Induction of p53-dependent Activation of the Human Proliferating Cell Nuclear Antigen Gene in Chromatin by Ionizing Radiation*

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A human fibroblast cell line with conditional p53 expression displayed a p53-dependent increase in both the protein and mRNA levels of proliferating cell nuclear antigen (PCNA) after exposure to ionizing radiation (IR). The combination of p53 induction and IR cooperated to activate a transiently expressed human PCNA promoter-reporter gene via a p53-responsive element. Chromatin immunoprecipitation assays with antibodies specific for p53 or p300/CREB-binding protein revealed specific p53-dependent enrichment of PCNA promoter sequences in immunoprecipitates of sheared chromatin prepared from irradiated cells. Maximal and specific association of acetylated histone H4 with the PCNA promoter also depended on p53 induction and exposure to IR. These data demonstrate p53 binding to a target site in the PCNA promoter, recruitment of p300/CREB-binding protein, and localized acetylation of histone H4 in an IR-dependent manner. These molecular events are likely to play a role in mediating activation of PCNA gene expression by p53 during the cellular response to DNA damage. The analyses indicate that the combination of p53 induction and IR activate the PCNA gene via mechanisms similar to that of p21/wild-type p53-activated factor but to a lesser extent. This differential regulation of PCNA and p21/wild-type p53-activated factor may establish the proper ratio of the two proteins to coordinate DNA repair with cell cycle arrest.

Cellular responses to DNA damage bear upon tumorigenesis and the development of anti-cancer therapies. The p53 tumor suppressor protein integrates many of the cellular responses to DNA damage (for recent reviews, see Refs. 1 and 2). p53 possesses sequence-specific DNA binding activity and primarily functions as a transcription factor that binds to degenerate response elements in a variety of target genes, including p21/WAF1,1 mouse double minute-2, and Bax (3). Activation of p53 target genes leads to either growth arrest and subsequent DNA repair or apoptosis depending on a number of variables including the extent of DNA damage (4). DNA-bound p53 recruits transcriptional co-activators that render localized remodeling of the chromatin structure to favor promoter access by the transcriptional machinery (5–7). To date, the best characterized p53 transcriptional co-activator is p300/CBP, which possesses intrinsic histone acetyltransferase activity and interacts with other proteins possessing histone acetyltransferase activity (8, 9). C-terminal acetylation of p53 by p300/CBP along with phosphorylation in the N terminus converts inert p53 to a transcriptionally active form (10, 11). p53-mediated transcriptional activation of the p21/WAF1 promoter correlates with p53 binding and p300/CBP-mediated acetylation of promoter-associated histones (12–14). The essential role of the TRRAP transcriptional co-activator in potentiating activation of the mouse double minute-2 promoter by p53 suggests that p53-associated transcriptional co-activators possess promoter selectivity (15).

The PCNA gene encodes a highly conserved protein that performs essential functions in DNA replication and DNA repair (16, 17). PCNA forms a trimer that encircles DNA and interacts with a number of proteins involved in DNA metabolism (18) and regulators of both the cell cycle (19, 20) and cell viability (21). Inhibition of PCNA expression in murine fibroblasts arrests cell growth (22), and PCNA overexpression in yeast blocks cell cycle progression (23) and in mammalian cells inhibits DNA repair (24, 25). Growth factors (26–28) and viral infection (29, 30) stimulate PCNA synthesis, and PCNA protein and mRNA levels fluctuate with the cell cycle (31). Both transcriptional (32, 33) and post-transcriptional (27, 34) mechanisms can account for changes in cellular PCNA levels.

The PCNA gene displays complex regulation by p53. In cells exposed to genotoxic stress, p53 expression can be correlated both directly (35–38) and inversely (39) with levels of PCNA. These seemingly conflicting results are supported by findings with transient expression assays, in which co-expression of p53 produces a variable response from a reporter construct fused downstream of the human PCNA promoter (see “Discussion”). Since assays of p53 function in transiently transfected cells may not correlate with p53-mediated regulation of a gene in native chromatin (40), more direct experiments are required to demonstrate regulation of the PCNA gene by p53. We showed previously that exposure of a rat fibroblast cell line to ionizing radiation (IR) increased endogenous PCNA mRNA levels and altered p53 binding to human PCNA promoter sequences in vitro (36). Moreover, induction of a transiently expressed human PCNA promoter-reporter construct by IR required an interaction between the p53 amino terminus and PCNA sequences.

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1 The abbreviations used are: WAF1, wild-type p53-activated factor; PCNA, proliferating cell nuclear antigen; ChIP, chromatin immunoprecipitation; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; IR, ionizing radiation; SSPE, sodium chloride, sodium phosphate, and EDTA; CAT, chloramphenicol acetyltransferase; FBS, fetal bovine serum.
activation p53-binding site (36). To address mechanisms of p53 promoter regulation in native chromatin by p53 and/or IR, we describe here results obtained with a human fibroblast cell line, TR9-7 cells, in which expression of wild-type p53 could be induced by withdrawal of tetracycline (41). This experimental model was used to establish regulation of the PCNA gene by p53 in vivo and, in conjunction with chromatin immunoprecipitation (ChIP) assay, to determine p53-binding site (36). To assess mechanisms of PCNA promoter regulation in native chromatin by p53 and/or IR, we describe here results obtained with a human fibroblast cell line, TR9-7 cells, in which expression of wild-type p53 could be induced by withdrawal of tetracycline (41). This experimental model was used to establish regulation of the PCNA gene by p53 in vivo and, in conjunction with chromatin immunoprecipitation (ChIP) assay, to determine p53-binding site (36).

EXPERIMENTAL PROCEDURES

Plasmids—The PCNA-CAT reporter constructs contained human PCNA promoter sequences—249 to +62 or +213 to +62 relative to the transcriptional initiation site (+1) fused upstream of the reporter sequences in pBACAT as previously described (42). Plasmid p53/S3-N3 expresses human wild type p53 from the cytomegalovirus immediate early promoter (43). pON260 expresses human wild type p53 from the cytomegalovirus immediate early promoter (44).

Cell Culture and Irradiation of Cells—TR9-7 cells (41) are a human fibroblast cell line that expresses human wild type p53 from a tetracycline-repressible promoter in a p53 null background. Cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) with 10% FBS (v/v), 100 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml hygromycin, 600 µg/ml Geneticin (Invitrogen), and 1 µg/ml tetracycline (Sigma) in a humidiﬁed incubator with 10% CO2. Lower concentrations of tetracycline in the medium (from 1 µg/ml to 0 µg/ml, ﬁnal concentration) were used to induce p53 expression when needed. Cells were exposed to IR at the indicated doses at a rate of 1.25 Grays/min from a cesium-137 source in a Gammacell 40 low dose rate irradiator (MDS Nordion, Inc., Kanata, Canada).

Antibodies—A sheep polyclonal antibody to p53 (Ab-7) and rabbit polyclonal antibody to p53/WAF1 (Ab-5) were purchased from Oncogene Research (Boston, MA). A mouse monoclonal antibody to PCNA (19F4) was provided by E. M. Tan (Scripps Clinic and Research Foundation, San Diego, CA). Mouse monoclonal antibodies to p53 (PAb1801 and PAb421) and a goat polyclonal to actin (I-19) were purchased from Santa Cruz Biotechnology. A rabbit polyclonal antibody to an N-terminal epitope of p300 (N-15) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A rabbit polyclonal antibody to acetylated histone H4 was purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). Control mouse IgG and normal rabbit serum were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Transient Transfection Assays—The PCNA-CAT reporter constructs were transfected into TR9-7 cells by the calcium phosphate method as previously described (36). Each transfection mixture contained 5 µg of the reporter plasmid. After transfection, the TR9-7 cells were grown in Dulbecco’s modiﬁed Eagle’s medium with 10% FBS and 1 µg/ml tetracycline for 18 h before transfer to fresh medium with different tetracycline concentrations (1, 0.1, and 0 µg/ml) for 24 h. Immediately after changing the medium, the transfected cells were mock-exposed or exposed to 20 Gray (Gy) IR at 2 Gy/min. Sixteen hours after the various treatments (Fig. 1A), the cells were ﬁxed in 1% formaldehyde and processed for ChIP as described below. 

Chromatin Immunoprecipitation Assays and Real Time PCR Analy- ses—TR9-7 cells were grown in Dulbecco’s modiﬁed Eagle’s medium containing 0.5% FBS and 1 µg/ml tetracycline for 48 h before changing the medium to Dulbecco’s modiﬁed Eagle’s medium with 10% FBS with or without tetracycline. Immediately after changing the medium, the cells were exposed to 20-gray IR. Sixteen hours after the various treatments (Fig. 1A), the cells were ﬁxed in 1% formaldehyde and processed for ChIP as described below. 

Acid Extraction of Histone and Immunoblot for Acetylated Histone

Northern Blot Analysis—TR9-7 cells underwent the treatments as depicted in Fig. 1A. Total cellular RNA was isolated with Ultraspec RNA (Biotech, Houston, TX) following the instructions provided. Northern blots were performed with a human PCNA cDNA, 1.0-kb mouse 36B4 cDNA, and 1.2-kb mouse PCNA cDNA, radiolabeled by random priming as described previously (47). Hybridization was carried out at 68 °C overnight for all three labeled probes in hybridization buffer (5× SSPE: 0.75 mM NaCl, 50 mM NaH2PO4, and 6 mM EDTA; 2% SDS; 5× Denhardt’s solution; 100 µg/ml single-stranded DNA). Washing conditions were twice for 15 min at room temperature with 2× SSPE/0.1% SDS, followed by once at 68 °C for 20 min, then twice in 0.2× SSPE, 0.1% SDS at 68 °C for 20 min each. After washing, the blot was wrapped in plastic wrap and exposed to Biomax x-ray ﬁlm (Eastman Kodak Co.) with an intensifying screen at −70 °C. The intensity of each mRNA signal was quantiﬁed by phosphor imager analyses (Fuji Photo Film Co., Kanagawa, Japan).

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FIG. 1. Increased PCNA and p21/WAF1 protein levels induced by p53 expression and IR. A, experimental protocol. The TR9-7 cells were serum-starved and then restored to normal serum followed by the various combinations of p53 induction and IR as depicted. B, induction of p53 by withdrawal of tetracycline. TR9-7 cells were treated as shown in A. The irradiated (IR+) or unirradiated (IR−) cells were harvested after 0, 8, 24, and 32 h of growth in media containing the indicated concentration of tetracycline. The cells were lysed directly in Laemmlı buffer, and equal amounts of protein from each sample were analyzed by immunoblotting with a p53-specific polyclonal antibody. C, p21/WAF1 and PCNA protein levels in irradiated (IR+) and unirradiated (IR−) TR9-7 cells after p53 induction. Total cellular proteins were isolated at the indicated times. Protein from each sample (50 μg; loaded on the gel as in B) was fractionated in a 12% polyacrylamide gel, followed by immunoblotting for p21/WAF1 (top two panels) or PCNA (middle two panels). An immunoblot for actin in each sample (bottom two panels) revealed comparable protein amounts in each lane. Densitometry was performed with correction to the actin loading control.

RESULTS

Cooperative Induction of PCNA Expression by p53 and Ionizing Radiation—TR9-7 cells, which express wild-type p53 from a tetracycline-repressible promoter (41), were used as an experimental model to provide a means of independently modulating p53 expression level and activating post-translational modifications of p53 by IR. The independent and cooperative roles of p53 induction and irradiation upon regulation of two important cell cycle regulatory proteins, p21/WAF1 and PCNA, were examined in these cells. The TR9-7 cells were incubated in low serum (0.5%) and 1 μg/ml tetracycline (no p53 induction) for 48 h (Fig. 1A). Then the cells were changed into fresh medium with serum (10%) containing 0, 0.1, or 1 μg/ml tetracycline to induce different levels of p53 expression. Immunoblotting assays postinduction revealed a 4–7-fold increase in p53 levels by 8 h in cells grown in the absence of tetracycline that was maintained through the 32-h time point (Fig. 1B). Immediately after changing the media, some cells were exposed to 20 grays of γ-irradiation. The effects of p53 expression and/or IR upon cellular levels of p21/WAF1 and PCNA were determined by immunoblotting total cell extracts from the untreated cells (0 h) and at 8, 24, and 32 h after the various treatments. In unirradiated cells without p53 induction, p21/WAF1 levels changed very little (Fig. 1C). Full p53 induction in unirradiated TR9-7 cells caused a gradual increase in levels of p21/WAF1, reaching a maximum at 24 h. Although exposure of the cells to IR alone produced an increase in p21/WAF1 levels (Fig. 1C, lanes 1, 2, 5, and 8), the combination of IR and p53 induction produced a larger increase (lane 10 versus lane 8). These data confirm p53-mediated regulation of a target gene in TR9-7 cells as shown previously (41) and indicate that IR and p53 cooperate to elevate cellular p21/WAF1 levels.

The effects of IR and p53 upon PCNA levels can be compared with the data for p21/WAF1 described above. In the absence of p53 induction, the levels of PCNA in unirradiated TR9-7 cells appeared higher at 24 h (Fig. 1C, lane 5), which probably reflects serum-stimulated entry into S phase. At the 24- and 32-h time points with p53 induction minus IR, PCNA levels in the serum-stimulated cells (lanes 7 and 10, respectively) appeared similar to that of serum-starved cells (lane 1). Independent of p53 induction, exposure to IR did not prevent the serum-induced increase in PCNA levels (lanes 5 and 8). In contrast to the effects of p53 induction in unirradiated cells, the combination of IR and p53 induction elevated PCNA levels at the 24- and 32-h time points (lanes 7 and 10). The augmented PCNA expression coincided with the increase in p21/WAF1 levels in the same cells. The increase of both p21/WAF1 and PCNA protein levels by p53 induction and IR is consistent with their physiological roles in the cellular response to DNA damage, participation in cell cycle arrest, and DNA repair. p53-associated elevation of PCNA protein levels at 24 and 32 h required exposure to IR and appeared to be limited relative to that of p21/WAF1.

A Northern blot was performed to determine the p21/WAF1 and PCNA mRNA levels in TR9-7 cells subjected to the above experimental protocol (see Fig. 1A). Exposure to IR or p53 induction elevated p21/WAF1 mRNA levels at 16 h over that of the serum-starved control cells (~2- and 6-fold, respectively (Fig. 2). Similar to the immunoblotting results, the combination of p53 induction and exposure to IR produced a maximal increase in p21/WAF1 mRNA levels, more than 14-fold. The
addition of serum alone did not affect p21/WAF1 mRNA levels. Some similarities and differences in the regulation of p21/WAF1 and PCNA mRNA levels were observed. Consistent with a serum response (26), the addition of serum alone elevated PCNA mRNA levels 2-fold over that of the serum-starved cells (Fig. 2). In the serum-stimulated cells, IR exposure or p53 induction elevated PCNA mRNA levels about 3- and 5-fold, respectively. Similar to p21/WAF1, the combination of p53 induction and IR maximally elevated PCNA mRNA levels 8-fold. With the exception of p53 induction alone, which did not increase PCNA protein levels but did increase PCNA mRNA levels, the Northern and immunoblotting assays revealed concordant regulation of the mRNAs and their respective proteins for p21/WAF1 and PCNA. Post-transcriptional regulation of PCNA expression, which may account for the disparity in PCNA mRNA and protein levels, has been previously reported (27, 34). These data indicate that the combination of p53 and IR induces maximal expression of both the p21/WAF1 and PCNA genes in serum-stimulated cells. The amplitude of induction of PCNA mRNA by the combination of p53 and IR is smaller relative to that of p21/WAF1 mRNA.

Cooperative Activation of the PCNA Promoter by p53 and Ionizing Radiation—We performed transient transfection assays with PCNA-CAT reporter constructs to investigate whether or not the cooperative roles of p53 and IR in induction of PCNA expression could be manifest at the transcriptional level (Fig. 3). Neither p53 expression nor IR independently had a major effect (less than 50% change) on expression from PCNA-CAT. Consistent with PCNA protein and mRNA expression, the combination of p53 induction and IR produced a 3-fold increase in PCNA promoter-directed CAT expression by the construct with an intact p53-binding site (−249 PCNA-CAT). The construct lacking the p53-binding site (−213 PCNA-CAT) failed to respond to the combination of p53 induction and irradiation. These observations show that the cooperative effects of p53 and IR on induction of PCNA expression occur, at least in part, at the transcriptional level.

p53 Binding and Recruitment of p300 to the p21/WAF1 and PCNA Genes in Irradiated TR9-7 Cells—Previous gel shift assays demonstrated that complexes containing p53 and p300 formed with oligonucleotides corresponding to the p53-binding site in the PCNA promoter (36). To examine the recruitment of p300 by p53 to the PCNA gene in vivo, we performed ChIP assays using antibodies specific for p53 and p300. Partially cross-linked, sheared chromatin was prepared from TR9-7 cells released from low serum in the presence or absence of p53 induction with or without exposure to IR, as described above (Fig. 1A). Real time PCR assays of the amplicons diagrammed in Fig. 4A were employed to quantify the amount of chromatin specifically immunoprecipitated with antibodies to p53 or p300. Restriction digestion (not shown) and melting curve analyses verified the identities of the p21/WAF1 (Fig. 4B) and PCNA (Fig. 4C) amplicons, thereby validating quantification of the PCR product by Sybr Green fluorescence. To quantify p53
binding, real time PCR amplification curves were established for the p21-p53 amplicon with the input sheared chromatin preparation and the material that was specifically immunoprecipitated with antibodies to p53 (Fig. 5A). Comparison of the threshold amplification value for each experimental sample to the threshold values for a titration of the input chromatin (data not shown) established the relative abundance of the immunoprecipitated material. The addition of serum or the combination of serum and IR had little effect on p53 binding to the p21/WAF1 gene at the 16-h time point (Fig. 5B). Only a slight increase, 1.6-fold, in p53 binding to the p21/WAF1 gene was observed upon p53 induction alone. The combination of p53 induction and exposure to IR dramatically enhanced p53 binding to the p21/WAF1 gene about 10-fold over that observed in cells without p53 induced.

Similar to the analyses of p21/WAF1, amplification curves were established for the PCNA-p53 amplicon with the input chromatin and the material immunoprecipitated with antibodies to p53 (Fig. 5C). A comparable, yet distinct, profile of p53 binding to the PCNA gene was observed in the ChIP assay. Exposure to IR or p53 induction produced a measurable increase in p53 binding to the PCNA gene, approximately a 2-fold increase over the control value for each one (Fig. 5D). As described for the p21/WAF1 gene, the combination of IR and p53 induction produced maximal p53 binding to the PCNA gene, about a 6-fold increase over the control value. Two control experiments indicated the specificity of the ChIP assays. First, ChIP assays of each sheared chromatin preparation with non-specific mouse IgG produced threshold values comparable with those with the 0-h control sample and the anti-p53 antibody (Fig. 5, A and C). Second, threshold values of p53-specific ChIP assays for an amplicon corresponding to the +1 site of the human β-globin promoter (no p53-binding site) and chromatin from the IR-p53 combination were above 32 cycles, similar to that of the mouse IgG ChIP results (data not shown). Taken together, these findings indicate that IR activates p53 binding to both the p21/WAF1 and PCNA genes with a magnitude of p53 recruitment to the p21/WAF gene greater than that for the PCNA gene.

The increase in p53 binding to both the PCNA and the p21 promoters in irradiated cells described above could be a consequence of the effects of IR upon the level of p53 protein or enhancement of the protein’s DNA-binding activity. Exposure of cells to IR induces a number of post-translational modifications of p53, including phosphorylation and acetylation that both stabilize the protein and convert it to a transcriptionally active form (50). Immunoblots of total cell extracts from unirradiated and irradiated cells with p53 induced did not reveal detectable differences in p53 protein levels (Fig. 6A). Apparently, the potent p53 induction in TR9-7 cells overcame the protein’s inherent instability mediated by human double minute-2 (51). Enhanced p53 binding to the p21/WAF1 and PCNA promoters in irradiated cells may be selective or a consequence of a general increase in DNA binding affinity. Immunoblots of protein recovered from chromatin immunoprecipitated with anti-p53 antibody from each experimental group did not show a general radiation-associated increase in p53 binding to chromatin (Fig. 6B). This finding indicates selective p53 binding to the p21/WAF1 and PCNA genes in irradiated cells.

Several reports have demonstrated that the p300/CPB potentiates transcriptional activation of p53-responsive promoters (5–7). ChIP assays were used to evaluate recruitment of p300 to the p53-binding sites in the PCNA and p21/WAF1 genes. The analyses were identical to the p53 binding assays described above with the exception that the specific antibody recognized p300. As described above, amplification curves for the p21-p53 amplicon were established, and comparison of threshold values to a standard curve of input chromatin served to quantify the amount of DNA immunoprecipitated with anti-p300 antibodies. Induction of p33 alone brought about a 4-fold increase in p300 binding to the p21/WAF1 gene (Fig. 7A). The combination of p53 induction and irradiation elicited the most p300 binding to the p21/WAF1 gene, about 7-fold higher than the 0-h control (Fig. 7A). ChIP analyses of p300 binding to the PCNA-p53 amplicon revealed similar results. p53 induction or the combination of p53 induction with irradiation increased the association of p300 with the PCNA promoter about 1.8- and 3-fold, respectively (Fig. 7B). Thus, the p53 and p300 ChIP results indicated that IR enhanced both p53 binding and p300 association to both the p21/WAF1 and the PCNA genes.

p53-dependent Acetylation of Promoter-associated Histone H4 in Irradiated TR9-7 Cells—The intrinsic histone acetyltransferase activity of p300 can promote chromatin decondensation via acetylation of core histones, which improves accessibility of the remodeled chromatin to the transcriptional machinery (8). Using antibodies specific for acetylated histones in the ChIP assay can evaluate the extent of histone acetylation (52). To this end, ChIP assays were performed on the sheared chromatin preparations described above with an antibody specific for acetylated histone H4. Real time PCR assays with primers flanking the transcription initiation sites of both the p21/WAF1 and the PCNA genes (p21-1 and PCNA-1; Fig. 4A) were used to quantify the amount of DNA co-immunoprecipitated with the antibody to acetylated histone H4. As described above, the real time PCR products were identified by restriction analyses and melting curves (not shown). Amplification curves of chromatin immunoprecipitated with an antibody to acetylated histone H4 were established for the p21/WAF1-1 and the PCNA-1 amplicons (data not shown). Again, real time PCR amplification of serial dilutions of sheared chromatin established a reference curve to quantify threshold values for both proximal promoter amplicons (data not shown). For p21-1, p53 induction alone caused a moderate 2.5-fold increase of histone H4 acetylation over that of the 0-h control, whereas neither serum stimulation nor irradiation alone produced a change in H4 acetylation (Fig. 8A). Similar to the patterns of p53 binding and recruitment of p300, the combination of p53 induction and irradiation maximally increased acetylation of histone H4 associated with the p21/WAF1 proximal promoter, a 10-fold elevation over the 0-h control (Fig. 8A). p53 induction alone moderately augmented acetylation of histone H4 associated with the PCNA gene, a 2.6-fold increase over that of the 0-h control (Fig. 8B). Acetylation of PCNA promoter-associated histone H4 climbed 7.5-fold in the irradiated cells with p53 induced. Although there were differences in magnitude, p53 induction correlated with acetylation of promoter-associated histone H4 on both the p21/WAF1 and the PCNA promoters. In addition to this similarity, serum stimulation induced a 2-fold increase in promoter-associated histone H4 acetylation relative to that of the serum-starved 0-h control (Fig. 8B) that did not correlate with p300 recruitment to the p53-binding site (see Fig. 7B). Exposure of the cells to IR abrogated this modest serum-stimulated elevation in PCNA promoter-associated histone H4 acetylation (Fig. 8B). Low background levels in control experiments with normal rabbit serum suggested a basal level of H4 acetylation on both the p21/WAF1 and the PCNA-proximal promoters in the serum-starved cells. ChIP assays with the β-globin proximal promoter did not show correlations between acetylation of histone H4 and p53 induction and/or exposure to IR (data not shown). Immunoblotting of histones from cells in each experimental group demonstrated that association of acetylated histone H4
with the p21/WAF1 and PCNA promoters was not due to a general increase in the abundance of acetylated histone H4 (Fig. 8C).

DISCUSSION

The data shown here demonstrate that exposure of human fibroblasts to IR increases both PCNA and p21/WAF1 mRNA levels in a p53-dependent manner. Moderate increases of both p21/WAF1 and PCNA mRNA expression by either p53 induction or IR alone relative to the two in combination indicate cooperative effects of IR and p53 upon expression of the two target genes. Both transcriptional and post-transcriptional mechanisms account for regulation of PCNA expression (27, 30), but the effects of p53 are primarily transcriptional. Regulation of the PCNA promoter by p53 appears to vary according to the assay system. The model used most frequently, transient co-expression of wild-type p53 with a PCNA promoter-reporter construct in p53 null cells, has demonstrated p53-mediated repression (53–55), no effect (56), or a variable concentration-dependent response (lower levels of p53 activate the PCNA promoter, whereas higher levels repress it) (42, 57). In the experiments described here, the combination of IR and p53 induction activates a transiently expressed PCNA-CAT re-

immunoprecipitated chromatin with a standard titration curve established the relative amount of DNA in each sample. The results were corrected for the amount of input chromatin (established by real time PCR) in each immunoprecipitation reaction. The graph shows the fold change in p53 binding to p21/WAF1 chromatin for the various treatments (with or without p53 induction, with or without IR), sheared chromatin was prepared from the cells. A pool of p53-specific monoclonal antibodies or nonspecific mouse IgG control (mIgG) was used to immunoprecipitate each sheared chromatin preparation. After reversal of the formaldehyde cross-linking, the amount of DNA in each immunoprecipitate was quantified by real time PCR. The graph shows the real time PCR amplification curves as fluorescence intensity versus cycle number for the p21-p53 amplicon (see Fig. 4A). Similar amplification profiles were generated for amplicons in each ChIP assay shown in Figs. 5, 7, and 8. B, p53 binding to the p21/WAF1 promoter. Comparison of the threshold values from real time amplification curves of
porter via a p53-responsive element (Fig. 3). However, transient reporter assays of p53 function may not be physiologically relevant (40, 58). Chromatin immunoprecipitation assays address p53-dependent regulation of the endogenous PCNA gene. The combination of IR and p53 increases p53 binding to the endogenous PCNA promoter with enhanced recruitment of p300 and acetylation of histone H4. These molecular events at the chromatin level correlate with the profiles of PCNA and p21/WAF1 mRNA expression upon p53 induction and exposure to IR. Little variation in the levels of actin protein (Fig. 1C), 36B4 mRNA (Fig. 2), and ChIP assays of the \( \beta \)-globin promoter (not shown) confirmed gene-specific responses to the combination IR and p53 induction. Activation of the PCNA gene by p53 and IR in TR9-7 cells compares favorably with that of the p21/WAF1 gene, albeit to a lesser extent. The results demonstrate p53-mediated transcriptional activation of the PCNA gene during the cellular response to DNA damage. Activation of the PCNA gene by p53 agrees with previous findings that the two proteins are co-expressed in cells exposed to genotoxic stress (35, 37, 38, 59, 60).

**Fig. 7.** Recruitment of p300 to the p21/WAF1 and PCNA promoters. A, binding of p300 to the p21 promoter; same as Fig. 4, except that association of p300 with the p21 gene was quantified by ChIP-real time PCR analyses with the p21-p53 amplicon after immunoprecipitation with a polyclonal antibody to p300. The graph shows the change in p300 recruitment associated with the indicated treatment (with or without p53 induction and with or without 20-gray IR) relative to that of untreated cells in low serum (-serum, -p53, -IR), which was arbitrarily set to 1. The results shown are the average \( \pm \) S.E. of two separate experiments assayed in duplicate by real time PCR. B, p300 binding to the PCNA promoter; same as in A, except that p300 recruitment to the PCNA promoter was assayed with the PCNA p53 amplicon (Fig. 3A).

**Fig. 8.** Acetylation of histone H4 associated with the p21/WAF1 and PCNA promoters. A, acetylation of histone H4 associated with the p21/WAF1 promoter. TR9-7 cells were treated as shown in Fig. 1. ChIP-real time PCR assays were used to assess acetylation of histone H4 associated with the p21/WAF1 promoter. The sheared chromatin was immunoprecipitated with an antibody specific for acetylated histone H4. The amount of DNA in each immunoprecipitate was quantified by real time PCR of the p21-p53 amplicon (see Fig. 4A). The graph shows the change in acetylated histone H4 associated with the p21/WAF1 promoter for the indicated treatment relative to the 0-h untreated control cells (-serum, -p53, -IR), which was arbitrarily set to 1. The results are the average \( \pm \) S.E. of two separate experiments assayed in duplicate by real time PCR. B, acetylation of histone H4 associated with the PCNA promoter; same as A, except that the amount of DNA immunoprecipitated with antibody to acetylated H4 from the sheared chromatin preparations was quantified by real time PCR with the PCNA p53 amplicon (see Fig. 4A). The graph shows the change in acetylation of the PCNA promoter-associated histone H4 for the indicated treatment relative to the 0-h control cells (-serum, -p53, -IR), which was arbitrarily set to 1. The results are the average \( \pm \) S.E. from two separate experiments assayed in duplicate by real time PCR. C, alteration of histone H4 acetylation by IR and p53. TR9-7 cells were treated the same as for ChIP assays. Histones were acid-extracted, followed by immunoblotting with a specific antibody to acetylated histone H4. Equivalent loading of the samples was verified by Ponceau S staining of the transferred blot. Lane 1, cells treated with the histone deacetylase inhibitor sodium butyrate to serve as a marker; lane 2, serum-starved cells; lane 3, cells restimulated with serum; lane 4, serum restimulation and IR; lane 5, serum restimulation and p53 induction; lane 6, serum restimulation, p53 induction, and IR.
Assays of p53 binding in vitro to short oligonucleotides support the model that p53 is expressed as a latent protein with the C-terminal domain inhibiting sequence-specific DNA binding (61). Deletion, post-translational modifications, binding of monoclonal antibody PAb421, or other perturbations of the C terminus increase the affinity of p53 binding to short oligonucleotides (61, 62). Analyses of p53 binding to long DNA probes (12) or to chromatin, in vitro (14, 63) or in vitro (12), have not supported the model that p53 exists in a latent form inactive for DNA binding. The results in Fig. 5 support the latent p53 model in that exposure of cells to IR enhances sequence-specific DNA binding by p53 in vitro. The IR-induced increase in sequence-specific binding by p53 was not due to an increase in protein levels or an increase in the general affinity of p53 for DNA, since immunoblotting revealed no detectable difference in either total or chromatin-bound p53 after exposure to IR (Fig. 6). Thus, expression of p53 that constitutively binds DNA at specific sites in vitro is not obligatory, and post-translational control of sequence-specific DNA binding activity to regulate p53 function can be achieved in cells. In cells exposed to genotoxic stress, those post-translational modifications that allow p53 accumulation may also activate DNA binding. In TR9-7 cells with inducible p53, protein accumulation can be achieved without activation. During embryogenesis, p53 is constitutively expressed at relatively high levels (64), and immunoblotting can readily detect p53 in rapidly proliferating mouse embryonic fibroblasts grown in culture.2 The latent p53 model provides an appealing hypothesis to explain how the rapidly proliferating embryonic cells tolerate abundant p53 expression.

p53-mediated regulation of PCNA expression displays differences in humans and rodents. Exposure of mice to IR fails to activate PCNA expression in a variety of tissues (65). Induction of the rat PCNA promoter in cells exposed to UV occurs in a p53-independent manner (66). No p53 binding site has been reported in the mouse PCNA promoter, and a site with only poor homology (50%) to the consensus p53 binding site has been identified in the rat PCNA promoter (55). In rat and mouse, p53-dependent repression, but not activation, of PCNA expression has been reported (55, 66, 67). In human cells exposed to genotoxic stress, p53 induces global genome repair, whereas most rodent tissues lack p53-inducible global genome repair (68). p53-mediated activation of PCNA for purposes of DNA repair in humans and the absence of this pathway in rodents appear to be consistent with the distinct responses to genotoxic stress in humans and rodents. Although rodents lack p53-inducible global genome repair, the correlation between reduced PCNA expression and the enhanced radiosensitivity of “wasted mice” suggests that constitutive levels of PCNA may be critical to survival of rodent cells exposed to genotoxic stress (69, 70).

The experiments described here show p53 binding in vitro to the p21/WAF1 and PCNA genes. However, binding of p53 to target sites in vitro can occur in the absence of transcriptional activation (14). Transient expression assays indicate that p53 cooperates with p300/CBP to activate the human PCNA promoter. Co-expression of the adenovirus E1A oncoprotein prevents p53-mediated activation of the PCNA promoter, and the N-terminal p300/CBP binding domain is essential for this activity of E1A (71, 72). Furthermore, co-transfection of a dominant negative mutant p300 inhibits activation of the PCNA promoter by p53 (71).3 In nuclear extracts prepared from irradiated cells, association of p300 with the PCNA p53-binding site in vitro correlates with activation of the PCNA gene by IR (36). Consistent with these findings, the data here show p53-dependent association of p300 with the PCNA promoter in vitro. p300 interacts with the PCNA protein and may be involved in DNA repair (73). In keeping with this function, exposure of cells to UV increases p300 binding to chromatin. This type of p300 association with chromatin is distinct from the results described here, which show that p300 binding is dependent, in part, upon p53 induction and through a p53-responsive element. Moreover, acetylation of PCNA promoter-associated histone H4 correlates with p300 interaction with the PCNA promoter.

Assays of lymphoblastoid cell lines exposed to IR demonstrate p53-dependent post-translational regulation of the subcellular distribution of PCNA with little effect of IR upon PCNA mRNA levels (74). In contrast, similar to the findings here, irradiation of peripheral blood lymphocytes induces a 2–3-fold increase in PCNA mRNA levels by 24 h after exposure (59). One possible explanation for this discrepancy is that regulation of PCNA mRNA levels by IR differs in quiescent versus cycling cells. In accord with this postulate, IR induces a form of p53 in S phase cells that is transcriptionally inactive (75). The higher levels of PCNA in cycling cells may be sufficient for DNA repair. p53-dependent activation may become obvious in the experiments described here because of the low levels of PCNA after 48 h of serum starvation.

Although the PCNA and p21/WAF1 genes display similar p53-dependent regulation in irradiated cells, the magnitude of PCNA induction is reduced relative to that of p21/WAF1. Data from other experiments not shown here indicate that the low affinity p53 binding site in the PCNA promoter determines transient activation by p53 relative to the high affinity upstream p53 binding site in the p21/WAF1 promoter.4 p21/WAF1 has been shown to selectively inhibit PCNA-dependent DNA replication but not PCNA-dependent DNA repair (76). Since p21/WAF1 inhibits DNA replication through direct PCNA binding (77), the relative ratios of the two proteins are important determinants of cell cycle progression. Overexpression of PCNA induced by HTLV-1 Tax protein inhibits DNA repair, which can be rescued by overexpression of p21/WAF1, but not a p21/WAF1 mutant that fails to bind PCNA (24, 25). The relatively reduced activation of the PCNA promoter by p53 probably serves to maintain the proper ratio of the two proteins to effect DNA repair and prevent cell cycle progression. Like p53-dependent activation of p53R2 (49), a ribonucleotide reductase subunit, activation of PCNA expression by p53 contributes to repair of DNA damage. The differential regulation of two DNA damage response effectors, p21/WAF1 and PCNA, by p53 may play a role in ensuring a prompt and appropriate growth arrest and DNA repair that is critical for maintenance of genomic stability in cells exposed to genotoxic stress.

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