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Impaired Ionizing Radiation-induced Activation of a Nuclear Signal Essential for Phosphorylation of c-Jun by Dually Phosphorylated c-Jun Amino-terminal Kinases in Ataxia Telangiectasia Fibroblasts*

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The c-Jun amino-terminal kinases (JNKs) participate in intracellular signaling in response to cytokines and cellular stresses. JNKs are activated by phosphorylation on two critical residues, the threonine 183 and tyrosine 185, within the TPY motif. The activated JNKs, in turn, phosphorylate the nuclear protein c-Jun, a major component of the transcription factor AP1. *In vitro* studies have revealed a defect in ionizing radiation-induced activation of the JNK signaling pathway in lymphoblastoid cells from individuals with ataxia telangiectasia (AT). However, the biochemical basis for this signaling defect is not clear. Here, we show that ionizing radiation induces the phosphorylation of endogenous c-Jun in normal fibroblasts but not in AT fibroblasts. The p46 isoforms of dually phosphorylated JNKs were detected in the nuclei of both normal and AT fibroblasts following exposure to ionizing radiation or sham radiation. However, c-Jun kinase activity was detected in normal cells but not in AT cells. Furthermore, an exogenous purified active JNK protein was able to phosphorylate endogenous c-Jun in nuclear extracts only of normal cells and only after the cells were irradiated. Electrophoretic mobility shift assays also showed that the ionizing radiation-induced increase in the DNA binding activity of AP1 observed in normal cells was absent or markedly reduced in AT cell lines. These data suggest that the defect in ionizing radiation-induced signaling through c-Jun in AT cells is the result of impaired function of an unknown nuclear protein or proteins that negatively regulate both JNK and c-Jun.

Ataxia telangiectasia (AT)[1] (1) is a human genetic disorder clinically characterized by neurodegeneration, defective immunological responses, and premature aging (1, 2). Cells from affected individuals show extreme sensitivity to ionizing radiation as well as defects in both oxidative stress responses and cell cycle regulation (2–4). The gene (*ATM*) that is mutated in AT patients has been isolated, and the encoded protein has been suggested to play roles in mitogenic signal transduction, meiotic recombination, and cell cycle control (5–7). Defects in intracellular signaling pathways in AT cells in response to ionizing radiation include impaired activation of the transcription factors NF-κB and c-Jun (8–10). The transcriptional activity of c-Jun is enhanced by phosphorylation of two serine residues (amino acids 63 and 73) in the NH_2-terminal trans activation domain by c-Jun NH_2-terminal kinase (JNK) in response to various stimuli including ionizing radiation, UV light, and tumor necrosis factor (11–15).

JNKs are a family of serine-threonine kinases that are related to the mitogen-activated protein kinase family (16–20). More than 10 isoforms of JNKs have been identified, encoded by three genes (*JNK1*, *JNK2*, and *JNK3*), with additional diversification resulting from alternative processing of transcripts (21–26). The JNK1 and JNK2 subfamilies each comprise four isoforms that result from the presence of alternative sequences within subdomains IX and X of the catalytic domain and alternative mRNA splicing at the extreme COOH terminus resulting in 46-kDa (p46) or 55-kDa (p55) proteins. The two members of the JNK3 subfamily (45–48 kDa and 54–57 kDa) possess extended NH_2-terminal sequences and alternatively spliced COOH termini (21). Whereas JNK1 and JNK2 are expressed in most cell types (26), expression of JNK3 appears limited to neuronal cells (21).

All JNKs possess a conserved TPY tripeptide motif in the kinase subdomain VIII (25), and their activation is mediated by phosphorylation of the threonine and tyrosine residues within this motif by the upstream JNK kinases MKK4 (27–29) and MKK7 (30). Among the known substrates of the JNKs are the transcription factors c-Jun (31), ATF2 (32), Elk1 (33), and p53 (34). Although all JNKs are capable of phosphorylating c-Jun on serine residues 63 and 73, isoforms exhibit different substrate binding specificities (21, 26, 35). The p21 Ras protein interacts specifically with both JNK and c-Jun (36). JNKs are stimulated in response to Ras activation, although the most marked physiological activation of JNK isozymes appears not to be mediated by Ras (22, 37). Cells deficient in the tyrosine kinase c-Abl fail to activate JNKs in response to ionizing radiation or alkylating agents (7, 38, 39), and the ATM protein has been shown to phosphorylate and activate c-Abl in response to DNA damage (2, 3).

Activation of the JNK signaling pathway by ionizing radiation is defective in AT lymphoid cells, but JNK activation in response to UV light or anisomycin remains intact in these cells (6). We have now investigated the biochemical events that underlie activation of c-Jun in response to ionizing radiation in normal human fibroblasts and have characterized the signaling defect in AT fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Antibodies specific for c-Jun phosphorylated on serines 63 or 73 were obtained from New England BioLabs; antibodies to AP1 (c-Jun) were from Transduction Laboratories; antibodies specific for

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[1] The abbreviations used are: AT, ataxia telangiectasia; JNK, c-Jun NH_2-terminal kinase; GST, glutathione *S*-transferase; Gy, gray(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

JNK phosphorylated on the threonine and tyrosine residues within the TPY motif were from Promega; and antibodies to JNK1 (C-17), JunD, or JunB were from Santa Cruz. A GST-c-Jun(1–89) fusion protein was obtained from New England BioLabs, and GST-c-Jun(1–79) was from Stratagene. The purified active dually phosphorylated form of JNK1α was supplied by T. Osterfield at New England BioLabs. T4 polynucleotide kinase and the double-stranded oligonucleotides AP1 and SP1 were from Promega. Poly(dI-dC):poly(dI-dC) was from Amersham Pharmacia Biotech, and precast polyacrylamide minigels (10–20%) were from Novex or Daiichi.

Cell Culture—Normal human fibroblasts (MRC5CV1) and AT fibroblasts (AT5BIVA, AT4BIVA, and AT3BIVA) were cultured at 37 °C under 5% CO_2 in Eagle's minimum essential medium supplemented with 15% (normal cells) or 20% (AT cells) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml). Hydrocortisone (5 μg/ml) was also included for AT cells. All of the cell lines used in this study were immortalized with simian virus 40 (40) and were checked for mycoplasma contamination every 6 months. Before exposure of cells to ionizing radiation or UV-C light, the culture medium was replaced with serum-free medium for 24 h. Irradiation was performed at room temperature using a JL Shepherd Mark I Radiator with a ^{137}Cs source emitting at a fixed dose rate of 3.83 Gy/min. UV-C light was delivered at a rate of 60 J/m^2 using the UV Stratalinker.

Subcellular Fractionation—Cells were washed three times with ice-cold phosphate-buffered saline, harvested by scraping from the culture dishes into ice-cold equilibrium buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, and protease inhibitors) (41), and disrupted by 100 strokes (pestle B) with a Dounce homogenizer. The nuclear fraction was isolated by centrifugation of the total cell homogenate at 800 $\times$ *g* for 5 min, resuspension of the resulting pellet in equilibrium buffer, and centrifugation of the resulting suspension at 600 $\times$ *g* for 15 min. The resulting nuclear pellet was washed two times and then solubilized with 1% CHAPS detergent on ice overnight. The cytosolic fraction was isolated by centrifugation of the 10,000 $\times$ *g* supernatant at 25,000 $\times$ *g* for 2 h. Protein concentration was determined with the Bradford protein assay (42), with bovine serum albumin as a standard.

Immunoblot Analysis—Subcellular fractions were denatured by boiling for 5 min in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis on 10–20% gels, after which the separated proteins were transferred to a polyvinylidene difluoride membrane. After transfer, the membrane was incubated with 5% nonfat dry milk in phosphate-buffered saline for 1 h at room temperature and then with primary antibodies overnight at 4 °C. Immune complexes were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Protein Kinase Assays—For immune complex kinase assays, nuclear or cytosolic proteins were incubated at 4 °C overnight with antibodies to JNK1 (C-17) and then for an additional 2 h in the presence of UltraLink Protein A/G Plus (Pierce). Immune complexes were washed four times with IgG binding buffer (Pierce), twice with equilibrium buffer, and once with kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, and 0.1 mM sodium orthovanadate). The complexes were then incubated for 15 min at 30 °C in 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; NEN Life Science Products), 50 μM ATP, and 2 μg of GST-c-Jun(1–79). The reaction was terminated by the addition of 5 $\times$ SDS sample buffer and boiling for 5 min, and proteins were resolved by SDS-polyacrylamide gel electrophoresis on 10–20% gels. The gels were stained with Coomassie Blue to confirm equal loading, dried, and analyzed by autoradiography. Alternatively, JNK activity was determined by incubation of nuclear proteins for 20 min at 30 °C with 4 μg of GST-c-Jun(1–89) in 20 μl of kinase buffer containing 20 μM ATP. Phosphorylated GST-c-Jun or endogenous c-Jun was detected by immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73.

Electrophoretic Mobility Shift Assay—A double-stranded oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) specific for AP1 binding was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at 37 °C for 30 min. The reaction was terminated by the addition of EDTA. Nuclear extracts (5 μg) were incubated for 10 min at room temperature with binding buffer (10 mM Tris-HCl, pH 7.5, poly(dI-dC):poly(dI-dC) (0.025 mg/ml), 5 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, and 4% glycerol) in a final volume of 11.5 μl and then for an additional 10 min at 4 °C to allow AP1 dimerization. After the addition of 1 μl of ^{32}P-labeled AP1 oligonucleotide (~35,000 cpm, 0.035 pmol), the reaction mixture was incubated for an additional 30 min at room temperature and then subjected to polyacrylamide gel electrophoresis on a 5% gel in 0.5 $\times$ Tris borate-EDTA buffer. Electrophoresis was continued until the dye front had migrated over 75% of the gel. A 50-fold excess of either unlabeled AP1 oligonucleotide or a control nonspecific oligonucleotide (SP1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′) or antibodies to c-Jun, to JunD, or to JunB were added to the dimerization reaction for determination of specific binding or "supershift" analysis, respectively.

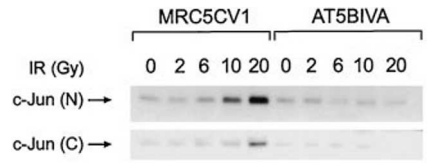

FIG. 1. **Effects of ionizing radiation on c-Jun expression in normal human fibroblasts (MRC5CV1) and AT cells (AT5BIVA).** Cells were exposed to the indicated doses of ionizing radiation (*IR*) and harvested 2 h later. Nuclear (*N*) and cytosolic (*C*) proteins were isolated and subjected to immunoblot analysis with antibodies to c-Jun.

RESULTS

Effects of Ionizing Radiation on c-Jun Expression and Phosphorylation—Regulation of the transcription factor AP1 is achieved by changes in the expression of members of the Jun and Fos families of proteins (43, 44) and by their post-translational modifications by phosphorylation (45). We first investigated the effects of ionizing radiation on c-Jun expression. MRC5CV1 (control), and AT5BIVA fibroblasts were exposed to various doses of ionizing radiation and harvested 2 h later. Nuclear and cytosolic proteins were then isolated and subjected to immunoblot analysis with antibodies to c-Jun. Expression of c-Jun was detected predominantly in the nuclear fractions of both cell types (Fig. 1). However, whereas ionizing radiation increased the amount of nuclear c-Jun in MRC5CV1 cells in a dose-dependent manner, with the maximal effect apparent at 20 Gy, it had no such effect on AT5BIVA cells (Fig. 1). The time course of the effect of ionizing radiation on c-Jun expression was then examined in MRC5CV1 cells and in AT fibroblasts (AT5BIVA, AT4BIVA, and AT3BIVA) derived from different patients (Fig. 2). The amount of c-Jun in the nucleus was increased within 30 min and reached a maximum within 2–4 h in MRC5CV1 cells, whereas no induction was observed in AT5BIVA or AT3BIVA cells. The amount of nuclear c-Jun in AT4BIVA cells was increased at 30 min and further increased at 1–2 h, although the extent of induction was much less than that apparent in MRC5CV1 cells.

We next determined the phosphorylation status of these serine residues after exposure of cells to ionizing radiation by performing immunoblot analysis with antibodies specific for c-Jun phosphorylated on either serine 63 or serine 73 (Fig. 2). In MRC5CV1 cells, ionizing radiation induced the phosphorylation of nuclear c-Jun on both serines 63 and 73 in a time-dependent manner. The phosphorylation of c-Jun on each of the two serine residues was apparent within 30 min and maximal within 2–4 h. In contrast, ionizing radiation did not induce phosphorylation of c-Jun in any of the AT cell lines. UV-C irradiation induced phosphorylation of c-Jun on both serine residues in both normal and AT cells (Fig. 2*C*), consistent with previous reports (6).

Effect of Ionizing Radiation on JNK Expression—To investigate whether the impaired c-Jun phosphorylation in AT cells was due to a defect in JNK expression, we examined the effect of ionizing radiation on JNK abundance in nuclei and cytosol by immunoblot analysis with polyclonal antibodies that recognize the COOH terminus of the p46 isoform of JNK1 (Fig. 3). Substantial amounts of p46 JNK isoforms were present in both nuclei and cytosol of both MRC5CV1 and AT cells, and ionizing radiation did not further increase JNK expression. Similar results were obtained by immunoblot analysis with antibody (New England Biolabs) to the full-length p55 isoform of JNK1

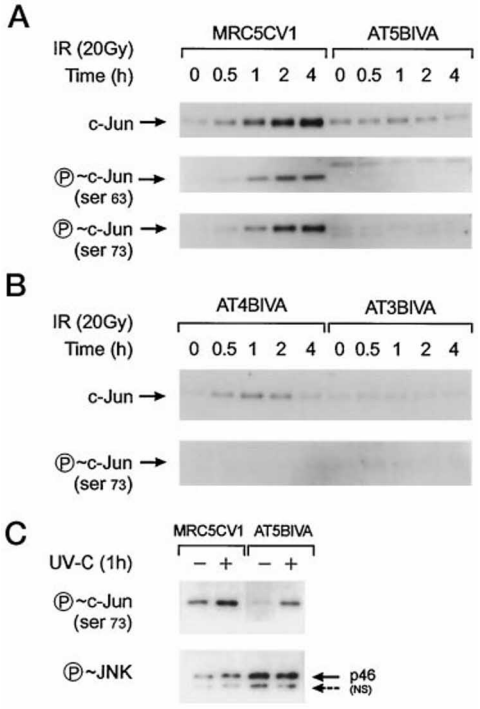

FIG. 2. **Effects of ionizing radiation and UV-C light on the expression and phosphorylation of c-Jun in normal human fibroblasts and AT cells.** MRC5CV1 and AT5BIVA cells (*A*) and AT4BIVA and AT3BIVA cells (*B*) were exposed to 20 Gy of ionizing radiation (*IR*) and harvested at the indicated times thereafter. Nuclear proteins were subjected to immunoblot analysis with antibodies specific for total c-Jun (both phosphorylated and nonphosphorylated forms; designated as c-Jun), for c-Jun phosphorylated on serine 63, and for c-Jun phosphorylated on serine 73. *C*, MRC5CV1 and AT5BIVA cells were exposed to 60 J/m^2 of UV-C light and harvested 1 h later, after which nuclear proteins were isolated and subjected to immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73 or antibodies specific for JNKs phosphorylated on threonine 183 and tyrosine 185.

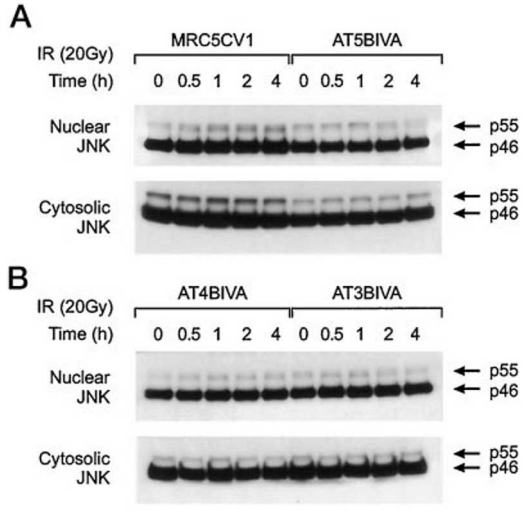

FIG. 3. **Effect of ionizing radiation on JNK expression.** MRC5CV1 and AT5BIVA cells (*A*) and AT4BIVA and AT3BIVA cells (*B*) were exposed to 20 Gy of ionizing radiation (*IR*) and harvested at the indicated times thereafter. Nuclear and cytosolic proteins were then subjected to immunoblot analysis with antibodies to the p46 isoform of JNK1.

(data not shown). The sequence of the peptide used for generation of the antibodies to the p46 isoform of JNK1 is 70, 59, 41, 82, and 53% identical to the corresponding sequences of p55 JNK1, p46 JNK2, p55 JNK2, p46 JNK3, and p55 JNK3, respectively (46); therefore, it is likely that p46 JNK1, which is a major JNK1 isoform (31), was preferentially detected in our

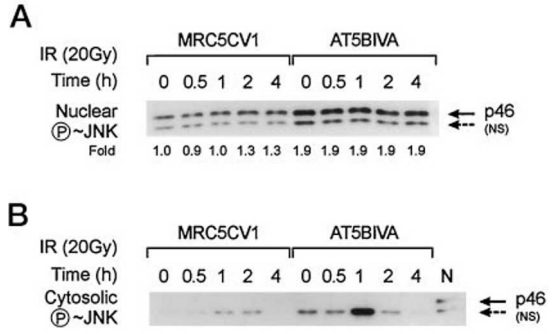

FIG. 4. **Effect of ionizing radiation on JNK phosphorylation.** MRC5CV1 and AT5BIVA cells were exposed to 20 Gy of ionizing radiation (*IR*) and harvested at the indicated times thereafter. Nuclear (*A*) and cytosloic (*B*) proteins were then subjected to immunoblot analysis with antibodies that recognize JNKs phosphorylated on threonine 183 and tyrosine 185. The dually phosphorylated p46 isoform of JNKs is indicated with a *solid arrow*. The *broken arrow* (*NS*) indicates an unknown immunoreactive protein of higher mobility than p46. The position of the phosphorylated JNK band for cytosolic proteins was clarified by electrophoresis of nuclear proteins from MRC5CV1 cells (*lane N*) on the same gel.

immunoblot analysis with these antibodies. The p55 isoform of JNK2 has been shown to exhibit a higher kinase activity and binding affinity for c-Jun than does p46 JNK1 (26). However, JNK2 protein expression was not detected in the cells used in the present study (data not shown).

Constitutive Expression of Dually Phosphorylated JNKs in Nuclei of MRC5CV1 and AT Cells—The activation of JNKs is mediated by phosphorylation of the threonine and tyrosine residues within the TPY motif (27–30). Therefore, we compared the phosphorylation patterns of JNKs in MRC5CV1 and AT5BIVA cells after exposure to ionizing radiation. Nuclear and cytosolic proteins were subjected to immunoblot analysis with antibodies specific for the active form of dually phosphorylated JNKs (Fig. 4). The p46 isoforms of dually phosphorylated JNKs were constitutively expressed in the nuclei but not in the cytosol of both MRC5CV1 and AT5BIVA cells. The levels of constitutively phosphorylated nuclear JNKs in the AT cells were greater than that in the control cells. Ionizing radiation did not change the abundance of dually phosphorylated JNKs in either cell type. Phosphorylated p55 JNK isoforms were not detected in control or AT cells exposed to ionizing radiation. The fact that they were detected in cells stimulated by $CdCl_2$ (data not shown) suggests that this observation was not the result of preferential selectivity of the antibodies for phosphorylated p46 isoforms. The subcellular localization and phosphorylation states of JNKs appear normal in AT cells.

Effect of Ionizing Radiation on JNK Activity—To examine directly the effect of ionizing radiation on JNK activity, we performed immune complex kinase assays using antibodies to JNK1 and GST-c-Jun(1–79) as the substrate. A 3-fold increase in nuclear JNK activity was observed 1 h following irradiation in MRC5CV1 cells but not in AT5BIVA cells (Fig. 5*A*). Whereas immune complexes derived from 10 μg of nuclear proteins from control cells were sufficient to demonstrate this increase in JNK activity, kinase activity was not detectable in immune complexes prepared from up to 100 μg of cytosolic proteins from irradiated MRC5CV1 cells (Fig. 5*A*). To exclude the possibility that the increase in nuclear JNK activity was due to the translocation of activated JNKs from the cytosol to the nucleus, we also measured kinase activities in extracts of cells prepared 30 min after irradiation. Again, no increases in cytosolic JNK activity were detected (data not shown).

The phosphorylation of c-Jun on serines 63 and 73 *in vitro* is catalyzed by all known JNK isoforms. However, different sub-

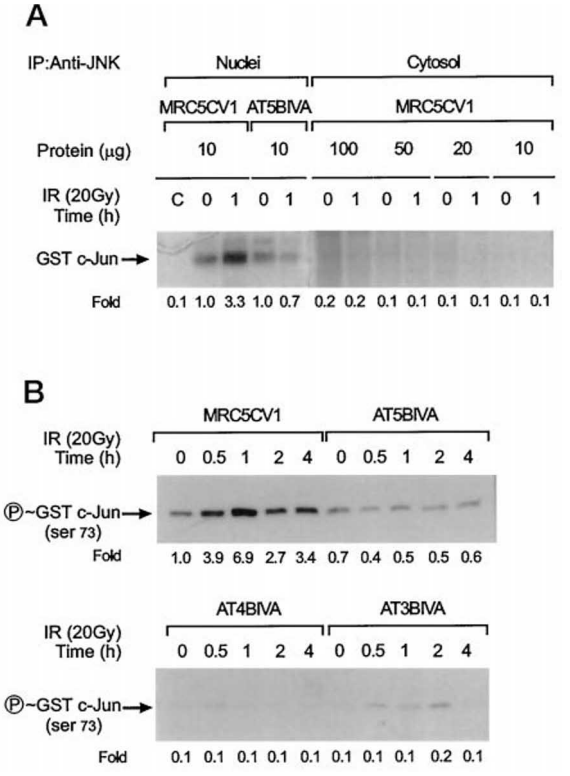

FIG. 5. **Effects of ionizing radiation on JNK activity in the nucleus and cytosol of normal human fibroblasts and AT cells.** Cells were exposed to 20 Gy of ionizing radiation (*IR*) and harvested at the indicated times thereafter. Nuclear and cytosolic proteins were isolated. *A*, immune complex kinase assay. Nuclear proteins (10 μg) or the indicated amounts of cytosolic proteins were subjected to immunoprecipitation (*IP*) with antibodies to JNK1, and the resulting precipitates were assayed for kinase activity with GST-c-Jun(1–79) as substrate. As a control (*lane C*), nuclear proteins from nonirradiated MRC5CV1 cells were subjected to immunoprecipitation with preimmune rabbit IgG. *B*, *in vitro* phosphorylation of GST-c-Jun by nuclear proteins. Nuclear proteins (10 μg) were incubated with GST-c-Jun(1–89) in a kinase reaction, and the phosphorylated substrate was detected by immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73.

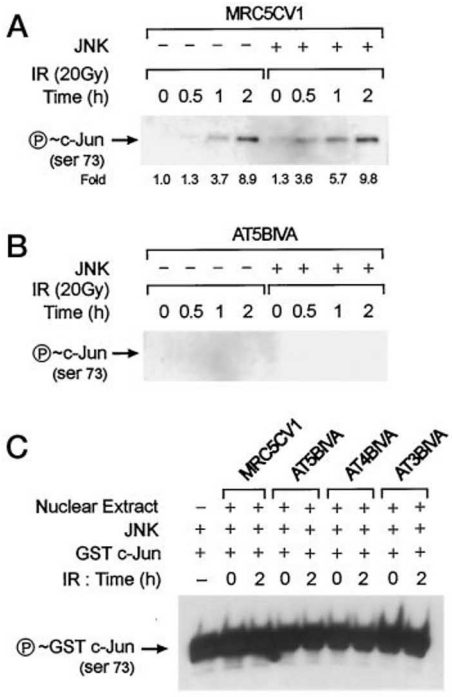

FIG. 6. **Effects of purified active JNK1α on the phosphorylation state of endogenous c-Jun and GST-c-Jun.** Either GST-c-Jun(1–89) or nuclear proteins isolated from MRC5CV1 cells (*A*) or AT5BIVA cells (*B*) at the indicated times after exposure to ionizing radiation (*IR*) were subjected to an *in vitro* phosphorylation reaction in the absence or presence of purified active JNK1α. *C*, *in vitro* phosphorylation of GST-c-Jun by purified active JNK1α was analyzed in the absence or presence of nuclear extracts prepared from cells at the indicated times after irradiation. Phosphorylated endogenous c-Jun (*A* and *B*) and GST-c-Jun (*C*) were detected by immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73.

sets of JNKs are thought to be selectively activated in cells depending on the signaling pathways triggered by a given stimulus (21). Because of the sequence homologies among JNK isoforms, antibodies are not strictly specific to a particular isoform. JNK activity following ionizing radiation in intact cells may represent isoform overlap. Furthermore, demonstration of phosphorylation of c-Jun on serines 63 and 73 by a phorbol 12-myristate 13-acetate-inducible 67-kDa protein indicates the existence of kinases other than JNKs that phosphorylate the NH_2-terminal region of c-Jun (47, 48).

We next examined the effect of ionizing radiation on total c-Jun NH_2-terminal kinase activity using kinase reactions containing nuclear proteins and GST-c-Jun(1–89). The phosphorylated GST-c-Jun was detected by immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73 (Fig. 5*B*). Induction of nuclear c-Jun kinase activity was apparent within 30 min of irradiation of MRC5CV1 cells, reached a maximum within 1 h, and remained increased after 2 and 4 h. In contrast, ionizing radiation had no effect on nuclear c-Jun kinase activity in any of the three AT cell lines. Similar results were obtained with a solid phase kinase assay in which GST-c-Jun(1–89) was immobilized on GSH-Sepharose beads (data not shown).

JNK activity following ionizing radiation was not observed in cytosolic fractions from MRC5CV1 or AT5BIVA cells using *in vitro* kinase assays with GST-c-Jun(1–89) (data not shown). Cytosolic JNK kinase activity was also measured using the maltose binding protein-JNK fusion protein (New England Biolabs) as a substrate, and no activity was observed following ionizing radiation of MRC5CV1 or AT5BIVA cells (data not shown). We interpret these data to support a c-Jun phosphorylating ionizing radiation-induced signaling pathway to be localized in the nucleus.

A Nuclear Signal Required for Ionizing Radiation-induced c-Jun Phosphorylation—To further investigate the mechanism by which phosphorylated JNK isoforms are regulated, we performed *in vitro* kinase assays using a purified active form of dually phosphorylated JNK1α and c-Jun in AT and normal cell nuclear extracts at various intervals after irradiation. Phosphorylated endogenous c-Jun was detected by immunoblot analysis with antibodies specific for c-Jun phosphorylated on serine 73 (Fig. 6). The dual phosphorylation of purified JNK1α was confirmed by immunoblot analysis with antibodies that recognize only dually phosphorylated JNKs (data not shown).

Purified active JNK1α potentiated phosphorylation of endogenous c-Jun in nuclear extracts prepared from MRC5CV1 cells 30 min or 1 h after irradiation. However, phosphorylation of endogenous c-Jun was not observed in nuclear extracts prepared from MRC5CV1 cells at time 0 (Fig. 6*A*). In AT cells, the active JNK1α did not phosphorylate endogenous c-Jun in nuclear extracts prepared from either irradiated or nonirradiated cells (Fig. 6*B*). The marked phosphorylation of GST c-Jun by purified active JNK1α was not inhibited by nuclear extracts prepared from either control (MRC5CV1) or AT cells (AT5BIVA, AT4BIVA, and AT3BIVA) immediately and 2 h following irradiation (Fig. 6*C*), indicating that a nonspecific inhibitor is not present in the nuclear extracts.

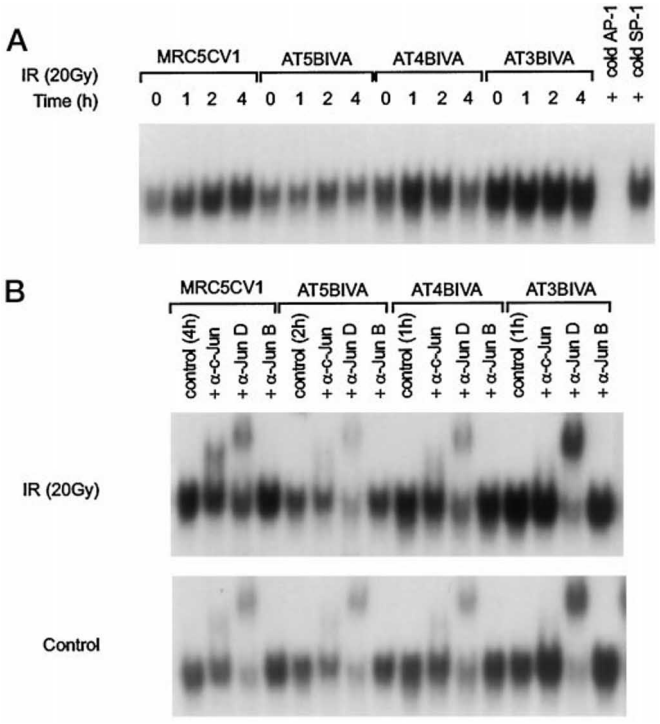

FIG. 7. **Effects of ionizing radiation on the DNA binding activity of AP1 in normal human fibroblasts and AT cells.** *A*, cells were harvested at the indicated times after exposure to ionizing radiation (20 Gy), and nuclear proteins (5 μg) were subjected to an electrophoretic mobility shift assay with a ^{32}P-labeled oligonucleotide containing the AP1 binding site. The specificity of binding was demonstrated with the use of nuclear proteins isolated from MRC5CV1 cells 4 h after irradiation and an excess of either unlabeled (cold) AP1 oligonucleotide or unlabeled SP1 (nonspecific) oligonucleotide. *B*, nuclear proteins isolated from nonirradiated (*Control*) or irradiated (*IR*) cells at the indicated times were subjected to supershift analysis with antibodies to c-Jun, to JunD, or to JunB.

Taken together, these data suggest that a nuclear signal induced by ionizing radiation is required to activate dually phosphorylated JNKs for c-Jun phosphorylation. However, ionizing radiation-induced activation of this nuclear signal is defective in AT fibroblasts.

Effect of Ionizing Radiation on DNA Binding Activity of AP1—The transcription factor AP1 is comprised of homo- or heterodimeric complexes formed by Fos and Jun families of proteins (49, 50). AP1 mediates transcriptional activation by interacting with its DNA target sequence, TGA(C/G)TCA, which is known as the TPA response element (51–56). Subsequently, many other promoter and enhancer regions of genes have been shown to contain AP1 binding sites.

We measured the DNA binding activity of AP1 in nuclear extracts of irradiated cells by electrophoretic mobility shift assay with a ^{32}P-end-labeled oligonucleotide containing the AP1 target sequence (Fig. 7*A*). Ionizing radiation induced an increase in the DNA binding activity of AP1 that was apparent for up to 4 h in MRC5CV1 cells. AT5BIVA and AT3BIVA cells showed no such increase in AP1 binding activity, whereas AT4BIVA cells showed a substantial increase that was apparent within 1 h of irradiation, consistent with the radiation-induced increase in c-Jun expression observed in these cells (Fig. 2*B*).

To determine the components of the AP1 binding complexes, we performed supershift assays with nuclear proteins isolated at the time of maximal binding activity for each cell line using antibodies to c-Jun, JunD, and JunB (Fig. 7*B*). Dimeric complexes containing c-Jun and JunD were induced within 4 h as compared with the control (sham irradiation) in MRC5CV1 cells. c-Jun did not appear to be a major component of AP1 in any of the AT cell lines; however, JunD was constitutively present in the binding complexes of all three lines. Complexes containing JunB were not detected either in MRC5CV1 or in AT fibroblasts. Immunoblot analysis of JunD and JunB supported these observations (data not shown). In addition, AP1 complexes containing c-Fos were not detected, apparently because of the low level of c-Fos expression in both normal and AT fibroblasts as determined by immunoblot analysis (data not shown).

DISCUSSION

Previous studies have shown that ionizing radiation-induced activation of the JNK signaling pathway is defective in lymphoblastoid cells derived from individuals with ataxia telangiectasia (6). Although ATM is believed to play a role in activation of this signaling pathway in response to ionizing radiation (2, 3, 57), the biochemical mechanism is not known. We have now characterized the biochemical events leading to the phosphorylation of c-Jun in response to ionizing radiation in normal human fibroblasts and have identified defects in this pathway in AT fibroblasts.

We have confirmed previous reports that ionizing radiation induces nuclear c-Jun kinase activity in normal human fibroblasts but not in AT cells (6). However, *in vitro* kinase activity does not always reflect kinase activity in cells. Our data demonstrate that ionizing radiation increases the phosphorylation of c-Jun on serines 63 and 73 in intact normal human fibroblasts but not in AT fibroblasts, whereas UV light radiation-induced phosphorylation on these residues of c-Jun is present in both cells. Interestingly, Devary *et al.* (13, 58) have shown, with the use of enucleated cells, that the UV light response does not require a signal generated in the nucleus and is likely to be initiated at or near the plasma membrane. These studies have also shown that UV light-mediated JNK activity occurs in cytoplasts. Taken together, these observations suggest that the ionizing radiation-mediated JNK signaling pathway through AP1 activation is independent of that mediated by UV radiation.

Members of the JNK kinase family are activated by dual phosphorylation on the threonine and tyrosine residues within the TPY motif catalyzed by cytosolic kinases MKK7 (30) or MKK4 (27–29). The dually phosphorylated JNKs translocate from the cytosol to the nucleus, where they phosphorylate the effector protein c-Jun. However, our results indicate that JNK activation by ionizing radiation takes place exclusively in the nucleus, independently of upstream cytosolic signaling pathways. This conclusion is based on our observations that: (i) an immune complex kinase assay with cytosolic extracts prepared from normal human fibroblasts did not show increase in JNK activity in response to ionizing radiation; (ii) phosphorylations of GST-c-Jun(1–89) or maltose binding protein-JNK fusion proteins *in vitro* by cytosolic proteins isolated from MRC5CV1 cells were unaffected by cell irradiation (data not shown); and (iii) dually phosphorylated JNKs were not detected in the cytosol of untreated or irradiated MRC5CV1 cells. Our data are consistent with recent reports indicating the existence of a mechanism for JNK activation that is independent of MKK4 (59–61).

We have shown that both normal and AT fibroblasts constitutively express dually phosphorylated JNKs in the nucleus. Indeed, the levels of nuclear phosphorylated JNKs were consistently greater for AT cells than for normal cells. Furthermore, ionizing radiation did not induce changes in the concentrations of dually phosphorylated JNKs in the nuclei of normal or AT fibroblasts. To address the apparent discrepancy between these observations and the fact that ionizing radiation induced phosphorylation on serines 63 and 73 of c-Jun in normal fibroblasts but not in AT cells, we investigated the effect of purified active JNK1α to nuclear extracts isolated from normal and AT fibroblasts at various times after irradiation. The ex-

ogenously active JNK1α was able to phosphorylate endogenous c-Jun only in nuclear extracts isolated from irradiated normal fibroblasts. These data show that both dually phosphorylated JNKs and c-Jun that are present in the nuclei of normal fibroblasts are subject to negative regulation that is relieved by exposure of cells to ionizing radiation.

In further support of this hypothesis, inhibition of radiation-induced signaling through AP1 activation has been previously demonstrated (62). Possible roles for p21 Waf1 (Cip1) (10), Hsp70 (63, 64), mitogen-activated protein kinase phosphatase (65), and IP1 (66) as inhibitors of JNK activity have also been suggested. In this model, the ATM protein is functionally associated with nuclear c-Abl, which is activated in response to ionizing radiation (2, 3), and overexpression of full-length ATM can normalize the radiosensitivity and impaired *in vitro* JNK activity in AT cells (57). On the basis of our data, we propose that ionizing radiation activates a nuclear signal that acts through ATM and c-Abl and enables dually phosphorylated JNKs to phosphorylate c-Jun by inducing the dissociation of a negative regulator such as p21 Waf1. Although increased total c-Jun was observed in AT4BIVA cells, DNA binding activities of AP1 are attributable mainly to JunD. However, JunD is known to be a poor substrate for JNKs (67).

A possible role for protein kinase C in c-Jun activation has also been suggested. Dephosphorylation of one or more serine residues in the C-cluster of c-Jun induced by the protein kinase C activator phorbol 12-myristate 13-acetate results in an increase in the DNA binding and trans-activation activity of c-Jun (68). Ionizing radiation induced protein kinase C expression in both MRC5CV1 and AT4BIVA cells (data not shown). Therefore, the ionizing radiation induced increases in total c-Jun expression and in the DNA binding activity of AP1 in AT4BIVA cells may be mediated by an increase in expression of protein kinase C.

In conclusion, ionizing radiation appears to activate a nuclear signal that relieves negative regulation of both JNKs and c-Jun in normal fibroblasts. Activation of this nuclear signal by ionizing radiation appears defective in AT cells.

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REFERENCES

1. Brown, L., and McCarthy, N. (1997) *Nature* **387,** 450–451
2. Baskaran, R., Wood, L. D., Whitaker, L. L., Cannoan, C. E., Morgan, S. E., Xu, Y., Balrow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M. B., and Wang, J. Y. J (1997) *Nature* **387,** 516–519
3. Shafman, T., Khanna, K. K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatel, M., Zhang, N., Watters, D., Egerton, M., Shiloh, Y., Kharbanda, S., Kufe, D., and Lavin, M. F. (1997) *Nature* **387,** 520–523
4. Jung, M., Zhang, Y., Lee, S., Dritschilo, A. (1995) *Science* **268,** 1619–1621
5. Minden, A., Lin, A., Smeal, T., Dérijard, B., Cobb, M., Davis, R., and Karin, M. (1994) *Mol. Cell. Biol.* **14,** 6683–6688
6. Shafman, T. D., Saleem, A., Kyriakis, J., Weichselbaum, R., Kharbanda, S., and Kufe, D. W. (1995) *Cancer Res.* **55,** 3242–3245
7. Kharbanda, S., Ren, R., Pandey, P., Shafman, T. D., Feller, S. M., Weichselbaum, R. R., and Kufe, D. W. (1995) *Nature* **376,** 785–788
8. Claret, F. X., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996) *Nature* **383,** 453–457
9. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) *Science* **277,** 693–696
10. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E. J. (1996) *Nature* **381,** 804–807
11. Smeal, T., Hibi, M., and Karin, M. (1994) *EMBO J.* **13,** 6006–6010
12. Binetruy, B., Smeal, T., and Karin, M. (1991) *Nature* **351,** 122–127
13. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) *Cell* **71,** 1081–1091
14. Radler-Pohl, A., Sachsenmaier, C., Gebel, S., Auer, H. P., Bruder, J. T., Rapp, U., Angel, P., Rahmsdorf, H. J., and Herrlich, P. (1993) *EMBO J.* **12,** 1005–1012
15. Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M., and Karin, M. (1991) *Nature* **354,** 494–496
16. Kyriakis, J. M., and Avruch, J. (1990) *J. Biol. Chem.* **265,** 17355–17363
17. Kyriakis, J. M., Brautigan, D. L., Ingebritsen, T. S., and Avruch, J. (1991) *J. Biol. Chem.* **266,** 10043–10046
18. Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Palech, S., Sanghera, J., and Avruch, J. (1992) *J. Biol. Chem.* **267,** 3325–3335
19. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
20. Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* **271,** 24313–24316
21. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Dérijard, B., and Davis, R. J. (1996) *EMBO J.* **15,** 2760–2770
22. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
23. Mohit, A. A., Martin, J. H., and Miller, C. A. (1995) *Neuron* **14,** 67–78
24. Kyriakis, J. M., Woodgett, J. R., and Avruch, J. (1995) *Ann. N. Y. Acad. Sci.* **766,** 303–319
25. Dérijard, B., Hibi, M., Wu, I., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76,** 1025–1037
26. Kallunki, T., Su, B., Tsigelny. I., Sluss, H. K., Dérijard, B., Moore, G., Davis, R., and Karin, M. (1994) *Genes Dev.* **8,** 2996–3007
27. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372,** 794–798
28. Dérijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and David, R. J. (1995) *Science* **267,** 682–685
29. Lin, A., Minden, A., Martinetto, H., Claret, F. X., Langer-Carter, C., Mercurio, F., Johnson, G. L., and Karin, M. (1995) *Science* **268,** 286–290
30. Tournier, C., Whitmarsh, A. J., Caranagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7337–7342
31. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7,** 2135–2148
32. Gupta, S., Campbell, D., Dérijard, B., and Davis R. J. (1995) *Science* **267,** 389–393
33. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) *Science* **269,** 403–407
34. Milne, D. M., Campbell, L. E., Campbell, D. G., and Meek, D. W. (1995) *J. Biol. Chem.* **270,** 5511–5518
35. Sluss, H. K., Barrett, T., Dérijard, B., and Davis, R. J. (1994) *Mol. Cell. Biol.* **14,** 8376–8384
36. Alder, V., Pincus, M. R., Brandt-Rauf, P. W., and Ronai, Z. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10585–10589
37. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) *Science* **226,** 1719–1723
38. Kharbanda, S., Yuan, Z., Weichselbaum, R., and Kufe, D. (1997) *Biochim. Biophys. Acta* **1333,** 1–7
39. Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., and Kufe, D. (1995) *J. Biol. Chem.* **270,** 30278–30281
40. Jung, M., Lee, S. A., Zhang, Y., and Dritschilo, A. (1997) *Int. J. Radiat. Oncol. Biol. Phys.* **37,** 417–422
41. Lee, S. A., Karaszkiewicz, J. W., and Anderson, W. B. (1992) *Cancer Res.* **52,** 3750–3759
42. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
43. Vogt, P. K., and Bos, T. J. (1990) *Adv. Cancer Res.* **55,** 1–35
44. Curran, T., Abate, C., Baker, S., Kerppola, T., and Xanthoudakis, S. (1993) *Adv. Second Messenger Phosphoprotein Res.* **28,** 271–277
45. Hunter, T., and Karin, M. (1992) *Cell* **70,** 375–387
46. Butterfield, L., Storey, B., Maas, L., and Heasley, L. E. (1997) *J. Biol. Chem.* **272,** 10110–10116
47. Alder, V., Polotskaya, A., Wagner, F., and Kraft, A. S. (1992) *J. Biol. Chem.* **267,** 17001–17005
48. Alder, V., Franklin, C. C., and Kraft, A. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5341–5345
49. Angel, P., and Karin, M. (1991) *Biochim. Biophys. Acta* **1072,** 129–157
50. Lallemand, D., Spyrou, G., Yaniv, M., and Pfarr, C. M. (1997) *Oncogene* **14,** 819–830
51. Haslinger, A., and Karin, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8572–8576
52. Karin, M., Haslinger, A., Heguy, A., Dietlin, T., and Cooke, T. (1987) *Mol. Cell. Biol.* **7,** 606–613
53. Lee, W., Haslinger, A., Karin, M., and Tjian, R. (1987) *Nature* **325,** 368–372
54. Lee, W., Mitchell, P., and Tjian, R. (1987) *Cell* **49,** 741–752
55. Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. (1987) *Mol. Cell. Biol.* **7,** 2256–2266
56. Piette, J., Hirai, S., and Yaniv, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3401–3405
57. Zhang, N., Chen, P., Khanna, K. K., Scott, S., Gatei, M., Kozlov, S., Watters, D., Spring, K., Yen, T., and Lavin, M. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8021–8026
58. Devary, Y., Rosette, C., DiDonato, J. A., and Karin, M. (1993) *Science* **261,** 1442–1445
59. Nehmé, A., Baskaran, R., Aebi, S., Fink, D., Nebel, S., Cenni, B., Wang, J. Y. J., Howell, S. B., and Christen, R. D. (1997) *Cancer Res.* **57,** 3253–3257
60. Yang, D., Tournier, C., Wysk, M., Lu, H.-T., Xu, J., Davis, R. J., and Flavell, R. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3004–3009
61. Nishina, H., Fischer, K. D., Radvanyl, L., Shahinian, A., Hakem, R., Ruble, E. A., Bernstein, A., Mark, T. W., Woodgett, J. R., and Penninger, J. M. (1997) *Nature* **385,** 350–353
62. Hallahan, D. E., Gius, D., Kuchibhotla, J., Sukhatme, V., Kufe, D. W., and Weichselbaum, R. R. (1993) *J. Biol. Chem.* **268,** 4903–4907
63. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *J. Biol. Chem.* **272,** 18033–18037
64. Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C., and Massie, B. (1997) *Mol. Cell. Biol.* **17,** 5317–5327
65. Hirsch, D. D., and Stork, P. J. S. (1997) *J. Biol. Chem.* **272,** 4568–4575
66. Auwerx, J., and Sassone-Corsi, P. (1991) *Cell* **64,** 983–993
67. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) *Cell* **87,** 929–939
68. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M., and Hunter, T. (1991) *Cell* **64,** 573–584