Ionizing Radiation Down-regulates p53 Protein in Primary Egr-1−/− Mouse Embryonic Fibroblast Cells Causing Enhanced Resistance to Apoptosis*

Received for publication, September 15, 2000, and in revised form, October 13, 2000
Published, JBC Papers in Press, October 16, 2000, DOI 10.1074/jbc.M008454200

In this study, we sought to investigate the mechanism of the proapoptotic function of Egr-1 in relation to p53 status in normal isogenic cell backgrounds by using primary MEF cells established from homozygous (Egr-1−/−) and heterozygous (Egr-1+/−) Egr-1 knock-out mice. Ionizing radiation caused significantly enhanced apoptosis in Egr-1−/− cells (22.8±6% of p < 0.0001) when compared with Egr-1+/− cells (3.5±3%). Radiation elevated p53 protein in Egr-1−/− cells in 3–6 h. However, in Egr-1−/− cells, the p53 protein was down-regulated 1 h after radiation and was completely degraded at the later time points. Radiation protein was down-regulated 1 h after radiation and was completely degraded at the later time points. Radiation elevated the p53-CAT activity in Egr-1−/− cells but not in Egr-1+/− cells. Interestingly, transient overexpression of EGR-1 in p53−/− MEF cells caused marginal induction of radiation-induced apoptosis when compared with p53+/+ MEF cells. Together, these results indicate that Egr-1 may transregulate p53, and both EGR-1 and p53 functions are essential to mediate radiation-induced apoptosis. Rb, an Egr-1 target gene, forms a trimeric complex with p53 and MDM2 to prevent MDM2-mediated p53 degradation. Low levels of Rb including hypophosphorylated forms were observed in Egr-1−/− MEF cells before and after radiation when compared with the levels observed in Egr-1+/− cells. Elevated amounts of the p53-MDM2 complex and low amounts of Rb-DM2 complex were observed in Egr-1−/− cells after radiation. Because of a reduction in Rb binding to MDM2 and an increase in MDM2 binding with p53, p53 is directly degraded by MDM2, and this leads to inactivation of the p53-mediated apoptotic pathway in Egr-1−/− MEF cells. Thus, the proapoptotic function of Egr-1 may involve the mediation of Rb protein that is essential to overcome the antiapoptotic function of MDM2 on p53.

The apoptotic pathways consist of an early component that includes molecular events specific for an inducer or a group of inducers and of downstream effector components common to diverse apoptotic signals (1). Apoptosis has also been reported in a variety of experimental tumor systems following exposure to radiation (2, 3). Ionizing radiation alters the expression of specific genes, the products of which may contribute to the events leading to apoptotic cell death. Ionizing radiation exposure is associated with activation of certain immediate-early genes that function as transcription factors (4). These include members of jun or fos and early growth response (EGR) gene families (5, 6).

The Egr gene family includes Egr-1 (7), Egr-2 (8), Egr-3 (9), Egr-4 (10), and the tumor suppressor, Wilms’ tumor gene product, WT1 (11, 12). The Egr family shows a high degree of homology in the amino acids constituting the zinc finger domain and binds to the same GC-rich consensus DNA sequence (13, 14). The Egr-1 gene product, EGR-1, is a nuclear protein that contains three zinc fingers of the C 2H2 subtype (15, 16). Structure-function mapping studies on EGR-1 protein suggest that the amino acids constituting the zinc finger motif confer DNA binding function, whereas the NH2-terminal amino acids confer transactivation function (16, 17). More recent studies have found that sequences diverging from the consensus may also bind EGR-1 (18, 19), thus having a broader spectrum of potential target genes. It is interesting to note that within this family of transcription factors, EGR-1 was found to be a positive activator of transcription, whereas WT1 is a transcriptional repressor, both acting via binding to the same GC-rich consensus sequence in reporter constructs (20–22). Depending on the cell type, EGR-1 may behave as a positive or negative regulator of gene transcription (16, 23, 24). The EGR-1 GC-rich consensus target sequence, 5′-GCC/GTG/GGC/CGC-3′ or 5′-TCC/TAT/CTTCT/CAC-3′, has been identified in the promoter regions of the following: (a) transcription factors, such as MYC and NUR77; (b) growth factors or their receptors, such as transforming growth factor-β1, TNF-α, PDGF-A (26), PDGF-B (27), insulin-like growth factor-II, fibroblast growth factor-β, or epidermal growth factor receptor (6, 7, 28, 29); (c) cell cycle regulators such as the retinoblastoma susceptibility gene Rb (30), cyclin D1 (31), e-Ki-ras (26), and p53 (32); and (d) thymidine kinase, an enzyme crucial in DNA biosynthesis (18) and MDR-1 (33).

It has also been speculated that x-ray induction of PDGF, transforming growth factor-β1, and TNF-α may be regulated by Egr-1 and c-jun (6). Apart from being a potential transcriptional regulator, Egr-1 has a radiation-inducible promoter. Through these distinct induction pathways, Egr-1 has been...
linked to signaling events initiating cell phenotypic response to radiation injury.

On the other hand, wild type p53 has been shown to be functionally necessary for growth inhibition and apoptosis following exposure to ionizing radiation, and p53 mutations have been reported to increase resistance to apoptosis (34). In our previous report, using melanoma cells, which contain wild type p53, a dose-dependent increase in EGR-1 expression with dose-dependent growth inhibition was observed when exposed to ionizing radiation (35). Transfectant melanoma cells stably expressing the dominant-negative mutant protein of EGR-1 showed significantly reduced (<50%) sensitivity to radiation-inducible growth inhibition, and this resistance was found to be dose-dependent. These observations suggest that the EGR-1 induction is involved in the regulation of radiation-inducible apoptosis despite the presence of wild type p53. Recently, we used a p53 null prostate cancer cell line (PC-3), which was found to be moderately resistant to ionizing radiation-inducible apoptosis (36). Western blot analysis and immunocytochemistry studies indicate that EGR-1 is induced in the PC-3 cells by ionizing radiation. Experiments with the Egr-1 dominant-negative mutant or Egr-1 overexpression suggest that Egr-1 function is required for the radiation-inducible apoptosis. Despite the absence of wild type functional p53 protein, the transfected cells expressing the dominant-negative mutant of EGR-1 were resistant to ionizing radiation, and cells overexpressing EGR-1 protein were sensitive to ionizing radiation. Our findings strongly suggested that the radiation-induced apoptotic response in PC-3 cells is elicited through up-regulation of TNF-α protein via EGR-1-mediated transactivation. Thus, EGR-1 is an important mediator of radiation responsiveness irrespective of p53 functional status. However, in a recent report, it was found that EGR-1 protein transactivates the promoter of p53 gene and up-regulates p53 mRNA and protein levels in response to apoptotic stimuli (32). This prompted us to investigate further the interactive role of Egr-1 with p53 during the process of apoptosis. We sought to investigate this mechanism in a normal cell background using isogenic normal primary cell cultures derived from mouse embryonic fibroblasts (MEF) with varied genomic status for Egr-1 gene (cells with both intact Egr-1 alleles, Egr-1+/+; cells with homozygous deletion of Egr-1 alleles, Egr-1−/−; and heterozygous deletion of one Egr-1 allele, Egr-1+/−). Based on findings from these isogenic normal cells with varied genomic status of Egr-1, we suggest that EGR-1 function is necessary for enhanced sensitivity to radiation-induced apoptosis and that the radiation-induced proapoptotic function of EGR-1 is directly mediated by the target genes p53 and Rb.

### MATERIALS AND METHODS

**Cell Culture**—Primary cultures of mouse embryonic fibroblast (MEF) cells from normal mice at passage 3 (kindly provided by Dr. Tyler Jacks, Howard Hughes Medical Institute) labeled as p53+/− were assumed to have two normal alleles of Egr-1 wild type (Egr-1+/+). Primary MEF cultures of cells at passage 0 from homozygous (Egr-1−/−) and heterozygous (Egr-1−/+ Egr-1−/−) Egr-1 knock-out mice (37) were grown in Dulbecco’s modified Eagle’s medium supplemented with 1% glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C and 5% CO2. Primary MEF cells at passage 3 containing homozygous deletion of wild type p53 gene (p53−/−) established from p53 knock-out mice were also grown in Dulbecco’s modified Eagle’s medium (kindly provided by Dr. Tyler Jacks, Howard Hughes Medical Institute).

**Plasmid Constructs**—The plasmid CMV-EGR-1, which encodes full-length EGR-1 protein, contains EGR-1 cDNA downstream of the CMV promoter in the vector pCB6 (21). Plasmid pCMV-WT1-EGR-1, which encodes a dominant-negative mutant of EGR-1, contains a WT1-EGR-1 chimera downstream of the CMV promoter in the vector pcB6 (21). The reporter construct, EBS-CAT, contains three EGR-1-binding sites (CGCCCCCGG) placed in tandem upstream of a minimal c-fos promoter and CAT cDNA. The p53-CAT construct (pAA-CAT), which contains 337-base pair (AraII-AwII) fragment (−441 to −104) of p53 promoter placed in front of CAT cDNA (38), was kindly provided by Dr. Moshe Oren, Weizmann Institute of Science, Israel. The EGR-1-binding site (TCC/TACC) on pAA-CAT was at −44 to −32. The Rb promoter region from −1343 to −1135 was generated from mouse genomic DNA template by PCR. The sense primer (5′-TTTTTTCTAGACGCTCTGGCCGCTAGTG-3′) and antisense primer (5′-AAAAAGCTTCCGCCGAGGG-3′) contained built-in sites (underlined) for XbaI and HindIII, respectively, and they generated a 236-base pair fragment of Rb promoter. The 236-base pair fragment of Rb promoter was cloned in pG-CAT, a vector for CAT reporter (BS-CAT). The control pCAT reporter vector was purchased from Promega.

**DNA Transfection and CAT Assays**—Transient transfections were performed by the calcium phosphate coprecipitation method as described previously (36). CAT assays were performed by thin layer chromatography as described previously (36).

**Irradiation**—A 100-kV industrial x-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was 3.85 Gy at a focus-surface distance of 20 cm.

**Quantitation of Apoptosis**—Apoptosis was quantified by TUNEL staining and flow cytometry. The ApopTag in situ apoptosis detection kit (Oncon, Gaithersburg, MD), that detects DNA strand breaks by terminal transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) was used as described (36). Briefly, cells were seeded in chamber slides, and the next day they were exposed to a 5-Gy dose of radiation. After 24 h, the DNA was labeled with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counter stained with propidium iodide and antifade. The stained specimen was observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, four experiments in total were performed, and ~1000 cells were counted in each experiment. For flow cytometry, cells were lifted by using nonenzymatic cell dissociation medium (Sigma), washed with phosphate-buffered saline, stained with Hoechst (Ho342) and mercocyanine (MC540), and analyzed by flow cytometry using a FACStar Plus cell sorter as described (36).

**23P-Reverse Transcriptase-Polymerase Chain Reaction (23P-RT-PCR) of p53 and Its Target Genes**—Total RNA was isolated from untreated and irradiated Egr-1−/+ and Egr-1−/− cells at various time intervals using TRIzol reagent (Life Technologies, Inc.). One μg of total RNA was reverse-transcribed into cDNA using oligo(dT) primers and reverse transcriptase in a 40-μl reaction mix as described previously (35). Radiation-induced mRNA expression of p53, p21WAF1/CIP1, mdm-2, and

| Gene     | Position | Primer sequence | GenBank™ accession number |
|----------|----------|-----------------|--------------------------|
| p53      | 178–188  | 5′-ACAGTCGGATATCAGCTCG-3′ (upper) | X01237                 |
|          | 429–453  | 5′-TTTTTTGAGAAGGGACAAAGATG-3′ (lower) | K01700                 |
| mdm-2    | 260–281  | 5′-AGGCTGTCAGACGAAGACTC-3′ (upper) | X58876                 |
|          | 550–570  | 5′-CTCTGCTCTACCTAGGGACTG-3′ (lower) | L22472                 |
| p21      | 102–123  | 5′-CCTGTTGATGATCGCCACTGTT-3′ (upper) | U24173                 |
|          | 450–470  | 5′-GGGAAATCTTCCGGCCCGCTC-3′ (lower) | M12481                 |
| bax      | 7–25     | 5′-GGGTCCGGGGGACGACGT-3′ (upper) | L22472                 |
|          | 486–506  | 5′-GGGGTCCGGGAGTGAAG-3′ (lower) | M12481                 |
| β-Actin  | 26–45    | 5′-TGGGGCGCTTCAGGACCA-3′ (upper) | L22472                 |
|          | 247–266  | 5′-TGGCCTTACCTGGCTCAGGGG-3′ (lower) | M12481                 |
Ionizing Radiation Induces EGR-1 Protein That Transactivates via the GC-rich Binding Site in Egr-1+/− and Egr-1+/+ MEF Cells—To determine whether irradiation causes induction of EGR-1 protein in Egr-1−/− and Egr-1+/− MEF cells, whole cell protein extracts were prepared from the cells at different time intervals after exposure to a 5-Gy dose of ionizing radiation and subjected to Western blot analysis. As shown in Fig. 1A, no detectable basal level of EGR-1 protein was found in untreated Egr-1+/− and Egr-1−/− cells. After exposure to 5-Gy dose of radiation, EGR-1 protein was induced at 30 min (10-fold) after the exposure (Fig. 1A) in Egr-1−/− cells. However, this induction was absent in Egr-1+/− cells.

To ascertain the EGR-1-dependent transactivation process in Egr-1−/−, Egr-1+/−, and Egr-1+/+ MEF cells, we performed transient transfections with the following: (a) only reporter construct EBS-CAT that contains three tandem EGR-1-binding sites; (b) EBS-CAT and an EGR-1 expression construct CMV-EGR-1; or (c) EBS-CAT and then exposed the cells to ionizing radiation. As seen in Fig. 1B, CAT activity was completely absent in basal and irradiated Egr-1−/− cells, whereas transient transfection with CMV-EGR-1 elevated the CAT activity. In Egr-1−/− and Egr-1+/− cells, ionizing radiation increased the relative CAT activity in an allelic dose-dependent manner. Similarly, Egr-1+/− cells showed slightly higher basal CAT activity as compared with Egr-1−/− cells. However, the CMV-EGR-1 construct caused an increase in CAT reporter activity irrespective of endogenous Egr-1 allelic status (Fig. 1B). These results confirmed that the EGR-1 protein is necessary for the transactivation of target genes containing the EGR-1-binding sequence.

Ionizing Radiation Caused Enhanced Cell Death in Egr-1−/− Cells—MEFs (Egr-1−/− and Egr-1+/− cells) were left untreated or irradiated at 5-Gy dose of ionizing radiation. TUNEL staining and flow cytometry were performed to determine the incidence of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 h of radiation was 3.5% in Egr-1−/− cells and 22.8% in Egr-1+/− cells (Fig. 2A). By flow cytometry assay using MC540 and Hoechst 3424 staining, the incidence of apoptosis after 48 h of radiation was 6.2% in Egr-1−/− cells and 53% in Egr-1+/− cells (Fig. 2B). Thus, ionizing radiation caused significantly enhanced apoptosis in Egr-1−/− cells (p < 0.0001) when compared with Egr-1+/− cells as demonstrated by TUNEL and flow cytometry assays. These observations suggest that despite the presence of wild type functional p53 gene in this normal cell background, MEFs with homozygous deletion of Egr-1 were resistant to ionizing radiation-inducible apoptosis.

High Basal Levels and Lack of Induction of p53, p21waf1/cip1, mdm-2, and bax mRNA by Ionizing Radiation in Egr-1−/− Cells—To ascertain whether radiation up-regulates p53 mRNA and the p53 target genes such as p21waf1/cip1, mdm-2, and bax, we performed 32P-RT-PCR using the RNA extracted from untreated and irradiated cultures at various time points. In Egr-1−/− cells, p53 mRNA was elevated after 15–30 min of irradiation (Fig. 3). Next, the cells were either left unexposed or exposed to a 5-Gy dose of ionizing radiation, and CAT activity was assayed and expressed as percent conversion of [14C]chloramphenicol to acetylated forms.

Fig. 1. EGR-1 is induced by ionizing radiation. A, EGR-1 protein induction detected by Western blot analysis. Whole cell protein extracts were prepared from Egr-1−/− and Egr-1+/− cells that were left untreated (UT) or exposed to a 5-Gy dose of ionizing radiation for various time intervals (hours) and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 or β-actin. B, Ionizing radiation and CMV-EGR-1 transactivate EBS-CAT reporter construct containing three tandem repeats of EGR-1-binding sites. MEF cells were transiently cotransfected with 4 μg of EBS-CAT or 4 μg of CMV-EGR-1. Next, the cells were either left unexposed or exposed to a 5-Gy dose of ionizing radiation, and CAT activity was assayed and expressed as percent conversion of [14C]chloramphenicol to acetylated forms.
transactivate the resistance to radiation. Thus, the loss of p53 protein in induced apoptosis. Absence of EGR-1 protein renders enhanced radiation resistance in Egr-1−/− cells due to loss of EGR-1-mediated transactivation in the R3, R4, and R5 compartments) in the irradiated population over the untreated population was 6.18 ± 0.02 in Egr-1−/− cells and 52.97 ± 3.23 in Egr-1−/− cells.

p21waf1/cip1, mdm-2, and bax were higher in Egr-1−/− cells as compared with Egr-1+/− cells. Recently, it was reported that EGR-1 protein directly binds to Rel homology domain in p65 (Rel A) subunit of NFκB complex (39), and p65 was found to transactivate the p53 promoter (40, 41). High basal levels of p53 and its target genes in Egr-1−/− MEFs (Fig. 3) may be attributed to induction of p53 promoter by elevated NFκB activity in the absence of Egr-1 function. Thus, absence of induction of these genes after radiation may have contributed to enhanced radiation resistance in Egr-1−/− cells.

Ionizing Radiation Caused Down-regulation of p53 Protein in Egr-1−/− MEF Cells—Western blot analysis was performed to examine whether exposure to ionizing radiation caused induction of p53 protein. Egr-1−/− and Egr-1+/− cells were either left untreated or exposed to a 5-Gy dose of ionizing radiation, and proteins were extracted at various time intervals and subjected to Western blot analysis for p53 protein. As seen in Fig. 4, a strong induction of p53 protein was noticed in Egr-1+/− cells after irradiation; after 3–6 h of radiation, p53 protein levels were increased about 5-fold in Egr-1−/− cells. However, in Egr-1−/− cells, the p53 protein was down-regulated after 1 h of radiation and reduced to <10% of basal levels at 6- and 12-h time points (Fig. 4). The above observations have ascertained the fact that EGR-1 protein is necessary to cause radiation-induced apoptosis. Absence of EGR-1 protein renders enhanced resistance to radiation. Thus, the loss of p53 protein in Egr-1−/− cells after radiation may have contributed to enhanced resistance to apoptosis.

Transfection of CMV-EGR-1 in Egr-1−/− Cells Led to Restoration of Sensitivity to Radiation-induced Apoptosis—To understand the regulation of p53 by Egr-1, we transiently transfected Egr-1−/− cells by using CMV-EGR-1 or vector-alone constructs. Transiently transfected cells were left untreated and irradiated, and then either total proteins were extracted for Western blot analysis of p53 protein or TUNEL was performed at 24 or 48 h after irradiation. In Egr-1−/− cells transfected with vector alone, p53 levels were down-regulated after irradiation (Fig. 5A), and these cells showed 8 or 11% cell death at 24 or 48 h after radiation, respectively (Fig. 5B). On the other hand, Egr-1−/− cells transfected with CMV-EGR-1 showed no down-regulation of p53 protein (Fig. 5A) and 10 or 38% cell death at 24 or 48 h after radiation, respectively (Fig. 5B).

We further performed p53-CAT reporter assays to understand whether the down-regulation of p53 protein after radiation was due to loss of EGR-1-mediated transactivation in Egr-1−/− cells. Basal and irradiated p53-CAT reporter activity was examined in Egr-1−/− and Egr-1+/− MEF cells. Both MEF cells showed basal p53-CAT activity; however, higher CAT activity was observed in Egr-1+/− cells than Egr-1−/− cells (Fig. 5C). Radiation elevated the p53-CAT activity in Egr-1−/− cells, whereas radiation caused no change in the p53-CAT activity in
Egr-1−/− cells (Fig. 5C). These results indicate that Egr-1 may transregulate p53. Together, Egr-1 is pivotal to mediate the apoptotic action by ionizing radiation.

Overexpression of CMV-EGR-1 in p53−/− MEF Cells Led to Modest Induction of Radiation-induced Apoptosis—Our data in Fig. 5C indicated lack of p53 promoter activity after radiation in Egr-1−/− cells, and this prompted us to understand precisely the cooperative role of Egr-1 and p53 in the regulation of radiation-induced apoptosis. We transiently transfected p53+/+ and p53−/− MEF cells by using CMV-EGR-1 or CMV-WT1-EGR-1 (dominant-negative mutant of EGR-1) or vector pCB6 constructs. Transiently transfected cells were left untreated and irradiated, and then TUNEL was performed 24 and 48 h after radiation (Fig. 6).

In p53+/+ MEF cells, overexpression of EGR-1 caused significant induction of cell death after 48 h when compared with p53−/− cells overexpressing vector alone. However, p53+/+ cells overexpressing dominant-negative mutant EGR-1 showed reduction in cell death when compared with p53−/− cells overexpressing vector alone (Fig. 6A).

Interestingly, overexpression of EGR-1 protein in p53−/− MEF cells caused modest induction of cell death after 48 h of
mechanism of p53 degradation in irradiated cells. We performed the following experiments to understand the role of Rb-mediated transactivation of EGR-1 and p53 proteins. Radiation-induced apoptosis was quantified by TUNEL assay using vector or CMV-EGR-1 or CMV-WT1-EGR-1-transfected p53+/− (A) and p53+/+ (B) MEF cells. Transfectant cells were left untreated (UT) or irradiated at 5-Gy dose of radiation and after 24 and 48 h were subjected to TUNEL analysis. Approximately 1000 cells in total were scored for TUNEL-positive cells in each experiment. Data shown are percent TUNEL-positive cells as a function of irradiation dose. Data represents a mean of three experiments. The error bars represent S.D.

Radiation when compared with p53−/− MEF cells overexpressing vector alone (Fig. 6B). Overexpression of dominant-negative mutant EGR-1 in p53−/− MEF cells showed reduction in radiation-induced cell death when compared with cells overexpressing vector alone. These data strongly suggest that both functional EGR-1 and p53 are essential to mediate radiation-induced apoptosis; however, absence of p53 may not contribute toward complete abrogation of EGR-1-mediated radiation-induced apoptosis.

Lack of a Hypophosphorylated Form of Rb Protein Leads to MDM2-mediated p53 Degradation in Egr-1−/− Cells—Recently, it was reported (30) that Rb regulates the stability of p53 through its consensus site on the p53 promoter. Since Rb critically regulates the stability of p53 protein, we hypothesized that the degradation of p53 protein after radiation in Egr-1−/− cells may due to loss of Egr-1-mediated transactivation of Rb gene. To test this hypothesis, we performed the following experiments to understand the mechanism of p53 degradation in irradiated Egr-1−/− cells.

First, to ascertain EGR-1-mediated induction of Rb, we performed Western blot analysis for Rb protein expression levels and Rb-CAT reporter assays in untreated and irradiated Egr-1+/− and Egr-1−/− MEF cells. Western blot analysis showed low levels of Rb including hypophosphorylated forms in Egr-1−/− MEF cells before and after radiation when compared with Egr-1+/− cells (Fig. 7A). Rb-CAT reporter assay also indicated low basal CAT activity in Egr-1+/− cells when compared with Egr-1−/− cells (Fig. 7B). After radiation, Rb-CAT activity was elevated to 2-fold in Egr-1+/− cells but not in Egr-1−/− cells (Fig. 7B). Recent studies have demonstrated that MDM2-p53 interaction directly targets p53 degradation (43). Since p53 was

![Fig. 6. Radiation-induced apoptosis depends on both functionally active EGR-1 and p53 proteins.](image)

![Fig. 7. Lack of hypophosphorylated form of Rb protein leads to MDM2-mediated p53 degradation in Egr-1−/− cells.](image)
degraded in irradiated Egr-1−/− cells, we performed Western blot analysis to determine the levels of MDM2 in untreated and irradiated Egr-1−/− and Egr-1+/− MEF cells. Interestingly, MDM2 levels were down-regulated after radiation in Egr-1−/− cells; on the other hand, MDM2 levels were up-regulated after radiation in Egr-1−/− cells (Fig. 7A).

Low levels of hypophosphorylated forms of Rb and low levels of MDM2 after radiation were evident in Egr-1−/− cells when compared with Egr-1+/− cells. Based on these observations, we hypothesized that p53 degradation in irradiated Egr-1−/− cells might be due to the presence of higher amounts of p53-MDM2-bound forms and relatively lower amounts of Rb bound to the p53-MDM2 complex (trimERIC complex of Rb-MDM2-p53). To test this hypothesis, we performed immunoprecipitation experiments followed by Western blot analysis with cell lysates from untreated and irradiated Egr-1−/− and Egr-1+/− MEF cells. Radiation caused high levels of Rb-MDM2 complex relative to p53-MDM2 complex in Egr-1−/− cells (Fig. 7C). By contrast, higher amounts of p53-MDM2 complex and lower amounts of Rb-MDM-2 complex were observed in Egr-1−/− cells after radiation (Fig. 7C). Thus, the degradation of p53 in Egr-1−/− cells after radiation may be due to diminished Rb binding to MDM2 and enhanced MDM2 binding to p53. Because of diminished Rb binding to MDM2, p53 is directly degraded by MDM2, and thus the p53-mediated apoptotic pathway in Egr-1−/− MEF cells is inactivated.

**Discussion**

Exposure to ionizing radiation is associated with the formation of reactive oxygen intermediates causing direct damage to DNA (44). These reactive oxygen intermediates target the sequence CC(AT)6GG to mediate the activation of EGR-1 (4). Previous studies from our laboratory (35) have suggested that despite the presence of wild type p53 background, inhibition of the expression or function of EGR-1 causes a diminution of radiation-induced growth inhibition in melanoma cells. In the absence of p53, radiation-induced apoptosis of prostate cancer cells was found to be mediated by EGR-1 via TNF-α transactivation (36). These results suggest that Egr-1 induction is involved in the radiation-induced signaling of the cascades of apoptosis pathway.

In this study, in contrast to Egr-1−/− MEF cells, Egr-1+/− MEF cells were significantly resistant to radiation-inducible apoptosis and showed no elevation of p53 protein after radiation. These observations indicate that radiation-induced EGR-1-mediated transactivation of downstream genes is essential for radiation sensitivity. Thus, in support of previous reports, the present study demonstrates that EGR-1 is the upstream mediator for the initiation of the radiation-induced signaling cascade leading to cell death.

The tumor suppressor gene p53 is a central mediator of apoptotic pathways in diverse model systems (45-48). The p53 protein can cause transcriptional up-regulation of a number of downstream genes, such as mdm-2, p21[waaln]cip1, bax, fas, apo1, insulin-like growth factor-binding protein-3, which are implicated in growth inhibition and apoptotic cell death (46-48). In this study, it was found that mRNA levels of p53, p21[waaln]cip1, mdm-2, and bax were elevated after irradiation in Egr-1+/− cells but not in Egr-1−/− cells. In addition, the basal levels of these mRNAs were high in Egr-1−/− MEF cells when compared with Egr-1−/− cells. Loss of radiation-induced elevation of p53 may be attributed to the loss of Egr-1-mediated transregulation of p53 in Egr-1−/− MEF cells, and this may have led to the loss of up-regulation of p53 target genes, p21[waaln]cip1, mdm-2, and bax.

Radiation caused degradation of p53 protein in Egr-1−/− cells, and this led to enhanced resistance to radiation-inducible apoptosis. Transient overexpression of EGR-1 protein in Egr-1−/− cells restored radiation sensitivity and stabilized the p53 protein levels. Thus, this observation suggests that EGR-1 protein is necessary for the up-regulation and the stability of p53 protein and radiation sensitivity. Moreover, radiation elevated the p53-CAT reporter activity in Egr-1−/− cells but not in Egr-1+/− cells. This observation is supported by a recent study that EGR-1 can directly bind with the p53 promoter at two consensus EGR-1-binding sites and induce the p53 mRNA and protein (32). Thus, Egr-1 is an important transregulator of p53.

A marginal induction of radiation-induced apoptosis observed in p53−/−/CMV-EGR-1 MEF transfectants when compared with p53+/−/CMV-EGR-1 MEF cells suggests that p53 played an important downstream role in regulation of Egr-1-mediated radiation-induced apoptosis. It also suggests that the absence of p53 may not contribute toward complete abrogation of EGR-1-mediated radiation-induced apoptosis. This is supported by our previous data that in p53 null prostate cancer cell line PC3, EGR-1 overexpression caused super induction of radiaosensitivity (36). The degree of induction of apoptosis was much higher in p53 null PC3 cells when compared with p53−/−/CMV-EGR-1 MEF transfectant cells in this study. The difference may be due to the tumor cell background versus the normal cell background. Thus, in the absence of p53, EGR-1 may mediate the proapoptotic action of radiation via TNF-α (36) or other downstream cell-death effector genes.

p53 can bind to the promoter region of MDM2 and activate its transcription, forming an autoregulation loop between the expression and function of p53 and MDM2 (49). It is also reported that MDM2-p53 interaction can target p53 for degradation (43). Rb can regulate the apoptotic function of p53 through binding to MDM2, thus preventing MDM2-mediated degradation of p53 (42). Rb can also prevent MDM2 from inhibiting p53-mediated apoptosis. In addition, Rb can protect p53 from MDM2-mediated degradation by forming a trimeric complex with p53 via binding to MDM2 (42). To understand further the mechanism of p53 degradation in irradiated Egr-1−/− MEF cells, we investigated the expression and functional interaction of Rb with p53 and MDM2 in Egr-1−/− and Egr-1+/− MEF cells. The rationale for analyzing the Rb function in this normal isogenic cell system is that (a) Rb regulates the apoptotic function of p53 by mitigating MDM2 mediated degradation (42) and (b) the Rb gene promoter contains EGR-1-binding sites that conform to the GC-rich consensus (30). Low expression levels of hypophosphorylated forms of Rb and decreased Rb-CAT reporter activity were found in Egr-1−/− MEF cells before and after irradiation when compared with Egr-1+/− MEF cells. Relatively higher levels of Rb-MDM2-bound complex and lower levels of p53-MDM2-bound complex were observed in irradiated Egr-1−/− MEF cells. In contrast, higher amounts of p53-MDM2 complex and low binding forms of the Rb-MDM2 complex were observed in Egr-1−/− cells. Lower amounts of the Rb-MDM2 complex along with higher amounts of p53-MDM2 in Egr-1−/− cells might have contributed to p53 degradation after radiation. Thus, apoptosis caused by ionizing radiation requires the induction of EGR-1 protein, which then transregulates the expression of p53 protein and also indirectly regulates the stability of p53 via Rb.

**Acknowledgments**—We thank Dr. Tyler Jacks for providing primary cell culture of p53+/- and p53−/- mouse embryonic fibroblasts and Dr. Moshe Oren for providing p53-CAT constructs.

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J. Biol. Chem. 2001, 276:3279-3286.
doi: 10.1074/jbc.M008454200 originally published online October 16, 2000

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