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Protein encoded by HSV-1 stimulation-related gene 1 (HSRG1) interacts with and inhibits SV40 large T antigen

H. X. Guo, W. Cun, L. D. Liu, S. Z. Dong, L. C. Wang,

C. H. Dong and Q. H. Li

Institute of Medical Biology, Chinese Academy of Medical Sciences, Kunming, 650118, China

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Abstract. Herpes simplex virus (HSV)-1 stimulation-related gene 1 (HSRG1) protein expression is induced in HSV-1 infected cells. We found that HSRG1 interacts with SV40 large T antigen (LT) in yeast two-hybrid assay and bimolecular fluorescence complementation (BiFC) assay. This interaction alters LT's regulation of the SV40 promoter and its ability to influence the cell cycle. Choramphenicol acetyl-transferase (CAT) assays revealed that initiation of gene transcription by LT is changed by HSRG1 expression. HSRG1 inhibits the ability of LT to activate SV40 late gene transcription. Further data indicate that the ability of LT protein to stimulate S-phase entry is also inhibited by the expression of HSRG1. The results of a colony-forming assay suggested that expression of HSRG1 in cells transfected by LT gene decreased the rate of colony formation. Yeast two-hybrid β-galactosidase assay revealed that amino acid residues 132–450 in LT bind HSRG1.

INTRODUCTION

Cellular proteins expressed in response to a virus binding to its specific receptor in the plasma membrane have been confirmed to be biologically functional in some cases (Boyle *et al*. 1999; Roggero *et al*. 2001). This is attributed to the fact that the interaction of virus and receptor is similar to the binding of a physiological ligand to its receptor (Nishigaki *et al*. 2000). Therefore, the transcriptional response of the cells to binding by a ligand is also capable of being induced by the virus binding to the same receptor (Richardson *et al*. 2003). Proteins encoded by genes transcribed in this process function in the cellular response to viral infection. In some cases, play a specific role in the virus life cycle (Ullrich *et al*. 2000), which, to some extent, might be classified as an intracellular innate antiviral response. This mechanism, however, needs further investigation.

Our previous work focused on the analysis of changes in gene expression induced by herpes simplex virus (HSV)-1 binding to human fibroblasts. We described several cDNA clones of genes and their encoded proteins (Li *et al*. 2002; Liu *et al*. 2002b; Dong *et al*. 2003; Li *et al*. 2004), which might be considered potential cellular innate immune response molecules. One of

Correspondence: Q. H. Li, Institute of Medical Biology, Chinese Academy of Medical Sciences, 379# Jiaoling Road, Kunming 650118, China. Tel.: +86-871-8335905; Fax: +86-871-8334483; E-mail: qihanlister@gmail.com
H. X. Guo and W. Cun contributed equally to this study.

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these proteins, HSV-1 stimulation-related gene (HSRG1), is a novel member of the SAND protein family, itself composed of 547 amino acids (Dong *et al*. 2003). Knowledge of the function of the SAND family, including HSRG1 protein, depends considerably upon work by Cottage *et al*. (2004), which suggests that conservation of the primary structure of this family's proteins implies a potential significance for the protein in vacuolar fusion of eukaryotic cells. Combined with this conclusion, our previous investigation suggests that the cytoplasmic location of HSRG1 and its TYR-phosphorylation site indicate a possible link between HSRG1 and signal transduction (Dong *et al*. 2003; Cottage *et al*. 2004). Depending on the fact that HSRG1 is induced in HSV-1 infection, it is possible to hypothesize that HSRG1 is involved in the process of vesicle fusion with lysosomes during HSV-1 penetration of the cell membrane (Cottage *et al*. 2004). However, how HSRG1 protein is involved in other functional processes in cells remains unclear.

SV40 is a monkey polyomavirus, which is also thought to be a potential human pathogen (Butel & Lednicky 1999; Li *et al*. 2002a; Vivaldi *et al*. 2003). As a tumour virus capable of inducing tumours in rodents and transforming some types of cells in culture, SV40 is a favoured model for mechanistic studies on cellular transcriptional regulation and tumorigenesis (Cacciotti *et al*. 2001) because of the function of the transforming protein large T antigen (LT) (Sullivan & Pipas 2002). LT interacts with a variety of cellular proteins including the tumour repressor proteins p53 and pRB (Kim *et al*. 2001). LT and p53 form a complex that inhibits p53 activity by preventing its binding to specific regulatory elements in the genome (Sheppard *et al*. 1999). As a multifunctional protein, LT is also capable of interacting with some essential transcription factors including the TATA box-binding protein (TBP), TBP-associated factors (TAFs), transcription enhancer factor-1 (TEF-1), transcription factor 2B (TFIIB) and activating enhancer-binding protein 2 (AP_2) (Casaz *et al*. 1991; Zhai *et al*. 1997; Damania *et al*. 1998). These interactions appear to be important for the activation of RNA polymerase I, polymerase II and polymerase III in cellular and viral transcription. Meanwhile, LT also plays a dominant role in SV40 infection, which includes regulating early viral gene transcription, improving late viral gene transcription (Khoury & May 1977; Hansen *et al*. 1981; Alwine & Picardi 1986), initiating viral DNA replication (Tegtmeyer 1972; Reed *et al*. 1975; Dornreiter *et al*. 1993), recruiting the DNA polymerase α-primase complex to the origin of replication and acting as a helicase (Wiekowski *et al*. 1988; Ramsperger & Stahl 1995; Ott *et al*. 2002). These activities of LT in viral infection depend upon its specific recognition of different promoter sequences in the SV40 genome and its interaction with other viral or cellular proteins (Wu *et al*. 2001). However, with such an important role in SV40 infection, LT might be a target of further cellular protein that could influence the viral infection cycle and viral transformation of host cells (Herbig *et al*. 1999).

In the present study, the interaction of HSRG1 and LT, observed in a yeast two-hybrid assay and confirmed in a bimolecular fluorescence complementation (BiFC) assay, alters LT's regulatory effect on the SV40 late promoters and its ability to influence cell growth. These results suggest that HSRG1 interacts with LT and inhibits its regulation of viral replication and host cell transformation, implying an unknown mechanism of intracellular innate antiviral response composed of a series of molecules induced by virus binding to receptors.

MATERIALS AND METHODS

Cells, viruses and plasmids

KMB17 human embryo fibroblasts (passage: 25–27, Institute of Medical Biology, Chinese Academy of Medical Sciences) and Vero cells (passage: 215–219, ATCC) were grown in

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Dulbecco's modified eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 2 ml L-glutamine (Sigma, St. Louis, MO, USA) and 10% (v/v) of foetal bovine serum (FBS, Gibco) in 5% CO_2 at 37 °C. SV40 was grown in Vero cells and titred in the same cells with plaque assay. Plasmids pcDNA (Invitrogen, Grand Island, NY, USA), pCAT (Invitrogen) and pGBK-T7 (Clontech, Palo Alto, CA, USA) were produced and purified according to standard protocols. Plasmids pcDNA-LT, pcDNA-hsrg1 and pCAT-S that contain the late promoter sequence of SV40 were constructed in this laboratory. Plasmids pHA that contain CMV promoter and N-terminal 1–154 amino acid of yellow fluorescent protein, pFLA that contains CMV promoter, and C terminal 155–238 amino acid of yellow fluorescent protein were provided kindly by Dr Kerppola's laboratory. The gene fragments of hsrg1, LT, p53 and Vp1 of the poliovirus genome were recombinated into these two vectors to produce plasmids of pHA-hsrg, pHA-p53, pHA-PVp1 and pFLA-T.

Yeast two-hybrid screen and β-galactosidase assay

A full-length cDNA of HSRG1 isolated from a cDNA library of KMB-17 cells bound by HSV1 was cloned into pGBK-T7 as a Gal4 DNA-binding domain fusion. This recombinant plasmid was used to screen the cDNA library and some viral proteins, including LT, which were then cloned into the pACT2 plasmid for the yeast two-hybrid assay. The positive clones were identified twice on synthetic dropout agar plates lacking leucine, tryptophan, histidine and adenine (QDO) and were cloned and sequenced. The β-galactosidase assay was performed to compare the relative strength of interaction between HSRG1 and selected proteins with the substrate o-nitrophenyl β-D-galactosidase (ONPG, Sigma). Gene segments encoding four domains of LT were synthesized by polymerase chain reaction (PCR) with the following primers: (1) 5′-ATGAATTCATGGATAAAGTTTTAAACAGAGAG-3′ and 5′-CTGAATTCTTATGTTTCAGGTTCAGGGGGGA-3′; (2) 5′-ATGAATTCATGGATAAAGTTTTAAACAGAGAG-3′ and 5′-CAGAATTCTTCTACCTTTCTCTTCTTTTTTGGA-3′; (3) 5′-CTGAATTCGAAGACCCCAAGGACTTTCCTT-3′ and 5′-GCGAATTCTAAAGCTTTCCCCCCACATAATT-3′; and (4) 5′-ACGAATTCTTAAATGTTAATTTGCCCTTGGACA-3′ and 5′-CTGAATTCTTATGTTTCAGGTTCAGGGGGG A-3′.

PCR products were cloned into pACT2 plasmids (Clontech) and were transfected into yeast Y187. These transfected Y187 clones were mated to AH109 transfected with pGBK-hsrg1. The fused clones were identified on QDO plates as previously described and were analysed by β-galactosidase assay according to a standard protocol. Transcriptional activity was reported in galactosidase units calculated from optical absorbance values at 420 nm. For the transfection of cultured mammalian cells, plasmids including pcDNA-hsrg1 and pcDNA-T were linearized by restriction enzyme digestion. The cells, including KMB-17 and Vero, were then transfected by electroporation as previously described (Baum *et al*. 1994). Control transfections were performed with pcDNA plasmid linearized by restriction enzyme. After transfection, the transfected cells were maintained in DMEM with 5% FBS for 24 h.

BiFC assay of HSRG1 and LT

Vero cells grown in glass plates in DMEM media supplemented with 1% (v/v) L-glutamine (Sigma) and 10% (v/v) FBS in 5% CO_2 at 37 °C to 90% confluence were transfected by pHA-hsrg and pFLA-T and were maintained for 12 h. After being replaced with fresh media, the transfected cells were continuously maintained for more than 24 h and 36 h. Yellow fluorescence protein (YFP) was observed with a fluorescence microscope (Nikon ECLIPSE E600) with B-2 A filter (450–490 nm excitation wavelength, 505 nm wavelength dichroic mirror and 520 nm wavelength barrier filter). Positive and negative controls were performed with pFLA-T and pHA-p53, and pFLA-T and pHA- PVp1 separately with the same protocol.

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Co-immunoprecipitation of HSRG1 and LT in Vero cells

Co-immunoprecipitation of HSRG1 and LT was performed according to a standard protocol. Vero cells grown in DMEM with 5% FBS to 90% confluence were washed twice with serum-free DMEM and were transfected with pcDNA-hsrg1. After transfection, the cells were recovered in DMEM supplemented with 5% FBS for 24 h and were incubated in methionine-free minimum essential medium for 1.5 h at 37 °C. The transfected cells were then infected with SV40 (m.o.i.: 0.5–1) and were maintained in the same media containing 50 μci/ml ^{35}S-methionine at 37 °C for more than 8 h. The cells were collected with a rubber policeman and were rinsed in cold phosphate buffer saline (PBS) (pH 7.4). They were then gently re-suspended in 5 vol of lysis buffer (Tris pH 7.5, 10 mM NP-40 0.05%, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM ethyleneglycol bis(2-aminoethyl-ether)teraacetic acid (EGTA), aprotinin, 20 mg/ml, 1 mM orthovanadate, 1 mM 4-[2-aminoethyl]-benzenosulphonyl fluoride (AEBSF) and leupeptin, 10 mg/ml) and were centrifuged at 350 ***g*** for 5 min at 4 °C. The supernatant corresponding to the cytoplasmic component was collected. The crude nuclear pellet was washed once in lysis buffer and twice in ice-cold cytoskeleton (CSK) buffer (10 mM piperazine-*N*,*N*9-bis (2-ethanesulphonic acid) pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, aprotinin, 20 mg/ml, 1 mM orthovanadate, 1 mM AEBSF and leupeptin, 10 mg/ml) by gentle re-suspension in 5 vol buffer and centrifugation at 350 ***g*** for 5 min at 4 °C. Nuclei were then re-suspended in CSK buffer containing 1 M sucrose and were centrifuged at 1200 ***g*** for 10 min at 4 °C. Co-immunoprecipitation of HSRG1 and LT was performed with this lysate and specific antibody against HSRG1 or LT as described by Ginsberg *et al*. (1994). The immune complexes adsorbed by protein A coupled to Sepharose 2B (Pharmacia) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gels were dried and were exposed to X-ray film.

Western blot of HSRG1 and LT

Cells transfected with pcDNA-hsrg or pcDNA-T were maintained in DMEM with 5% FBS for more than 36 h and were lysed in buffer (150 mm NaCl, 1% NP-40, 0.1% SDS, 50 mm Tris-HCl, pH 7.5) at 4 °C. The samples of supernatant were run in 12% SDS-PAGE gels and were transferred to nitric cellulose membrane. Western blotting of HSRG1 or LT was performed with specific antibodies against HSRG1 or LT, according to the standard protocol.

CAT transcription assays pCAT-S, pcDNA-T and pcDNA-hsrg1 were used to transfect Vero cells either separately or together. At 24 h after transfection, the cells were harvested and were pelleted in cold PBS for washing (×2). The pelleted cells were lysed in three cycles of freezing and thawing in 200 μl of 250 mM Tris-HCl, pH 7.8. Cell debris was removed by centrifugation at 15 000 ***g*** for 5 min at 4 °C. Finally, 100 μi of ^{3}H-chloramphenicol was added to the supernatant for assay of CAT activity at 37 °C for 1 h. After extraction with xylene, the incorporated radio-activity was measured in a scintillation counter. CAT activity was established using serial CAT enzyme (Promega) dilutions from 0.1 to 0.003125 units. One unit (U) was defined as the amount of enzyme required to transfer 1 nmol of acetate to chloramphenicol in 1 min at 37 °C.

Cytometry of transfected human fibroblasts

KMB-17 cells plated in six-well plates at 90% confluence in DMEM supplemented with 10% FBS were transiently transfected with pcDNA, pcDNA-T and/or pcDNA-hsrg1. At 12, 24, 36, 48, 60 and 72 h post-transfection, the cells were trypsinized, washed in PBS and fixed in 70% ethanol at 4 °C for 30 min. The treated cells were analysed by flow cytometry.

Colony-forming assay

Vero cells grown to 95% confluence in DMEM supplemented with 10% FBS were transfected with pcDNA-T and with pcDNA as control. The transfected cells were grown in selective

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Table 1. Isolated genes, coding proteins interacting with HSRG1 from yeast trap

No.	Gene	Accession no.
11	Cyclin T2	NM-00124
43	Flotillin1	BC001146
70	SR-A1	NM-021228
112	Not known Protein	AW 307598
145	SV40 large T Antigen	NP-043127

medium containing 800 μg/ml G418 for cloning of positive cells expressing LT protein. After identification of LT antibody with Western blot (data not shown), the cells expressing LT stably were grown in the same medium containing 400 μg/ml G418 for two more generations. Transfection with pcDNA-hsrg1 was then performed in these cells according to the procedure described previously. After 36 h of transfection, the cells were trypsinized and diluted to 100, 500, 1000 and 1500 cells/ml in DMEM for subculturing in six-well plates according to the standard protocol. The colonies formed in agar media were counted after 10 days.

RESULTS

Interaction of HSRG1 and LT

Our previous work revealed that HSRG1 induced by HSV-1 binding to cells probably functions in cellular signal transduction and viral gene transcription (Dong *et al*. 2003). As a member of the SAND protein family with conserved primary structure in eukaryotic species, HSRG1 should be investigated further for its possible interaction with other components in cellular signal transduction and viral gene transcription. We cloned HSRG1 in pGBK-T7 and expressed it as a GAL-HSRG1 fusion protein to screen for interacting proteins in a yeast two-hybrid system. In the screened cDNA library of human liver and viral transcriptional proteins, which includes RAP94 of Vaccinia, LT of SV40, E2A of adenovirus and ICP22 of HSVI, at least four proteins were identified as interacting with HSRG1 (Table 1). Protein number 112 is a novel protein, which will be described elsewhere. Protein numbers 11, 43 and 70 have been studied by other workers. For this reason, LT became the subject of this present work. In comparison with positive and negative controls, the β-galactosidase assay confirmed the specific interaction of HSRG1 and LT (Fig. 1a), and suggested that the HSRG1-interacting region of LT is mainly located in amino acid residues 131–450 (Fig. 1b).

Identification of the interaction between HSRG1 and LT of SV40 *in vivo*

Depending upon the result of the yeast two-hybrid experiment, the biochemical interaction between HSRG1 and LT was predicted and identified in BiFC assay. This approach is based on the complementation between two non-fluorescent fragments of YFP when they are brought together by interactions between proteins fused to each fragment, and is effective in determining the interaction of proteins by visualization of fluorescence on protein complex in living cells (Hu *et al*. 2002; Hu & Kerppola 2003). The result of fluorescence images of Vero cells co-transfected with pHA-hsrg and pFLA-T indicates an interaction between the expressed HSRG1 and LT in Vero cells. The protein complex was localized predominantly in nuclear regions (Fig. 2). Compared

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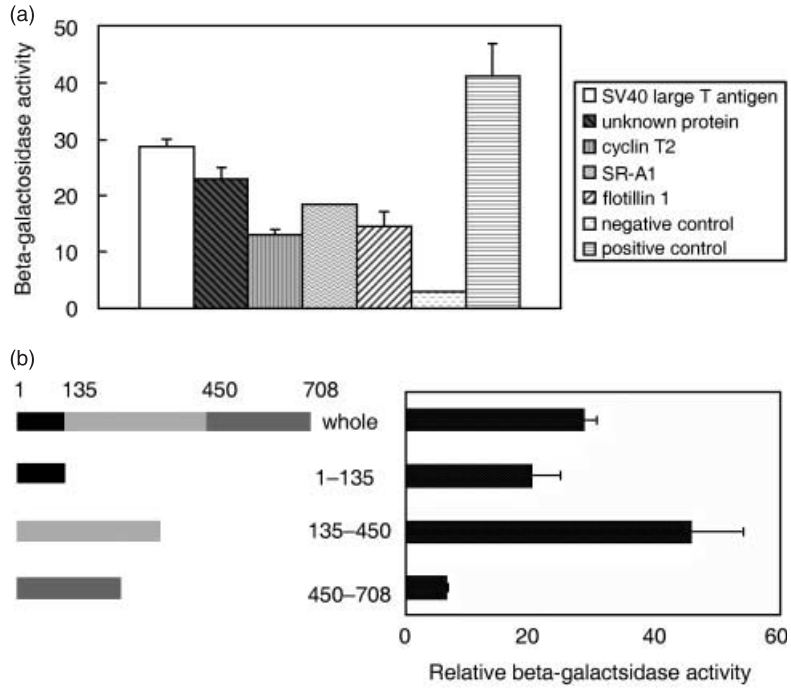

Figure 1. Binding assay of the proteins and HSRG1 and mapping the region of SV40 large T antigen interaction with HSRG1. (a) Fusion yeast strains selected through QDO plate screening in two-hybrid system were grown overnight in YPD media. The cells were collected and re-suspended quantitatively in 100 μl Z buffer. After several repetitions of freeze/thaw cycles, 0.7 ml Z buffer + β-mercaptoethanol and ONPG were added for further incubation at 30 °C. Yellow colouration was developed and measured at A420. The positive control was set with the fusion yeast containing pGBK-p53 and pACT-LT. The negative control was set with the fusion yeast containing pGBK-Lam and pACT-LT. β-galactosidase units were calculated using the formula: β-galactosidase units = $1000 \times A420/(t \times V \times A600)$. T = elapsed time of incubation (min); V = 0.1 ml × concentration factor; A600 = A600 of 1 ml of culture. (b) The plasmids encoding SV40 large T antigen amino acid residues 1–135, 135–450 and 450–708 were constructed and transfected into yeast Y187. These transfected Y187 clones were fused with AH109 transfected with pGBK-hsrg1. These fused clones were identified on QDO plates and their β-galactosidase activity was analysed.

with the positive control of the interaction between LT and p53 located in nucleus and the negative control of pHA-PVp1 (Vp1 protein of poliovirus) and pFLA-T without fluorescence in cells, this result suggests that HSRG1 binds LT probably as the latter moves into the nucleus.

The interaction of HSRG1 and LT in cells infected by SV40

Although the results of the yeast two-hybrid experiments, β-galactosidase assay and BiFC assay suggested that HSRG1 interacts with LT, further supportive data concerning their relations are needed in order to explore the biological significance of this interaction *in vivo*. For this purpose, co-immunoprecipitation of the two proteins was attempted with specific antibodies against HSRG1 and LT. After verification of HSRG1 expression in Vero cells transfected by pcDNA-hsrg1 (Fig. 3a), the cells were infected with SV40. The nuclei and cytoplasmic extract of these infected cells were used in immunoprecipitation with specific antibodies against HSRG1 and LT. In the nuclear fraction, both HSRG1 and LT antibodies were able to co-precipitate HSRG1 and LT (Fig. 3b). However, HSRG1 being precipitated by its antibody from the cytoplasmic fraction of uninfected cells (Fig. 3b) is consistent with our previous description (Dong *et al*. 2003). In

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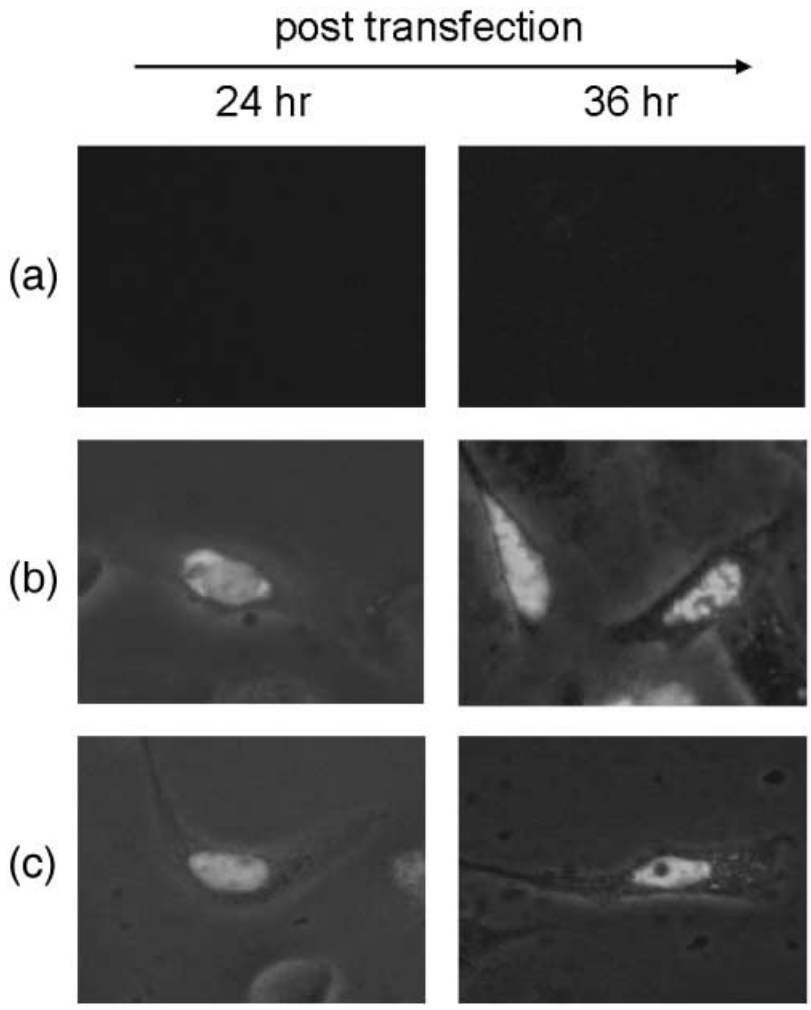

Figure 2. Fluorescence images of Vero cells cotransfected with pHA-hsrg and pFLA-T. (a) Vero cells transfected with pHA-pVp1 and pFLA-T indicated no visualization of protein interaction. (b) Cells visualized fluorescence at 24 h and 36 h after transfection of pHA-p53 and pFLA-T, indicating positive control of protein interaction. (c) Cells visualized fluorescence at 24 h and 36 h after transfection of pHA-HSRG and pFLA-T, indicating the interaction between these two proteins.

combination with the BiFC experiment, this result might suggest an *in vivo* biological significance of the interaction between HSRG1 and LT.

HSRG1 impacts the transcriptional regulatory ability of LT

LT is one of the two early non-structural protein genes and is a multifunctional protein (Damania *et al*. 1998) required for initiating viral DNA synthesis (Sladek *et al*. 2000). To investigate the functional significance of HSRG1's interaction with LT, an SV40 late transcriptional promoter was inserted into pCAT to serve as a reporter plasmid in the CAT assay. This was used to transfect Vero cells with pcDNA-hsrg1 and/or pcDNA-T in order to analyse how the interaction of HSRG1 and LT affects LT's ability to regulate viral transcription. The result of the CAT assay

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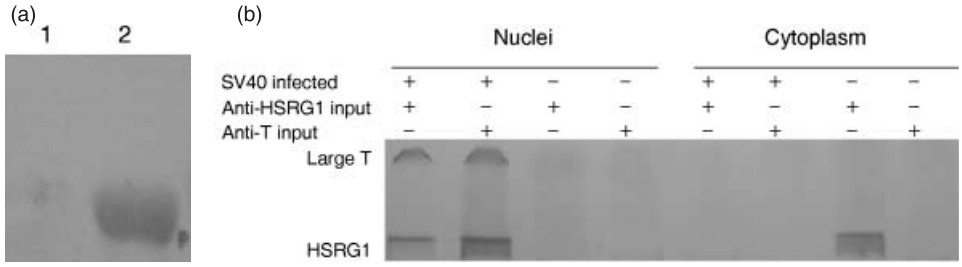

Figure 3. Co-immunoprecipitation of lysates from cells transfected with pcDNA-hsrg1 and infected with SV40, with HSRG1 antibody and large T protein antibody. (a) Western blot of HSRG1 expressed in Vero cells transfected by pcDNA-hsrg as described. 1 = Negative control of Vero cells; 2 = Vero cells transfected by pcDNA-hsrg. (b) Co-immunoprecipitation of HSRG1 and LT. Vero cells transfected with pcDNA-hsrg1 and infected (+) or not (–) with SV40 were fractionated as described in the Materials and Methods section. Proteins from each fraction were analysed by co-immunoprecipitation using the anti-HSRG1 polyclonal antiserum and the anti-SV40 T antibody.

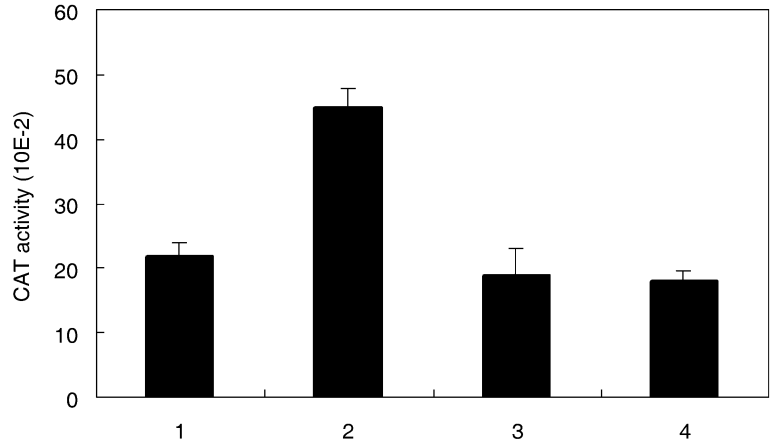

Figure 4. Interaction of HSRG1 with T antigen *in vivo* decreases the level of induction of CAT activity. 1 = CHO cells transfected with pCAT-S and pcDNA; 2 = CHO cells transfected with pCAT-S and pcDNA-LT; 3 = CHO cells transfected with pCAT-S and pcDNA-hsrg1; 4 = CHO cells transfected with pCAT-S, pcDNA-LT and pcDNA-hsrg1. Each transcription reaction contained the same quantity of each plasmid. Error bars indicate the standard deviation of the mean ($n = 4$).

clearly indicates that transcription of the CAT gene controlled by the late promoter of SV40 in cells transfected by pcDNA-hsrg1 and pcDNA-T decreased compared with that in cells transfected with pcDNA-T only (Fig. 4). On the contrary, the transcription of CAT controlled by SV40 late promoter in cells transfected only by pcDNA-hsrg1 does not show any change relative to the positive control, implying that the changes in CAT expression are caused by the interaction of HSRG1 with LT rather than an effect of HSRG1 on the SV40 promoter. This result suggests HSRG1's capability of changing LT's regulation on viral transcription.

HSRG1 inhibits the ability of LT to subvert cell growth control

LT is capable of stimulating host cells to enter the S phase and to undergo DNA synthesis by interacting with several cellular proteins to form complexes (Dornreiter *et al*. 1993; Sladek *et al*. 2000). This effect of LT on cell growth activation is effected through binding to cellular tumour

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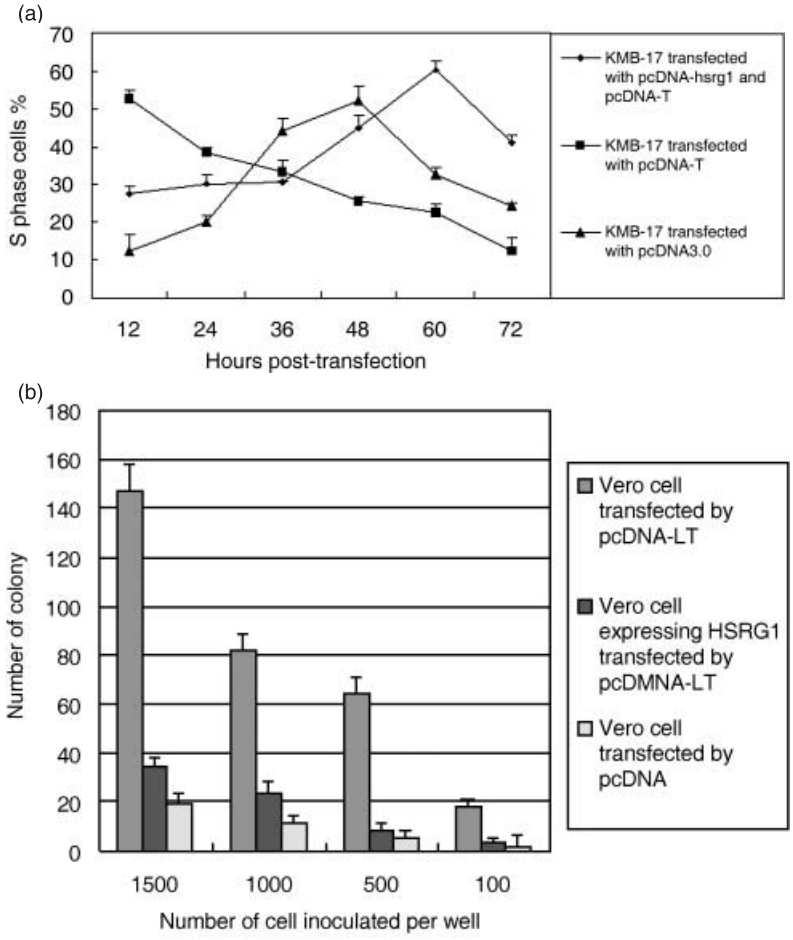

Figure 5. Expression of HSRG1 impairs the ability of the large T protein to affect the cell cycle and HSRG1 inhibits the transforming ability of SV40 large T protein *in vivo*. (a) KMB-17 cells were transfected with pcDNA-T, pcDNA-hsrg1 or pcDNA3.0. The samples were collected and assayed by flow cytometry at 12, 24, 36, 48, 60 and 72 h. (b) Vero cells that expressed HSRG1 protein stably were transfected with pcDNA-LT and pcDNA, respectively. After 36 h of transfection, the cells were trypsinized and diluted to 100, 500, 1000 and 1500 cells/ml in DMEM for subculturing in 6-well plates according to standard protocols. The colonies formed in agar media were counted after 10 days. Data were collected from experiments and were repeated three times.

suppressor proteins, which include p53, pRb, p107 and p130/pRb2, to release cell cycle arrest mediated by these proteins (Stubdal *et al*. 1996; Lin & Decaprio 2003). Cell cycle analysis of human fibroblasts transfected by pcDNA-LT and/or pcDNA-hsrg1 has indicated that expression of HSRG1 is capable of altering LT's ability to stimulate cells to enter into the S phase. As observed in Fig. 5(a), LT is able to stimulate cells to enter into the S phase earlier than control cells. However, this ability is weakened with the presence of HSRG1 (Fig. 5a). The colony-forming assay revealed that HSRG1 expressed in Vero cells and confirmed by Western blot is capable of interfering with the transforming ability of LT (Fig. 5b). After Vero cells expressing HSRG1 protein were transfected by pcDNA-LT, the rate of colony forming of the cells was decreased compared with control cells transfected by pcDNA-LT. This also suggests that HSRG1 interacts with LT and inhibits its function *in vivo*.

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DISCUSSION

Much has been recently published concerning protein molecules induced in the process of viral infection (Liu *et al*. 2002; Dong *et al*. 2003). These proteins function in various cellular activities and in the viral replication cycle (Scholle *et al*. 2004), and might be involved in an intracellular antiviral response such as the action of interferon. As a complex DNA virus, HSV-1 is capable of inducing complex cellular responses during infection (Ray & Enquist 2004), which might bring about the transient opportunity for a change in the cellular microenvironment to affect viral replication (Lomonte & Everett 1999). As part of the immediate–early gene response induced by HSV-1 binding to human embryo fibroblasts, HSRG1 is a member of the highly conserved SAND protein family of eukaryotes. It is necessary to further explore its function in human fibroblasts depending upon its possible interaction with the cellular or viral proteins. Our previous data suggested that this protein is probably involved in cellular signal transduction and viral transcription based on its cytoplasmic localization, primary structure and phosphorylated form in cells (Dong *et al*. 2003). Our identification of a specific interaction of HSRG1 and LT in the yeast two-hybrid system and BiFC assay also provides a possible clue for further investigation of HSRG1's function. Immunoprecipitation by LT or HSRG1 antibody in the nuclear extract of transfected Vero cells by pcDNA-hsrg and infection with SV40 confirmed this interaction (Fig. 3). However, our previous work that indicated a cytoplasmic location of HSRG1 and the fact that LT functions in the nucleus, suggest that it would be interesting to investigate the mechanism of HSRG1 and LT interaction. Depending upon the results of the BiFC assay and co-immunoprecipitation, it is probably safe to predict that HSRG1 localized normally in the cytoplasm meets and binds LT in the cytoplasm. Then, with the leading nuclear localization signal of LT, the complex of HSRG1 and LT moves into the nucleus. The two-hybrid interaction assay also suggested that LT interacts with HSRG1 amino acid residues 132–450 (Fig. 1b), which include the DNA binding, p53 binding and part of the *pol* α binding domains. This result suggests, to some extent, that HSRG1 is capable of influencing LT's transcriptional activity through binding to this domain of LT.

LT, an essential viral replication factor, can regulate viral early and late transcription in SV40 infection (Khoury & May 1977; Hansen *et al*. 1981; Alwine & Picardi 1986). LT also serves as a transcriptional activator of cellular promoters through direct interaction with components of transcription machines including TBP, TAPs and more to regulate cellular or viral transcription (Johnston *et al*. 1996). Combined with the ability to inhibit p53, LT is able to drive cells into the S phase (Sladek *et al*. 2000). In this study, several properties of LT are used as indicators to show how HSRG1 interacts with LT. In the CAT assay, the transcription of CAT linked to the SV40 promoter is strongly increased by LT (Fig. 4, panel 2), revealing the ability of LT to improve viral late gene transcription. However, this function of LT is gravely weakened by the expression of HSRG1 in cells (Fig. 4, panel 4). This result shows that interaction of LT and HSRG1 greatly represses the ability of LT to activate transcription of the SV40 late promoter. In comparison with the control of HSRG1 alone, which confirms no effect of HSRG1 on the transcription of SV40 late promoter (Fig. 4, panel 3), these data suggest that interaction of HSRG1 changes LT's promoter transcriptional regulation. The ability of LT to drive cell growth is also inhibited by the expression of HSRG1, which includes the change of LT's ability to stimulating cell entry into the S phase and of cell colony formation (Fig. 5a,b). As we know, LT is capable of transforming some types of cells through abrogation of cell cycle control, thereby enhancing cell proliferation. Our results are supportive of this fact by exhibiting LT's stimulating human fibroblast entry into the S phase 20 h earlier than performed by control cells. Interestingly, the ability of HSRG1 to interact with LT and to release its stimulation of cell proliferation implies a possible defensive mechanism against virus infection of a cell. This is understandable

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that, to some extent, virus binding to a receptor mimics the process of physiological ligand binding and can induce cellular responses composed of various molecules, some of which exert innate antiviral activities (such as interferon). However, the expression of HSRG1 in host cells could block the specific bioactivity of LT, suggesting a unique significance of HSRG1 in such an intracellular antiviral response. Whether HSRG1 works as a member of the systematic innate responses or merely as a specific molecule with antiviral characteristics needs further investigation. Although we do not know the physiological role of HSRG1 as a cellular protein, the results observed in this study indeed suggest that such an interesting interaction between HSRG1 induced in viral infection and LT could provide a possible means of interference with viral replication through the specific interaction of a cell protein with a viral protein.

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REFERENCES

Alwine JC, Picardi J (1986) Activity of simian virus 40 late promoter elements in the absence of large T antigen: evidence for repression of late gene expression. *J. Virol*. **60**, 400–404.

Baum C, Forster P, Hegewisch-Becker S, Harbers K (1994) An optimized electroporation protocol applicable to a wide range of cell lines. *Biotechniques* **17**, 1058–1062.

Boyle KA, Pietropaolo RL, Compton T (1999) Engagement of the cellular recept protein B of human cytomegalovirus activates the interferon-responsive pathway. *Mol. Cell Biol*. **19**, 3607–3613.

Butel JS, Lednicky JA (1999) Cell and molecular biology of simian virus 40: implications for human infections and disease. *J. Natl. Cancer Inst*. **91**, 99–134.

Cacciotti P, Libener R, Betta P, Martini F, Porta C, Procopio A, Strizzi L, Penengo L, Tognon M, Mutti L, Gaudino G (2001) SV40 replication in human mesothelial cells induces HGF/Met receptor activation: a model for viral-related carcinogenesis of human malignant mesothelioma. *Proc. Natl. Acad. Sci. USA* **98**, 12032–12037.

Casaz. P, Sundseth R, Hansen U (1991) Trans-activation of the simian virus 40 late promoter by large T antigen requires binding sites for the cellular transcription factor TEF-1. *J. Virol*. **65**, 6535–6543.

Cottage A, Mullan L, Portela MB, Hellen E, Carver T, Patel S, Vavouri T, Elgar G, Edwards YJ (2004) Molecular characterisation of the SAND protein family: a study based on comparative genomics, structural bioinformatics and phylogeny. *Cell. Mol. Biol. Lett*. **9**, 739–753.

Damania B, Mital R, Alwine JC (1998) Simian virus 40 large T antigen interacts with human TFIIB-related factor and small nuclear RNA-activating protein complex for transcriptional activation of TATA-containing polymerase III promoters. *Mol. Cell. Biol*. **18**, 1331–1338.

Dong S, Dong C, Liu L, Che Y, Sun M, Hu F, Li J, Li Q (2003) Identification of a novel human sand family protein in human fibroblasts induced by herpes simplex virus 1 binding. *Acta Virol*. **47**, 27–32.

Dornreiter I, Copeland WC, Wang TS (1993) Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase alpha with large T antigen. Mol. *Mol. Cell. Biol*. **13**, 809–820.

Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, Decaprio JA, Lawrence JB, Livingston DM (1994) E2F4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev*. **8**, 2665–2679.

Hansen U, Tenen DG, Livingston DM, Sharp PA (1981) T antigen repression of SV40 early transcription from two promoters. *Cell* **27**, 603–613.

Herbig U, Weisshart K, Taneja P, Fanning E (1999) Interaction of the transcription factor TFIID with simian virus 40 (SV40) large T antigen interferes with replication of SV40 DNA in vitro. *J. Virol*. **73**, 1099–1107.

Hu CD, Kerppola TK (2003) Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat. Biotechnol*. **21**, 539–545.

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Hu CD, Chinenov Y, Kerppola TK (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798.

Johnston SD, Yu XM, Mertz JE (1996) The major transcriptional transactivation domain of simian virus 40 large T antigen associates nonconcurrently with multiple components of the transcriptional pre-initiation complex. *J. Virol.* **70**, 1191–1202.

Khoury G, May E (1977) Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T-antigen. *J. Virol.* **23**, 167–176.

Kim HY, Ahn BY, Cho Y (2001) Structural basis for the inactivation of retinoblastoma tumor suppressor of by SV40 large T antigen. *EMBO J.* **20**, 295–304.

Li R, Branton MH, Tanawallanacharoen S, Falk RA, Jennette JC, Kopp JB (2002a) Molecular identification of SV40 infection in human subject and possible association with kidney siseases. *J. Am. Soc. Nephrol.* **13**, 2320–2330.

Li QH, Zhao HL, Jiang L, Che YC, Dong CH, Wang LC, Wang J, Liu LD (2002b) An SR-protein induced by HSVI binding to cells functioning as a splicing inhibitor of viral pre-mRNA. *J. Mol. Biol.* **316**, 887–894.

Li JF, Liu LD, Ma SH, Che YC, Wang LC, Dong CH, Zhao HL, Liao Y, Li H (2004) HTRP-an immediate-early gene product induced by HSV1 infection in human embryo fibroblasts, is involved in cellular co-repressors. *J. Biochem.* **136**, 169–176.

Lin JY, Decaprio JA (2003) SV40 large T antigen promotes dephosphorylation of p130. *J. Biol. Chem.* **278**, 46482–46487.

Liu L, Dong C, Dong S, Li Q (2002) Cloning, expression and analysis of a novel gene induced by the HSV-I binding to fibroblast. *J. Northwest Uni.* **32**, 10–16.

Lomonte P, Everett RD (1999) Herpes simplex virus type 1 immediate-early protein vmw110 inhibits progression of cells through mitosis and from G1 into S phase of the cell cycle. *J. Virol.* **73**, 9456–9946.

Nishigaki K, Hanson C, Ohashi T, Thompson D, Muszynski K, Ruscetti S (2000) Erythroid cells rendered erythropoietin independent by infection with friend spleen focus-forming virus show constitutive activation of phosphatidylinositol 3-kinase and akt kinase: involvement of insulin receptor substrate-related adapter proteins. *J. Virol.* **74**, 3037–3045.

Ott RD, Rehfuess C, Podust VN, Clark JE, Fanning E (2002) Role of the p68 subunit of human DNA polymerase α-primase in simian virus 40 DNA replication. *Mol. Cell. Biol.* **22**, 5669–5678.

Ramsperger U, Stahl H (1995) Unwinding of chromatin by the SV40 large T antigen DNA helicase. *EMBO J.* **14**, 3215–3225.

Ray N, Enquist LW (2004) The transcriptional response of a common permissive cell type to infection by two diverse alpha herpesviruses. *J. Virol.* **78**, 3489–3501.

Reed SI, Ferguson J, Davis RW, Stark GR (1975) T antigen binds to simian virus 40 DNA at the origin of replication. *Proc. Natl. Acad. Sci. USA* **72**, 1605–1609.

Richardson C, Fielding C, Rowe M, Brennan P (2003) Epstein–Barr virus regulates STAT1 through latent membrane protein 1. *J. Virol.* **77**, 4439–4443.

Roggero R, Robert-Hebmann V, Harrington S, Roland J, Vergne L, Jaleco S, Devaux C, Biard-Piechaczyk M (2001) Binding of human immunodeficiency virus type 1 gp120 to CXCR4 induces mitochondrial transmembrane depolarization and cytochrome c-mediated apoptosis independently of fas signaling. *J. Virol.* **75**, 7637–7650.

Scholle F, Li K, Bodola F, Ikeda M, Luxon BA, Lemon SM (2004) Virus–host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. *J. Virol.* **78**, 1513–1524.

Sheppard HM, Corneillie SI, Espiritu C, Gatti A, Liu X (1999) New insights into the mechanism of inhibition of p53 by simian virus 40 large T antigen. Mol. *Mol. Cell. Biol.* **19**, 2746–2753.

Sladek TL, Laffin J, Lehman JM, Jacobberger JW (2000) A subset of cells expressing SV40 large T antigen contain elevated p53 levels and have an altered cell cycle phenotype. *Cell Prolif.* **33**, 115–125.

Stubdal H, Zalvide J, Decaprio JA (1996) Simian virus 40 large T antigen alters the phosphorylation state of the RB-related proteins p130 and p107. *J. Virol.* **70**, 2781–2788.

Sullivan CS, Pipas JM (2002) T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis. *Microbiol. Mol. Biol. Rev.* **66**, 179–202.

Tegtmeyer P (1972) Simian virus 40 deoxyribonucleic acid synthesis: the viral replication. *J. Virol.* **10**, 591–598.

Ullrich CK, Groopman JE, Ganju RK (2000) HIV gp120- and gp160-induced apoptosis in cultured endothelial cells is mediated by caspases. *Blood* **96**, 1438–1442.

Vivaldi A, Pacini F, Martini F, Iaccheri L, Pezzetti F, Elisei R, Pinchera A, Faviana P, Basolo F, Tognon M (2003) Simian virus 40-like sequences from early and late regions in human thyroid tumors of different histotypes. *J. Clin. Endocrinol. Metab.* **88**, 892–899.

Wiekowski M, Schwarz. MW, Stahl H (1988) Simian virus 40 large T antigen DNA helicase. *J. Biol. Chem.* **263**, 436–442.

Wu C, Roy R, Simmons DT (2001) Role of single DNA binding activity of T antigen in simian virus 40 DNA replication. *J. Virol.* **75**, 2839–2847.

Zhai W, Tuan JA, Comai L (1997) SV40 large T antigen binds to the TBP-TAF (I) complex SL1 and coactivates ribosomal RNA transcription. *Genes Dev.* **11**, 1605–1617.

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