Elevated Expression of Ribosomal Protein Genes L37, RPP-1, and S2 in the Presence of Mutant p53

W. Troy Loging and David Reisman

Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

Abstract

The wild-type p53 protein is a DNA-binding transcription factor that activates genes such as p21, MDM2, GADD45, and Bax that are required for the regulation of cell cycle progression or apoptosis in response to DNA damage. Mutant forms of p53, which are transforming oncogenes and are expressed at high levels in tumor cells, generally have a reduced binding affinity for the consensus DNA sequence. Interestingly, some p53 mutants that are no longer effective at binding to the consensus DNA sequence and transactivating promoters containing this target site have acquired the ability to transform cells in culture, in part through their ability to transactivate promoters of a number of genes that are not targets of the wild-type protein. Certain p53 mutants are therefore considered to be gain-of-function mutants and appear to be promoting proliferation or transforming cells through their ability to alter the expression of novel sets of genes. Our goal is to identify genes that have altered expression in the presence of a specific mutant p53 (Arg to Trp mutation at codon 248) protein. Through examining differential gene expression in cells devoid of p53 expression and in cells that express high levels of mutant p53 protein, we have identified three ribosomal protein genes that have elevated expression in response to mutant p53. Consistent with these findings, the overexpression of a number of ribosomal protein genes in human tumors and evidence for their contribution to oncogenic transformation have been reported previously, although the mechanism leading to this overexpression has remained elusive. We show results that indicate that expression of these specific ribosomal protein genes is increased in the presence of the R248W p53 mutant, which provides a mechanism for their overexpression in human tumors.

Introduction

The wt p53 gene encodes a DNA-binding transcription factor that functions as a negative growth regulator (1, 2) that controls entry into the G1-S and G2-M phases of the cell cycle. Conditions of cell stress, such as DNA damage and hypoxia (3, 4), lead to p53 induction. p53 transactivates a number of different genes and, depending on which genes are induced, the expression of p53 can have a number of different effects on the cell cycle. For example, G1 arrest is thought to occur in part through the induction of p21. DNA damage repair is thought to occur through the induction of GADD45, and apoptosis is thought to occur through the induction of Bax (5).

Mutations in the DNA-binding domain of p53 have been found in more than 60% of all human tumors examined, making mutation in p53 the most commonly observed mutation in human cancers. One of the most commonly occurring mutations in p53 is the Arg to Trp mutation at codon 248 (R248W), and recent findings using X-ray crystallography suggest that this, as well as other mutations, affects DNA binding without affecting the overall structure of the protein. The positively charged side chain of Arg248 on the wt protein interacts with the negatively charged phosphodiester backbone of the target DNA sequence. Other amino acids, such as Arg175 and Asp281, provide conformational stability through interactions with other amino acids on p53 (6). Therefore, p53 mutants can be placed into two general categories: (a) those affecting DNA contact; and (b) those affecting the the conformation required for DNA contact to take place.

Recent findings have suggested that in some cases, the process by which mutant p53 leads to cell transformation is through a gain of function. It appears that loss or inactivation of p53 often may not necessarily be sufficient for transformation, because the majority of common tumor types are found to contain missense mutations rather than premature terminations or deletions in the gene (7). The presence of mutant p53 may coincide with the expression of novel sets of downstream genes (8). Expression of a number of mutant p53 proteins has in fact been shown to alter the transcription of genes such as the multidrug resistance gene (MDR1; Ref. 9), c-myc (10), the HIV type 1 long terminal repeat (11, 12), and the human epidermal growth factor receptor (13). We hypothesize that the gain-of-function activity of mutant p53 seen in a variety of tumor cells expressing mutant p53 is due to its ability to alter the expression of genes that may contribute to increased proliferation or to the transformed phenotype.

Because altered gene expression is known to occur in presence of mutant p53, and these changes may play a role in the process of oncogenic transformation, it is important to identify genes that may be targets of mutant p53. We therefore...
sought to develop a system in which genes that are expressed in the presence of mutant p53 could be isolated. Through subtractive screening of a cDNA library derived from cells expressing the R248W p53 mutant, we observed elevated expression of several mRNAs transcripts. Three cDNAs that we have isolated contain high homology to mRNAs encoding ribosomal proteins L37, RPP-1, and S2. Interestingly, a number of recent studies have shown that these ribosomal protein genes are expressed at high levels in colon tumors. Our results indicate that the expression of these specific ribosomal protein genes is increased in the presence of the R248W p53 mutant and thus may provide a mechanism for their overexpression in human tumors.

Materials and Methods

Cell Culture and Transfections. 10(3) cells (provided by Dr. Arnold Levine, Princeton, University, Princeton, NJ) are murine fibroblasts that are devoid of endogenous p53 expression due to point mutations in the gene that introduce a premature translation stop codon at amino acid 173. 10(3) cells and derivatives of 10(3) expressing mutant p53 were maintained in DMEM containing 10% FBS, 2 mM glutamine, 100 μg/ml streptomycin, and 100 μg/ml penicillin. All Burkitt’s lymphoma-derived cell lines and early-passage EBV-nontransformed lymphoblastoid cell lines (LCLs; provided by Dr. Bill Sugden, University of Wisconsin, Madison, WI) were grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml glutamine, and 100 μg/ml penicillin.

All human colon carcinoma-derived R248W p53 mutant was placed under the control of the CMV promoter on a plasmid that also expresses resistance to the drug G418. Cells at 30–40% confluence were transfected with this expression vector by calcium phosphate coprecipitation. Twenty-four h after transfection, the cells were shifted to media containing 500 μg/ml G418. After approximately 2–3 weeks of selection, viable clones of cells were picked and expanded into cell lines by continual growth in selective media. For chloramphenicol acetyltransferase assays, extracts were prepared at 48 h after transfection and assayed for chloramphenicol acetyltransferase activity using equivalent amounts of protein in a 1-h reaction at 37°C in the presence of acetyl-CoA and [14C]chloramphenicol. The products were separated by TLC and subjected to autoradiography.

Western Blot Analysis/Western Transfer. Cells were washed twice in PBS, and total cell extracts were prepared by resuspending the pellet in 100–150 μl of sample buffer [70 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 3% SDS, and 0.01% bromphenol blue]. Equal amounts of protein from each sample were separated by electrophoresis and transferred to Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham). Human p53 was detected by incubation of the membrane with anti-p53 pAb421 monoclonal antibody for 1 h, followed by washing and subsequent incubation with horseradish peroxidase-conjugated sheep antimouse immunoglobulin. Proteins were visualized by chemiluminescence (Amersham) and exposure to Kodak X-ray film from 15 s to 1 h. The molecular weights were determined by prestained standards (Bio-Rad). Equivalent protein loading was verified by staining the gel with Coomassie Blue after transfer.

cDNA Library Formation and cDNA Recovery. Total RNA was prepared from CM248.2 cell pellets by acid-guanidium thiocyanate/phenol-chloroform extraction using TRI-Reagent (Molecular Research Center, Inc.). mRNA was isolated by oligo(dT) cellulose column chromatography. cDNA was generated by reverse transcription using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). After ligation of EcoRI adapters, the cDNA was ligated into a Lambda Zap vector (Stratagene) to obtain 6.0 × 10^5 independent cDNA clones. The Lambda ZAP vector used to create the cDNA library contained T3/T7 primer regions flanking the cDNA cloning site. Therefore, we exploited this feature to isolate the cDNA inserts by PCR using T3/T7 primers. This allowed for amplification of the inserts from the vector and their subsequent purification by agarose gel electrophoresis.

Subtractive Hybridization. cDNA probes enriched for CM248.2-specific sequences were obtained through subtractive hybridization. Subtracted cDNA probes were obtained by photobiotinylatation of 10 μg of polyadenylated mRNA isolated from the p53-null cell line 10(3). One μg of single-stranded cDNA generated from polyadenylated mRNA and isolated from the mutant p53-expressing cells, CM248.2, was hybridized to 10 μg of biotinylated 10(3) mRNA for 24 h at 65°C. The cDNA-mRNA hybrid was eliminated after the addition of streptavidin by phenol-chloroform extraction. The remaining cDNA, calculated to be 4–5% of the initial input mRNA, was labeled with α-32P by random priming.

Northern Blot Analysis/Northern Transfer Analysis. Total RNA was prepared from cell pellets by acid-guanidium thiocyanate/phenol-chloroform extraction using TRI-Reagent (Molecular Research Center, Inc.). Ten μg of RNA were denatured with formaldehyde-formamide and separated in a 1% agarose/formaldehyde gel. RNA was then transferred to BioTrace NT membrane (Gelman Sciences). The blot was hybridized with [α-32P]dATP-labeled probes. After multiple washings at 55°C with 2× saline-sodium phosphate-EDTA, signals were detected using a Storm 860 PhosphorImager (General Dynamics).

Results

We sought to isolate genes that are overexpressed in the presence of the R248W p53 mutant through subtractive screening of a cDNA library generated from cells expressing this p53 mutant. The parental cell line that was used in these experiments is an immortalized, p53-null, murine fibroblast cell line known as 10(3). The 10(3) cells are ideally suited for these experiments because, although they are immortalized, they do not express a transformed phenotype (14). To establish derivatives of the 10(3) cells expressing mutant p53, we carried out stable transfections with a mammalian expression vector containing p53 with an Arg to Trp mutation at codon 248 expressed from the CMV promoter. After transfection and selection for G418 resistance, 32 G418-resistant clones were obtained. The p53 expression in these cells was assayed by Western blot analysis using anti-p53 antibodies. Of the 32 clones generated, 12 were found to express mutant p53 (Inset Fig. 1). Three of the mutant p53-expressing clones, labeled CM248.2, CM248.6, and CM248.9, were used in growth assays along with the parental 10(3) line. All three p53-expressing cell lines showed a considerable enhancement in growth rates when compared with 10(3). The doubling time of the 10(3) cell line was found to be approximately 36 h at logarithmic growth as compared with 18 h for CM248.2 and CM248.9 clones (Fig. 1). The CMV248.6 cells had an intermediate doubling time of approximately 24 h. The number of cells at saturation density was found to be higher in the CM248.2 and CM248.9 clones than in the 10(3) cell line. These results indicate that, consistent with previously reported results, the 10(3) cells...
expressing high levels of mutant p53 have increased growth potential.

The ability of mutant but not wt p53 to alter gene expression of the multidrug resistance gene (MDR1) represents a gain-of-function activity that appears to be essential for transformation by certain mutants of p53. Therefore, to determine whether the mutant p53 expressed in the CM248.2 cells coincided with expression of the MDR1 gene, the CM248.2 cell line was assayed for MDR1 expression with the pMDR-CAT reporter. In cells lacking p53 (parental 10(3) cells), there was a very low level of MDR1 expression. In contrast, in the CM248.2 cells, which express the mutant p53 protein, transcription of the MDR1 gene was enhanced approximately 5–7-fold (data not shown), indicating that these cells have altered gene expression attributable to the mutant p53-dependent gain-of-function activity.

Having established altered gene expression in cell lines that express the R248W mutant p53 allele, we screened for other cellular genes whose expression may be altered in the presence of this mutant. To do this, we prepared a cDNA library in bacteriophage lambda having approximately 600,000 independent cDNA clones from mRNA derived from the CM248.2 cell line. We used this cell line because it was found to express the highest levels of mutant p53 protein and demonstrated a growth advantage over the parental 10(3) cells. This cDNA library, which we predicted would contain cDNA clones representing genes induced in the presence of mutant p53, was then screened by hybridization to a subtracted cDNA probe enriched for cDNAs specific for the CM248.2 cells. This subtracted cDNA probe was prepared by generating single-strand cDNA from the CM248.2 cells and then hybridizing this cDNA to an excess of in vitro biotinylated mRNA from parental p53-negative 10(3) cells. The hybrids were treated with streptavidin and removed by phenol-chloroform extraction. By this method, we routinely eliminated approximately 95% of the starting cDNA material that represents transcripts common to both cell lines. The remaining single-stranded cDNA molecules therefore represent a population that is highly enriched for cDNAs that are unique to the cells expressing the p53 mutant. This material was labeled and used as a probe to screen the cDNA library. Twenty individual plaques that showed hybridization were picked and plaque-purified. During the final round of plaque purification, duplicate filters were probed with labeled cDNA derived from 10(3) and CM248.2 cells. Only those candidates that showed elevated levels of hybridization to cDNA derived from CM248.2 cells were picked for further analysis.

The plaque-purified cDNA clones were isolated by PCR, labeled with 32P, and used to probe Northern blots containing RNA isolated from the parental 10(3) cells and the p53-expressing CM248.2 cells. Three of the cDNA clones isolated showed up to 5-fold elevated expression in CM248.2 cells (Fig. 2, A and C). These clones, labeled Bri4, Bri5, and Bri8, were subcloned, sequenced, and compared to known sequences within the Genetics Computer Group (GCG) databases. Bri4 was found to be close to 100% homologous to the acidic RPP-1 mRNA from mouse (accession number U29402), Bri5 was found to be homologous to ribosomal protein L37 mRNA from rat (accession number S79981), and Bri8 was found to be homologous to ribosomal protein S2 mRNA from rat (accession number X57432). Interestingly, all three of these ribosomal protein genes have been shown in previous studies to be overexpressed in transformed cells derived from human tumor biopsies (15–18). As a control to ask whether the elevated expression of these three genes was due to a general elevated expression of numerous ribosomal protein genes, we tested whether the gene encoding the large 60S ribosomal subunit protein L24 was also expressed at an elevated level in CM248.2 cells. As shown in Fig. 2R, L24 mRNA was expressed at approximately equal levels in 10(3) and CM248.2 cells. These results indicate that the elevated expression of RPP-1, L37, and S2 is not due to an overall general increase in the expression of ribosomal protein genes.

To test whether RPP-1, L37, and S2 genes are expressed at elevated levels in cells derived from human tumors that express mutant p53, we carried out Northern transfer analysis on RNA derived from a small panel of immortalized nontumorigenic human B-cell lines and Burkitt’s lymphoma cell lines. Our previous work has determined that mutant p53 is expressed at elevated levels in a number of Burkitt’s lymphoma cell lines and that the elevated p53 gene expression is due in part to elevated transcription of the mutant p53 gene (19). As shown in Fig. 3, all three mRNAs were in fact present at elevated levels in the Burkitt’s lymphoma cell lines (Namalwa and BL-30) when compared with nontransformed EBV-immortalized B-cell lines (LCL-3 and LCL-4). These results are consistent with the results presented above that the RPP-1, L37, and S2 genes have increased gene expression in the presence of mutant p53 protein.
Discussion

We have initiated a search for genes that are activated by an oncogenic form of the p53 tumor suppressor. Mutant p53 clearly contributes to the transformed phenotype because it can transform primary rat embryo fibroblasts in cooperation with the activated ras oncogene (20–22) and because it is tumorigenic in transgenic mice when expressed at high levels (23). However, this transforming activity in tissue culture assays may be due to the ability of mutant p53 to function as a trans-dominant negative repressor of wt p53 functions (24). A gain of transforming function by mutant p53, on the other hand, has been observed by introducing mutant p53 into immortalized cell lines lacking p53. A number of groups have shown that the introduction of mutant p53 into p53-negative immortalized cells can increase their growth rates in culture and converted them into cells that are tumorigenic in vivo (14, 25). One particular p53 mutant at codon 172 can cooperate with the neu/ERBB2 oncogene to promote mammary tumorigenesis in transgenic mice (26). These findings indicate that at least some forms of mutant p53 acquire novel functions that may contribute to oncogenic transformation.

The ability of mutant p53 to enter the nucleus and trans-activate gene expression has been shown to be required for its transforming activity. Missense mutations are rarely if ever observed in either the NH2-terminal transactivation domain of the protein or the nuclear localization signals. In fact, mutations in the nuclear localization signals destroy the transforming activity of mutant p53 (27). Here we report that three ribosomal protein genes, L37, RPP-1, and S2, are expressed at elevated levels in cells expressing the R248W p53 allele. The expression of these mRNAs was shown to be elevated 3–5-fold in comparison to the p53-null parental cell line. Furthermore, all three were found to be expressed at elevated levels in Burkitt’s lymphoma cell lines overexpressing mutant p53. We are now in the process of determining the mechanism of induction of these genes by p53.

Interestingly, this is not the first report that these ribosomal protein genes are overexpressed in human tumors. L37 overexpression was first identified in colon carcinomas (15), a tumor type in which there is a high frequency of expression of the mutant (R248W) p53. In the same study, the acidic ribosomal phosphoprotein P0, a gene having high homology to the acidic RPP-1, was found to be overexpressed predominantly in both colonic and hepatocellular carcinomas. Furthermore, using the newly described SAGE technique to examine gene expression in human colon tumors, Vogelstein and colleagues found...
linked to ribosomal protein gene expression are intriguing and suggest that p53 may be linked to ribosome activity or regulation in human tumors through the modulation of a subset of ribosome-related genes. The mechanism by which mutant p53 contributes to activation of ribosomal protein genes is currently being addressed.

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References


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