Identification of two novel functional p53 responsive elements in the herpes simplex virus-1 genome

Jui-Cheng Hsieh *, Ryan Kuta, Courtney R. Armour, Paul E. Boehmer

Department of Basic Medical Sciences, University of Arizona College of Medicine-Phoenix, 425 North 5th Street, Phoenix, AZ 85004-2157, USA

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Analysis of the herpes simplex virus-1 (HSV-1) genome reveals two candidate p53 responsive elements (p53RE), located in proximity to the replication origins oriC and oriS, referred to as p53RE-L and p53RE-S, respectively. The sequences of p53RE-L and p53RE-S conform to the p53 consensus site and are present in HSV-1 strains KOS, 17, and F. p53 binds to both elements in vitro and in virus-infected cells. Both p53RE-L and p53RE-S are capable of conferring p53-dependent transcriptional activation onto a heterologous reporter gene. Importantly, expression of the essential immediate early viral transactivator ICP4 and the essential DNA replication protein ICP8, that are adjacent to p53RE-S and p53RE-L, are repressed in a p53-dependent manner. Taken together, this study identifies two novel functional p53RE in the HSV-1 genome and suggests a complex mechanism of viral gene regulation by p53 which may determine progression of the lytic viral replication cycle or the establishment of latency.

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Introduction

The genome of human herpes simplex virus-1 (HSV-1) is a linear double-stranded DNA of ~152-kbp containing two types of DNA replication origin: one copy of oriC, located within the unique long region, and two copies of oriS, located in the c repeats flanking the unique short region (Boehmer and Lehman, 1997). oriC is flanked by the promoters of the essential DNA replication genes UL29 and UL30, encoding the single-strand DNA binding protein (ICP8) and DNA polymerase, respectively. In contrast, oriS is flanked by the promoters of the immediate early (IE) genes ICP4 and ICP22 or ICP47, that are critical for the viral life cycle (Rajcani and Durmanova, 2000). Although the functions of many HSV-1 gene products have been extensively studied, the molecular mechanisms governing their expression are not fully understood, particularly their differential regulation during lytic replication and latency.

Human p53 is a phospho-protein composed of several structural and functional domains, including an N-terminal transactivation domain, a central DNA-binding domain, a tetramerization domain, and a C-terminal regulatory domain (Somassundaram and El-dieery, 2000). Biochemically, p53 acts as a transcription factor capable of mediating both activation and repression of gene expression. p53 tetramers bind to specific DNA elements in the promoter regions of target genes leading to activation or repression of gene expression (Riley et al., 2008). Crystallographic analysis has revealed a loop–sheet–helix motif and two large loops within the DNA-binding domain of p53 that mediate interactions with both the major and minor grooves of DNA (Cho et al., 1994). Binding of p53 to DNA occurs either after post-translational modification or when it forms a complex with other essential nuclear proteins (Funk et al., 1992). Expression of p53 is induced in response to diverse stimuli including: DNA damage, hypoxia, oncogene activation, and viral infection, whereupon it executes a plethora of functions to control the dynamic pattern of gene expression required for maintaining a normal cell-cycle (Beckerman and Prives, 2010). Biologically, p53 functions as a tumor suppressor, acting as the “guardian of the genome” (Lane, 1992) and the “cellular gatekeeper” (Levine, 1997) by regulating a number of downstream target genes involved in cell-cycle regulation, DNA repair, cell–cell interaction, immune response, and apoptosis (Collot-Teixeria et al., 2004). Despite these important roles, mutations in p53 are not lethal (Donehower et al., 1992). Rather, p53 mutations are the most common genetic abnormality found in human cancer including colon, breast, and lung cancers (Levine, 1993). Through its multiple actions, p53 has also been documented to be a key regulator of viral infections (Lazo and Santos, 2011).

Earlier studies have shown that p53 is not only recruited to nuclear HSV-1 DNA replication compartments, but also colocalizes with the essential viral DNA replication protein ICP8...
(Wilcock and Lane, 1991). This redistribution of p53 is specific, since other host nuclear proteins including p68, lamin B, nucleophosmin, and Ki67 do not redistribute during HSV-1 infection. Also, Maruzuru et al. (2013) recently reported that p53 can play both a positive and negative role in HSV-1 replication at different stages by regulating ICP27 and ICP0 gene expression. However, the molecular basis of these effects was not explored in their report. These observations and the fact that p53 is a transcription factor with DNA binding activity (Pavletich et al., 1993), prompted us to hypothesize that a p53 responsive element(s) (RE) may exist in the HSV-1 genome, allowing p53 to exert transcriptional control over viral gene expression and/or DNA replication. Therefore, in this study, we sought to identify p53RE in the HSV-1 genome. Here we identify two candidate p53RE, located in the vicinity of the HSV-1 DNA replication origins oriL and oriS. Further, we show that p53 binds to both these sites in vitro and in vivo. In addition, we demonstrate that these novel p53RE can confer transcriptional control onto a heterologous reporter gene. Importantly, we find that p53 affects the expression of two essential viral proteins (ICP4 and ICP8) that are adjacent to these p53RE. The presence of functional p53RE in the HSV-1 genome has implications for a direct role of p53 in controlling HSV-1 replication.

Results

Identification of two putative p53-responsive elements in the HSV-1 genome

To test the hypothesis that p53 may directly interact with the HSV-1 genome via p53RE to potentially control viral gene expression and/or DNA replication, we performed an in silico search for conserved p53 binding sites in the HSV-1 genome. The consensus p53RE contain two copies of a 10 bp binding motif 5′-RRR[AC/AT][TT/AC]YYY, in which the core motif is underlined and R is a purine and Y is a pyrimidine, separated by a spacer of 0–13 bp (El-Deiry et al., 1992). These binding motifs can occur as direct or inverted repeats, which dictates the symmetry with which the p53 tetramer occupies the site (Ma et al., 2007). As shown in Fig. 1A, the analysis revealed two candidate p53RE: one in the vicinity of oriL and the other in the c repeats, in the vicinity of oriS, henceforth designated p53RE-L and p53RE-S, respectively. In HSV-1 strain KOS, p53RE-L is located at nucleotides 62608–62630, while the two p53RE-S are located at nucleotides 131119–131144 and 146713–146738. The sequence of p53RE-L, 5′-AAACAGGGCCCTTGCAATGCCCG, contains a pair of perfect 5′-C(A/T)[T/A]CAG-3′ p53RE core motifs with a 3 bp spacer (indicated in lower case). In contrast, the sequence of the two p53RE-S, 5′-GAATTACCGGCG-gaccGTTGATGCT-3′, contains an extra G in the second core motif and a 5 bp spacer. A precedent for an extra G exists in the p53RE of caspase 10 (CASP10) (Margalit et al., 2006). A detailed mapping of the HSV-1 p53RE relative to the viral replication origin domains and promoter elements is shown in Fig. 1A. Importantly, both p53RE-L and p53RE-S are present in identical locations with 100% sequence identity in HSV-1 strains F, 17 and KOS.

Fig. 1B shows a comparison and sequence alignment between p53RE-L and p53RE-S and other well characterized p53RE, including those associated with the HSV-1 TK (Yuan et al., 1993), BCL6 (Margalit et al., 2006), ABCA1 (Johnson et al., 2001), GADD45A (Holland et al., 1993), p21 (Saramaki et al., 2006), MDM2 (Zauberaman et al., 1995), VDR (Maruyama et al., 2006), p73 (Chen et al., 2001), CASP6 (MacLachlan and El-Deiry, 2002), and CASP10 (Rikhof et al., 2003) genes, and hepatitis B virus (Orl et al., 1998). It should also be noted that albe1 not the subject of the current studies, our analysis indicates that candidate p53RE exist in other herpesvirus family members including Varicella Zoster Virus, Epstein Barr virus and cytomegalovirus (data not shown). These findings, together with the reports cited above, suggest that p53 may play a role in regulating HSV-1 replication. In this regard, it is noteworthy to mention that the HSV-1 p53RE are flanked by important viral genes: in the case of p53RE-L the essential early genes encoding the DNA polymerase (UL30) and ICP8 (UL29), and in the case of p53RE-S the critical IE genes ICP4, and ICP22 or ICP47 (Fig. 1A).

p53 binds to HSV-1 p53RE in vitro and in vivo

Electrophoretic mobility shift assays (EMSA) were utilized to examine the ability of p53 to bind 32P-labeled oligonucleotides containing the p53RE-L and p53RE-S sequences. The GADD45A p53RE (5′ GAACATGCTAAGCATGCGT) were used as a positive control. Initially, we evaluated the ability of purified Escherichia coli expressed p53 to bind the probes. The results show that purified p53 can indeed bind directly to the GADD45A, p53RE-L and p53RE-S oligonucleotides (Fig. 2A, lanes 2, 4, and 6). As indicated, two complexes, designated I and II, were formed with GADD45A and p53RE-L, while only complex II was evident with p53RE-S. While these results do not address the affinity with which p53 interacts with these sites, they nevertheless demonstrate that these sites are recognized in the absence of any other co-factors and despite the fact that p53 may lack some of the typical mammalian post-translational modifications that may affect binding. Subsequently, to evaluate the recognition of these sites by p53 in the context of any possible co-factors, we performed EMSA with cell extracts. Hence, in order to obtain functional p53 protein along with any necessary co-factors, p53 was overexpressed in mammalian COS-7 cells, followed by preparation of whole cell lysates. Immunoblot analysis demonstrates robust expression of p53 in both mock-transfected and to a higher level in p53-transfected COS-7 cell lysates used for EMSA (Fig. 2B). Fig. 2C shows that lysates incubated with the GADD45A p53RE, p53RE-L and p53RE-S probes led to the formation of the same complexes (I and II) observed with purified p53 (Fig. 2C, lanes 2, 6, and 10). Notably, both complexes I and II were observed with p53RE-S. To ascertain that complexes I and II contain p53, EMSA was performed in the presence of p53-specific monoclonal antibodies, either DO-1 or PAb1801, which recognize residues 11–25 and 32–79 of human p53, respectively. The addition of DO-1 had no discernible effect on complexes I and II formation with either GADD45A p53RE-L and p53RE-S (Fig. 2C, lanes 3, 7, and 11). In contrast, in the presence of PAb1801, complex I formation was enhanced, whereas complex II formation was dramatically inhibited with all three p53RE (Fig. 2C, lanes 4, 8, and 12). No apparent ternary antibody–p53–DNA complex was formed with either antibody as evidenced by the lack of a supershifted species. While the formation of such ternary complexes is typically used to evaluate the specificity of a protein–DNA complex, prior studies with particular p53 antibodies support our findings that ternary complexes may not form. Rather, consistent with our results, studies with the Cdc25C and the 3′-p21 p53RE found that PAb1801 and PAb421 inhibited complex formation (Resnick-Silverman et al., 1980), while p53 binding to the MDR1 promoter p53RE was enhanced by PAb240, PAb246 and PAb1620 (Johnson et al., 2001). Hence, our observation of specific PAb1801-induced changes in the dynamics of complexes I and II formation demonstrates that p53 is part of these complexes. Taken together, our results indicate that p53 binds to both p53RE-L and p53RE-S, and based on the formation of two complexes with varying sensitivity to antibody, that the conformation of the p53 protomer on these two elements may be different. To further characterize the specificity of complexes I and II, a competition assay was performed using unlabeled GADD45A,
p53RE-L and p53RE-S oligonucleotides as specific competitors to compete with the labeled probes for p53 binding. Complexes I and II formed with GADD45A p53RE-L or p53RE-S were all markedly reduced by a 50-fold excess of each of the unlabeled specific competitors (Fig. 2D, lanes 3–5, 8–10, and 13–15). Slight differences were noted in the effectiveness of the three unlabeled oligonucleotides when competing for binding with p53RE-L, with the homologous p53RE-L being the most effective and the p53RE-S being the least effective (compare Fig. 2D, lanes 8–10). EMSA was also performed using radiolabeled p53RE-L and p53RE-S probes containing C to A and G to T substitutions at both ends of the core motif (5′-C(A/T)(A/T)G-3′). EMSA with these mutated p53RE displayed significantly reduced complex formation (Fig. 2E, lanes 3, 4, 7, and 8), thus confirming the specificity and crucial role of these bases in the binding by p53. Together, the results indicate that p53 binds specifically to p53RE-L and p53RE-S.

We proceeded to examine whether p53 was capable of binding to p53RE-L and p53RE-S in vivo in HSV-1-infected Vero cells using ChIP assays. The results show that both p53RE-L and p53RE-S sequences are co-precipitated by the p53-specific antibody DO-1 antibody (Fig. 3A, lanes 6 and 12), but not by normal rat IgG (Fig. 3A, lane 5). No PCR product was detected in control precipitations lacking antibody (Fig. 3A, lanes 1–3 and 7–9). In a further control to demonstrate the specificity of p53 binding to p53RE-L and p53RE-S, using a primer pair corresponding to a non-p53RE-containing region of the UL30 gene, no amplification was detected following immunoprecipitation with the DO-1 antibody (Fig. 3B). Taken together, these results provide evidence that p53 binds to p53RE-L and p53RE-S in HSV-1 infected cells.

p53RE-L and p53RE-S confer p53-dependent reporter gene activation

Since p53 binds to both p53RE-L and p53RE-S in vitro and in vivo, these sequences might function to regulate viral gene expression. To investigate whether p53RE-L and p53RE-S can function as bona fide p53RE, luciferase reporter gene vectors were constructed that contain wild-type and mutant variants of p53RE-L or p53RE-S. The mutations engineered into p53RE-L and
p53RE-S were identical to those that disrupt p53 binding in EMSA. A vector containing the GADD45A p53RE was used as a positive control while the non-recombinant plasmid was used to determine baseline reporter gene activity. Each of the reporter plasmids, including one encoding Renilla luciferase as an internal control, were co-transfected with a p53 expression plasmid into four recipient cell lines: African green monkey COS-7 fibroblast-like kidney cells, human colon cancer p53 knock-out cells HCT116 (p53−/−), human embryonic kidney cells HEK293, and African green monkey kidney epithelial Vero cells. The results from dual luciferase assays of cell lysates indicate that both p53RE-L and p53RE-S were capable of conferring p53-dependent luciferase reporter gene activity in COS-7 cells (Fig. 4A). However, reporter gene activation was significantly more robust with p53RE-S (~40-fold increase) than with p53RE-L (~3-fold activation). In the presence of 5-fluorouracil (5-FU), a DNA damaging agent and p53 co-activator (Bunz et al., 1999), reporter gene activity was enhanced in all cases, with p53RE-S mediating higher activity than p53RE-L as observed in the absence of 5-FU. Consistent with the results obtained by EMSA, mutant p53RE-L and p53RE-S, exhibited baseline reporter gene activity, thus confirming the importance of the C and G residues in the core motifs of p53RE-L and p53RE-S.
Similar co-transfections were performed in the p53 knock-out cells HCT116 (p53\(^{−/−}\)), HEK293 cells, and Vero cells. In the absence of p53, no reporter gene activation was observed in HCT116 (p53\(^{−/−}\)) cells (Fig. 4B). However, co-transfection with p53 led to \(~255\)- and \(~47\)-fold increases in reporter gene activity with p53RE-L and p53RE-S, respectively (Fig. 4B). Similarly, there were \(~30\)- and \(~2146\)-fold increases in p53 dependent activation of reporter gene activity by p53RE-L and p53RE-S in HEK293 cells (Fig. 4C), and corresponding increases of \(~32\)- and \(~221\)-fold in Vero cells (Fig. 4D). The differential activity of p53RE-L and p53RE-S between cell lines observed in these assays suggests that a cell or tissue specific factor may influence the action of p53 on the HSV-1 p53RE. Nonetheless, these results indicate that p53RE-L and p53RE-S are functional p53RE, each of which is sufficient to confer p53-dependent transcriptional activation on a luciferase reporter gene.

**p53 represses p53RE proximal viral genes**

To gain initial insight into whether p53 affects viral gene expression in a manner that may be mediated via the HSV-1 p53RE, we utilized quantitative real-time PCR and immunoblotting to examine the levels of ICP4 and ICP8, representing genes proximal to p53RE-S and p53RE-L, respectively. Fig. 5 shows that transcript levels of both ICP4 and ICP8 are significantly reduced in HSV-1 infected cells expressing p53. Thus, in p53 knock-out HCT116 (p53\(^{−/−}\)) cells, their expression is \(~5\)-fold higher than in the parental (p53\(^{+/+}\)) cells that express normal levels of p53 (Fig. 5A). Similarly, in HeLa cells ICP4 and ICP8 expression are \(~20\)-fold higher than in cells which over-express p53 via transfection (Fig. 5B). These findings are substantiated by immunoblotting which shows that ICP4 and ICP8 protein levels are reduced when p53 is overexpressed (Fig. 6). Thus, HEK293 cells infected with HSV-1 show robust expression of both ICP4 (both at 6 and 24 h post-infection) and ICP8 (at 24 h post-infection) (Fig. 6, lanes...
was used as a loading control.

50

observed in HCT116 (p53

Materials and methods. (A) p53

transfection with p53 (Fig. 6, lanes 3 and 6).

Fig. 5. Repression of ICP4 and ICP8 gene expression by p53. Quantitative real-time PCR analysis of ICP4 and ICP8 gene expression was performed as described in Materials and methods. (A) p53−/− HCT116 (solid) and parental p53+/+ (empty) cells. (B) Mock-transfected (solid) and p53-transfected (empty) HeLa cells. The results obtained for ICP4 and ICP8 were normalized to human glyceraldehyde phosphate dehydrogenase expression, and are shown relative to the values observed in HCT116 (p53−/−) (A) and mock-transfected HeLa cells (B) which were assigned values of 1.0. Each bar represents the average of 3 independent experiments ± standard deviation.

Fig. 6. Repression of ICP4 and ICP8 protein expression by p53. Immunoblot showing ICP4, ICP8, p53 and β-actin levels in mock-infected/transfected and HSV-1-infected/p53-transfected HEK293 cells. Where indicated, cells (3 × 10⁵) were transfected with 2 μg pC53-SN. 24 h post-transfection cells were infected with HSV-1 at a moi of 10 as indicated. Cells were harvested at the indicated times post-infection. 20 μg of cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting as described in Materials and methods. Each panel represents the area of the gel corresponding to ICP4, ICP8, p53 and β-actin as indicated. β-actin was used as a loading control.

2 and 5) while their expression is barely detectable upon cotransfection with p53 (Fig. 6, lanes 3 and 6). β-actin was used as a loading control (Fig. 6, bottom panel). Collectively, these data indicate that p53 has a repressive effect on ICP4 and ICP8.

Discussion

The molecular mechanisms by which p53 regulates target genes are complicated and remain elusive. The mechanisms for p53 action appear to fall into two general categories: p53RE-dependent and p53RE-independent. In the first case, p53 mediates transcriptional control by binding to p53RE as a tetramer and subsequently recruiting diverse transcriptional co-regulators in response to distinct genetic stimuli. Alternatively, p53 is reported to regulate target genes via protein–protein interactions or in response to post-translational modification including phosphorylation, acetylation, ubiquitination, and/or methylation (Beckerman and Prives, 2010).

To date, over one hundred and sixty-two p53RE have been published and about a quarter of them were found to be repressive elements (Riley et al., 2008; Wang et al., 2009). Due to the degeneracy of the p53 binding motif, each p53RE displays variations in both the core motif sequence and in the spacer length. Most p53RE exist in the promoters and introns of cellular target genes. p53 is known to have differential effects on several viral systems. Not considering our current findings, only three other conserved p53RE have been characterized in viral systems including in the enhancer of hepatitis B virus (5’ TTGCATGTATAAGGCTGCTTGGATTTGC) (Ori et al., 1998), in the non-coding region of human papillomavirus 77 (5’ AACAGTTGGACTGGAATCCCG (Purdie et al., 1999), and in the HSV-1 thymidine kinase gene promoter (5’ TGGCTTCCGTGGACTGGAATCCCG) (Yuan et al., 1993).

Additionally, it has been reported that p53 can bind to atypical viral DNA elements such as the GC boxes of the simian virus 40 early promoter (Bargonetti et al., 1991) and the long terminal repeat of human immunodeficiency virus-1 (Guadagno et al., 1995). The case of human immunodeficiency virus-1 is particularly unusual in that its p53RE (5’ GCCCTGACGTGTCCCC) is not only located within the Tax-responsive element but also overlaps with a cAMP-responsive element. Moreover, during infection by human immunodeficiency virus-1, cytomegalovirus, Ab-murine leukemia virus, adenovirus, Epstein Barr virus, rift valley fever virus, and simian virus 40, it has been observed that p53 may undergo extensive post-translational modifications and participate in protein–protein interactions that affect viral protein expression (Lazo and Santos, 2011). These actions of p53 in viral infection not only display great mechanistic diversity, but may also provide crucial insight into virus–host cell interactions.

Here we provide evidence for two novel types of functional p53RE in a viral genome, namely that of HSV-1. The HSV-1 p53RE are located in the vicinity of the replication origins oriL and oriS. In HSV-1 strain KOS p53RE-L is situated at nucleotides 62608–62630 within the UL29–UL30 intergenic region, while p53RE-S is located in the c repeats at nucleotides 131119–131144 and 146713–146738, flanked by the critical IE genes ICP4 and ICP22 or ICP47. Analysis of the sequences of p53RE-L (5’ AAAAAAGGCCGCTGCAATGCGG) and p53RE-S (5’ GAACATGCGGCGGCTGCAATGCGG) reveals that p53RE-L represents a perfect match to the consensus with a 3 bp spacer between the two core p53 binding motifs, whereas p53RE-S has an extra G in the second motif and a 5 bp spacer.

We observed binding of p53 to both p53RE-L and p53RE-S, in vitro using EMSA, and in HSV-1-infected cells in vivo via ChIP. Moreover, both HSV-1 p53RE are functional based on their ability to activate reporter gene expression. Based on our EMSA results, p53 binds to p53RE-L with a higher apparent affinity than to p53RE-S. Despite the apparently lower affinity for p53RE-S and its divergence from the consensus, in all four cell lines tested, p53RE-S was significantly more active in the reporter gene assay than p53RE-L. Moreover, we found that reporter gene activation by p53RE-L and p53RE-S was markedly cell type-dependent, with the highest activity observed for p53RE-L in HCT116 (p53−/−) cells.
and p53RE-S in HEK293 cells. Together, these data suggest that the length of the spacer may influence p53RE activity, but that there may also exist cell-type specific interactions with host proteins that may affect the ability of p53 to exert its biologic actions. While p53RE-L and p53RE-S are closest to the UL30 and ICP4 transcription start sites, respectively, it is yet to be established which genes might be the actual targets for regulation by p53 and whether they are activated or repressed. Therefore, the possible effects of p53 on the expression of UL29, UL30, ICP4, ICP22 or ICP47 and other genes warrants further study. Moreover, given their location, effects of these p53RE on the initiation of HSV-1 DNA replication at or1L and/or or1S should also be entertained.

In this regard, our initial data indicate that in several cell lines, p53 represses the expression of ICP8 and ICP4, that are proximal to p53RE-L and p53RE-S, respectively. While further studies to delineate the underlying mechanisms are necessary, the findings raise the interesting notion that p53 has the ability to shut down the expression of critical viral regulatory proteins and thereby prevent a lytic replication cycle to perhaps favor the establishment of latency. Our results are consistent with a previous report by Yuan et al. (1993) in which the p53RE in the HSV-1 thymidine kinase gene promoter binds p53 and is involved in the repression of thymidine kinase gene expression.

Several groups have demonstrated that ectopic p53 expression can result in cell-cycle arrest or apoptosis via generally established pathways (Amundson et al., 1998; Smith et al., 2003) including in the following cell lines: the myeloid leukemia line M1 (Yonish-Rouach et al., 1991), osteosarcoma lines (Diller et al., 1990), colorectal carcinoma lines SW480 and SW837 (Baker et al., 1990), post-mitotic neurons (Slack et al., 1996), and HeLa (Haupt et al., 1995). In the case of HeLa cells, two distinct p53-dependent apoptotic pathways have been proposed, either relying on p53RE-specific transactivation or independent of p53RE sequences (Haupt et al., 1995). So while it is possible that ectopic expression of p53 in our transient co-transfections may have elicited such responses, albeit without performing a quantitative evaluation for apoptosis or cell-cycle markers, no morphological features associated with cell death were observed by microscopic examination of COS-7, HEK293, HeLa, Vero, and HT116 cell lines under our experimental conditions. Therefore, in our transient co-transfections, using the luciferase reporter gene assay with the empty reporter plasmid (pLucMCS) and the p53RE as negative and positive controls, respectively, under the conditions, the differential effects observed on reporter gene activity should be attributed uniquely to the HSV-1 p53RE.

Additional mechanism for regulating HSV-1 IE gene expression. Several groups have demonstrated that ectopic p53 expression mediated by the viral p53RE, providing initial insight into the possible effects of p53 on the expression of vital HSV-1 functions and therefore on its replication. While the present data provide some molecular evidence to support a regulatory role for p53 in HSV-1 infection, further studies assessing the functional role of these elements and the underlying mechanisms, taking into account their genomic context and their potential interaction with other transcription factors, are clearly merited.

**Materials and methods**

**Reagents**

p53 specific antibodies DO-1 and PAb1801 were purchased from Santa Cruz Biotechnology, Dallas, TX and Delta Biolabs, Gilroy, CA, respectively. Peroxidase-conjugated goat anti-mouse IgG, β-actin antibody and ICP4 antibody were from Santa Cruz Biotechnology. ICP8 antibody was as previously described (White and Boehmer, 1999). Cells were transfected using the Express-\(\text{In}\) transfection reagent (Thermo Scientific, Lafayette, CO). Plasmids totaling 1.0 μg were diluted into 25 μl serum-free medium and mixed with 25 μl serum-free medium containing 3 μl Express-\(\text{In}\) reagent. Following a 30 min incubation at room temperature, the DNA and Express-\(\text{In}\) mixtures were added to cells and incubated for 48 h. Protein concentrations were determined by the BCA assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s directions.

**Cell culture and virus**

COS-7, HeLa and Vero cells were grown in DMEM containing 10% fetal bovine serum (FBS). Parental (p53\(^{+/+}\)) and p53 knockout (p53\(^{-/-}\)) HT116 cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum. HEK293 cells were

<table>
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<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>p53 Genotype</th>
<th>Expressed antigen</th>
<th>Effects of antigen expression</th>
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<tr>
<td>COS-7</td>
<td>African green monkey kidney fibroblast</td>
<td>Wild-type</td>
<td>SV40 T antigen</td>
<td>SV40 T antigen inhibits p53 binding to DNA and reduces p53-mediated transcriptional activity (Jiang et al., 1993).</td>
</tr>
<tr>
<td>HT116 (p53(^{+/+}))</td>
<td>Human colorectal carcinoma</td>
<td>Wild-type</td>
<td>N/A</td>
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<tr>
<td>HT116 (p53(^{-/-}))</td>
<td>Human colorectal carcinoma</td>
<td>Knock-out</td>
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<td>N/A</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney epithelium</td>
<td>Wild-type</td>
<td>HPV-18 E6</td>
<td>E6 inhibits p53 DNA binding and affects its phosphorylation (Ajay et al., 2012; Thomas et al., 1993; Thomas et al., 1996).</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>Wild-type</td>
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The current understanding regarding the expression of the five HSV-1 IE genes, ICP0, ICP4, ICP22, ICP27, and ICP47, includes the dependency on the viral transactivator VP16, a component of the viral tegument protein that forms a transcriptional regulatory complex with Host Cell Factor-1 and cellular protein Oct-1 to bind to consensus 5'-TAATGARAT sequences in IE gene promoters (Wysocka and Herr, 2003; Kristie et al., 2009). Our discovery that p53RE-S is located between ICP4 and ICP22, may provide a novel additional mechanism for regulating HSV-1 IE gene expression.

In conclusion, we have demonstrated the presence of two novel functional p53RE in the vicinity of the replication origins in the HSV-1 genome. Importantly, we also show that p53 represses the expression of ICP4 and ICP8 which are in direct proximity to p53-RE-S and p53RE-L, respectively, presumably in a mechanism mediated by the viral p53RE, providing initial insight into the possible effects of p53 on the expression of vital HSV-1 functions and therefore on its replication. While the present data provide some molecular evidence to support a regulatory role for p53 in HSV-1 infection, further studies assessing the functional role of these elements and the underlying mechanisms, taking into account their genomic context and their potential interaction with other transcription factors, are clearly merited.

**Table 1**

Pertinent properties of cell lines used in this study.
maintained in MEM containing 10% FBS. The experiments performed in these studies used HSV-1 strain KOS.

Plasmid construction

The following synthetic oligonucleotides and their complements were obtained from IDT DNA (Coralville, IA): GADD45A p53RE (5’ AGCTTGAACCATGCTAAGCTGTCGTA), p53RE-E-L (5’ AGCT-TAAAGAGGGCCCTTGACATGGCCGA), p53RE-S (5’ AGCTTGAAC-TAGCCGGACCCGGTCAAGTCTTA), mutant p53RE-L (5’ AATGCTAACAGACGGCAATTGCGGA) and mutant p53RE-S (5’ AGCTTGAATAATCCGGACCAGGCTAAGTCTTA). Base substitutions relative to the wild-type sequences of p53RE-L and p53RE-S are shown in italics. Each oligonucleotide also contained terminal bases corresponding to the overhangs of HindIII and BglII at the 5’ and 3’ ends, respectively. Oligonucleotide pairs were annealed and inserted into the HindIII and BglII sites of pLuc-MCS (Stratagene Corp., La Jolla, CA). The integrity of each resulting reporter plasmid was confirmed by DNA sequencing. The p53 expression vector, pC53SN, was a generous gift from Dr. Curtis Harris at the National Cancer Institute (Huo et al., 2001).

Immunoblotting

For immunoblot analyses, cultured cells were harvested, washed three times with PBS, and resuspended in KEDT-0.3 buffer. After sonication, lysates were centrifuged at 13,000 rpm for 15 min at 4°C and supernatants collected. The specified amounts of cell lysate were resolved by 0.1% SDS, 7.5% PAGE, followed by transfer to Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA). The resulting membrane was blocked with 5% milk in TBST for 1 h and incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h at room temperature. Visualization of antibody complexes was performed using an ECL kit according to the manufacturer instructions (GE Healthcare, Pittsburgh, PA).

p53 protein expression and purification

The p53 expression vector pFLAG-p53 (a generous gift from Dr. Robert C. Roeder, Rockefeller University, New York, NY (Gu and Roeder, 1997)) was introduced into E. coli BL21(plysS). p53 expression was induced with 0.3 mM isopropyl β-D-thiogalactoside at 37°C for 3 h and the cell pellet suspended in extraction buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 15 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A) with sonication. p53 was immunoprecipitated with anti.Flag M2-agarose beads (Sigma-Aldrich, St. Louis, MO) and centrifugation for 1 min at 3000 rpm. The beads were washed twice with TBS (50 mM Tris–HCl pH 7.4, 150 mM NaCl) and p53 eluted by incubating the beads for 1 h at 4°C with TBS containing 150 ng/μL 1 × Flag peptide (Sigma-Aldrich, St. Louis, MO). The purity and authenticity of the eluted protein were confirmed by Coomassie Blue staining and immunoblotting with the p53-specific monoclonal antibody DO-1 (data not shown).

Electrophoretic mobility shift assay

Synthetic double-stranded p53RE oligonucleotides corresponding to the sequences above were labeled with [α-32P]dATP (3000 Ci/mmol) with the Klenow fragment of DNA polymerase (Thermo Scientific, Pittsburgh, PA). EMSA was performed with 150 ng of purified p53 or 3 μg of whole cell extract with the p53-SN expression vector, as indicated. Cells were harvested, washed three times with phosphate-buffer saline (PBS) (136 mM NaCl, 26 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2), and resuspended in KEDT-0.3 buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 300 mM KCl, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 15 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A). After sonication, lysates were centrifuged at 16,000 rpm for 30 min at 4°C. Supernatants were collected, divided into small aliquots, and stored at −80°C. For the EMSA, purified p53 or lysate was combined with DNA binding buffer (10 mM Tris–HCl, pH 7.6, 100 mM KCl, 2 μg of bovine serum albumin, 1 μg of poly(dI:dC)) and incubated with 1 ng 32P-labeled probe for 30 min in a 20 μl reaction volume. The reaction mixtures were resolved through 4% non-denaturing TBE-polyacrylamide gels, dried, and exposed for autoradiography.

Chromatin immunoprecipitation assay

Vero cells were seeded at 1.5 × 106 cells/100 mm dish and transfected with pC53-SN. Thirty-six hour post-transfection, cells were infected with HSV-1 for 3 h at a multiplicity of infection (moi) of 3. Mock-transfected/infected cells were used as a negative control. The ChIP assay was performed using the EMD Millipore ChIP assay kit (Billerica, MA) essentially according to the manufacturer’s instructions. In brief, cells were cross-linked with formaldehyde for 10 min at 37°C. The cross-linking was terminated by the addition of glycine followed by 5 min at 37°C. Cells were harvested and lysed. After 10 min on ice, the mixtures were sonicated 10 times at 30 s on ice, to shear the DNA to lengths between 200 and 1000 bp. The sonicated mixtures were centrifuged at 13,000 rpm at 4°C, and the supernatants transferred to new tubes. To reduce non-specific background binding, the mixtures were precleared with salmon sperm DNA/protein A/G-agarose beads for 1 h at 4°C with rotation. The precleared chromatin supernatant was collected by centrifugation. Ten percent of the precleared chromatin was used as input control for subsequent PCR. The precleared chromatin was then incubated overnight at 4°C with rotation with 2 μg of p53-specific DO-1 antibody or 2 μg of goat anti-rat IgG. Immunoprecipitation was achieved by adding salmon sperm DNA/protein A/G-agarose beads for 90 min at 4°C with rotation. Immune complexes were obtained by repeated centrifugation and washing. Bound chromatin was eluted and protein–DNA cross-links were reversed by heating at 65°C overnight. Following proteinase K digestion, DNA was extracted with phenol–chloroform and precipitated with glyceroph. PCR (35 cycles: denaturing at 95°C for 3 min, annealing at 54°C for 30 s, and extension at 72°C for 1 min) was performed to detect p53RE-L (forward primer 5’-ACCGAAGTTAAACCGGGCCCGC and reverse primer 5’-TTGTCCCGAACGTTCCCTA), p53RE-S (forward primer 5’-GGCAGCTAGCCTGAGTTCA and reverse primer 5’-GCCAGGACGACCGTCA) and a non-specific target corresponding to nucleotides 64416–64680 in the UL30 gene (forward primer 5’-GCTACAGGTTAAGCCCTA and reverse primer 5’-AGGCCTTTAAGAGGCGGCTAGT) to generate products of 200, 220 and 265 bp, respectively. PCR products were detected by electrophoresis through 2% agarose gels followed by ethidium bromide staining.

Transcriptional activity assay

The specified mammalian cell lines were seeded in 24-well plates at a density of 8 × 104 cells/well in 1 ml of the appropriate medium plus 10% fetal bovine serum. After 6 h, cells were co-transfected with 250 ng of the reporter plasmid to be tested, along with 1 ng pRL-CMV, 250 ng pC53-SN and 500 ng of pTF18U plasmid as carrier DNA. Transfected cells were incubated for 48 h with or without 5-FU (1 μM) treatment. Thereafter, cells
were washed twice with PBS and lysed with 100 µl passive lysis buffer (Dual luciferase assay kit, Promega Corp., Madison, WI). Firefly and Renilla luciferase activities were measured sequentially for each lysate using a Sirius Luminometer (Pforzheim, Germany) and a Dual Luciferase assay kit (Promega Corp., Madison, WI) as per the manufacturer’s instructions. The ratio of firefly to Renilla luciferase activity was calculated to normalize for transfection efficiency.

Quantitative real-time PCR

HCT116 (parental (p53−/−) and p53 knock-out (p53−/−)) and HeLa cells were seeded at 6 × 10⁵ cells/60 mm plate and infected with HSV-1 at moi of 1. Total RNA were extracted using an Aurum total RNA Mini kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. First-strand cDNA were synthesized from 2.0 μg total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. Two microliters of each cDNA synthesis reaction was used as template in 20 μl PCR reactions containing 10 μl PerfeCTa SYBR Green Fast Mix (Quanta biosciences, Gaithersburg, MD) and 1 μl primers (18 μM). Reactions were performed in 96-well PCR plates using a 7500 Fast detection system (Applied Biosystems Inc., Foster City, CA). Reactions were performed with the following primer pairs: human glyceraldehyde phosphate dehydrogenase forward primer 5′-ACACCTTGATCTGCGAAGGC and reverse primer 5′-CAGGGATGTTGCTGGAGACG; HSV-1 IF14 forward primer 5′-GCAAGCTACATACGACC and reverse primer 5′-GATCCGCCCTCGCGCTTCGTCC; HSV-1 UL29 forward primer 5′-GTTGTCGGTTGAGCATCAG and reverse primer 5′-TCCCGGCGAGGCTC-3′. All results were normalized to the activity observed with human glyceraldehyde phosphate dehydrogenase.

Acknowledgments

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References


