Productive human herpesvirus 6 infection causes aberrant accumulation of p53 and prevents apoptosis

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p53 plays an important role in tumour suppression in cells exposed to some genotoxic stresses. We found that the p53 protein level was increased in a variety of cell lines infected with human herpesvirus 6 (HHV-6). Because the elevation in p53 began very soon after infection (4 h) and did not occur with UV-inactivated virus infection, it appeared to require the expression of one or more viral immediate-early (IE) genes. To elucidate the mechanism of p53 induction, we investigated its regulation at the protein level. Pulse–chase analysis showed that the stability of p53 increased in HHV-6-infected cells. In addition, the ubiquitination of p53 decreased after infection, indicating that the stability of p53 was increased through deubiquitination. We showed by confocal microscopy that the additional p53 mainly localized to the cytoplasm and that p53 was retained in the cytoplasm even after UV irradiation, but that it translocated into the nucleus in mock-infected cells. Furthermore, DNA fragmentation analysis, a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay and annexin V staining showed that infected cells were resistant to UV-induced apoptosis. These results lead us to propose that HHV-6 has a mechanism for retaining p53 within the cytoplasm and protects the infected cells from apoptosis.

INTRODUCTION

The tumour-suppressor protein p53 plays a central role in regulating cell growth and death. Normally, p53 is present in a latent state and is maintained at low levels by rapid degradation through ubiquitination, which is mediated by the MDM2 RING finger protein (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). However, once cells are exposed to genotoxic stresses, UV or γ-ray irradiation or chemicals that cause DNA damage, p53 becomes stabilized and activated via multiple post-translational modifications (Ashcroft et al., 1999, 2000; Blattner et al., 1999; Dumaz & Meek, 1999; Shieh et al., 1997). The active p53 protein accumulates in the nucleus and acts as a transcription factor for its target genes. This leads either to cell-cycle arrest, which allows more time for DNA repair, or to apoptosis, which eliminates damaged cells. In this way, p53 functions as ‘the guardian of the genome’ by preventing the accumulation of genetic alterations. Therefore, every infectious agent that requires the replication of its own genome in the host nucleus has to overcome the barrier presented by p53.

Human herpesviruses (HHVs) are large, enveloped DNA viruses that carry a double-stranded DNA genome of approximately 120–230 kbp. On the basis of a diverse collection of in vivo and in vitro biological properties, HHVs are divided into three subgroups: alpha, beta and gamma (McGeoch, 1989, 1990; Roizmann et al., 1992). Human herpesvirus 6 (HHV-6) is a ubiquitous betaherpesvirus that was first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disorders (Salahuddin et al., 1986) and acquired immunodeficiency syndrome (Josephs et al., 1986; Tedder et al., 1987). HHV-6 utilizes the cellular CD46 molecule as an entry receptor (Santoro et al., 1999), predominantly infects and replicates in CD4+ lymphocytes (Lusso et al., 1988; Takahashi et al., 1989) and may establish latency in the monocyte/macrophage lineage (Kondo et al., 1991).

Alterations in the level, function and localization of p53 by HHVs have been reported in a number of studies. Among the HHVs shown to affect p53 are Epstein–Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), which are gammaherpesviruses associated with tumorigenesis. Like small DNA tumour viruses, they encode proteins that target and inactivate p53: for EBV, this protein is BZLF1.
(Zhang et al., 1994); for KSHV, these proteins are LANA (Friborg et al., 1999), LANA2 (Rivas et al., 2001), vIRF (Nakamura et al., 2001; Seo et al., 2001) and K-bZIP (Park et al., 2000). Both herpes simplex virus (HSV) and human cytomegalovirus (HCMV), which represent alpha- and betaherpesviruses, respectively, stabilize p53 by their immediate-early (IE) gene products: infected cell protein zero (ICP0) for HSV (Hobbs & DeLuca, 1999) and IE1 (Muganda et al., 1994) and IE2 (Bonin & McDougall, 1997; Muganda et al., 1994; Speir et al., 1994) for HCMV. In addition, the HCMV mtrII oncoprotein (UL111a) has also been reported to interact with p53 and down-regulate its transcriptional activity (Muralidhar et al., 1996). For HHV-6, it has been reported that the transcriptional activity of p53 is repressed by its interaction with the DR7 protein in NIH3T3 cells transfected with the Sall-L fragment of the HHV-6 genome (Kashanchi et al., 1997). However, the behaviour of p53 in the context of a viral infection remains to be investigated.

In this paper, we focused on changes in the level and localization of p53 caused by HHV-6 infection. We demonstrated that the p53 protein level increased in a variety of cell lines infected with HHV-6 and that this was due both to the increased synthesis of p53 and to its stabilization through debiquitination. These changes were induced at a very early stage of infection and most likely involved the expression of viral IE genes. Confocal microscopy showed a cytoplasmic rather than nuclear localization of p53 and documented that p53 did not translocate to the nucleus even after UV irradiation. Furthermore, HHV-6-infected cells were resistant to UV-induced apoptosis, which was independent of viral IE proteins. Taken together, these data suggest the possibility that HHV-6 employs some mechanism that inhibits the nuclear localization of p53 following UV irradiation and the consequent apoptosis.

### METHODS

#### Cells, viruses and infection.
Human T-lymphoblastoid cell lines MolT-3, HSB-2 and MT-4, erythroleukaemia K562 cells and promyelocytic leukaemia HL60 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS). Myelomonocytic leukaemia U937 cells, human embryonic lung (HEL) fibroblasts and hepatocytic carcinoma HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% FCS. Umbilical cord blood mononuclear cells (CBMCs) were collected and stimulated prior to infection as described previously (Takemoto et al., 2001).

HHV-6A strain U1102 and HHV-6B strain HST were propagated in CBMCs until their cytopathic effects were maximal. The cells were lysed by freezing and thawing once. Cell debris was removed by centrifugation at 1500 g for 5 min. The supernatants were used for virus infections.

For the infection of cells with HHV-6, 2 × 10⁶ cells were collected by centrifugation, resuspended in 1 ml of the virus stock described above and incubated at 37°C for 1 h. Mock- or HHV-6-infected cells were cultured in the appropriate medium as described above.

#### Antibodies.

p53 was detected by Western blotting with a monoclonal antibody (mAb) (DO-1) (Santa Cruz Biotechnology) at a 1:2000 dilution and by indirect immunofluorescence with a goat polyclonal antibody (FL-393) (Santa Cruz Biotechnology) used at a 1:200 dilution. mAbs against Rb (G3-245) (BD Biosciences) and α-tubulin (B-5-1-2) (Sigma) were used at a 1:500 and 1:10000 dilution, respectively.

To detect HHV-6B early and late antigens, the mAbs OHV-2, against the viral DNA replication compartment, and OHV-3, against glycoprotein H (gH), were used at a 1:100 dilution. For viral IE antigens, rabbit polyclonal antisera specific to HHV-6B IE1 and IE2 were used at a 1:1000 dilution. The mAbs OHV-2 (Mori et al., 2000) and OHV-3 (Takeda et al., 1997) and the rabbit polyclonal antiserum against IE1 (Mori et al., 2002) have been previously described. We expressed part of the HHV-6 U66 ORF in bacteria as a glutathione S-transferase fusion protein and raised U66-specific antisera in rabbits to characterize the IE2 protein. U27 mAb was generated by immunizing mice with a recombinant U27 protein according to the method described elsewhere (Mori et al., 2002). An immunogen was prepared as follows. A PCR product generated with the primers U27C334F (5’-AGTCAATTCTTTAGGAAAAGTTGTTTCTTCGAC-3’) and U27C1129R (5’-AGTGATGTCGACTATCTCTGTCTCTTAGGATTGGAGC-3’) was digested with EcoRI and Sall and inserted into a pMAL-c2 vector (New England Biolabs) at these sites. A maltose-binding protein fusion protein, MBP-U27, expressed in Escherichia coli, was purified with amylose resin (New England Biolabs) and used for immunization. In the same way, we also generated U95 mAb using the primers U95C1180F (5’-AGTCTGAAATTTCAAGAGAAAATGTGTTCTCTCGAC-3’) and U95C2061R (5’-AGTCTGACTCTGCTGGAAGTCTCTCATGACG-3’).

#### Western blot analysis.

Cells were resuspended in Laemmli reducing sample buffer (LRSB) (50 mM Tris, pH 6-8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.5% bromophenol blue) at a concentration of 10⁶ cells (µl buffer)⁻¹, then boiled for 5 min. Equal volumes of whole-cell lysate were loaded on to SDS-polyacrylamide gels, fractionated by electrophoresis and Western blotted on to PVDF membranes (Bio-Rad). The blots were blocked with 5% skimmed milk in Tris-buffered saline (TBS) overnight at 4°C, washed twice briefly with TBST (TBS containing 0.02% Tween 20) and incubated for 1 h at room temperature with primary antibody in TBS containing 3% BSA. The blots were then washed three times with TBST, incubated for 1 h at room temperature with a 1:2500 dilution of the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham-Pharmacia) in TBS containing 3% skimmed milk, washed three times with TBST and developed using an ECL detection kit (Amersham-Pharmacia) according to the manufacturer’s instruction. The images on the blot were directly quantified by densitometry using a FluorChem IS-8000 imaging system (Alpha Innotech).

To detect the ubiquitinated forms of p53 according to a previously described method (Ling et al., 2003; Xirodimas et al., 2001; Zaika et al., 1999), MolT-3 cells infected with HHV-6B strain HST were treated with 0.5 µM MG132 (Peptide Institute), an inhibitor of the 26S proteasome, for the indicated 6 h periods, harvested after treatment and subjected to Western blot analysis.

#### Plasmids and transient transfection.

The HHV-6 U95 expression plasmid, pcDNA-U95, was generated as described previously (Takemoto et al., 2001) and the HHV-6 IE1 cDNA clone was obtained in the same way as U95. Briefly, the Pst-digested fragment from pSTY03 (Isegawa et al., 1999) was 5’-end-labelled with [³²P]ATP and used as a probe to screen the IE1 cDNA clone from a cDNA library of HHV-6 strain HST-infected cells (Mori et al., 1998). The full-length IE1 cDNA clone whose sequence had been determined was excised from the library vector as a NotI fragment and inserted into pCEP4 at a NcoI site. The HCMV major IEs (MIEs) expression plasmid, pSV2neo-IE1B, was a generous gift from Tsuchiya Murayama (Kagoshima University).

MT-4 cells (2 × 10⁶) were transfected with 1 µg of viral IE gene
expression plasmids using lipofectamine plus (Invitrogen) according to the manufacturers instructions and subjected to Western blotting and an immunofluorescence assay (IFA) 36 h post-transfection.

**Pulse-chase and immunoprecipitation experiments.** Molt-3 cells (3 x 10^6) were either mock infected or infected with HHV-6B strain HST, then pulsed at 24 h post-infection (p.i.) with 100 µCi [35S]methionine ml^-1 for 1 h, washed twice with serum-free medium and cultured in medium containing unlabelled 1-methionine for 0, 2, 4, 6, 8 or 16 h of chase. The cells were lysed in RIPA buffer (10 mM Tris, pH 7-4, 1% NP-40, 0-1% SDS, 0-1% sodium deoxycholate, 0-15 M NaCl, 1 mM EDTA and 2 µg each of leupeptin, aprotinin and pepstatin ml^-1) for 1 h at 4°C, then spun in a centrifuge. The supernatants of the cell lysates were pre-incubated with protein G-Sepharose, which had previously been blocked with 0.1% FCS to reduce non-specific binding of proteins to Sepharose beads. Subsequently, the supernatants were incubated with anti-p53 or α-tubulin mAb-bound protein G-Sepharose at 4°C overnight. The Sepharose beads were rinsed four times with RIPA buffer, resuspended in LRSB and boiled for 5 min. The immunoprecipitated proteins were separated by 10% SDS-PAGE and treated with Enlight (Mo Bi Tec). The gel was dried and exposed to a BAS-III imaging plate (Fujifilm). The autoradiogram was imaged and quantified with a BAS 2000 II Bio-imaging analyser (Fujix). The percentage stability was calculated as the value relative to that of each protein chased for 0 h, which was defined as 100%.

**Indirect immunofluorescence assay.** Cells were plated on glass coverslips, fixed in cold acetone for 5 min and incubated for 1 h at room temperature with the primary antibody in PBS containing 3% BSA. The coverslips were then washed with PBST for 5 min, followed by a wash with PBS for 5 min and incubated for 30 min at room temperature with the appropriate secondary antibody labelled with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Dako). After washing as above, the coverslips were mounted in glycerol and examined by fluorescence microscopy. The confocal images were captured using a Zeiss LSM410 confocal microscope and software provided by the manufacturer.

**Exposure of viruses and cells to UV irradiation.** To inactivate HHV-6B strain HST by UV light, 1 ml of an HST stock was plated in a 6 cm diameter dish and irradiated at 2500 J m^-2. To confirm the inactivation of the virus, an IFA detected no IE1 gene expression at 24 h p.i., indicating that there was no virus survival.

To induce DNA damage and subsequent p53-dependent apoptosis in Molt-3 cells infected with strain HST, cells at 6 or 24 h p.i. were concentrated to 2 x 10^6 cells ml^-1 in culture medium, irradiated at 60 J m^-2 and cultured at a concentration of 4 x 10^4 cells ml^-1 for 12 h. The induction of apoptosis was analysed by the detection of DNA fragmentation following electrophoresis, a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay and annexin V staining to visualize phosphatidylserine externalized in the early apoptotic phase.

**DNA extraction and electrophoresis.** Cells (5 x 10^5) exposed to UV irradiation were collected, resuspended in 200 µl of PBS(-) containing 0.5% SDS and 500 µg proteinase K ml^-1, and incubated for 1 h at 55°C. RNase A (50 µg) was added and incubation at 55°C was continued for 1 h. Samples were mixed with 300 µl sodium iodide solution (6 M NaI, 26 mM Tris, pH 8-0, 13 mM EDTA, 0.5% sodium-N-laurylsarcosinate) containing 3 µl Ethacin mate (Nippogene) and incubated for 15 min at 60°C. DNA was precipitated with 2-propanol, dried and resuspended in TE (10 mM Tris, pH 8-0, 1 mM EDTA). The extracted DNA was subjected to electrophoresis in a 2% agarose gel and visualized by UV light after staining with ethidium bromide.

**TUNEL assay.** Cells (10^6) exposed to UV irradiation were collected after 12 h and subjected to the TUNEL assay according to the manufacturer’s instructions (in situ cell death detection kit; Boehringer) except for the fixation step (70% ethanol at -20°C for 1 h). Samples were analysed on a FACSCalibur (CELLQuest software; BD Biosciences).

**Annexin V staining.** Cells (10^6) exposed to UV irradiation were collected after 6 h and subjected to annexin V staining, according to the manufacturer’s instructions (Annexin V-FITC apoptosis detection kit; BioVision). Samples were analysed on a FACSCalibur.

**RESULTS**

**The p53 level increases in a variety of cell types infected with HHV-6.** Human umbilical CBMCs were infected with HHV-6A strain U1102 or HHV-6B strain HST, harvested at 24 and 48 h p.i. and subjected to Western blot analysis. As shown in Fig. 1(A), the p53 level was significantly increased in cells infected with either strain. To determine whether the increase in p53 was induced in the HHV-6-infected cells or in the neighbouring, uninfected cells, we performed an indirect IFA. Double staining of p53 and the HHV-6 IE gene product IE1 revealed that the elevated level of p53 was in the HHV-6 IE1-positive cells at 48 h p.i. (Fig. 1B). The increase in the p53 level was induced in the HHV-6-infected cells but not in the others. p53 increased to a similar degree in the T-cell lines Molt-3 and HSB-2, which were infected with strain U1102 (Fig. 1C). In contrast, strain HST caused the p53 increase only in the Molt-3 T-cell line (Fig. 1C), but the failure of HST to increase the p53 level in HSB-2 cells can be explained by its inability to enter the cell.

To test whether the elevation of p53 by HHV-6 infection was limited to certain cell types, other cell lines infected with strain U1102 were analysed. U937 and HEL cells infected with strain U1102 showed increased p53 levels, comparable with those observed in the T-cell lines, at 24 and 48 h p.i. (Fig. 1D). Smaller increases in the level of p53 were observed in K562, HL60 and HepG2 cells following U1102 infection (Fig. 1D). These results indicated that the elevation in p53 is not restricted to certain cell types but occurs in a variety of cells infected with HHV-6.

**The p53 level increases at the initial stage of HHV-6 infection.** To determine the time point of the increase in p53 level, total cell extracts from HST-infected Molt-3 cells harvested at 0, 4, 8, 16, 24, 48 and 72 h p.i. were analysed by Western blotting. In these HST-infected cells, the p53 level began to increase by 4 h p.i., reached a maximum at 48 h p.i. and remained high until 72 h p.i. (Fig. 2A and B). In contrast, the level and hyperphosphorylated form of another tumour suppressor protein, Rb, was constant throughout the course of the HST infection.

Because the p53 elevation occurred at a very early stage of HHV-6 infection and since only the IE genes are expressed at this time, as shown in Fig. 2(C), the increase in p53 was most likely due to the effect of either virion proteins or IE
gene expression. To determine which viral factors were responsible for the p53 elevation, we infected Molt-3 cells with strain HST that had either been inactivated with UV irradiation at 60 J m\(^{-2}\) or left untreated. At 24 h p.i., Molt-3 cells infected with UV-irradiated virus, which failed to show detectable HHV-6 IE antigens (Fig. 2D, lower photograph), did not exhibit elevated levels of p53 (Fig. 2D, lane 4), in contrast to cells infected with untreated virus (Fig. 2D, lane 2). This indicated that virion proteins are not responsible for the increase in p53 level. An additional study of the effects of transiently transfecting MT-4 cells with the HHV-6 IE genes IE1 and U95 revealed that neither of these genes affected the p53 level (Fig. 2E, lanes 2 and 3), while HCMV MIE genes elevated the endogenous p53 level slightly (Fig. 2E, lane 4), suggesting that these two genes, IE1 and U95, are insufficient for the increase in the p53 level and that other IE genes are also required. The expression of IE1 and the U95 was confirmed by IFA, as shown in Fig. 2E (lower photographs).

These observations suggest the involvement of one or more IE genes in the elevation of p53 levels in HHV-6-infected cells.

**HHV-6 infection induces the stabilization and synthesis of p53**

p53 function is controlled through several mechanisms, one of the most effective being the regulation of protein stability. Numerous studies have shown the involvement of multiple pathways in p53 stabilization. To test whether the elevation of p53 in HHV-6-infected cells is dependent on the regulation of the protein’s stability, we performed a pulse–chase radiolabelling experiment in Molt-3 cells infected with strain HST, at 24 h p.i. p53 protein pulse-labelled with \[^{35}\text{S}\]methionine was extracted and immunoprecipitated with DO-1-conjugated protein G–Sepharose beads after chase periods of 0, 2, 4, 6, 8 and 16 h. As shown in Fig. 3(A) and (B), a slight increase in the amount of p53 was observed in both mock- and HHV-6-infected cells after a 2 h chase, but from the 4 h chase to the 16 h chase, the stability of the p53 protein in the HHV-6-infected cells was approximately twofold higher than in the mock-infected cells at every time point. In contrast, the stability of \(\alpha\)-tubulin showed no significant difference between mock- and HHV-6-infected cells. These results indicated that the stability of p53 increases in HHV-6-infected cells. In addition, HHV-6-infected cells that were pulse-labelled for 1 h (and chased for 0 h) exhibited a 1.4-fold higher amount of p53 protein than mock-infected cells, indicating that the synthesis of p53 protein was enhanced in the HHV-6-infected cells. These results suggest that the elevation of p53 in HHV-6-infected cells is due to increases in both its stability and synthesis.

**p53 is deubiquitinated in HHV-6-infected cells**

Ubiquitination serves as a crucial signal for specific protein degradation and controls a variety of cellular processes. Numerous studies have shown that, like many other proteins, p53 is regulated in a ubiquitin-dependent manner. To elucidate the molecular mechanism of the p53 stabilization induced by HHV-6 infection, we studied whether p53’s state of ubiquitination was altered by HHV-6 infection. When infected cells were treated with MG132, an inhibitor of the 26S proteasome, for 6 h, a high level of polyubiquitinated...
p53 was detected (Fig. 4A). This p53 ubiquitination gradually decreased as the infection with HHV-6 proceeded, for up to 12 h p.i., after which point it recovered slightly (Fig. 4A). Nevertheless, the amount of p53 ubiquitination after 12 h p.i. was one-half that of mock-infected cells (Fig. 4B, lower graph); this stabilization in p53 level is probably dependent on the balance between the recovery of the p53 ubiquitination and the increased synthesis of p53. From these results, we concluded that the stabilization of p53 induced by HHV-6 infection is due to p53 deubiquitination, but the mechanism and the causative HHV-6 protein(s) remain to be elucidated.

**p53 is retained in the cytoplasm in HHV-6-infected cells**

Because the activity of p53 as a tumour suppressor mainly depends on its ability to induce the transcription of its responsive genes, such as p21WAF1 (el-Deiry et al., 1993), GADD45 (Kastan et al., 1992; Lu & Lane, 1993) and Bax (Miyashita & Reed, 1995), it is important for p53 to translocate into the nucleus in response to DNA-damaging stimuli. Data from the IFA in which HHV-6-infected cells were triple stained for p53, HHV-6 IE1 and DNA (Fig. 1B) showed that most of the p53 accumulated in the cytoplasm.
We investigated the intracellular distribution of p53 in the HHV-6-infected cells in detail. At 36 h p.i., Molt-3 cells infected with strain HST were fixed, double stained with anti-p53 goat polyclonal antibodies (FL393) and mAbs against viral antigens OHV-2 and -3 and observed using a confocal laser-scanning microscope. Whereas p53 was close to the detection limit in mock-infected cells (Fig. 5A, a), it was readily detected in the cytoplasm of the infected cells and its staining patterns resembled the endoplasmic reticulum and Golgi apparatus (Fig. 5A, b and c). Double staining for viral antigens revealed that p53 mainly colocalized with OHV-3 (gH) (Fig. 5A, c) but not with OHV-2 (early nuclear antigen) (Fig. 5A, b), indicating that p53 is localized to the cytoplasm in HHV-6-infected cells. Next, we tested whether p53 moves from its cytoplasmic localization and translocates into the nucleus in response to UV irradiation in the context of HHV-6 infection. HST-infected Molt-3 cells were exposed to UV light at 24 h p.i., fixed after 12 h and subjected to an IFA. Whereas a number of mock-infected cells showed nuclear accumulation of p53 following UV irradiation (Fig. 5B, a), HST-infected cells still exhibited the cytoplasmically localized p53 (Fig. 5B, b and c). These data suggested that HHV-6 employs some mechanism that actively retains p53 within the cytoplasm, which might explain the functional defect of p53 in the experiments described above.

**HHV-6-infected cells are resistant to UV-induced apoptosis**

The inability of p53 to translocate to the nucleus following UV irradiation led us to suspect that the infected cells’ sensitivity to apoptosis induced by p53 might be altered. To address the possibility that HHV-6 can protect the infected...
cells from p53-dependent apoptosis, HST-infected Molt-3 cells were exposed to UV light at 6 and 24 h p.i. and DNA was extracted from the cells 12 h after irradiation. Mock-infected cells exposed to lethal doses of UV light underwent rapid p53-dependent apoptosis as determined by DNA fragmentation (Fig. 6A). Intriguingly, HHV-6-infected cells at 6 h p.i. exhibited the same degree of DNA fragmentation as mock-infected cells, despite the expression of MIE proteins (Fig. 2C), indicating that these two MIE gene products could not block the p53-mediated apoptosis, which HCMV MIEs also fail to do (Zhu et al., 1995). However, HST-infected cells exposed to UV light at 24 h p.i. showed little fragmented DNA, equal to the level in non-irradiated cells (Fig. 6A).

A TUNEL assay was then performed to assess the anti-apoptotic effect of HHV-6 infection. As shown in Fig. 6(B), the number of TUNEL-positive HST-infected Molt-3 cells was unchanged, even after exposure to UV light, whereas the histogram of uninfected cells clearly shifted following UV irradiation (55 % TUNEL positive versus 6 % of control). Although the levels of TUNEL staining of the infected cells were higher than those of the uninfected and non-irradiated control cells, the DNA extracted from the infected cells showed no fragmentation (Fig. 6A), showing that these higher levels of TUNEL positivity did not represent apoptosis. The most likely reason for this result is that the TUNEL staining was influenced by the increased number of DNA ends derived from the viral genomic DNA synthesized in the nucleus.

Finally, we confirmed the prevention of apoptosis by HHV-6 infection using annexin V staining. The levels of apoptosis (percentage of annexin-V-positive and propidium iodide-negative cells) of mock- and HST-infected Molt-3 cells at 30 h p.i. were only 2.87 % and 3.55 %, respectively, without any treatment (Fig. 6C). In contrast, the level of apoptosis of the mock-infected cells caused by UV irradiation (60 J m⁻²) had increased to 29.62 % by 6 h post-irradiation; that of the HST-infected cells at 24 h p.i. was only 9.41 %, demonstrating that the HST-infected cells are indeed resistant to UV-induced apoptosis. Taken together, these findings imply that HHV-6 protects infected cells against the p53-dependent apoptosis induced by UV irradiation by inhibiting p53 from translocating to the nucleus.

**DISCUSSION**

In response to a broad range of stresses, including DNA damage, hypoxia and heat, the tumour-suppressor protein p53 is rapidly stabilized through multiple post-translational modifications that reduce its degradation via the ubiquitin-proteasomal pathway (Ashcroft et al., 1999, 2000; Blattner et al., 1999; Dumaz & Meek, 1999; Shieh et al., 1997).

In the present study, we demonstrated that the p53 level increased in Molt-3 cells soon after infection (4 h p.i.) with HHV-6 strain HST, but did not increase in Molt-3 cells infected with UV-inactivated virus, suggesting the possibility that viral IE gene expression is involved. In addition, a pulse-chase experiment revealed that the increase in p53 level was due to increases in both the synthesis and the stabilization of the protein. When cells were radiolabelled for 1 h, the labelled p53 protein in HST-infected Molt-3 cells was higher by 1.4-fold after a 0 h chase and also higher by twofold at every time point examined after a 4 h chase than in mock-infected cells, indicating that both synthesis and stabilization of p53 were upregulated in the infected cells. The extremely small amount of protein (Fig. 3A, 16 h chase in mock-infected cells) may be due to loss of material as a result of cell death by the time of the 16 h chase. It is reported that HCMV stabilizes p53 but does not increase its synthesis (Fortunato & Spector, 1998) and that the p53 increase caused by HSV-1 ICP0 is independent of the transcription of p53 (Hobbs & DeLuca, 1999). In this study, we showed that HHV-6 has the same effect on the regulation of p53, i.e. stabilization, as other herpesviruses, but also a different effect – an increase in p53 synthesis.

We next addressed the state of ubiquitination of p53 and observed the reduced level of ubiquitinated p53 in HST-infected Molt-3 cells soon after infection (Fig. 4). Little has been reported to date on the possibility that viral infection causes the stabilization of cellular proteins through deubiquitination, whereas there are many reports of viruses promoting the ubiquitination and subsequent degradation of specific cellular proteins to optimize the conditions of the cell for its replication. Recently, p53 was shown to interact directly with herpesvirus-associated ubiquitin-specific protease (HAUSP). HAUSP is known to interact with HSV-1 ICP0, to co-localize with the PML nuclear body and to stabilize p53 through its intrinsic deubiquitinating enzyme activity (Li et al., 2002). It is possible that herpesviruses exploit cellular enzymes such as the ubiquitin-specific processing protease (UBP) to stabilize specific cellular proteins that are required for efficient virus replication, but no evidence currently exists to support this scenario.

Normally, p53 is activated in response to various genotoxic stresses, including UV irradiation, and accumulates in the nucleus, as shown in Fig. 5(B, a). However, in HHV-6-infected cells, the cytoplasmic localization of p53 was unchanged, even when the infected cells were irradiated with UV light at 24 h p.i. (Fig. 5B, b and c). This result led us to hypothesize that a viral protein may directly bind p53 and inhibit its nuclear localization. One possible candidate for preventing p53-dependent apoptosis is DR7, which has been reported to bind to and inactivate p53 (Kashanchi et al., 1997).

More intriguing is that the localization of p53 in cells infected with HHV-6 is quite different from that seen in cells infected with other HHVs. During productive infection for all the subgroups of HHVs, namely HSV (Wilcock & Lane, 1991; Zhong & Hayward, 1997), HCMV (Fortunato & Spector, 1998) and KSHV (Katano et al., 2001), p53 has been reported to co-localize with virus replication compartments in the nucleus. The recruitment of p53 into these virus
replication foci leads us to speculate that these viruses exploit p53 for their efficient replication, but no evidence currently exists to support this assumption. Although there are reports that HCMV sequesters p53 in the cytoplasm in infected multinucleated giant cells, p53 has also been shown to co-localize with IE84 protein in the nucleus in single
nuclear non-giant cells that are thought to be in the early phase of infection (Kovacs et al., 1996; Wang et al., 2001). In contrast, in HHV-6-infected cells, p53 predominantly accumulated in the cytoplasm and co-localized with gK, but not with the early nuclear antigen labelled by OHV-2, which is a putative component of the virus replication compartment. In addition, there is no evidence that p53 accumulates in the nucleus at any stage of HHV-6 infection (data not shown). If p53 plays some positive role in the replication of other HHVs, it will be of interest to find out whether HHV-6 employs a replication mechanism that does not require the recruitment of p53, unlike other herpesviruses.

Here we have demonstrated that the p53-dependent apoptosis induced by UV irradiation was blocked at 24 h p.i. (Fig. 6). To determine whether UV-induced apoptosis in

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**Fig. 5.** Subcellular localization of the p53 protein in HHV-6-infected Molt-3 cells. (A) Uninfected (a) or HST-infected (b, c) Molt-3 cells were fixed at 36 h p.i., double stained with an anti-p53 goat polyclonal antibody (FL393; FITC) and mAbs OHV-2 (b; TRITC) and OHV-3 (c; TRITC) and analysed by confocal microscopy. Cells were counterstained with Hoechst 33342 to localize the nucleus (a; DAPI). (B) Uninfected (a) or HST-infected (b, c) Molt-3 cells were exposed to UV light at 24 h p.i., fixed after 12 h and double stained following the same protocol as for (A) (see Methods).

**Fig. 6.** HHV-6 protects cells against UV-induced apoptosis. (A) Molt-3 cells infected with strain HST were either exposed to a lethal dose of UV light (60 J m\(^{-2}\)) at 6 and 24 h p.i., or were not exposed, and were cultured for an additional 12 h. DNA extracted according to the method of Hirt was analysed by gel electrophoresis for the presence of fragmented DNA. (B) TUNEL assay of mock and HST-infected cells irradiated with UV light (60 J m\(^{-2}\)) at 24 h p.i. Twelve hours after irradiation, the cells were subjected to TUNEL staining. The flow cytometric histograms for the mock- and HST-infected cells are shown in the left and right panels; the unshaded and the shaded histograms represent the number of TUNEL-positive cells in the unirradiated and UV-irradiated conditions, respectively. (C) Annexin V staining was performed to confirm the prevention of apoptosis by HHV-6 infection. HST-infected Molt-3 cells at 24 h p.i. were irradiated with UV light (60 J m\(^{-2}\)), stained with annexin V–FITC and propidium iodide at 6 h post-irradiation and analysed on a FACSCalibur. The number on each quarter panel shows the percentage of 10 000 cells in the panel, and the annexin V-positive and propidium iodide-negative (lower right) quadrant shows apoptotic cells.
Molt-3 cells was actually mediated by p53 activation, the number of cells that were positive for the nuclear accumulation of p53 was counted in three fields. Approximately 43% of the cells were positive for nuclear accumulation of p53 (data not shown), indicating that UV irradiation induces p53-dependent apoptosis in Molt-3 cells, when their 55% TUNEL-positive labelling is taken into account (Fig. 6B).

Whereas the elevation of the p53 level occurred at the IE stage, the block of apoptosis was not observed until the late stage, indicating that these two effects of HHV-6 infection on p53 are carried out by different viral proteins.

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