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Epidermal Growth Factor Receptor Dependence of Radiation-induced Transcription Factor Activation in Human Breast Carcinoma Cells

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Ionizing radiation (1–5 Gy) activates the epidermal growth factor receptor (EGFR), a major effector of the p42/44 mitogen-activated protein kinase (MAPK) pathway. MAPK and its downstream effector, p90 ribosomal S6 kinase (p90RSK), phosphorylate transcription factors involved in cell proliferation. To establish the role of the EGFR/MAPK pathway in radiation-induced transcription factor activation, MDA-MB-231 human breast carcinoma cells were examined using specific inhibitors of signaling pathways. Gel-shift analysis revealed three different profile groups: 1) transcription factors that responded to both radiation (2 Gy) and epidermal growth factor (EGF) (CREB, Egr, Ets, and Stat3); 2) factors that responded to radiation, but not EGF (C/EBP and Stat1); and 3) those that did not respond significantly to either radiation or EGF (AP-1 and Myc). Within groups 1 and 2, a two- to fivefold maximum stimulation of binding activity was observed at 30–60 min after irradiation. Interestingly, only transcription factors that responded to EGF had radiation responses significantly inhibited by the EGFR tyrosine kinase inhibitor, AG1478; these responses were also abrogated by farnesyltransferase inhibitor (FTI) or PD98059, inhibitors of Ras and MEK1/2, respectively. Moreover, radiation-induced increases in CREB and p90RSK phosphorylation and activation of Stat3 and Egr-1 reporter constructs by radiation were all abolished by AG1478. These data demonstrate a distinct radiation response profile at the transcriptional level that is dependent on enhanced EGFR/Ras/MAPK signaling.

INTRODUCTION

The epidermal growth factor receptor (EGFR or ErbB1) is a member of the ErbB family of receptor Tyr kinases (RTK). These transmembrane proteins are activated by extracellular ligands of the epidermal growth factor (EGF) family, resulting in a cascade of cytoplasmic signaling events. More recently, clinically relevant doses of ionizing radiation in the 1- to 5-Gy dose range can activate EGFR, apparently mimicking GF effects (Goldkorn *et al.*, 1997; Schmidt-Ullrich *et al.*, 1997; Bowers *et al.*, 2001). A consequence of ligand- or radiation-induced stimulation of the EGFR is the activation of the p42/44 mitogen-activated protein kinase (MAPK) cascade (Contessa *et al.*, 1999; Dent *et al.*, 1999; Reardon *et al.*, 1999). We have previously demonstrated that single and repeated radiation exposures can induce a cellular proliferative response in vitro (Kavanagh *et al.*, 1995; Contessa *et al.*, 1999) that is blocked by selective inhibition of EGFR Tyr phosphorylation. This cytoprotective response is likely to represent the underlying mechanism of accelerated repopulation in tumors (Withers *et al.*, 1988), implicating radiation-induced activation of EGFR as the initiating event (Reardon *et al.*, 1999; Schmidt-Ullrich *et al.*, 1999). This conclusion is supported by the findings that inhibition of EGFR function by overexpression of dominant negative EGFR-CD533 or cell treatments with monoclonal antibodies against EGFR (C225) or small molecule ErbB tyrosine kinase inhibitors (CI-1033 and Iressa) results in tumor cell radiosensitization; similar effects are seen when the EGFR downstream effector MEK1/2 is inhibited by exposure of cells to PD98059 (Contessa *et al.*, 1999; Reardon *et al.*, 1999; Saleh *et al.*, 1999; Mendelsohn and Baselga, 2000; Rao *et al.*, 2000; Lammering *et al.*, 2001). However, the molecular mechanisms of radiosensitization through EGFR/MAPK inhibition have to be elucidated in more detail. Considering the role of the EGFR/

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MAPK cascade on accelerated proliferation, we have focused on mechanisms that are involved in the modulation of transcriptional events associated with EGFR and MAPK activation and cellular proliferation control. The mechanistic relationship between radiation-induced signals along the EGFR/MAPK cascade and specific transcriptional events was established through the use of specific functional inhibitors of EGFR, Ras, and MAPK.

Currently, a link between MAPK activation and the phosphorylation and activation of transcription factors involved in proliferation and cell growth is established by the finding that MAPK activates p90 ribosomal S6 kinase (p90RSK; De Cesare *et al.*, 1998; Frodin and Gammeltoft, 1999; Smith *et al.* 1999). MAPK has also been reported to activate several growth-related transcription factors, without mention of p90RSK (Davis, 1995; Lewis *et al.*, 1998). To establish transcription factor response profiles and their dependence on EGFR- and MAPK-mediated signaling, we have selected the following transcription factors for analysis: AP-1, CREB, C/EBP, Egr, Ets, Myc, Stat3, and Stat1 (Davis, 1995; Lewis *et al.*, 1998; McCubrey *et al.*, 2000). The panel was also based on previous reports identifying their response to ionizing radiation (Hallahan *et al.*, 1991; Wilson *et al.*, 1993; Sahijdak *et al.*, 1994; Borovitskaya *et al.*, 1996).

MATERIALS AND METHODS

Reagents

Unless specified otherwise, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 medium and LipofectAMINE PLUS reagent were obtained from Life Technologies (Carlsbad, CA), and fetal bovine serum was purchased from HyClone (Logan, UT). AG1478, PD98059, and farnesyltransferase inhibitor I (FTI), specific inhibitors of EGFR, MEK 1/2, and Ras, respectively, were purchased from Calbiochem (La Jolla, CA). Bradford protein assay reagents were obtained from Bio-Rad (Hercules, CA). All gel shift oligonucleotides and supershift antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and poly dI-dC was obtained from Pharmacia (Piscataway, NJ). Primary antibodies against CREB (also used for CREB supershift), phospho-CREB (Ser 133), p90RSK (Ser 381), or β-actin and horseradish peroxidase-linked secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell Treatments and Irradiation

Culture of MDA-MB-231 human breast carcinoma cells has been previously described (Bowers *et al.*, 2001). Briefly, cells were obtained from American Type Tissue Collection (ATTC, Rockville, MD) and were cultured in RPMI-1640 medium + 5% fetal bovine serum (RPMI/5FBS) + penicillin/streptomycin. Cells were plated for 4 d and then serum-starved overnight (16–18 h, RPMI/0.5%FBS), to achieve 80–90% confluency the day of experiments. Stock solutions of AG1478, PD98059, FTI, and epidermal growth factor (EGF) were stored in aliquots at −20°C. Before irradiation, cells were treated with 5 μM AG1478 (Levitzki and Gazit, 1995) or 10 μM PD98059 (Dudley *et al.*, 1995) at 37°C for 1 h each or 50 μM FTI for 18 h (Manne *et al.*, 1995). Positive controls included treating cells with EGF at 10 ng/ml for various times. Cultures were then exposed to 2 Gy of ionizing radiation at a dose rate of 1.6–1.8 Gy/min (depending on the month of calibration) using a ^{60}Co source, then incubated at 37°C.

Transient Transfections

Plasmid DNA containing Stat3 binding sites with a chloramphenicol acetyl transferase (CAT) reporter gene (3X SIE-CAT) was generously donated by Dr. Timothy Schaefer (Schaefer *et al.*, 2000). A similar reporter construct containing 3X Egr-1 binding sites was provided by Dr. Frank J. Rauscher III. The β-galactosidase reporter plasmid, RSVβgalBSH, was described previously (Valerie *et al.*, 1988). Transient transfections were performed using the LipofectAMINE PLUS reagent. Reporter construct (1 μg) was cotransfected with 1 μg of the β-galactosidase plasmid per dish, using optimum LipofectAMINE PLUS conditions described by the manufacturer. Three hours after transfection in serum-free, antibiotic-free medium, complete medium was added, and dishes were incubated at 37°C for 24 h. Cells were then incubated in RPMI/0.5% FBS for 18 h, treated with radiation or EGF, and incubated at 37°C for various times.

Preparation of Nuclear Extracts

Cells were rinsed twice with ice-cold PBS (all subsequent conditions were ice-cold), removed with a rubber cell scraper in 1 ml PBS, and pelleted in 1.5-ml tubes at 500 × *g* for 5 min using a microcentrifuge. After two more PBS rinses, cells were resuspended in hypotonic buffer I (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 2 mM Mg acetate, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) at 3× pellet volume. Cells were then centrifuged at 1000 × *g* for 3 min, resuspended in 3× pellet volume buffer I, and incubated on ice for 10 min. Cells were disrupted by 10 passes through a 27 G1/2 needle, and the extent of nuclear isolation was monitored microscopically. Nuclei were centrifuged for 5 min at 1000 × *g*, then resuspended in 3× pellet volume hypertonic buffer II (same as buffer I, except 400 mM KCl with 20% glycerol), and kept on ice for 10 min. Samples were centrifuged at 12,000 × *g* for 5 min, and the supernatant (nuclear extracts) was aliquoted and frozen at −80°C. Protein amounts were measured using the Bradford protein assay.

Gel Shift Assays

Gel shift oligonucleotides specific for transcription factor families were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook *et al.*, 1989). Each nuclear extract (5 μg) was mixed with 1 μl of ^{32}P-labeled oligonucleotide probe (~0.1 ng), 1 μl of 0.5 μg/μl poly dI-dC, and buffer II (see above) to make a total volume of 16 μl. Samples were incubated at room temperature for 30 min. Supershift antibodies (2 μg) were added immediately after oligonucleotide/extract incubation and incubated for an additional 15–20 min. Four microliters of 5× Ficoll buffer (20% Ficoll, 10 mM HEPES, 250 mM KCl, 5 mM EDTA, 5 mM DTT, and 1.25 mg/ml bovine serum albumin) was added to each reaction, and 20-μl samples were fractionated on a 5% polyacrylamide-TBE gel at 120 V for 1 h. Dried gels were analyzed by autoradiography.

Western Blotting

Equal amounts of protein were loaded onto 6 or 10% SDS-polyacrylamide gels. Protein was then transferred electrophoretically onto nitrocellulose membranes. Membranes were probed with primary antibodies against CREB, phospho-CREB (Ser 133), p90RSK (Ser 381), or β-actin and horseradish peroxidase-linked secondary antibody according to the manufacturer's instructions. Blots were analyzed by chemiluminescence detection, autoradiography, and densitometry.

CAT and β-Galactosidase Assays

CAT and β-galactosidase (β-gal) assays were performed as previously described (Valerie *et al.*, 1988). Briefly, equal amounts of extracts were incubated with cocktail containing ^{14}C-chloramphenicol and acetyl-CoA. Reactions were extracted with ethyl acetate and spotted onto TLC plates. Plates were then placed in a tank with 95% chloroform-5% methanol for 45 min and exposed overnight to x-ray film. For β-gal assays, equal amounts of protein and resorufin were added to wells, and β-gal activity was measured using a fluorime-

Table 1. EGFR-dependence of transcriptional radiation responses: summary of gel-shift analyses for MDA-MB-231 cells

Transcription factor	Response to 2 Gy, 60 min after radiation	Response to 10 ng/ml EGF 30 min treat.	Inhibition of 2 Gy response by AG1478 (%)	Inhibition of 2 Gy response by FTI (%)	Inhibition of 2 Gy response by PD98059 (%)
CREB	+	+	78	81	100
Egr[a]	+	+	80	86	98
Ets	+	+	50	92	75
Stat3	+	+	92	100	95
C/EBP	+	−[c]	18[b]	50	60
Stat1	+	−	6[b]	33[b]	45[b]
AP-1	−	−	N/A	N/A	N/A
Myc	−	−	N/A	N/A	N/A

All maximum increases and percent inhibition were statistically significant ($p < 0.05$, $n = 3$ experiments), unless otherwise indicated.

[a] Egr was measured at 30 min after radiation, to correspond with the peak activity.

[b] Difference between 2 Gy and 2 Gy plus inhibitor was not statistically significant ($p > 0.05$).

[c] −, Difference between 2 Gy and untreated control was not statistically significant ($p > 0.05$).

ter. CAT activity, calculated by densitometry as % (converted)/(converted + unconverted), was then normalized for the β-gal transfection efficiency.

Statistical Analysis

A two-tailed Student's *t* test was used to determine statistical significance for $n = 3$ independent experiments. $p < 0.05$, as calculated using Sigma-Plot software, was considered statistically significant.

RESULTS

Radiation-induced Binding of Nuclear Protein to Transcription Factor Consensus Sequences

Previous reports have described radiation-induced increases in transcription factor binding to oligonucleotide consensus sequences, as well as increases in expression of the genes encoding these transcription factors, in mammalian cancer cells (Hallahan *et al.*, 1991; Wilson *et al.*, 1993; Sahijdak *et al.*, 1994). However, most of these studies were performed using doses >4 Gy, leading to <50% cell survival. The focus of the present study was to define response profiles of transcriptional responses in their dependence of radiation-induced EGFR and MAPK activation. These experiments focused on gel-shift assays to test for transcription factor activation in MDA-MB-231 cells in response to a radiation dose of 2 Gy, permitting >80% of the cells to furnish EGFR-induced cytoprotective responses.

Radiation-induced significant increases in DNA binding were found for six of eight transcription factors and are summarized in Table 1. The time-dependence profiles for the induction of transcription factor binding by ionizing radiation are shown in Figure 1. No significant changes were observed in the binding of AP-1 or Myc at 30, 60, or 120 min after irradiation; thus, these two transcription factors were not further examined. A time-dependent maximum 3.4-fold increase in the binding of CREB was measured 60 min after irradiation. Similar increases of C/EBP, Ets, Stat1, and Stat3 ranged from 2.6- to 4.6-fold 60 min after irradiation. A maximum 2.0-fold increase occurred with Egr within 30 min of irradiation.

The Role of EGFR in the Radiation-induced Transcription Factor Binding

Previously, we demonstrated that the EGFR is activated by ionizing radiation (1–5 Gy) in several cell lines including MDA-MB-231 mammary carcinoma cells (Schmidt-Ullrich *et al.*, 1997; Dent *et al.*, 1999; Bowers *et al.*, 2001). The radiation-induced increase in EGFR Tyr phosphorylation was completely blocked by the EGFR-specific tyrphostin, AG1478 (Levitzki and Gazit, 1995). Herein, we use AG1478 as an EGFR inhibitor in experiments that measured the radiation-induced binding of nuclear protein to DNA consensus sequences specific for various transcription factor families. Because our previous studies showed that treatment of MDA-MB-231 cells with 10 ng/ml EGF induced both EGFR Tyr phosphorylation and MAPK activation, we have used EGF as a positive control to further test the role of EGFR in these transcriptional responses.

The effects of AG1478 on the radiation-induced maximum responses in C/EBP, CREB, Egr, Ets, Stat1, and Stat3 binding are shown in Figure 2, and the percent inhibition is listed in Table 1. The radiation-induced increase in CREB binding was significantly inhibited by AG1478, at 60 min after radiation. The maximum fold-increases in Egr and Stat3 binding after irradiation were inhibited by AG1478 by ≥80% (Table 1). AG1478 caused partial but significant ($p < 0.05$) inhibition of radiation-induced Ets binding. No significant inhibition by AG1478 was observed for radiation-induced C/EBP or Stat1 binding. Treatment of cells with EGF for 30 min before nuclear extraction resulted in increased binding of CREB, Egr, Ets, and Stat3 (Figure 3). EGF treatment did not stimulate significant binding of AP-1, C/EBP, Myc, or Stat1; interestingly, the binding of these transcription factors was either not significantly inhibited by AG1478 (if radiation-induced) or did not respond to radiation at all. The results from Figures 2 and 3, which are summarized in Table 1, provide evidence that the radiation-induced responses involve the EGFR. The six transcription factors for which radiation responses were observed were chosen for further experimentation, testing the role of Ras and

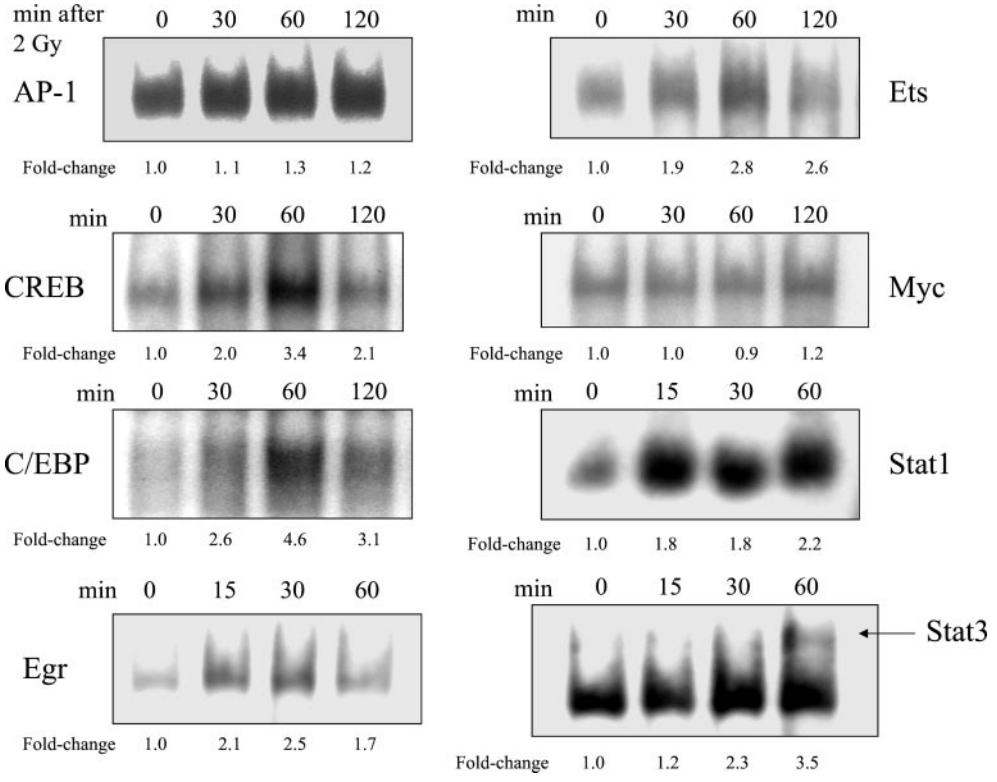

Figure 1. Time-dependent increases in the radiation-induced binding of MDA-MB-231 nuclear extracts to transcription factor consensus sequences. The experimental protocol is described in the legend of Figure 2, except AG1478 treatment was not used in these experiments. Fold-changes represent the mean of three independent experiments.

MAPK as important intermediates between EGFR and transcription factors.

Effect of Ras Inhibition on Radiation-induced Transcription Factor Binding Responses

Several studies have demonstrated that EGFR can activate the Ras/MAPK pathway through cellular exchange factors (Daub *et al.*, 1996; Rojas *et al.*, 1996; O'Bryan *et al.*, 1998). Ras function can be blocked by inhibiting Ras farnesylation, which prevents translocation of Ras to the plasma membrane (Suy *et al.*, 1997). We have previously shown that radiation-induced MAPK activation in MDA-MB-231 cells can be completely inhibited by pretreating cells with FTI (Reardon *et al.*, 1999). In this study, Ras was inhibited by treatment of MDA-MB-231 cells with 50 μM FTI for 18 h before irradiation. The effects of FTI treatment on the binding of transcription factors that responded to radiation are shown in Figure 4, and the corresponding percent inhibition is given in Table 1. The maximum radiation responses of CREB, Egr, Ets, and Stat3, which were each at least twofold above control levels for each transcription factor, were abolished by pretreatment with FTI. Radiation responses for C/EBP and Stat1 were inhibited with FTI by 50 and 33%, respectively, although inhibition of Stat1 was not statistically significant ($p > 0.05$).

Effect of MAPK Pathway Inhibition on Radiation-Induced Transcription Factor Binding

A transient activation of MAPK in MDA-MB-231 cells was observed with radiation in our previous studies, which was inhibited by either PD98059 (MEK 1/2 inhibitor) or AG1478 (Schmidt-Ullrich *et al.*, 1997; Dent *et al.*, 1999; Bowers *et al.*, 2001). To test the role of MAPK activation in radiation-induced transcription factor binding, PD98059 was used to block the MEK/MAPK pathway (Dudley *et al.*, 1995). The effects of PD98059 on the binding of transcription factors that responded to radiation are shown in Figure 5 and Table 1. The maximum radiation response of CREB, Egr, and Stat3 binding was abolished by PD98059; significant abrogation was also observed for Ets because of PD98059 treatment. The response of C/EBP to radiation was significantly inhibited (60%, $p < 0.05$) with PD98059, whereas inhibition of Stat1 was not statistically significant.

To further characterize the transcription factors that were dependent on the EGFR or MAPK, supershift antibodies were added to gel-shift reactions. Results of supershift analysis (Figure 6) demonstrated that Egr-1, Ets-2, and C/EBP-β were probably involved in the Egr, Ets, and C/EBP gel-shift responses, respectively. The CREB and Stat3 supershift antibodies used recognize all forms of these two transcription factors; these antibodies also resulted in supershift effects. No clear supershift response was observed using antibodies against C/EBP-α.

Radiation-induced Phosphorylation of CREB and p90RSK

CREB and p90RSK are activated via phosphorylation at specific amino acid residues. CREB activation occurs upon phosphorylation of Ser 133 (Xing *et al.*, 1996) and p90RSK at Ser 381 (Dalby *et al.*, 1998) in response to growth factors. To

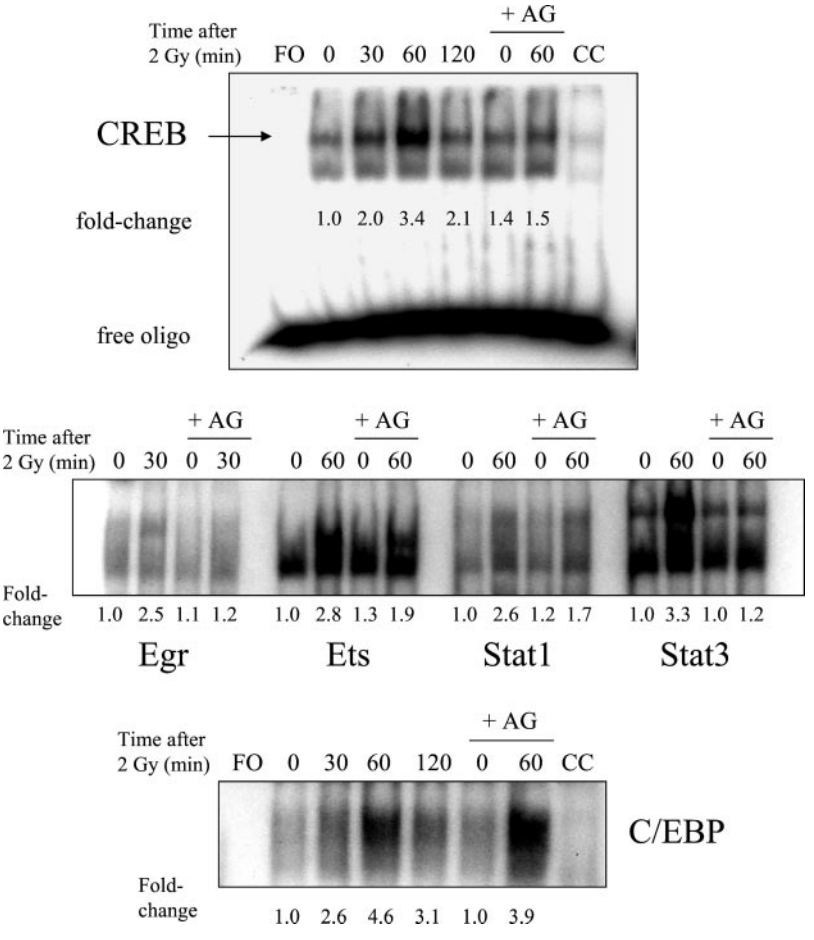

Figure 2. Inhibition of nuclear extract binding to transcription factor consensus sequences by AG1478 (AG). Cells were preincubated with 5 μM AG1478 for 1 h, irradiated with 2 Gy, and nuclear extracts were isolated at various times. Extracts were incubated with ^{32}P-labeled consensus oligonucleotides, run on TBE-PAGE gels, and autoradiography was performed. The free oligo (FO) samples were incubated without nuclear extract. A 20-fold excess of nonradioactive oligonucleotides were added to the 60 min sample as a "cold" competitor (CC). Fold-changes represent the mean of three independent experiments.

test whether phosphorylation-dependent pathways are involved in the radiation response, extracts were probed with antibodies specific for these phosphorylated proteins. A time-dependent increase in the phosphorylation of CREB was observed, with a maximum of 3.1 $\pm$ 0.3 observed at 60 min postirradiation (Figure 7). Under the same conditions, Western blot analysis of the same blots using antibodies against nonphosphorylated CREB (the same antibodies used for CREB supershifts) showed no significant changes in CREB protein levels. AG1478 completely inhibited the radiation-induced increase in CREB phosphorylation. The fold-changes and timing are similar to those measured by gel-shift; thus, these experiments suggest that increased phosphorylation of CREB at Ser 133 may be involved in the radiation-induced binding of CREB to the CRE consensus sequence, which is present in the promoter region of several genes involved in cellular proliferation.

Because CREB activation has been shown to be regulated by p90RSK, the radiation response of p90RSK was examined. Western blot analyses were used in a similar manner as with phospho-CREB, but using antibodies against the phosphorylated form of p90RSK (Ser 381). A time-dependent increase in the radiation response of p90RSK was observed, which reached a maximum of 2.0 $\pm$ 0.3 at 30 min after irradiation (Figure 8A). Probing of the same blots with antibodies against β-actin showed no significant difference in protein loading. To investigate whether radiation-induced p90RSK phosphorylation was dependent on EGFR or MAPK activation, cells were incubated with AG1478 or PD98059 for 1 h before irradiation. Treatment with either inhibitor resulted in abrogation of the radiation-induced increase in p90RSK phosphorylation (Figure 8B). Thus, the data suggests that radiation-induced activation of p90RSK is dependent on EGFR and MAPK activation, which is expected since MAPK activates CREB through this mediator kinase (Xing *et al.*, 1996; DeCesare *et al.*, 1998; Andrisani, 1999).

Activation of Egr-1– and Stat3-directed Reporter Genes by Radiation

To test whether the observed changes in transcriptional activation by radiation could be verified using a functional assay, reporter constructs containing transcription factor binding sites for Egr-1 or Stat3 were transfected into MDA-MB-231 cells. The transfected plasmids contain a chloramphenicol acetyl transferase (CAT) gene; therefore, CAT assays were used to determine the relative levels of transcriptional activation. Because cellular CAT activity can take several hours to accumulate, incubation of cells for 30–60 min after irradiation did not significantly change the activity of either Egr-1 or Stat3. However, when cells were incubated for 3 or 6 h postirradia-

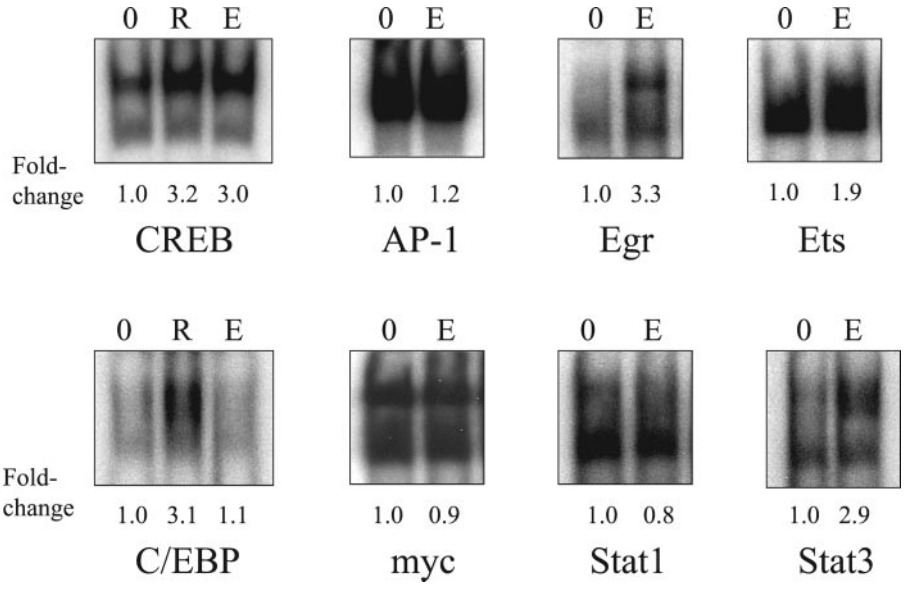

Figure 3. Effect of epidermal growth factor (EGF) treatment on the binding of nuclear extracts to transcription factor consensus sequences. EGF treatments (10 ng/ml) were for 30 min at 37°C before nuclei extraction. For irradiated samples, nuclei were extracted 60 min after a 2-Gy dose. The rest of the protocol is the same as described in the legend of Figure 2. 0, untreated control; R, treatment with radiation alone; E, treatment with EGF alone.

tion immediately before protein extraction, increases in CAT activity were observed with Egr-1 (Figure 9A) and Stat3 (Figure 9B). At 6 h after irradiation, the fold-activations observed for Egr-1 and Stat3 were 2.9 ± 0.5 and 2.0 ± 0.1, respectively. Incubation with EGF resulted in a 1.6-fold activation of Egr-1 or Stat3. When cells were preincubated with AG1478 for 1 h before irradiation, with the drug present for an additional 1 h after radiation treatment, the radiation-induced increases in Egr-1 and Stat3 activities were abolished. These data support that the radiation-induced increases in nuclear protein binding to Egr and Stat3 consensus sequences corresponded to an increase in transcriptional activity.

DISCUSSION

The study of transcription factor activation after irradiation of MDA-MB-231 mammary carcinoma cells with therapeutically applied doses of 2 Gy yielded three distinct response profiles. Because we are interested in tracking the radiation-induced changes in transcriptional regulation to upstream signaling events, we have used small molecule inhibitors with specificity for EGFR (AG1478), Ras (FTI), and MEK (PD98059), the latter being used to block the MAPK pathway. Of eight transcription factors tested, six are activated by ionizing radiation, and only four of those six respond to the physiological EGFR ligand EGF. As is illustrated by the

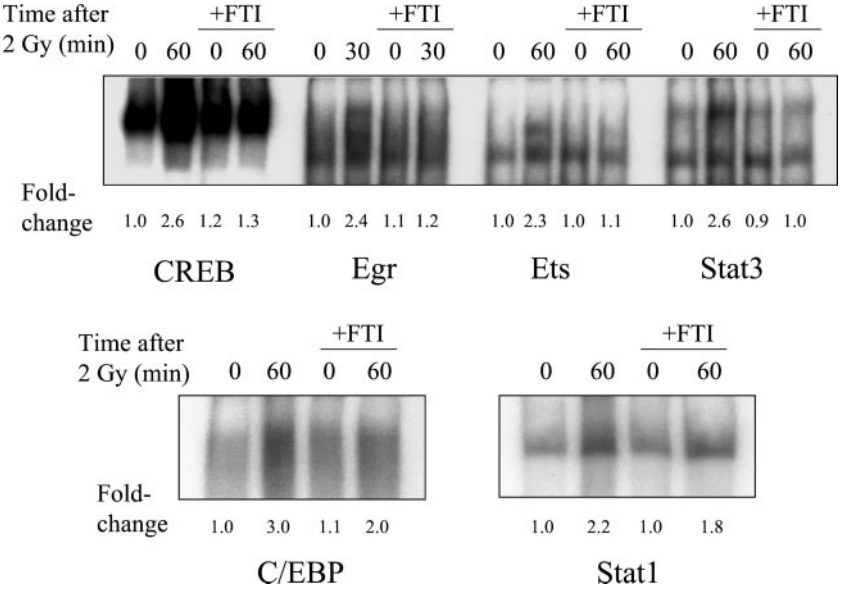

Figure 4. Effect of farnesyltransferase inhibitor I (FTI) on the radiation-induced maximum increase in binding of nuclear extracts to transcription factor consensus sequences. Cells were treated with 50 μM FTI for 18 h before irradiation, and drug was present until protein extraction. Otherwise, the protocol is the same as described in the legend of Figure 2. Fold-changes represent the mean of three independent experiments.

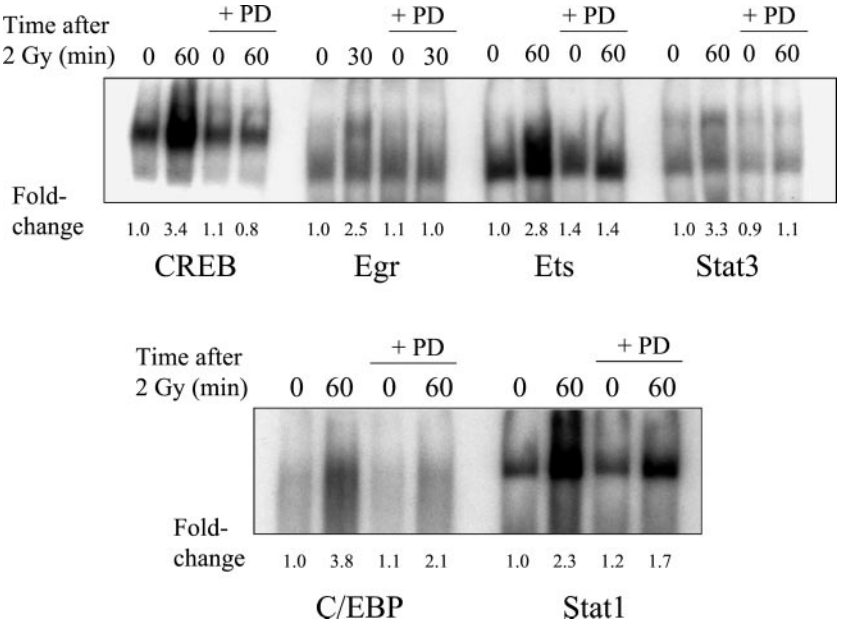

Figure 5. Effect of PD98059 (PD) on the radiation-induced maximum increase in binding of nuclear extracts to transcription factor consensus sequences. Cells were treated with 10 μM PD98059 for 1 h before irradiation, and drug was present until protein extraction. Otherwise, the protocol is the same as described in the legend of Figure 2. Fold-changes represent the mean of three independent experiments.

summary of our results in Table 1 and the diagram in Figure 10, the first group of transcription factors, CREB, Egr, Ets, and Stat3, respond equally to radiation and EGF and are quantitatively inhibited by all three inhibitors. This provides another line of evidence that ionizing radiation, like EGF, acts through EGFR activation as a result of Tyr phosphorylation, as described in previous studies from our laboratory (Schmidt-Ullrich *et al.*, 1997; Contessa *et al.*, 1999; Dent *et al.*, 1999; Reardon *et al.*, 1999; Bowers *et al.*, 2001; Lammering *et al.*, 2001). The EGFR signals are transmitted through Ras and MAPK as intermediate signal components (Figure 10). The second group of transcription factors, C/EBP and Stat1, is activated by radiation but not EGF, suggesting that the activation signal does not originate from EGFR. This conclusion is supported by our inability to inhibit the radiation-induced activation of C/EBP and Stat1 with AG1478. Fi-

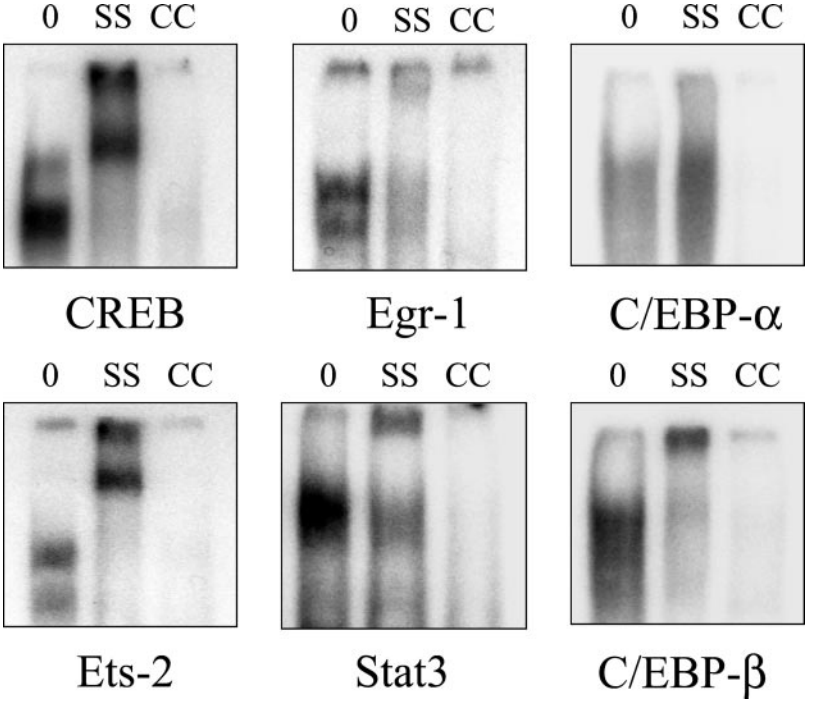

Figure 6. Gel-shift supershift and cold-competitor controls for transcription factors dependent on the EGFR (CREB, Egr, Ets, and Stat3) or MAPK (C/EBP). Control nuclear extracts (0) were from untreated cells. Supershift antibodies against CREB (all forms), Stat3, Egr-1, Ets-2, C/EBP-α, and C/EBP-β were added to reactions after nuclear extract incubation with the CREB, Stat3, Egr, Ets, and C/EBP oligonucleotides, respectively (SS). A 20-fold excess of nonradioactive oligonucleotides were added to nuclear extracts as a "cold" competitor (CC).

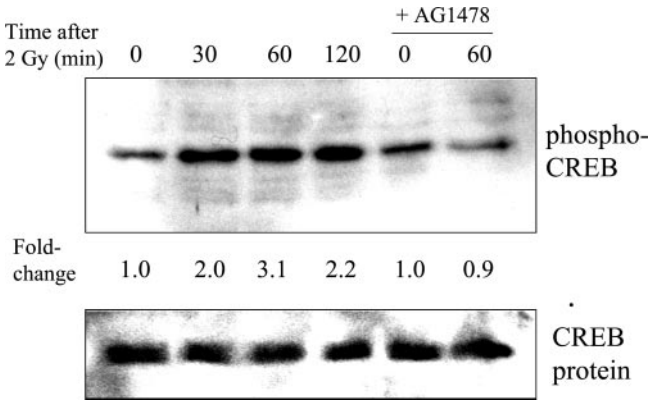

Figure 7. Time-dependent radiation-induced increase in phospho-CREB phosphorylation and inhibition of this effect by AG1478 (1 h pretreatment). Cells were irradiated with 2 Gy, incubated for various times, and extracts were analyzed by Western blotting using phospho-specific antibodies. The bottom panel shows the same blot probed for nonphosphorylated CREB. Fold-changes represent the mean of three independent experiments; maximum fold-increases and inhibition by AG1478 were statistically significant ($p < 0.05$).

nally, the third category of transcription factors, AP-1 and Myc, did not demonstrate an activation response to radiation or EGF, suggesting that there may be high constitutive activity or that higher radiation doses may be required (Wilson *et al.*, 1993; Sahijdak *et al.*, 1994).

Importantly, we provide direct experimental evidence that the radiation-induced activation of EGFR is converted into distinct transcriptional responses. The relative timing of these events is consistent with an initiating activation step of receptor Tyr kinases, EGFR, and/or other ErbB species, within 1–5 min of irradiation. Downstream signaling through Ras and other intermediates leads to another critical event of MAPK activation, reflecting a cytoprotective response including proliferation and enhanced biosynthesis (Reardon *et al.*, 1999; Schmidt-Ullrich *et al.*, 1999). MAPK activation occurs 5–15 min after irradiation of cells (Contessa *et al.*, 1999; Reardon *et al.*, 1999; Bowers *et al.*, 2001). The current report complements the cellular radiation response timetable by demonstrating EGFR/Ras/MAPK-dependent peak activation of transcription factors 30–60 min after irradiation (Figure 10). The transcription factors dominantly dependent on radiation-induced EGFR activation, CREB, Egr, Ets, and Stat3, have been previously shown to be regulated by EGFR and MAPK and are involved in transcriptional regulation of cell growth and proliferation genes (Davis, 1995; Ceresa et al., 1997; McCarthy *et al.*, 1997; De Cesare et al., 1998; Hodge *et al.*, 1998; Lewis *et al.*, 1998; McCubrey *et al.*, 2000; Song and Grandis, 2000). In addition, we have previously shown that either dominant-negative EGFR (EGFR-CD533) or the MEK inhibitor PD98059 can interfere with mammary carcinoma cell proliferation after exposure to ionizing radiation (Contessa *et al.*, 1999; Reardon *et al.* 1999).

Previous studies have demonstrated increased nuclear protein binding to transcription factor consensus sequences or enhanced expression of genes encoding transcription factors after radiation exposures of mammalian cells. The radiation responses varied and required, as in the case of AP-1, relatively high doses of 4.5–10 Gy for modest increase in nuclear protein binding to AP-1 consensus sequences (Wilson *et al.*, 1993; Sahijdak *et al.*, 1994). The radiation doses are particularly relevant, because there is a dramatic difference in cell survival between the 2-Gy dose used in this study and the higher doses reported in previous studies. Similar conflicting data exist for c-Myc for which, in positive experiments, increased mRNA levels have been reported several hours after irradiation (Wilson *et al.*, 1993; Borovitskaya *et al.*, 1996). DNA binding was stimulated for oligonucleotides containing the CREB consensus site 4 h after 4.5 Gy (Sahijdak *et al.*, 1994), and enhanced Egr-1 expression was also reported after irradiation (Hallahan *et al.*, 1991). Thus, the results of this study are in general agreement with previous reports but demonstrate that these transcriptional responses occur at low doses of 2 Gy and can be traced to defined radiation-induced upstream signaling events. This was demonstrated by the quantitative ablation of transcription factor responses when EGFR and MEK1/2 were inhibited with AG1478 and PD98059, respectively, at concentrations highly specific for these molecular targets. The interpretation of our results may be more difficult for the FTI because, besides the established target Ras (which includes both H-ras and K-ras submembers), other potential targets have been reported, such as Rho (Lebowitz and Prendergast, 1998). The involvement of H-ras, K-ras, and/or Rho in these radiation-induced transcriptional responses is currently being investigated in more detail in our laboratory.

Radiation-induced increases in CREB, Egr, Ets, and Stat3 binding were quantitatively abrogated by inhibitors of EGFR, Ras, and MEK 1/2. These gel shift data were confirmed by studies with monoclonal antibodies against phosphorylated CREB (Ser 133) and by experiments using Egr-1 and Stat3 reporter constructs; all of these radiation-induced increases were blocked by AG1478. However, because there is evidence that phosphorylation of CREB at Ser 133 occurs after CREB is bound to the CRE (Ionescu *et al.*, 2001), the cause and effect relationship of radiation-induced transcription factor binding to transcription factor phosphorylation requires further investigation. Independent data showed that the activation of CREB by phosphorylation at Ser 133 depended on the MAPK/p90RSK pathway (Xing *et al.*, 1996; DeCesare *et al.*, 1998; Andrisani, 1999). We also demonstrated that p90RSK phosphorylation increases after radiation and were blocked by AG1478 and PD98059. These data support our finding that transcription factor activation by radiation occurs through MAPK, based on previous studies that establish the dependence of CREB activation on MAPK/p90RSK (Xing *et al.*, 1996; DeCesare *et al.*, 1998; Andrisani, 1999). Activation of the EGFR can activate CREB (DeCesare *et al.*, 1998; Andrisani, 1999), and we have confirmed this using EGF as a positive control. In turn, CREB regulates several genes involved in cellular proliferation including cyclin A (Desdouets *et al.*, 1995; Beier *et al.*, 2000), cyclin D1 (Beier *et al.*, 1999a, 1999b; Sabbah *et al.*, 1999), proliferating cell nuclear antigen (PCNA; Huang *et al.*, 1994; Lee and Mathews, 1997), c-fos (DeCesare *et al.*, 1998; Andrisani, 1999), and cyclooxygenase-2 (COX-2; Tang *et al.*, 2001; Figure 10).

Activation of the EGFR/Ras/MAPK cascade can also stimulate the activation of Egr-1 (Hodge *et al.*, 1998; Liu *et al.*, 2000; McCubrey *et al.*, 2000; Mechtcheriakova *et al.*, 2001). This is

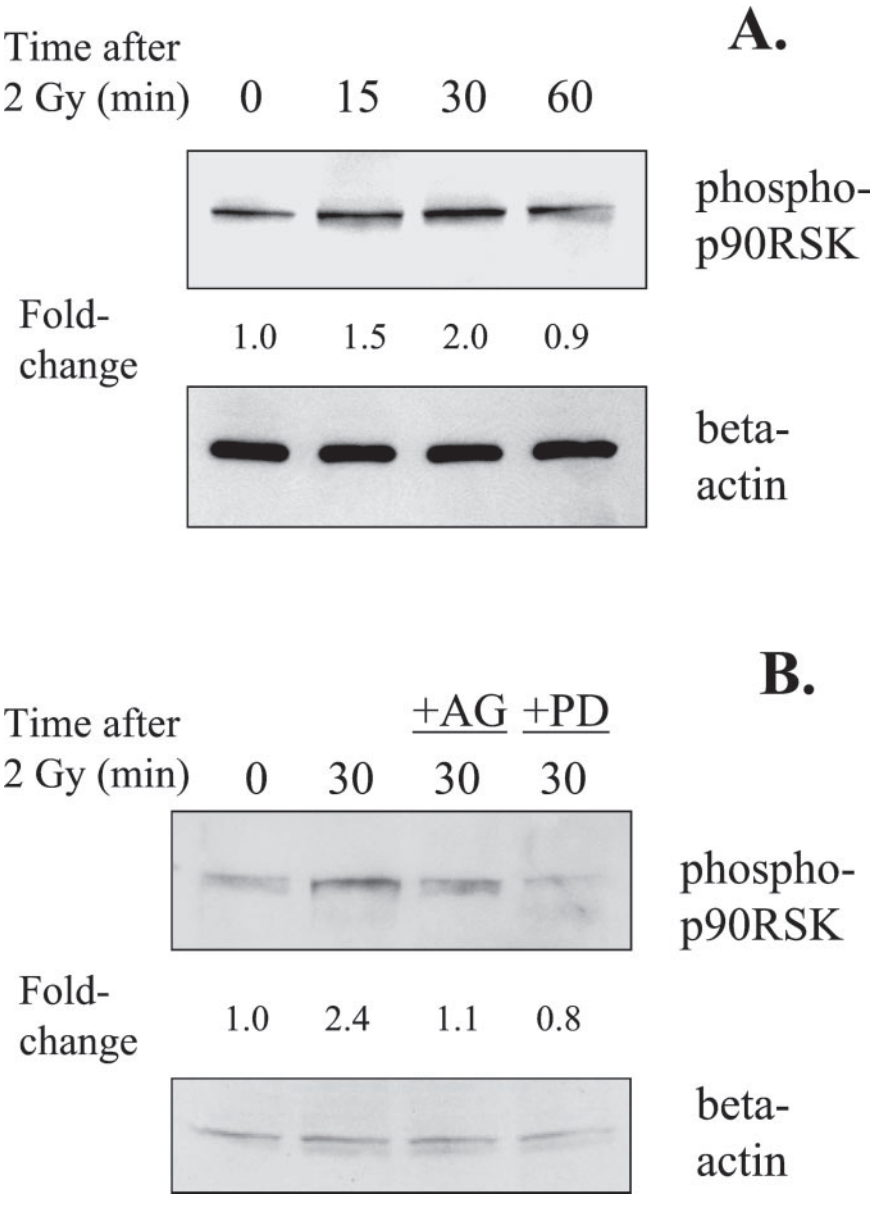

Figure 8. (A) Time-dependent radiation-induced increase in p90RSK phosphorylation. Cells were irradiated with 2 Gy and incubated for various times, and extracts were analyzed by Western blotting. The top panel shows phospho-p90RSK, and the bottom panel shows the same blot probed for β-actin. (B) Inhibition of p90RSK phosphorylation at 30 min after irradiation by either AG1478 or PD98059. Fold-changes represent the mean of three independent experiments. The fold-change at 30 min and inhibition by either compound were significant ($p < 0.05$).

consistent with our results that Egr was activated by EGF and that the radiation-induced Egr response was inhibited by either AG1478 or PD98059; these results were verified in a more direct functional assay using an Egr-1 reporter construct. As CREB, Egr-1 has been shown to regulate transcription of genes involved in cellular growth and proliferation, including basic fibroblast growth factor (bFGF; Biesiada *et al.*, 1996), platelet-derived growth factor (PDGF), and transforming growth factor-β (Liu *et al.*, 2000), and cyclin D1 (Guillemot *et al.*, 2001; Yan *et al.*, 1997; Figure 10). Our data showing radiation-induced Ets activation and its inhibition by AG1478 and PD98059 are consistent with previous reports that MAPK signaling can regulate Ets2 (McCarthy *et al.*, 1997; Park *et al.*, 2000). The Ets consensus binding site used in our experiments is shared by multiple Ets family members; this is the most likely reason for partial (50%) inhibition by AG1478 (Table 1). Ets-2 is involved in regulation of proliferation regulatory genes including $p21^{Cip\text{-}1/WAF1}$ (Beier *et al.*, 1999a, 1999b; Park *et al.*, 2000), cyclin A (Wen *et al.*, 1995), and cyclin D1 (Albanese *et al.*, 1995). The inhibition of radiation-induced Stat3 binding by PD98059 and FTI is consistent with previous findings demonstrating that Stat3 phosphorylation is dependent on Ras/MAPK signaling (Ceresa *et al.*, 1997). However, Stat3 phosphorylation is also regulated by the Janus kinases (JAK; Park *et al.*, 1996; Song and Grandis, 2000); in addition, Stat3 can be activated by EGFR (Park *et al.*, 1996; Shen *et al.*, 2001; Song and Grandis, 2000), which is demonstrated by inhibition of the radiation response with the EGFR inhibitor AG1478 in gel shift and Stat3 reporter construct experiments. Again, Stat3 regulates the transcription of genes involved in cellular proliferation control, including increased expression of Bcl-X_L (Song and Grandis, 2000; Shen *et al.*, 2001), c-fos (Davis, 1995), $p21^{Cip\text{-}1/WAF1}$, and cyclin D1 (Sinibaldi *et al.*, 2000).

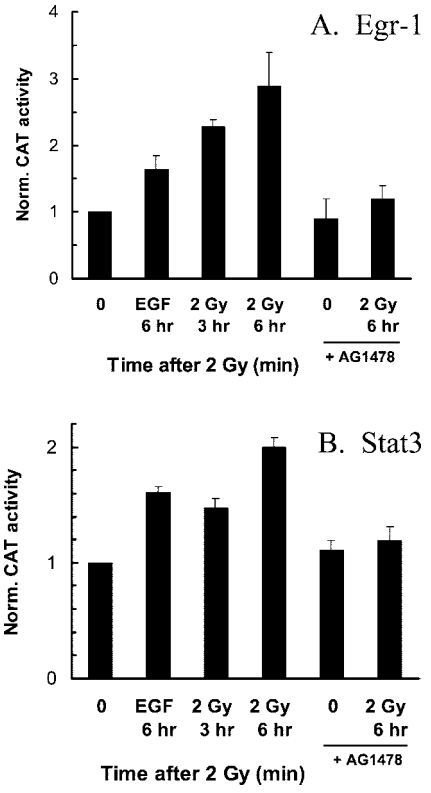

Figure 9. Radiation-induced increase in reporter construct activation. Cells were cotransfected with reporter constructs containing transcription factor binding sites, and β-galactosidase reporter constructs (to obtain transfection efficiencies values for normalization). Cells were irradiated or EGF-treated and incubated at 37°C for 3 or 6 h. AG1478 was present for 1 h before and after irradiation. Error bars, ±SEM of three independent experiments. Maximum fold-changes and inhibition by AG1478 were statistically significant ($p < 0.05$). (A) Egr-1 reporter construct; (B) Stat3 reporter construct.

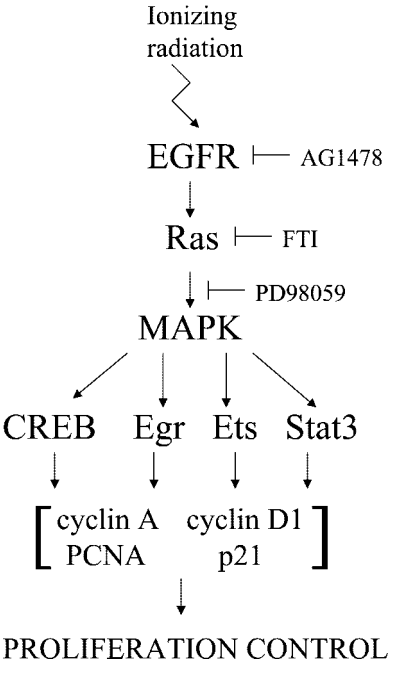

Figure 10. A schematic representation of the effects of EGFR, MAPK, and Ras inhibition on radiation-induced transcription factor activation and proliferation control. The events that are shown downstream of transcription factors in this model are referenced in the DISCUSSION; each transcription factor regulates one or more of the indicated proteins that are involved in proliferation control.

Radiation-induced increases in C/EBP and Stat1 were not significantly inhibited by AG1478, consistent with our finding that these transcription factors were not stimulated by EGF. Thus, the initial signaling events for radiation-induced C/EBP and Stat1 remain unknown but are likely to originate upstream of Ras. Nonreceptor Tyr kinases, such as Src (Tice *et al.*, 1999), are currently examined as potential candidates. Previous reports show that some C/EBP family members are regulated by MAPK activity (Davis, 1995; Park *et al.*, 2000), in agreement with our result that C/EBP was significantly inhibited (60%) by PD98059. Stat1 activation by radiation was not significantly inhibited by PD98059 or FTI, suggesting the lack of a key role of MAPK and Ras in this response. Phosphorylation of Stat1 is mediated primarily by the Janus kinases (JAK; Song and Grandis, 2000), but the p38 pathway may also be involved (Goh *et al.*, 1999). Our data fail to show a significant effect of radiation or EGF on either AP-1 or myc. These two transcription factors are phosphorylated by the c-jun N-terminal kinase (JNK), although the p42/p44 MAPK has also been shown to play a role (Davis, 1995; Lewis *et al.*, 1998). Alternatively, the lack of radiation responses may be due to constitutive activation of AP-1 and Myc in MDA-MB-231 cells that express mutated K-Ras (Kozma *et al.*, 1987).

In summary, we have demonstrated three different response profiles of transcription factors that are activated by ionizing radiation in human carcinoma. These transcriptional responses have been linked to the EGFR/Ras/MAPK pathway using specific, small molecule inhibitors. We have previously demonstrated that radiation causes activation of the EGFR and MAPK pathways in MDA-MB-231 cells and that inhibition of EGFR or MAPK function can decrease cellular proliferation and survival after radiation exposure (Contessa *et al.*, 1999; Reardon *et al.*, 1999; Schmidt-Ullrich *et al.*, 1999). Because radiation-induced transcriptional activation is dependent on EGFR and MAPK, the target genes involved in cellular proliferation and their regulation by these transcription factors are likely to enhance our understanding of cellular protective responses to ionizing radiation at the molecular level.

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