrated equal loading of the lanes.

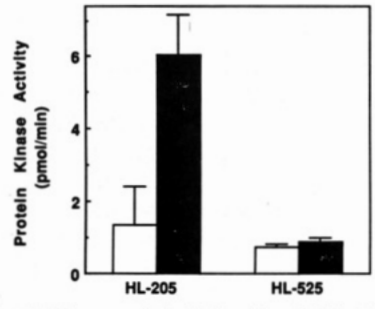

FIGURE 4: Protein kinase activity in irradiated HL-205 and HL-525 cells. Cells (10^5) were exposed to 20-Gy ionizing radiation and harvested at 30 s. Extracts were assayed for phosphorylation of a synthetic PKC substrate derived from myelin basic protein. The results represent the mean ± SEM of three experiments. Open bars, control cells; solid bars, irradiated cells.

ionizing radiation is associated with increased phosphorylation of a synthetic PKC substrate derived from myelin basic protein (Hallahan et al., 1991b). Similar findings were obtained with HL-205 cells (Figure 4). In contrast, there was no detectable effect of X-rays on phosphorylation of this substrate in HL-525 cells (Figure 4). Taken together with the finding that c-jun expression is superinduced by ionizing radiation in HL-525 cells, these results also suggested that X-ray-induced c-jun expression is mediated by events independent of PKCα and PKCβ.

Ionizing Radiation Regulates c-jun Expression by Transcriptional and Posttranscriptional Mechanisms in HL-525 Cells. In order to further define the mechanisms responsible for induction of c-jun expression in HL-525 cells, we first performed nuclear run-on assays to determine the effects of

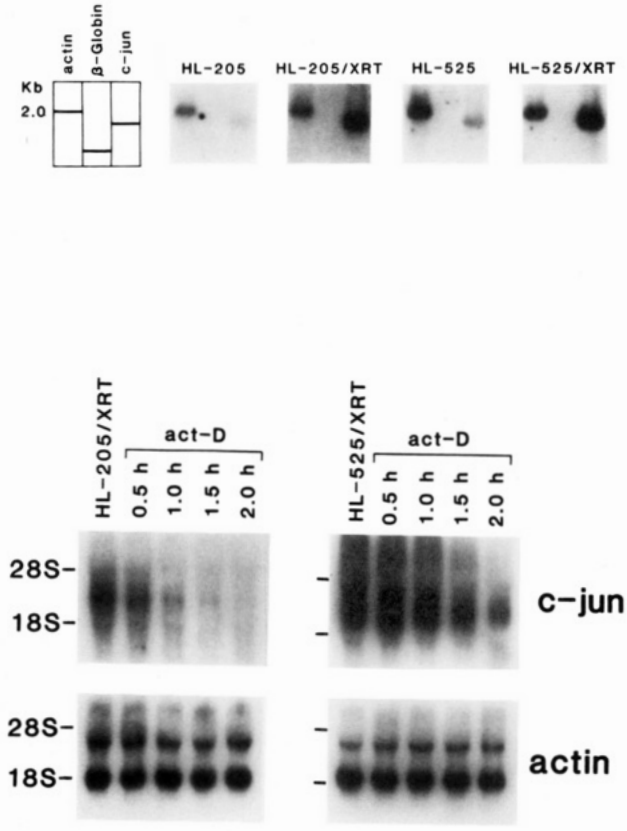

FIGURE 6: Stability of c-jun transcripts in irradiated HL-205 and HL-525 cells. Cells were exposed to 20-Gy ionizing radiation (XRT), and after 3 h, actinomycin D (act-D; 5 μg/mL) was added. RNA (20 μg) was isolated at the indicated times after addition of actinomycin D and hybridized to the labeled c-jun and actin probes.

ionizing radiation on rates of c-jun transcription. Similar studies were conducted in HL-205 cells for comparative purposes. The actin gene (positive control) was constitutively transcribed in HL-205 cells, while there was no detectable transcription of the β-globin gene (negative control) (Figure 5). Similar patterns were observed in HL-525 cells (Figure 5). Transcription of the c-jun gene was detectable at low levels in both cell types. Moreover, X-ray treatment of the HL-205 and HL-525 lines resulted in a 16- and 18-fold stimulation in the rate of c-jun transcription, respectively (Figure 5). These findings suggested that other mechanisms, perhaps at the posttranscriptional level, contributed to the higher levels of c-jun expression following treatment of HL-525 cells. In order to address this issue, we studied the stability of c-jun mRNA after treatment with actinomycin D to inhibit further transcription. The half-life of c-jun transcripts in X-ray-treated HL-205 cells was 31 min (Figure 6). In contrast, stability of these transcripts in irradiated HL-525 cells was increased over 3-fold with a half-life of 106 min (Figure 6). Taken together, these results indicated that transcriptional activation of the c-jun gene by X-rays is similar in both cell types and that higher levels of c-jun expression in the HL-525 variant are related to differences in posttranscriptional control.

ROIs Induce c-jun Expression by a Protein Kinase-Dependent Mechanism. The finding that ionizing radiation induces c-jun expression in the HL-525 cells which exhibit an altered response of this gene to TPA suggested that PKC-independent pathways may mediate this effect of X-rays. In this context, ionizing radiation is known to induce the formation of ROIs (Hall, 1988). Moreover, recent studies have demonstrated that H_2O_2, another agent that acts through production of ROIs, activates the c-jun gene in HeLa cells (Devary

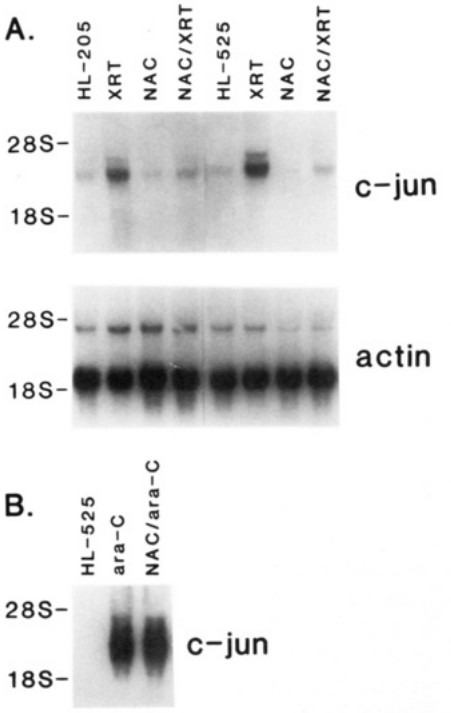

FIGURE 7: NAC inhibits the induction of c-jun expression by ionizing radiation. Cells were exposed to 20-Gy ionizing radiation (XRT) and/or 30 mM NAC. The NAC was added 30 min prior to irradiation and included in the medium for an additional 3 h. Cells treated with NAC alone were harvested after 3.5 h. Total cellular RNA (20 μg) was hybridized to the c-jun and actin probes.

et al., 1991). The effects of ROIs in cells are counteracted by the antioxidant *N*-acetyl-L-cysteine (NAC) (Roederer et al., 1990; Staal et al., 1990). The exposure of HL-205 cells to 30 mM NAC had little if any effect on constitutive levels of c-jun transcripts (Figure 7A). However, this agent inhibited X-ray-induced increases in c-jun expression by over 95% (Figure 7A). Similar findings were obtained in the HL-525 cells (Figure 7A). These inhibitory effects of NAC on X-ray-induced c-jun expression were mediated by a block in transcriptional activation of the c-jun gene (data not shown). In contrast, NAC had no detectable effect on induction of the c-jun gene in HL-525 cells by 1-β-D-arabinofuranosylcytosine (ara-C; Figure 7B), another DNA-damaging agent which incorporates into the DNA strand (Kharbanda et al., 1990, 1991). These findings indicated that NAC is a specific inhibitor of X-ray-induced c-jun transcription, presumably through its effects on ROIs.

Previous studies have demonstrated that the induction of c-jun expression by ionizing radiation in wild-type HL-60 cells is inhibited by prolonged pretreatment with TPA and nonspecific inhibitors of PKC (Hallahan et al., 1991a). Since HL-525 cells are resistant to TPA-induced PKC-mediated signal events and c-jun induction, we reasoned that prolonged exposure to TPA would have little if any effect on activation of c-jun expression by ionizing radiation. However, while treatment of HL-525 cells with TPA alone for 36–39 h had no detectable effect on c-jun mRNA levels, pretreatment with this agent blocked X-ray-induced increases in c-jun expression by over 80% (Figure 8A). Similar studies were performed with bryostatin, an agent distinct from TPA that also transiently activates PKC (Stone et al., 1988). Exposure of HL-525 cells to bryostatin alone for 36–39 h also had no effect on c-jun mRNA levels (Figure 8B). Moreover, treatment with this agent before irradiation inhibited the induction of c-jun transcripts by 66% (Figure 8B). Since

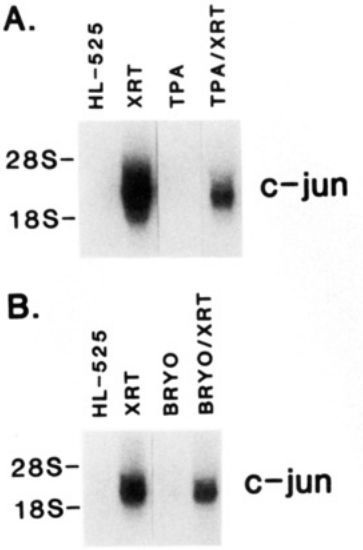

FIGURE 8: Pretreatment with TPA or bryostatin inhibits induction of c-jun expression by ionizing radiation. HL-525 cells were pretreated with 32 nM TPA (A) or 10 nM bryostatin (BRYO) (B) for 36 h and then exposed to ionizing radiation (XRT). Total cellular RNA was isolated 3 h later and hybridized to the c-jun probe. Actin signals demonstrated equal loading of the lanes.

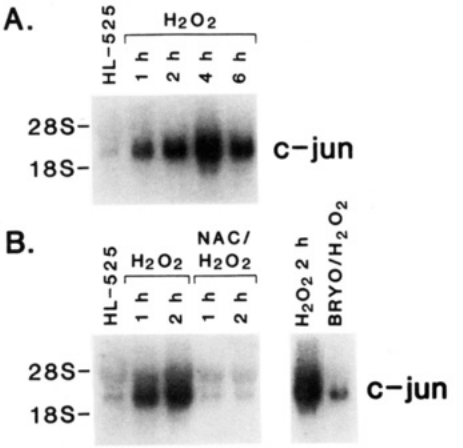

FIGURE 9: Induction of c-jun expression by H_2O_2 is inhibited by NAC and bryostatin. (A) HL-525 cells were exposed to H_2O_2 for the indicated times. (B) Cells were treated with NAC for 0.5 h before addition of 50 μM H_2O_2 for 1 or 2 h. Cells were also treated with bryostatin for 36 h before addition of H_2O_2 for 2 h. Total cellular RNA (20 μg) was hybridized to the c-jun probe. Actin hybridization demonstrated equal loading of the lanes.

H_2O_2 also acts as a DNA-damaging agent through the production of ROIs, we performed similar experiments in HL-525 cells treated with this agent. H_2O_2 transiently increased c-jun mRNA levels in these cells (Figure 9A). The increase in c-jun expression was detectable at 1 h and reached maximal levels at 4 h (Figure 9A). This effect of H_2O_2 was completely blocked by NAC (Figure 9B). Furthermore, pretreatment of the HL-525 cells with bryostatin to down-modulate PKC resulted in inhibition of H_2O_2-induced gene expression (Figure 9B). Similar findings were obtained following TPA pretreatment (data not shown). These findings suggested that the induction of c-jun expression by H_2O_2, as well as ionizing radiation, is mediated by a cascade involving the formation of ROIs and activation of a protein kinase sensitive to down-regulation by TPA and bryostatin. In order to confirm the involvement of a protein kinase in ROI-mediated induction of the c-jun gene, we treated HL-525 cells with H7, a nonspecific inhibitor of PKC (Hidaka et al., 1984). H7 completely blocked X-ray- and H_2O_2-induced increases in

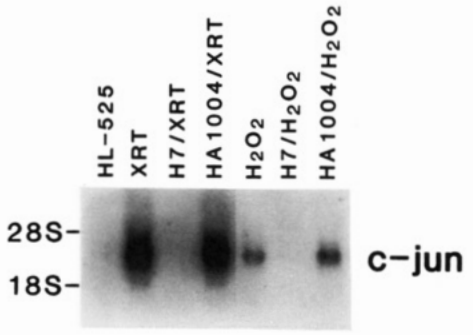

FIGURE 10: H7, but not HA1004, inhibits induction of c-jun expression by ionizing radiation and H_2O_2. HL-525 cells were pretreated with 50 μM H7 or HA1004 for 0.5 h before exposure to 20-Gy ionizing radiation (XRT) or the addition of 50 μM H_2O_2. After 3 h, total cellular RNA (20 μg) was isolated and hybridized to the c-jun probe. Hybridization to the actin probe demonstrated equal loading of the lanes.

c-jun transcripts (Figure 10). In contrast, HA1004, a more selective inhibitor of cyclic nucleotide-dependent kinases (Hidaka et al., 1984), had no detectable effect (Figure 10).

DISCUSSION

Previous studies have suggested that the induction of c-jun expression by ionizing radiation is mediated by a PKC-dependent mechanism (Hallahan et al., 1991a). Prolonged treatment with TPA to down-regulate PKC results in marked attenuation of the c-jun gene by X-rays. Moreover, X-ray-induced c-jun expression is inhibited by H7, a nonspecific inhibitor of PKC, but not by HA1004, a more selective inhibitor of cyclic nucleotide-dependent protein kinases (Hallahan et al., 1991a). Consequently, we expected that cell lines, such as HL-525, which are deficient in PKC-mediated signaling would respond to ionizing radiation with an attenuated induction of c-jun expression. In this context, the activation of TNF gene expression in X-ray-treated HL-525 cells is diminished compared to TPA-responsive HL-60 lines (Hallahan et al., 1991b). Furthermore, the present finding that treatment of HL-525 cells with TPA is associated with an attenuated c-jun response supports a defect in PKC-mediated events that control c-jun expression. Nonetheless, HL-525 cells responded to ionizing radiation with an increase in c-jun expression which was more pronounced than that obtained in HL-205 cells. The results demonstrate that this increase in c-jun mRNA levels is regulated by activation of c-jun transcription as well as by a prolongation in the half-life of these transcripts. This posttranscriptional regulation of c-jun may be related to the presence of AT-rich regions in the untranslated region of this gene (Hattori et al., 1988). These regions presumably serve as recognition sites for degradation by labile RNases (Shaw & Kamen, 1986). Whatever the mechanisms, the finding that stability of the c-jun transcript is increased in X-ray-treated HL-525 cells is thus in certain respects similar to that observed in TPA-treated HL-60 cells (Sherman et al., 1990b). Other members of the jun/fos family (jun-B, jun-D, c-fos, fos-B) were also induced following X-ray exposure of the HL-525 variant, while treatment of these cells with TPA resulted in little if any effect on expression of these genes. These findings indicated that ionizing radiation increases jun/fos expression through signaling pathways distinct from those activated during induction of these genes in TPA-treated cells. Moreover, the finding that X-ray-induced TNF gene expression is attenuated in HL-525 cells (Hallahan et al., 1991b) suggests that ionizing radiation induces the c-jun and TNF genes by distinct signaling pathways.

NAC counteracts the effects of oxidative stress by scavenging ROIs and increasing intracellular glutathione (GSH) (Aruoma et al., 1989; Burgunder et al., 1989). Previous studies have demonstrated that NAC is a potent inhibitor of phorbol ester-induced activation of the HIV-1 long-terminal repeat (Roederer et al., 1990). This antioxidant has also been found to inhibit activation of the nuclear factor κB (NF-κB) by phorbol esters and other agents such as H_2O_2 (Staal et al., 1990; Schreck et al., 1991). The available findings suggest that ROIs activate NF-κB by inducing the release of the inhibitory subunit IκB (Schreck et al., 1991). ROIs are also formed during the treatment of cells with ionizing radiation (Hall, 1988). The cellular response to this agent may thus involve ROI-induced activation of transcription factors and thereby longer term effects on gene expression. Indeed, recent work has demonstrated that DNA binding activity and expression of NF-κB are induced following exposure to ionizing radiation (Brach et al., 1991). However, ROIs have extremely short half-lives, and while DNA binding of NF-κB is rapidly increased in X-ray-treated cells, it is not clear whether this effect is directly or indirectly related to the formation of oxygen radicals. The results of the present studies with NAC suggest that ROIs also contribute to the activation of c-jun transcription by ionizing radiation. Moreover, exposure to H_2O_2 was associated with increased c-jun expression, and this effect was similarly inhibited by NAC. While these findings might be attributed to nonspecific inhibition of c-jun expression, NAC had no detectable effect on ara-C-induced increases in c-jun transcripts. Ara-C damages DNA by incorporation into elongating strands and is not known to mediate its cytotoxic effects through ROIs (Ohno et al., 1988). Of interest, the cellular response to ara-C also includes activation of NF-κB (Brach et al., 1992). Since ROIs damage DNA in irradiated and H_2O_2-treated cells, this damage may represent the event in common with diverse DNA-damaging agents, such as ara-C, UV light, alkylating agents, and etoposide, which also induce c-jun transcription (Devary et al., 1991; Kharbanda et al., 1990, 1991; Rubin et al., 1991).

The basis for the lack of PKC redistribution in TPA-treated HL-525 cells is unclear (Homma et al., 1986). Nonetheless, translocation of PKC from the cytosol to the cell membrane may be necessary for certain TPA-induced signaling events, such as induction of c-jun expression. However, it is not known whether translocation to the cell membrane is necessary for activation of each of the different PKC isoforms. The present results demonstrate that prolonged treatment of the HL-525 variant with TPA blocks X-ray-induced increases in c-jun expression. This finding lends support to the involvement of a PKC-dependent mechanism. The HL-525 variant expresses relatively low levels of PKCα and PKCβ compared to HL-205 cells. Thus, other PKC isozymes which are sensitive to down-regulation by TPA may be responsible for transducing signals which confer X-ray inducibility of the c-jun gene. Alternatively, prolonged TPA treatment could cause down-regulation of other PKC-independent signaling pathways involved in induction of c-jun by ionizing radiation. We have previously shown that X-ray treatment of HL-60 cells is associated with stimulation of a PKC-like activity (Hallahan et al., 1991b). In contrast, increases in this activity were undetectable following irradiation of the HL-525 variant. Further studies are therefore needed to determine which protein kinase(s) is (are) responsible for transducing signals that confer X-ray inducibility of the c-jun gene.

Recent studies have demonstrated that reduction–oxidation (redox) changes regulate the affinity of c-jun binding to DNA

(Abate et al., 1990; Bannister et al., 1991). Reduction of certain cysteine residues in the leucine zipper and adjacent basic region is required for association with the AP-1 site. Since oxidation of these cysteines inhibits the DNA binding activity of c-jun (Abate et al., 1990; Bannister et al., 1991) and c-jun transcription is positively autoregulated by Jun/AP-1 (Angel et al., 1988a), it is unlikely that induction of c-jun expression by ionizing radiation or H_2O_2 is directly through the formation of ROIs. Indeed, the present results suggest that ROIs induce c-jun expression by a protein kinase-dependent mechanism. In this regard, the nonspecific protein kinase inhibitor H7 blocked induction of c-jun by both ionizing radiation and H_2O_2. Moreover, X-ray- and H_2O_2-induced c-jun expression was abrogated by prolonged exposure to TPA or bryostatin. While this exposure to TPA or bryostatin may inhibit cellular events unrelated to PKC, these results, taken together with the H7 findings, support the involvement of a protein kinase. One potential explanation for these findings is that ROIs directly increase a protein kinase activity and thereby c-jun transcription. Alternatively, since ROIs are known to damage DNA, this damage may be responsible for activation of a protein kinase and then c-jun expression. Indeed, the demonstration that other agents, such as ara-C, damage DNA in the absence of ROI formation suggests that alterations in chromatin structure initiate a signaling cascade which includes protein kinase activation and induction of the c-jun gene.

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