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Adenovirus 2 E1B-55K protein relieves p53-mediated transcriptional repression of the survivin and MAP4 promoters

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Abstract **It is well established that adenovirus E1B-55K protein functions as an inhibitor of the tumor suppressor protein p53 by binding and inactivating p53 as a transcriptional activator protein. Here we show that the adenovirus 2 E1B-55K protein also blocks p53 as a transcriptional repressor protein of the survivin and the MAP4 promoters. The repression is dependent on the ability of E1B-55K to bind to p53 and is enhanced by coexpression of the adenovirus E4orf6 protein. Overexpression of the transcriptional corepressor protein Sin3A partially relieves the inhibitory effect of E1B-55K, suggesting that E1B-55K blocks p53 functions by interfering with the Sin3 complex.**

Key words: Adenovirus; E1B-55K; Survivin; Sin3A

1. Introduction

The adenovirus (Ad) E1B-55K protein is a multifunctional phosphoprotein that serves several critical functions during lytic virus growth (reviewed in [1]). Since the discovery of E1B-55K as a binding partner to the human tumor suppressor protein p53 [2], a main focus in E1B-55K research has been on the functional consequence of this interaction on different p53-regulated processes. It is well documented that E1B-55K binding to the amino-terminal transactivation domain of p53 functionally inactivates p53 as a transcription activator of different p53-dependent promoters [3,4].

The p53 protein is a nuclear transcription factor that is latent in normal cells, but becomes activated by a variety of stress signals. Following induction p53 upregulates a set of genes, such as p21, GADD45, cyclin G and Bax, that can promote cell death or growth arrest (reviewed in [5]). More recently, p53 has been shown to repress transcription of a number of genes, including bcl-2, MAP4 and survivin, all of which have been implicated as proteins opposing apoptosis [6,7].

Compared to the wealth of knowledge about p53 as a transcriptional activator protein, limited data are available on how p53 inhibits transcription. The recent observation that p53 associates with the Sin3 corepressor complex may be of significance in order to explain how p53 inhibits transcription [8]. The Sin3 complex is a transcriptional repressor complex, consisting of the Sin3A, HDAC1, and HDAC2 proteins, which regulate chromatin structure by histone deacetylation [9]. The interaction between p53 and Sin3A targets histone deacetylases to the promoter of a p53-repressed gene, thereby creating a chromatin environment that is unfavorable for transcription initiation [8].

We have previously shown that the adenovirus E1B-55K protein interacts with HDAC1 and Sin3A [10]. The novel findings on p53-mediated transcription repression by recruitment of the Sin3 complex prompted us to test the possibility that E1B-55K might interfere with p53-mediated repression of transcription through the Sin3 complex.

As a model substrate for a repressed promoter, we selected the human survivin promoter. Recent studies have shown that survivin transcription can be repressed by p53 through recruitment of the Sin3 protein and by changes in chromatin structure that affect promoter accessibility [7,11].

Here we report that the Ad2 E1B-55K protein can relieve p53-mediated transcriptional repression of the survivin and the MAP4 promoters. Furthermore, we show that the effect is dependent on the ability of E1B-55K to bind to p53. The capacity of the E1B-55K protein to relieve p53-mediated transcriptional repression of the survivin promoter was enhanced by coexpression of the adenovirus E4orf6 protein. We also show that overexpression of the Sin3A protein partially opposed the effect of E1B-55K on the survivin promoter, suggesting that E1B-55K may accomplish this by interfering with the Sin3 complex.

2. Materials and methods

2.1. Plasmids

Plasmid maps and sequences are available on request. Plasmid pCMVE1B-55K was generated by cleavage of pBSE1B-55K [10] with *Hin*cII and ligation of *Bam*HI linkers to the 3′ ends of the E1B-55K sequence. The E1B-55K sequence was subsequently recloned into the pCMVNeoBam plasmid [12] as a *Bam*HI/*Bam*HI fragment. Plasmid pCH110, encoding β-galactosidase, was purchased from Amersham Pharmacia Biotech. The point mutations inserted into plasmids pCMVE1B-55K, pCGp53 and pGEXp53 were generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The MAP4-Luc plasmid was kindly provided by Dr. Maureen Murphy. Plasmids SpII-Luc, pCMVE4orf6, pcDNAmSin3A, pCGp53(wt) and cyclin G-Luc have been described previously [7,13–16].

2.2. Transfections and luciferase assay

H1299 cells were seeded in 35 mm plates at 1.5×10^5 cells per plate and allowed to grow overnight. Transfections were done in duplicate in several independent experiments, using the FuGene6 (Roche) transfection reagent according to the manufacturer's protocol. The amount of DNA used is specified in the figure legends, except for pCH110,

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which was constantly used at 1 μg per transfection. After 36 h of incubation, luciferase assay and β-galactosidase assay were performed using the Luciferase Assay System (Promega) or Galacto-Star System (Applied Biosystems), respectively. The reactions were analyzed on Luminoscan (Labsystems). Luciferase activity was normalized to total protein level and to β-galactosidase activity.

2.3. In vitro and in vivo binding assays

H1299 cells, transfected with 2 μg of pCMVE1B-55K plasmids, were lysed 36 h after transfection in buffer J (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% NP40) and passed through a 21G needle. Equal amounts of the lysates were precleared by incubation with glutathione *S*-transferase (GST) bound to glutathione Sepharose beads. Approximately 1 μg of GST-p53 or GST-p53P27Y proteins bound to glutathione Sepharose beads were incubated with 500 μg of precleared H1299 lysates for 1 h. The beads were washed four times with buffer J and eluted proteins were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blotting was performed using an anti-E1B-55K (2A6) [17] or an anti-p53 (mAb421) [15] antibody. Coimmunoprecipitations with an anti-Sin3A antibody and purification of GST fusion proteins were done as described previously [10].

3. Results

3.1. E1B-55K relieves p53-mediated transcriptional repression of the survivin promoter

It is well established that E1B-55K binds and inhibits p53 as a transcriptional activator protein [4]. Here we tested the hypothesis that E1B-55K interaction with p53 also blocks p53 as a transcriptional repressor protein. For this experiment, H1299 cells were transfected with a survivin luciferase reporter construct and plasmids encoding the p53 and E1B-55K proteins. In agreement with previous results [7], the wild-type p53 protein repressed survivin promoter activity (Fig. 1A, lane 2). However, cotransfection of increasing amounts of a plasmid encoding the E1B-55K protein relieved the p53-mediated repression, in a concentration-dependent manner (Fig. 1A, lanes 4 and 6). The same amounts of the E1B-55K plasmid used in the survivin promoter assay were also able to repress p53-activated transcription of the cyclin G promoter (Fig. 1B, lanes 4 and 6). This result is important as it shows that under the same experimental conditions, E1B-55K blocks p53 as both a repressor and activator of transcription.

Collectively our results show that E1B-55K, in addition to blocking p53 as a transcriptional activator protein, can also relieve p53-mediated transcriptional repression of the survivin promoter.

3.2. E1B-55K binding to p53 is necessary to block p53 as a transcriptional repressor protein

To show that the E1B-55K effect is dependent on binding to p53, we tested the p53 point mutant protein, p53(P27Y), which has a reduced binding capacity to the E1B-55K protein [18]. As shown in Fig. 1A, lane 3, the p53(P27Y) protein showed similar repressor activity as wild-type p53 on the survivin promoter. However, coexpression of E1B-55K did not significantly relieve the repressive effect of p53(P27Y) (lanes 5 and 7), suggesting that E1B-55K has to make physical contact with p53 to inactivate it as a transcriptional repressor protein. A mutation changing the conserved histidine 260 to alanine has been shown to drastically reduce the Ad5 E1B-55K protein binding to p53 [19]. Therefore, we created the corresponding point mutation in Ad2 E1B-55K (mutant E1B-55K(H259A)). Another point mutant, E1B-55K(Y443A),

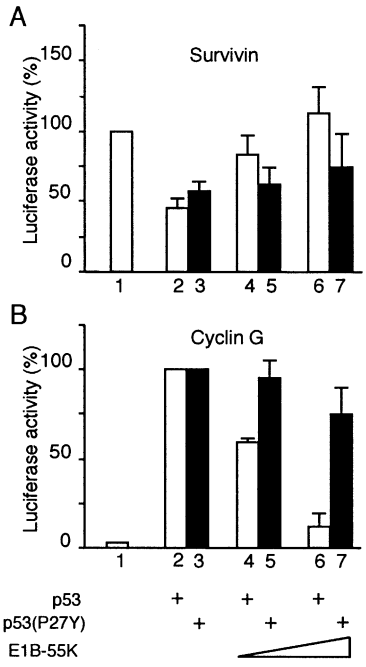

Fig. 1. E1B-55K relieves p53-mediated transcriptional repression of the survivin promoter. A: H1299 cells transfected with 1 μg of the survivin promoter construct SpII, 10 ng p53(wt) or p53(P27Y) and increasing amounts (10–50 ng) of E1B-55K(wt) plasmids. The activity of SpII in the absence of p53 was taken as 100%. B: H1299 cells transfected with 100 ng of the cyclin G promoter construct and the same amounts of plasmids encoding p53 and E1B-55K as in A. The activity of the cyclin G luciferase in the presence of p53 or p53(P27Y) was taken as 100%.

was used as a control, to show the specificity of the H259A mutation. The results from H1299 transfected cells showed that the E1B55K(H259A) protein was impaired in its capacity to relieve p53-mediated repression of the survivin promoter (Fig. 2A, compare lanes 3 and 4). However, the C-terminal point mutant Y443A was essentially as efficient as the wild-type E1B-55K protein in inactivating p53 as transcriptional repressor protein (Fig. 2A, lane 5). Interestingly, the same E1B-55K point mutants showed a difference in activity when tested on the p53-activated cyclin G promoter. As shown in Fig. 2B, E1B-55K(H259A) had a reduced capacity to block p53-activated transcription compared to wild-type E1B-55K, whereas mutant Y443A was essentially as effective as the wild-type E1B-55K protein (Fig. 2B, lane 4). Thus, it is noteworthy that the effect of the E1B-55K(H259A) protein was more dramatic on the survivin promoter compared to the cyclin G promoter. We also tested if E1B-55K can interfere with another p53 repressed promoter. To that end, we used the MAP4 promoter, which has been shown to be repressed by p53 expression [8]. Similarly to survivin promoter, the E1B-55K expression opposed p53-mediated repression of the MAP4 promoter in a p53-binding-dependent manner (Fig. 2C). All E1B-55K proteins were expressed at comparable levels (Fig. 2D).

To study the binding capacity of E1B-55K(H259A) to p53, we incubated purified recombinant GST-p53 or GST-p53(P27Y) proteins with cell lysates prepared from H1299 cells transfected with E1B-55K encoding plasmids. As shown in Fig. 3A, the E1B-55K(wt) protein showed a strong interaction with GST-p53 (lane 2). In contrast, the E1B-

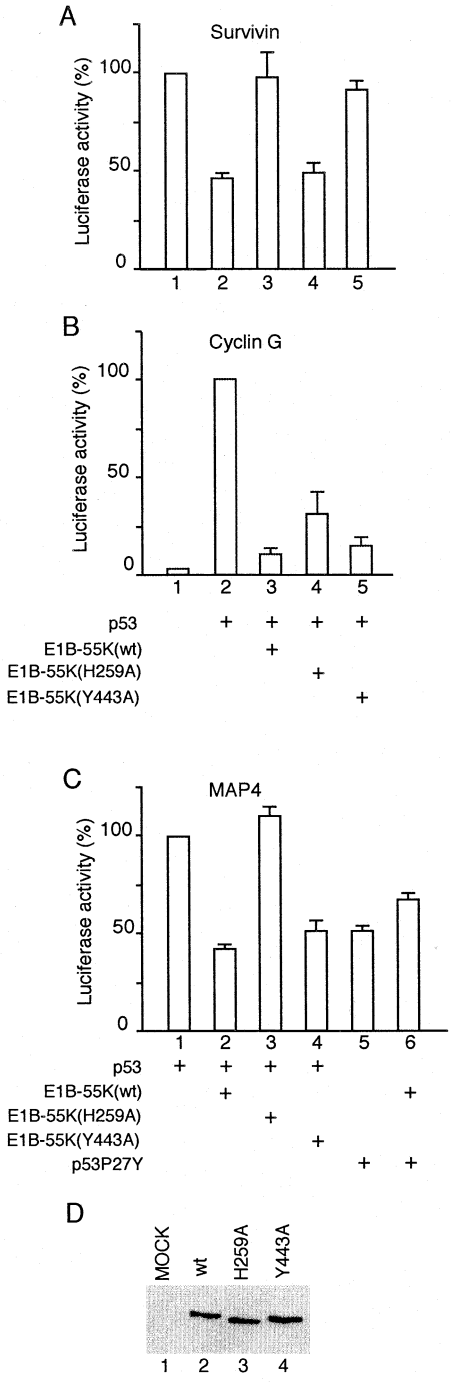

Fig. 2. E1B-55K binding to p53 is a prerequisite to relieve the p53-mediated repression of survivin and MAP4 promoter activity. A: H1299 cells were transfected with 1 μg of the survivin promoter construct SpII, 10 ng of p53 and 50 ng of E1B-55K encoding plasmids. The activity of SpII in the absence of p53 was taken as 100%. B: H1299 cells transfected with 100 ng of the cyclin G promoter construct and the same amounts of p53 and E1B-55K encoding plasmids as in A. The activity of the cyclin G promoter in the presence of p53 was taken as 100%. C: H1299 cells transfected with 1 μg of MAP4-Luc, 20 ng of p53 or p53P27Y and 100 ng of E1B-55K or E1B55K(H259A) encoding plasmids. The activity of MAP4-Luc in the absence of p53 was taken as 100%. D: Western blot showing the expression of E1B-55K proteins, detected with an anti-E1B-55K antibody.

55K(H259A) protein did not bind to GST-p53 under our experimental conditions (compare lanes 2 and 5). The specificity of the interactions was confirmed with GST-p53(P27Y) protein, which did not show detectable binding to E1B-55K(wt) or E1B-55K(H259A) (Fig. 3A, lanes 3 and 6). This result is in agreement with our finding that E1B-55K(H259A) was essentially non-functional as an inhibitor of p53-mediated repression of survivin and MAP4 promoter activity (Fig. 2A,C).

3.3. *Sin3A overexpression partially abolishes the inhibitory effect of E1B-55K on p53-mediated repression of the survivin promoter*

Previous studies have shown that p53 binds the Sin3A protein [8]. In addition, we have shown that E1B-55K also interacts with the Sin3A protein [10]. To test if the E1B-55K(H259A) mutant protein was able bind the Sin3A protein, H1299 cells were transfected with the E1B-55K(wt) and the E1B-55K(H259A) constructs. Cell lysates were immunoprecipitated with an anti-Sin3A antibody and interacting E1B-55K proteins were detected in a Western blot using an antibody directed against E1B-55K. As shown in Fig. 3B, the endogenous Sin3A protein interacted efficiently with both the E1B-55K(wt) and the E1B-55K(H259A) proteins (lanes 5 and 6). The specificity of the interactions was confirmed by immunoprecipitating the same lysates with an anti-Gal4 antibody, which did not coimmunoprecipitate the E1B-55K proteins (lanes 8 and 9).

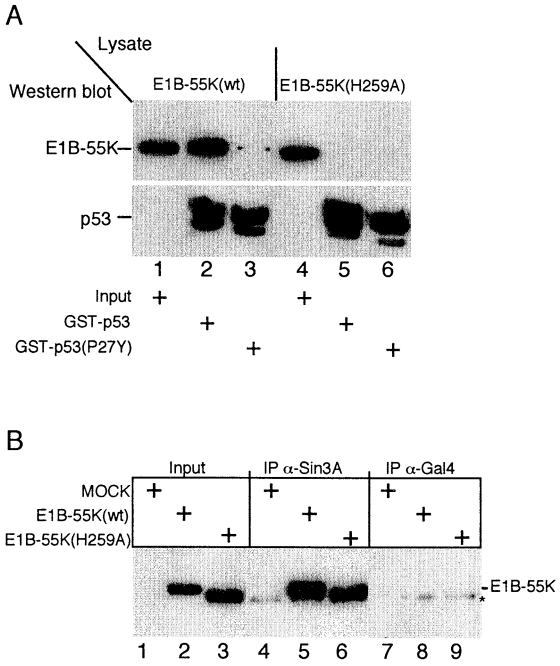

Fig. 3. E1B-55K(H259A) is deficient in p53 binding, but not binding to the Sin3A protein. A: H1299 cells expressing E1B-55K(wt) or E1B-55K(H259A) proteins were lysed and incubated with purified GST-p53 (lanes 2 and 5) or GST-p53(P27Y) (lanes 3 and 6). Bound proteins were resolved by 10% SDS–PAGE and Western blot analyses performed with anti-E1B-55K or anti-p53 antibodies. B: H1299 cells transfected with E1B-55K(wt) or E1B-55K(H259A) encoding plasmids were lysed and coimmunoprecipitations were done using an anti-Sin3A (lanes 4–6) or an anti-Gal4 antibody (lanes 7–9). Inputs (lanes 1–3), represent 1/50 of the cell extracts used for coimmunoprecipitations. The asterisk indicates the migration of the antibody heavy chain.

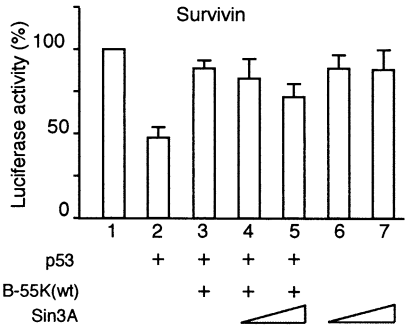

Fig. 4. Sin3A overexpression partially abolishes the effect of E1B-55K as a suppressor of p53-mediated repression of survivin promoter activity. H1299 cells were transfected with plasmids encoding the survivin luciferase promoter construct (SpII, 1 μg), p53(wt) (10 ng), E1B-55K (50 ng) and increasing amounts (100–250 ng) of Sin3A encoding plasmids. The activity of SpII in the absence of p53 was taken as 100%.

Potentially, the suppressive effect of E1B-55K on p53-mediated repression of the survivin promoter might result from a sequestering of the Sin3A protein by E1B-55K. If the effect of E1B-55K on p53-repressed survivin promoter activity was due to an interference with Sin3A, then overexpression of the Sin3A protein would be predicted to counteract the effect of E1B-55K. To test this hypothesis, H1299 cells were cotransfected with the survivin reporter construct and plasmids encoding p53, E1B-55K and Sin3A proteins. Interestingly, coexpression of the Sin3A protein reduced the E1B-55K activating effect and resulted in partial restoration of the repressor activity to p53 (Fig. 4, compare lanes 3 and 5). We have also observed a similar result by transiently overexpressing the HDAC1 protein (data not shown).

Collectively, these results suggest that E1B-55K may target the Sin3 complex to regulate p53-mediated transcriptional repression of the survivin promoter.

3.4. *E4orf6 augments the effect of E1B-55K on p53-mediated repression of the survivin promoter*

Like the E1B-55K protein, the adenovirus E4orf6 protein has been shown to bind to p53 and block its activity as a transcriptional activator protein [20]. We therefore decided to test whether the E4orf6 protein also had an effect on p53-repressed transcription. As shown in Fig. 5A, the E4orf6 protein, alone, was unable to relieve p53-mediated repression of the survivin promoter (lanes 6 and 7). However, in combination with E1B-55K, the E4orf6 protein amplified the effect of E1B-55K (lanes 4 and 5). The same experimental set-up was used to study the effect of E4orf6 on p53-activated transcription of the cyclin G promoter (Fig. 5B). However, coexpression of E4orf6, alone or together with E1B-55K, did not significantly repress cyclin G promoter activity (lanes 4–7).

Taken together, these data suggest that E4orf6 strengthens the effect of E1B-55K on p53-repressed transcription, but does not have a significant effect on p53-activated transcription, in our assay system.

4. Discussion

Here we show that the Ad2 E1B-55K protein blocks p53 both as a transcriptional activator and as a transcriptional repressor protein. Interestingly, both effects require that E1B-55K interacts with the amino-terminal transactivation domain of p53. However, a closer inspection of the data suggests a small, but potentially important, difference in results. Thus, E1B-55K binding to p53 appeared to be essential for E1B-55K to function as an inhibitor of p53 as a transcriptional repressor protein. The mutation in E1B-55K [E1B-55K(H259A)], which disrupts protein–protein interactions, was essentially unable to relieve p53 as repressor protein of both survivin and MAP4 promoter activity (Fig. 2A,C). In contrast, the same mutant protein was only partially defective in blocking p53 as a transcriptional activator of the cyclin G promoter (Fig. 2B). This finding is in line with previous experiments showing that E1B-55K does not simply inactivate p53 as a transcriptional activator protein by masking the transactivation domain [21]. Thus, a physical interaction between E1B-55K and p53 appeared to be most important for E1B-55K to function as an inhibitor of p53-mediated repression of transcription.

The activity of p53 as a transcriptional repressor protein has previously been suggested to result from a recruitment of histone deacetylases in the Sin3 complex to the promoter of a repressed gene [7,8]. In addition, we have shown that the E1B-55K protein interacts with HDAC1 and Sin3A [10]. Therefore, we hypothesized that the blocking activity of E1B-55K on p53 as a transcriptional repressor protein could be due to the fact that both proteins might compete for the binding to the Sin3A protein. Indeed, transient overexpression of the Sin3A protein partially abrogated the repressive effect

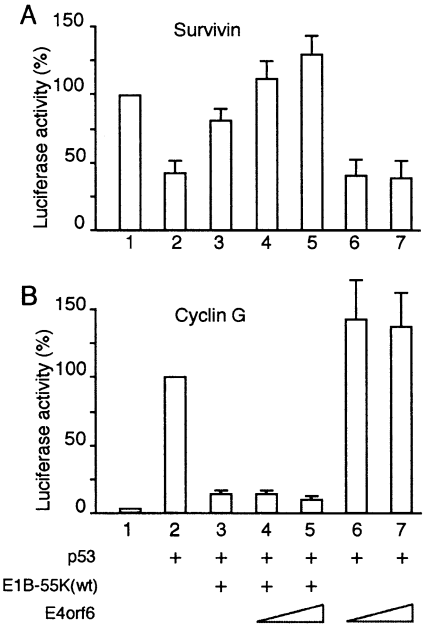

Fig. 5. E4orf6 augments the effect of E1B-55K as a suppressor of p53-mediated repression of survivin promoter activity. A: H1299 cells were transfected with the survivin luciferase promoter construct (SpII, 1 μg), 10 ng of p53, 50 ng of E1B-55K and increasing amounts (10–50 ng) of E4orf6 encoding plasmids. The activity of SpII in the absence of p53 was taken as 100%. B: H1299 cells were transfected with 100 ng of the cyclin G promoter construct and the same amounts of plasmids encoding p53, E1B-55K and E4orf6 as in A. The activity of the cyclin G promoter in the presence of p53 was taken as 100%.

of E1B-55K on p53-mediated repression of the survivin promoter (Fig. 4).

It is likely that p53 inhibits the survivin promoter in more ways than by the recruitment of the Sin3 complex. The survivin promoter contains numerous Sp1, putative p53 and E2F1 binding sites [7,22]. In a recent study, Hoffman and coworkers have proposed a model, whereby p53-recruited Sin3 complex generates a chromatin conformation, which will impair E2F1 binding/transactivation of the survivin promoter [7]. Although an attractive model, the p53 binding to the survivin promoter has been reported to be controversial and may depend on the experimental system [11]. It should be noted that we have been unable to detect binding of p53 to the survivin promoter in chromatin immunoprecipitation assays (data not shown). The repressive effect of p53 on the survivin promoter may also be indirect and dependent on p53 activating synthesis of a repressor protein [7].

The adenovirus E4orf6 protein has previously been reported to bind p53 and inhibit p53 as a transcriptional activator protein [20,23]. However, we found that under our experimental conditions transient E4orf6 expression had no effect on either p53-repressed or -activated transcription (Fig. 5A,B). In contrast, our data showed that the E4orf6 protein specifically augmented the opposing effect of E1B-55K on p53-mediated repression of survivin transcription. This effect was selective since E4orf6 did not impinge on the activity of E1B-55K as an inhibitor of p53-mediated activation of cyclin G transcription (Fig. 5B). Although previous studies have shown that the E1B-55K/E4orf6 protein complex causes p53 ubiquitination and degradation [24], it appears unlikely that this is the major effect seen under our experimental conditions. Thus, if the E1B-55K/E4orf6 complex acted by degrading the p53 protein one would have expected to see an effect of E1B-55K and E4orf6 coexpression, not only on p53-mediated transcriptional repression (Fig. 5A), but also on p53-mediated transcriptional activation (Fig. 5B). It is possible that the E1B-55K/E4orf6 complex works in concert to enhance survivin transcription by an alternative, but unknown mechanism.

What is the potential physiological significance of E1B-55K regulating survivin gene expression? Survivin is a bifunctional protein that inhibits apoptosis and promotes cell division (reviewed in [25]). One possible explanation is that E1B-55K counteracts p53-induced apoptosis by upregulating expression of the anti-apoptotic survivin protein. Interestingly, in a microarray study survivin transcription was shown to be enhanced at early times of a wild-type adenovirus infection (Zhao, Granberg, Elfineh, Pettersson and Svensson, submitted). Furthermore, a recent study shows that the HIV-1 Vpr protein stimulates transcription from the survivin promoter [26]. Collectively, these results point toward the possibility that viral control of survivin gene expression may be a significant mechanism contributing to viral inhibition of apoptosis.

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