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Experimental Cell Research 305 (2005) 10–22

Experimental Cell Research

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Binding sequence-dependent regulation of the human proliferating cell nuclear antigen promoter by p53

Bin Shan, Gilbert F. Morris*

Programs in Molecular and Cellular Biology and Lung Biology, Department of Pathology, Tulane Cancer Center and Tulane/Xavier Center for Bioenvironmental Research, Tulane University Health Sciences Center, SL-79, 1430 Tulane Avenue, New Orleans, LA 70112-2699, USA

Received 7 May 2004, revised version received 7 September 2004
Available online 24 January 2005

Abstract

Exposure of a lung epithelial cell line to ionizing radiation (IR) arrests cell cycle progression through 48 h post-exposure. Coincidentally, IR differentially activates expression of the cell cycle inhibitor, p21/WAF1, and the DNA replication protein, proliferating cell nuclear antigen (PCNA). p21/WAF1 mRNA levels remain elevated through 48 h post-exposure to IR, while PCNA mRNA levels increase transiently at early times. Since p21/WAF1 inhibits DNA replication by directly binding PCNA, the relative levels of the two proteins can determine cell cycle progression. The PCNA p53-binding site displayed reduced p53 binding affinity in vitro relative to the distal p21/WAF1 p53-binding site. Substitution of the p21/WAF1 site for the resident p53-binding site in the PCNA promoter altered the responses to increasing amounts of p53 or IR in transient expression assays. The p21/WAF1 p53-binding site sustained activation of the chimeric PCNA promoter under conditions (high p53 levels or high dose IR) that the PCNA p53-binding site did not. Binding site-specific regulation by wild-type p53 was not observed with mutant p53 harboring a serine to alanine change at amino acid 46. Limited activation of the PCNA promoter by p53 and its modified forms would restrict the amount of PCNA made available for DNA repair.

Keywords: p53; PCNA; Ionizing radiation; Transcription regulation; Promoter; DNA-binding site; DNA damage; p21/WAF1

Introduction

Mutations of the p53 tumor suppressor gene occur in more than 50% of human cancers [1], and p53 null mice develop tumors by 9 months [2]. Wild-type p53 prevents propagation of genetic mutations from parental to daughter cells by regulating cell cycle progression, DNA repair, and cell viability [3]. p53 primarily functions as a transcription factor [4] that binds to target DNA sequences that consist of a pair of ten nucleotide repeats, 5′-RRRC(A/TA/T)GYYY-3′, with R for any purine and Y for any pyrimidine [5]. Upon stress, p53 undergoes a series of post-translational modifications, which lead to increased stability and a gain of transcriptional activity [6,7]. Target genes that mediate p53-dependent cellular responses can be divided into three categories. One group encodes proteins that induce growth arrest, such as p21/WAF1 [8] and 14-3-3σ [9]. A second family of p53 target genes encodes proteins that regulate the apoptotic response to genotoxic insults, including bax [10], AIP1 [11], PUMA [12], and DR5 [13]. Mounting evidence suggests selective activation of one of these two families by p53 depends on cell type, type of DNA damaging agent, and severity of DNA damage [14–17]. A third group of p53 activated genes expresses proteins involved in DNA repair, and examples of this group are p53R2, a DNA repair-specific ribonucleotide reductase regulatory subunit [18], and GADD45 [19].

One of the p53 target genes, PCNA, encodes a protein that functions in both DNA replication and repair [20]. Accordingly, PCNA is expressed in cells exposed to mitogens [21,22] or genotoxic stress [23,24]. A body of evidence indicates that p53 can activate or repress tran-

* Corresponding author. Fax: +1 504 588 5707.
E-mail address: gmorris2@tulane.edu (G.F. Morris).

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doi:10.1016/j.yexcr.2004.09.033

scription of the PCNA gene [25–29]. The various roles of PCNA and the large number of PCNA interacting proteins [20] suggest that modulation of PCNA levels by p53 may integrate various cellular responses to stress. Although mechanisms of transcriptional activation or repression by p53 have been addressed [3,17], the mechanisms of dual regulation of a single target gene by p53 remain largely undefined. The p53 protein levels and the relative DNA binding affinity for various responsive elements are critical determinants of differential activation of target genes by p53 [30]. The binding affinity of p53 for specific sequences can be modulated in several ways [6,7]. Phosphorylation at serine 315 in the C terminus of p53 by cyclin-dependent kinases stimulates sequence-specific DNA binding activity and differentially alters the affinity of p53 for binding specific sites [31]. A mouse monoclonal antibody that binds to an epitope in the C-terminus of p53, PAb421, enhances p53 binding to the high affinity upstream p53 site in the p21/WAF1 promoter in vitro, but diminishes p53 binding to a lower affinity downstream site [32] or to a p53 site in the bax promoter [33]. PCNA and p21/WAF1 are regulated differentially by p53 in irradiated cells with activation of p21/WAF1 greater than that of PCNA [34]. Since p21/WAF1 binds PCNA and thereby inhibits its function in DNA replication [35], the relative levels of these two p53 target proteins can affect cell cycle progression. Differential regulation of PCNA and p21/WAF1 expression by p53 likely contributes to a properly coordinated cellular response to DNA damage. The experiments described here address whether or not the specific p53-binding sequence limits activation of the PCNA promoter by p53.

Materials and methods

Plasmids

The PCNA-CAT reporter constructs contained the full-length human PCNA promoter sequences −1265 to +62 relative to the transcription initiation site (+1), fused upstream of the chloramphenicol acetyltransferase (CAT) reporter sequences in pCAT-Basic (Promega, Madison, WI). Briefly, both the PCNA-CAT in pBACAT [36] and the pCAT-Basic vector were double digested with *Xho*I and *Nco*I. The *Xho*I–*Nco*I digest of pCAT basic releases a small fragment encompassing the upstream portion of the CAT coding sequence. The *Xho*I–*Nco*I fragment from PCNA-CAT with the promoter and upstream portion of CAT was then inserted into the *Xho*I–*Nco*I-digested pCAT-Basic vector by ligation. The PCNA-CAT reporter in pCAT-Basic harboring the resident p53-binding sequences (−236 to −217) was designated as PCNA-CATwt. Two variant PCNA-CAT reporter constructs were generated by site-directed PCR mutagenesis (see Fig. 4A). In PCNA-CATp21, the resident p53-binding site was replaced by the p53 binding sequences from the p21/WAF1 5′ site. PCNA-CATmt was generated by mutating all the essential fourth G/C nucleotides [29] within the p53-binding site. Briefly, DNA fragments that harbor the p21 5′ p53-binding site (Fig. 3A, p21 5′) or a mutated PCNA p53-binding site (Fig. 3A, MT) were generated by PCR of sequences −296 to −205 of the PCNA-CATwt template with the following primers: forward primer for PCNA-CATp21 and PCNA-CATmt, GAGGCT GAGGAGCCACC (−296 to −280); reverse primer for PCNA-CATp21, AACCCGGCCGCAGAACATG-TCCCAACATGTTGGGA GATC (−205 to −243); reverse primer for PCNA-CATmt AACCCGGCCGCAGAATAAA-TCCGGGTA TATGTGG (−205 to −238). The PCNA-CATwt construct and the PCR products were digested with *Bst*XI (−278) and *Bst*ZI (−210) followed by gel-purification to generate cohesive ends for ligation. The PCR products were substituted for the wild-type sequence in PCNA-CAT by ligation of the complementary ends. Plasmid pC53-SN3 expresses human wild-type p53 from the CMV promoter [37]. The p53S46A mutant was generated by mutating the serine 46 residue to alanine using site-directed PCR mutagenesis as described elsewhere [11] with Quickchange purchased from Stratagene (La Jolla, CA). pON260 expresses β-galactosidase from the cytomegalovirus (CMV) immediate early promoter [38]. pSuper.p53, a plasmid that expresses a short hairpin RNA that inactivates p53 mRNA [39], and the empty vector control plasmid, pSuper, were purchased from Oligoengine, Inc. (Seattle, WA).

Antibodies

p53-specific mouse monoclonal antibodies, PAb1801 and PAb421, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Oncogene Research (Boston, MA), respectively. A p53-specific sheep polyclonal antibody, Ab-7, and a rabbit polyclonal antibody to p21/WAF1, Ab-5, were purchased from Oncogene Research. A rabbit polyclonal antibody specific for p53 with serine 46 phosphorylated, Phospho-p53 (Ser46), was purchased from Signaling Technologies (Beverly, MA). An actin-specific goat polyclonal antibody, I-19, was purchased from Santa Cruz Biotechnology.

Cell culture and irradiation

HeLa cells, a human cervical carcinoma cell line, H1299 cells, a human large cell lung carcinoma cell line, and A549 cells, a human lung adenocarcinoma cell line, were purchased from ATCC. All cells were grown in DMEM (Sigma, St. Louis, MO) with 10% FBS (vol/vol), 100 μg/ml penicillin, and 100 μg/ml streptomycin. A549 cells were exposed to ionizing radiation at a rate of 1.1 Gy/min from a ^{137}Cs source in a Gammacell 40 low-dose-rate research irradiator (MDS Nordian, Inc., Kanata, Ontario, Canada).