Human herpesvirus 6B induces phosphorylation of p53 in its regulatory domain by a CK2- and p38-independent pathway

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Here, we demonstrate that human herpesvirus 6B (HHV-6B) infection upregulates the tumour suppressor p53 and induces phosphorylation of p53 at Ser392. Interestingly, phosphorylation at the equivalent site has previously been shown to correlate with p53 tumour suppression in murine models. Although the signalling pathways leading to Ser392 phosphorylation are poorly understood, they seem to include casein kinase 2 (CK2), double-stranded RNA-activated protein kinase (PKR), p38 or cyclin-dependent kinase 9 (Cdk9). By using column chromatography and in vitro kinase assays, CK2 and p38, but not PKR or Cdk9, eluted in column fractions that phosphorylated p53 at Ser392. However, treatment of cells with neither the CK2 and Cdk9 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) nor p38 kinase inhibitors reduced HHV-6B-induced Ser392 phosphorylation significantly. Knockdown of the CK2α subunit or p38α by small interfering RNA had no effect on HHV-6B-induced phosphorylation of p53 at Ser392. Thus, HHV-6B induces p53 Ser392 phosphorylation by an atypical pathway independent of CK2 and p38 kinases, whereas mitogen-activated protein (MAP) kinase signalling pathways are involved in viral replication.

INTRODUCTION

Human herpesvirus 6B (HHV-6B) is a roseolovirus (family Herpesviridae, subfamily Betaherpesvirinae), a genus that also includes HHV-7 and the closely related HHV-6A. Human cytomegalovirus (HCMV) (Speir et al., 1994; Muralidhar et al., 1996; Tsai et al., 1996; Bonin & McDougall, 1997; Castillo et al., 2005) and possibly HHV-6A (Kashanchi et al., 1997; Takemoto et al., 2005) and HHV-6B (De Bolle et al., 2004; Takemoto et al., 2004; Øster et al., 2005) interfere with the tumour suppressor protein p53. The major functions of p53 are associated with maintaining the integrity of the genome by inducing cell-cycle arrest, senescence or apoptosis (Jin & Levine, 2001). Tight regulation of p53, e.g. by post-translational modifications in the N- and C-terminal regions, is required, as more than 100 different target genes are under the control of p53 (Resnick et al., 2005). Phosphorylation, mediated by various different kinases, is a reversible way of regulating p53 functions. A number of phosphorylation sites have been identified in p53, together with an even longer list of kinases capable of phosphorylating p53 at specific sites. Significant redundancies are found, in that several different kinases can phosphorylate the same site on p53.

Like HCMV (Castillo et al., 2005), HHV-6B is capable of inducing phosphorylation in the N terminus of p53 at Ser15 (Øster et al., 2005). Other human herpesviruses may also interfere with p53 functions. Herpes simplex virus 1 (HSV-1) induces phosphorylation of p53 at Ser15 and 20 (Boutell & Everett, 2004; Shirata et al., 2005), and Epstein–Barr virus (EBV) induces phosphorylation at Ser6, 9, 15, 33, 46, 315 and 392 (Mauser et al., 2002; Kudoh et al., 2005). Nevertheless, knowledge regarding human herpesvirus-induced p53 phosphorylation is limited (Collot-Teixeira et al., 2004), and even less is known about the kinases responsible for the virus-induced phosphorylation events on p53.

In the C-terminal domain, nuclear-localization signals mediate translocation of p53 to the nucleus (Shaursly et al., 1990; Liang et al., 1998). Post-translational modifications of this region modulate the specific activity of p53 in vivo and, in unstressed cells, the C-terminal domain maintains p53 in a non-DNA-binding form (Levine, 1997; Hupp et al., 2000). Conversely, phosphorylation at Ser392 in this domain is involved in stabilization of tetramer formation and enhanced DNA binding (Hupp et al., 1992; Hupp & Lane, 1994; Sakaguchi et al., 1997). The evolutionary conservation from bony fish to humans reflects the importance of this phosphorylation site (Keller et al., 2001), which also has been designated the ‘CK2 site’, named after the first identified kinase capable of phosphorylating p53 at Ser392 (Meek et al., 1990).

CK2 is a ubiquitously distributed protein kinase with more than 100 physiological targets (Litchfield, 2003). It is
composed of two regulatory β subunits and two catalytic α and/or α’ subunits. The α and α’ subunits have significant homology in the N-terminal region, and may have complementary functions (Hanna et al., 1995). Knockout of CK2β is lethal in mice, due to complete abrogation of CK2 functions (Buchou et al., 2003). In fact, CK2 was considered the only Ser392 kinase for several years until the discovery of two additional Ser392 kinases: double-stranded RNA (dsRNA)-activated protein kinase (PKR) (Cuddihy et al., 1999) and mitogen-activated protein (MAP) kinase p38 (Huang et al., 1999; Keller et al., 1999). PKR is activated by dsRNA and is involved in the regulation of several signal-transduction pathways in the cell. In particular, PKR is known for its role in the innate response to virus infections, as an inducer of interferons (Langland et al., 2006). Via its N-terminal region, PKR can bind to the C terminus of p53 and phosphorylate p53 at Ser392 in vitro (Cuddihy et al., 1999). The p38 MAP kinase regulates multiple processes in the cell (Bradham & McClay, 2006) and may phosphorylate p53 at Ser33, 46 and 392 in response to UV radiation (Bulavin et al., 1999; Huang et al., 1999; Keller et al., 1999). Indeed, p38 has been proposed to be one of the most important mediators of UV-induced Ser392 phosphorylation (Huang et al., 1999; Keller et al., 1999).

Most recently, cyclin-dependent kinase 9 (Cdk9) has been identified as a p53 Ser392 kinase (Claudio et al., 2006; Radhakrishnan & Gartel, 2006). Cdk9 is a Cdc2-related serine–threonine kinase that, unlike most other Cdc2-related kinases, is not involved in cell-cycle regulation (de Falco & Giordano, 1998). By phosphorylation of RNA polymerase II, Cdk9 plays an important role in transcriptional elongation (de Falco & Giordano, 1998). Cdk9 binds p53 (de Falco & Giordano, 1998; Claudio et al., 2006), among other proteins, and phosphorylates it at Ser392 by direct interaction between the N-terminal region of Cdk9 and the C-terminal domain of p53 (Claudio et al., 2006). Phosphorylated p53 activates cdk9 gene expression, hence forming a feedback loop. Cdk9 also phosphorylates p53 at Ser33 and 315, although the function of these post-translational modifications remains to be determined (Radhakrishnan & Gartel, 2006). Regardless of the kinases that modify this Ser392 site (Ser389 is the equivalent in the mouse), it is known that Ser389 mutation to a non-phosphorylatable codon renders mice prone to carcinogen-induced bladder cancer and UV-induced skin cancer (Bruins et al., 2004; Hoogervorst et al., 2005).

Many distinct stresses induce p53 activation via phosphorylation changes, although the signals that activate p53 are only beginning to be defined. Some of the key signals induced by DNA damage that are thought to signal to p53 include DNA-strand breaks, DNA-repair adducts, stalled transcription complexes and thymidine dimers. How other stresses, such as hypoxia, signal to p53 has not been defined, although perturbation of metabolites may trigger p53 activation. The recent observations that DNA and RNA viruses activate p53 highlight a possible mechanism for the evolution of the p53 checkpoint response and provide a physiological model to define how the p53 system is integrated into immune-system responses to stress. We have reported previously that HHV-6B, the causative agent of the childhood disease exanthema subitum, induces phosphorylation of p53 in the N-terminal region at Ser15 and Ser20. Mutation of the Ser20 site (Ser23 in mouse) sensitizes animals to B-cell lymphoma (MacPherson et al., 2004), highlighting the importance of this kinase site in tumour suppression. The role of p53 during HHV-6B infection remains to be defined, yet p53 has been identified as a critical factor in the antiviral defence of the host cell (Takaoka et al., 2003). Recently, it has been noted that HHV-6B-induced cell-cycle arrest occurs independently of p53, despite an aberrant accumulation of p53. In fact, cells lacking functional p53 were reported to arrest at an earlier time point than cells containing p53 (Oster et al., 2006).

Here, we report that HHV-6B infection induces p53 Ser392 phosphorylation. By using a combination of column fractionation and in vitro kinase assays, small interfering RNA (siRNA) knockdown of protein kinases, and inhibitors of kinase activity, we have excluded the known p53 Ser392 kinases from performing this modification, indicating that HHV-6B induces p53 Ser392 phosphorylation by a novel pathway. Due to the importance of Ser392 phosphorylation in damage-induced tumour suppression by p53 and the physiological relevance of a p53 sensor in the antiviral response, these data highlight a critical pathway, further study of which will shed light on how cells use p53 to sense and respond to viral infection.

**METHODS**

**Cells and viruses.** The human epithelial cell line HCT116, a gift from B. Vogelstein and K. W. Kinzler, Johns Hopkins University School of Medicine, Baltimore, MD, USA, was grown in McCoy’s medium supplemented with extra glutamine (0.146 g l⁻¹), 10 mM HEPES, streptomycin (0.2 g l⁻¹), penicillin (0.2 IU l⁻¹) and 10 % heat-inactivated fetal bovine serum. The T-cell line MOLT3 was grown in Iscove’s modified Dulbecco’s medium supplemented with 10 % heat-inactivated fetal bovine serum, glutamine (0.292 g l⁻¹), 10 mM HEPES, streptomycin (0.2 g l⁻¹) and penicillin (0.2 IU l⁻¹). HHV-6B (strain PL-1) was produced in MOLT3 cells (Oster & Hällsberg, 2002). Virus was collected from supernatant of HHV-6B-infected MOLT3 cells and concentrated 20-fold by centrifugation. First, supernatant was cleared from cell debris by centrifugation at 4500 g for 1 h and subsequently centrifuged at 100 000 g for 1 h. The titre of the virus stock was expressed in TCID₅₀ as determined by the Reed–Muench method (Reed & Muench, 1938) on 0.1 × 10⁶ cells in 200 μl medium. Under these conditions, the titre of the virus stock was 3200 TCID₅₀. Infection was performed with 2 × 10⁶ MOLT3 cells ml⁻¹ with a 10-fold dilution of the virus stock.

**Cell lysis and immunoblotting.** Whole-cell extracts were prepared by using 1 × lysis buffer, purchased from Cell Signaling Technology, supplemented with 1 mM PMSF, 5 mM NaF and Complete mini protease inhibitor (Roche Diagnostics) in a concentration defined by the manufacturer’s recommendations. Whole-cell extracts used for column fractions were prepared with 1 × lysis buffer, further
supplemented with 0.5 M NaCl to extract CK2 from the chromatin. Lysates were centrifuged first at 2600 g for 5 min then at 20 000 g for 10 min, and whole-cell extracts were frozen immediately at −70 °C. Proteins were separated in XT Criterion 10 % gels (Bio-Rad) using XT MES running buffer (Bio-Rad) and subsequently transferred to nitrocellulose membranes. The following primary antibodies were used: p53 (DO-7), 1: 500 (Biosource); p53 Ser392 (FP3 2.1), 1: 2000 (monoclonal antibody; Blaydes et al., 2000a, b); ERK p44/p42, 1: 500; PARP, 1: 1000; p-p38 (Thr180/Tyr182), 1: 1000 (all from Cell Signaling Technology); CK2α (1A9D), 1: 500 (Calbiochem); CK2α′ (C-20) sc-6481, 1: 200; CK2β (6D5) sc-12739, 1: 500; PKR (D-20) sc-708, 1: 600; p38 (H-147) sc-7149, 1: 500; Chk1 (G-4) sc-8408, 1: 500; Chk2 (A-12) sc-5278, 1: 500; Gsk9 (D-7) sc-13130, 1: 500; MNK1 (C-20) sc-6965, 1: 1000; and GAPDH (FL-335) sc-25778, 1: 2000 (all from Santa Cruz Biotechnology); DAP kinase (610290), 1: 1000 (BD Biosciences); ZIP1 (Ab2057), 1: 1000 (Abcam). An HHV-6B nuclear protein was visualized by using anti-herpes virus type 6 antibody 7C7; Argene), diluted 1 : 400. The secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse (1: 2000), swine anti-rabbit (1: 2000) or rabbit anti-goat (1: 1000) from DAKO. All antibodies were diluted in 5 % skimmed milk in TBS/Tween 20 (0.1 %). Immunoblots were developed by using enhanced chemiluminescence.

**Column fractionation.** Lysates from 108 uninfected or HHV-6B-infected cells in 2 ml lysis buffer were treated with 1250 U benzonase (Novagen) for 30 min on ice, and passed through a syringe with a 0.6 mm needle to shear the DNA. When fluid, lysates were centrifuged at 2600 g for 5 min. The supernatants were diluted 100 fold to 1 ml fraction and kinase buffer [50 mM KCl, 50 mM HEPES (pH 7.5), 50 mM MgCl2, 1 mM DTT, 0.2 mM EDTA and 100 μM ATP] in a final volume of 20 μl. The reaction mixture was incubated for 30 min at 30 °C and reactions were terminated by adding 30 μl SDS sample buffer. To detect phosphorylation at Ser392, 10 μl kinase reaction was used for immunoblotting.

**In vitro kinase assay.** The column fractions were tested for Ser392 kinase activity in an in vitro kinase assay, performed with 50 ng human recombinant p53 protein tetramers, 2 μl fraction and kinase buffer [50 mM KCl, 50 mM HEPES (pH 8.0), 1 mM dithiothreitol (DTT) and 1 mM benzamidine] and applied to a 5 ml Q Sepharose Fast Flow Anion Exchanger column (Sigma–Aldrich) that was equilibrated with buffer A. The protein was eluted by a linear gradient of 0–100 % buffer B [10 % glycerol, 20 mM HEPES, (pH 8.0), 1 M KCl, 1 mM DTT and 1 mM benzamidine] over 10 column vols, followed by 100 % buffer B for 2 column vols. Fractions (1 ml) were collected. Every other eluted fraction was used for the detection of kinases by immunoblotting and for detection of kinase activity in an in vitro kinase assay.

**Inhibitor experiments.** Kinase inhibitors were included in the medium of uninfected and HHV-6B-infected MOLT3 cells, concomitantly with infection, for 48 h at the following concentrations: 20 μM CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma), 1 mM PKR inhibitor (Calbiochem), 20 μM p38 inhibitor SB202190 (Sigma–Aldrich), 20 μM p38 inhibitor SB239063 (Sigma–Aldrich), 50 μM MEK inhibitor PD98059 (Sigma), 40 μM ERK inhibitor A6355 (Sigma), 10 μM MNK1 inhibitor (Calbiochem), 1 μM INK inhibitor (Alexis).

**siRNA.** CK2β, p38 and MNK1 siRNA SMART Pools, purchased from Dharmacon, were used to silence CK2β, p38 or MNK1, respectively. HCT116 cells were transfected with 100 nM CK2β siRNA, 200 nM p38 siRNA or 200 nM MNK1 siRNA by using Nucleofector (Amaxa, Program D-32) according to the manufacturer’s protocol. Control cells were transfected with transfection reagents without siRNA. After 24 or 48 h incubation, the transfected cells were infected with a 1:10 dilution of HHV-6B stock and incubated for a further 48 h.

**RESULTS**

**HHV-6B induces phosphorylation of p53 in its regulatory domain**

Although p53 may play an important role in antiviral defence (Takaoka et al., 2003), little is known about the crosstalk between HHV-6B and p53. During HHV-6B infection, cells accumulate significant amounts of p53, and the rate of accumulation depends on cell type as well as virus titre used for infection (unpublished observation) (De Bolle et al., 2004; Takemoto et al., 2004; Øster et al., 2005). HHV-6B infection of the human epithelial cell line HCT116 has previously been characterized regarding accumulation of p53, cell-cycle arrest and synctium formation (Øster et al., 2006). HHV-6B-induced accumulation, concomitant with an increased DNA-binding activity, of p53 (Øster et al., 2006) prompted us to examine the potential phosphorylation of the p53 C-terminal regulatory domain following HHV-6B infection.

Epithelial cells were infected by HHV-6B and examined for p53 Ser392 phosphorylation by using a phosphorylation-specific antibody. At 28 h post-infection (p.i.), a significant amount of p53 Ser392 phosphorylation could be detected (Fig. 1). To investigate which kinases were involved in this reaction, whole-cell lysates were applied to a Sepharose Q column and fractions were eluted at various KCl concentrations (0–1 M). By using recombinant p53 as the substrate, the fractions were investigated for Ser392 kinase activity by using a phospespecific antibody recognizing p53 phosphorylated at Ser392. One major peak in fractions 26–32 could be observed in the eluates from uninfected and HHV-6B-infected cells (Fig. 2). In the infected cells, an additional peak was observed in fractions 14–18, suggesting that HHV-6B may induce kinase activity that is absent in uninfected cells.

![Fig. 1. HHV-6B infection induces phosphorylation in the regulatory domain of p53. HCT116 cells were infected with HHV-6B and lysates were prepared after 16, 28 and 40 h infection. Western blotting was performed with antibodies against the indicated proteins.](http://vir.sgmjournals.org)
HHV-6B-induced p53 Ser392 phosphorylation is CK2-independent

To identify the HHV-6B-induced p53 Ser392 kinase, Western blots of the protein eluates were probed with an antibody against CK2, the kinase traditionally known to phosphorylate p53 at Ser392. However, although the presence of CK2 correlated with some fractions containing Ser392 kinase activity, it did not co-elute with the highest Ser392 kinase activity (Fig. 2). Moreover, incubation of cells in the presence of the CK2 inhibitor DRB did not abolish p53 Ser392 phosphorylation (Fig. 3a).

To establish the role of CK2 during HHV-6B-induced phosphorylation of p53 Ser392, CK2 was knocked down by siRNA against the CK2β subunit. Despite the long half-life of CK2 (Seeber et al., 2005), CK2β siRNA at a concentration of 60–100 nM reduced the level of the CK2β subunit markedly following 48 h incubation (Fig. 3b). When HHV-6B-infected cells were transfected with CK2β siRNA, the level of the CK2β subunit was reduced in uninfected cells, as well as in infected cells (Fig. 3c). However, no corresponding reduction in HHV-6B-induced p53 Ser392 phosphorylation could be observed, which supported the observation that p53 Ser392 phosphorylation was maintained in the presence of the CK2 inhibitor DRB. Interestingly, knockdown of CK2β also affected expression of the α and α′ subunits, as an increase in the level of the CK2α subunit could be observed, whereas the level of the CK2α′ subunit decreased. Similarly, Seeber et al. (2005) reported a decrease in the expression of CK2β when depleting cells for CK2β, whereas CK2α′ depletion did not affect CK2β expression.

HHV-6B-induced p53 Ser392 phosphorylation is p38α/β-independent

HHV-6B-induced p53 Ser392 phosphorylation was not cell type-specific and could also be demonstrated in MOLT3 cells (Fig. 4a). When applying whole-cell extracts from MOLT3 cells to a Sepharose Q column, the major p53 Ser392 phosphorylation peak could be detected in exactly the same fractions as in HCT116 cells (Fig. 4b). In whole-cell extracts, p53 Ser392 phosphorylation could only be detected in infected cells, and not in uninfected cells. However, in the cell-free in vitro kinase assay, regulation of

Fig. 2. Fractions with p53 Ser392 in vitro kinase activity co-elute with CK2α′. Lysates of uninfected and HHV-6B-infected HCT116 cells were fractionated on a Q Sepharose Fast Flow Anion Exchanger column and assayed for in vitro kinase activity with recombinant p53 as the substrate. An aliquot of the in vitro kinase reaction was analysed for the presence of p53 (input) and p53 Ser392 phosphorylation, and the column fractions were analysed for the presence of CK2α′ by Western blotting.

Fig. 3. CK2 inhibition or knockdown does not abolish p53 Ser392 phosphorylation. (a) HCT116 cells were infected in the presence or absence of the CK2 inhibitor DRB. Lysates were examined by Western blotting with antibodies against the indicated proteins. (b) HCT116 cells were transfected with or without siRNA against CK2β for 48 h. Lysates were examined by Western blotting with antibodies against the indicated proteins. (c) HCT116 cells were transfected with or without siRNA (100 nM) against CK2β for 48 h and subsequently incubated in the presence or absence of HHV-6B for 48 h. Lysates were examined by Western blotting with antibodies against the indicated proteins.
the p53 Ser392 kinase is not recapitulated, and significant phosphorylation at Ser392 was detected in the column fractions from uninfected as well as infected cells (Figs 2 and 4b). The minor peak, found only in whole-cell extracts from HHV-6B-infected cells, was comparable between the two cell types, although it seemed to be more distinct in the HCT116 cells.

We next examined the other identified p53 Ser392 kinases, PKR, Cdk9 and p38. The fractions containing PKR correlated with neither the major nor the minor p53 Ser392 phosphorylation peak. Cdk9 correlated with the tail of the minor peak, although the fraction with the majority of kinase activity had virtually no detectable Cdk9 (Fig. 4b). In addition, as shown in Fig. 3(a), incubation of cells in the presence of DRB, a pharmacological inhibitor of Cdk9 as well as CK2, did not abrogate phosphorylation of p53 at Ser392. These observations suggested that Cdk9 was not a major kinase mediating HHV-6B-induced phosphorylation of p53 at Ser392.

In contrast to Cdk9, p38 co-eluted with the major p53 Ser392 phosphorylation peak in fraction 28 and 30 (Fig. 4b). Supporting an involvement of p38, HHV-6B infection also led to phosphorylation of Ser33, a known target for p38 kinase (Fig. 4c). To follow up on this finding, inhibitors against members of the MAP kinase pathway, p38, ERK and JNK, and a specific PKR inhibitor were used to examine the role of these kinases in HHV-6B-induced phosphorylation of p53 at Ser392 (Fig. 5a, b). However, the p38 inhibitor SB202190 and a MEK1/2 inhibitor did not reduce phosphorylation of p53 at Ser392, whereas phosphorylation at this particular site was enhanced in the presence of inhibitors against JNK and PKR.

Fig. 4. The p38 MAP kinase co-elutes with p53 Ser392 kinase activity. (a) HHV-6B-induced phosphorylation of p53 Ser392 in MOLT3 cells. Cells were treated with or without HHV-6B for 16, 28 or 40 h, lysed and analysed by Western blotting with antibodies against the indicated proteins. (b) Lysates of uninfected and HHV-6B-infected MOLT3 cells were fractionated on a Q Sepharose Fast Flow Anion Exchanger column and assayed for in vitro kinase activity with recombinant p53 as the substrate. Column fractions and aliquots of the in vitro kinase reactions were analysed by Western blotting with antibodies against the indicated proteins. (c) MOLT3 cells were infected by HHV-6B for 4, 24 and 48 h. Lysates were examined by Western blotting with antibodies against the indicated proteins.
The MEK1/2 inhibitor is known to inhibit ERK activation (Dudley et al., 1995) and, although the MEK1/2 inhibitor did not affect p53 Ser392 phosphorylation, the column fractions were also tested for the presence of ERK. In contrast to the striking correlation between fractions containing p53 phosphorylated at Ser392 and p38, ERK came out in fractions 20 and 22 only, which are fractions that correlate with neither of the two Ser392 peaks (Fig. 4b).

Although it did not affect p53 Ser392 phosphorylation, the p38 inhibitor reduced the expression of a viral 116 kDa nuclear protein, detected by using the 7C7 antibody raised against this protein. The reduced expression of the viral protein was not a consequence of apoptosis, as measured by the cleavage of poly(ADP-ribose) polymerase (PARP). In contrast, the MEK1/2 inhibitor induced apoptosis, and this was also seen when inhibitors of p38 were combined with inhibitors of MEK1/2, JNK or PKR.

We included an additional p38 inhibitor (SB239063) and an ERK inhibitor (Fig. 5b). The concerted action of the p38 and ERK inhibitors led to a significant reduction in expression of the viral nuclear protein 7C7. As these kinases both phosphorylate MNK1, we examined the significance of an MNK1 inhibitor for p53 Ser392 phosphorylation. The MNK1 inhibitor reduced the presence of 7C7, whereas p53 and p53 Ser392 phosphorylation levels were unaffected. The SB239063 p38 inhibitor did not inhibit p53 Ser392 phosphorylation, but rather enhanced it. We therefore attempted to knock down the p38 kinase by using siRNA (Fig. 5c and d). In the presence of 200 nM siRNA, the level of p38 was reduced significantly, but this had no impact on p53 Ser392 phosphorylation. Likewise,

Fig. 5. Inhibition of p38α/β or MEK1/2 does not prevent HHV-6B-induced p53 Ser392 phosphorylation. (a) MOLT3 cells were infected by HHV-6B for 48 h in the presence or absence of inhibitors of the kinase activity of p38α/β, MEK1/2, JNK or PKR. (b) MOLT3 cells were infected by HHV-6B for 24 h in the presence or absence of inhibitors of the kinase activity of p38, ERK or MNK1. (c) HCT116 cells were transfected with or without siRNA against p38 for 72 h. (d) HCT116 cells were transfected with or without siRNA (200 nM) against p38 for 24 h and incubated in the presence or absence of HHV-6B for 48 h. (e) HCT116 cells were transfected with or without siRNA (200 nM) against MNK1 for 24 h and incubated in the presence or absence of HHV-6B for 48 h. Lysates were examined by Western blotting with antibodies against the indicated proteins.
MNK1 siRNA was unable to prevent p53 Ser392 phosphorylation (Fig. 5e).

To compare the p38 inhibitors directly, we examined their impact on the expression of p53, p53 Ser392 and p53 Ser33 and on the phosphorylation of p38 at Thr180/Tyr182 during HHV-6B infection. Surprisingly, the SB202190 p38 inhibitor induced the phosphorylation of p38 itself significantly, whereas SB239063 induced a barely detectable level of p38 phosphorylation. However, SB239063 enhanced the phosphorylation of p53 on Ser392 and Ser33 (Fig. 6a). The induction of p38 phosphorylation by SB202190 suggested that HHV-6B might induce p38 phosphorylation. To address this further, a dose-dependent SB202190 inhibitor experiment was performed. This demonstrated that SB202190 induced p38 phosphorylation in HHV-6B-infected cells to a much higher level than it did in uninfected cells. As no p38 phosphorylation was detectable at 48 h.p.i. in the absence of p38 inhibitor (Fig. 6b), this experiment also suggested that the SB202190 p38 inhibitor prevents p38 dephosphorylation.

To examine directly whether HHV-6B infection induced p38 phosphorylation, we investigated lysates at 0.5, 1, 2 and 3 h.p.i. (Fig. 6c). Although variable, minor phosphorylation could be detected at approximately 2 h.p.i. Importantly, this phosphorylation was invariably reduced to below the level observed in uninfected cells at 3 h.p.i. This suggested that HHV-6B infection resulted in first phosphorylation, and subsequently in dephosphorylation, of p38.

As CK2, PKR, Cdk9 or p38 did not appear to be the kinases responsible for phosphorylation of p53 at Ser392 during HHV-6B infection, we went on to test the column fractions of the MOLT3 protein eluates by Western blotting with antibodies against Chk1, Chk2, Zip1 and DAP kinase. None of these kinases co-eluted with the Ser392 kinase activity (data not shown), further suggesting the involvement of a novel kinase.

**DISCUSSION**

The functions of p53 are regulated by post-translational modifications at sites predominantly found in the N- and C-terminal regions of p53. Specific combinations of phosphorylations at multiple sites of p53 direct the interaction of p53 with other proteins, which in turn affect the stability, localization, DNA binding- and transcriptional activity of p53.

Several herpesviruses, such as HSV (Boutell & Everett, 2004), EBV (Mauser et al., 2002), HCMV (Castillo et al., 2005) and HHV-6B (Øster et al., 2005), have been shown to induce phosphorylations at multiple sites in p53. Only two viruses, EBV and human T-cell lymphotropic virus type 1, have previously been reported to induce phosphorylation of human p53 at Ser392 (Pise-Masison et al., 1998; Mauser et al., 2002). The kinases responsible for virus-induced phosphorylation at Ser392 have never been described. Here, we report that HHV-6B induces...
phosphorylation of p53 at Ser392 independently of CK2 and p38. Phosphorylation of p53 at Ser392 is known to stabilize tetramer formation (Sakaguchi et al., 1997) and enhance the DNA-binding ability of p53 (Hupp et al., 1992). This correlates well with the enhanced DNA-binding function that is observed during HHV-6B-infection (Øster et al., 2005).

Current knowledge of the interrelationship between p53 and HHV-6B is still limited. During HHV-6B infection, p53 is phosphorylated at Ser15, 20, 33 and 392 (Øster et al., 2005). Importantly, although the significance of these phosphorylations is not yet clear, two of the sites, Ser20 and 392, are known as tumour-suppressor sites (Hoogervorst et al., 2005; Bruins et al., 2004; MacPherson et al., 2004). Any potential for HHV-6B to act as an oncogenic virus has yet to be demonstrated. The closely related virus HHV-6A encodes a protein, DR7, that, when injected into nude mice, causes fibrosarcoma. The DR7 protein can bind directly to p53 and abolish p53-dependent transcription (Kashanchi et al., 1997). In addition, DNA from HHV-6B, in particular, but also from HHV-6A, has been detected in Hodgkin’s lymphoma and oral carcinoma (Braun et al., 1997; Kashanchi et al., 1997).

Only a few pathways are known to activate p53. Hence, the existence of virus-induced stress-activated pathways that lead to phosphorylation of p53 may be of great importance. None of these HHV-6B-induced pathways has been identified to date. CK2, Cdk9, PKR and p38/z’/b have been shown to phosphorylate p53 at Ser392. Of these, p38/z’/b eluted in approximately the same fractions as the p53 Ser392 kinase. Previously, p38 has been shown to phosphorylate p53 at Ser389 in mice, the equivalent of human Ser392, in response to UV radiation (Huang et al., 1999). The consequences of UV-induced p38-mediated phosphorylation at this site are enhanced DNA binding and transcriptional activity of p53 (Huang et al., 1999). Interestingly, Keller et al. (1999) could not completely abolish Ser389 phosphorylation by using a p38/z’/b-specific inhibitor, even at high concentrations, suggesting that additional kinases were involved. By using imidazole compounds inhibiting p38/z’/b, we were unable to prevent HHV-6B-induced p53 Ser392 phosphorylation. Likewise, p38 knockdown by siRNA had no impact on this phosphorylation. This would not, however, rule out p38 entirely, as the δ isoform, expressed by T cells, is not inhibited by the imidazole compounds used.

The EBV protein BZLF1 has been reported to enhance phosphorylation of p53 at serines 6, 9, 15, 33, 46, 315 and 392 (Mauzer et al., 2002) and increase the activity of p38 and c-Jun N-terminal kinases (Adamson et al., 2000). Although p38 is capable of phosphorylating p53 at Ser33, 46 and 392 (Bulavin et al., 1999; Huang et al., 1999; Keller et al., 1999), whether p38 is involved in the phosphorylation of any of these sites during EBV infection remains to be determined. Nevertheless, it is clear that p38 plays an important role in disrupting EBV latency (Adamson et al., 2000). BZLF1 and BRLF1 are two immediate-early EBV proteins, either of which can activate latent infection to the lytic form. Disruption of EBV latency by BRLF1 is prevented by inhibitors of p38 or JNK (Adamson et al., 2000). Another human herpesvirus, Kaposi’s sarcoma-associated herpesvirus (HHV-8), has been reported to activate the ERK, p38 and JNK MAP kinase pathways during primary infection (Naranatt et al., 2003; Xie et al., 2005), and the activation of these pathways is essential for HHV-8 infection (Pan et al., 2006). Inhibitors of all three MAP kinase pathways reduce HHV-8 infectivity by reducing lytic replication and, as a result, the yield of infectious virions (Pan et al., 2006).

HHV-6B may also exploit the cellular MAP kinase pathways during infection, as the presence of an inhibitor of p38 reduced the level of the 116 kDa viral nuclear protein significantly, as detected by Western blotting. When the p38 inhibitor was combined with either the MEK1/2 inhibitor or the ERK inhibitor, the level of the 116 kDa viral nuclear protein was reduced markedly. The reduction in viral protein may reflect reduced HHV-6B infectivity. Surprisingly, the phosphorylation of p38 increased in the presence of the SB202190 inhibitor during HHV-6B infection. This suggests that HHV-6B infection may induce p38 kinases (MAPKK), but that the tendency to phosphorylate p38 is counteracted by dephosphatases. Further experiments are required to elucidate the interactions between the MAP kinase pathways, HHV-6B and p53 during HHV-6B infection.

In summary, our experiments demonstrate that HHV-6B induces a novel pathway of p53 Ser392 phosphorylation independent of CK2, Cdk9, PKR and p38/z’/b.

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