Murine gammaherpesvirus 68 ORF20 induces cell-cycle arrest in G2 by inhibiting the Cdc2–cyclin B complex

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The objective of this work was to identify novel viral ‘evasion’ genes without homology in the database through functional assays. Using this approach, the ‘unassigned’, conserved murine gammaherpesvirus ORF20 gene was shown to localize in the nucleus and to induce cell-cycle arrest followed by apoptosis in both mouse and human cells. Such growth-arrested cells did not express phospho-histone H3, demonstrating that the virus protein caused arrest at the G2 stage of the cell cycle. To characterize the mechanism further, Western blots of ORF20-recombinant lentivirus-infected cells were developed with antibodies to cyclin B1, Cdc2 and phospho-Tyr-15-Cdc2. This analysis revealed a relative increase in cyclin B and phospho-Tyr-15-Cdc2, from 24 to 72 h after infection with recombinant lentivirus. The demonstration that Cdc2 is in its inactive phosphorylated form and the clearly increased levels of cyclin B indicated that the virus gene blocks the progression of cells into mitosis by acting at the level of the Cdc2–cyclin B complex. To confirm this result, the Cdc2–cyclin B complex in ORF20-expressing cells was shown to be essentially without kinase activity. As the ORF20 gene is conserved in all herpesviruses, it may be presumed to have evolved to fulfil an important, as yet undefined, biological role in host-cell modification.

INTRODUCTION

Manipulation of cell-cycle regulation is a commonly employed strategy of viruses for achieving a favourable cellular environment. Indeed, the study of such strategies not only has contributed to our understanding of virus replication, but may also have the potential to reveal novel details of cellular protein interactions in the regulation of the eukaryotic cell cycle. For example, there are early and immediate-early herpesvirus proteins that delay cell-cycle progression or cause arrest at the G1 stage (Cayrol & Flemington, 1996; Izumiya et al., 2003; Jean et al., 2001; Lu & Shenk, 1999). A number of viruses target the critical regulators of G2/M progression, the cyclins and cyclin-dependent kinases or their regulators (Friborg et al., 1999; Groschel & Bushman, 2005; He et al., 1995; Park et al., 2000). The expression of cyclin B oscillates throughout the cell cycle, beginning to accumulate during S phase and reaching its maximum at the G2/M phase. Activation of the Cdc2–cyclin B complex for entry into mitosis is regulated by dephosphorylating Cdc2 on Thr-14 and Tyr-15. In this work, we describe a ‘non-assigned’ gene of murine gammaherpesvirus 68 (MHV68), which has evolved into a host-modifying gene causing inactivation of the Cdc2–cyclin B complex.

MHV68 is a particularly useful laboratory model for the study of gammaherpesviruses such as Epstein–Barr virus and Kaposi’s sarcoma associated herpesviruses (Virgin et al., 2001; Simas & Efstathiou, 1998). Originally isolated from wild rodents, it is capable of infecting laboratory strains of mice, thus providing a system to study gammaherpesvirus infection and pathogenesis (Sunil-Chandra et al., 1992a). After intranasal infection, MHV68 establishes a productive infection in lung epithelial cells followed by a latent infection of spleen B cells, macrophages, dendritic cells and lung epithelial cells (Flano et al., 2000; Sunil-Chandra et al., 1992b; Weck et al., 1999). In addition, MHV68 replicates efficiently in vitro, thereby permitting the study of early events in virus infection and replication.

Although the genome of MHV68 contains genes unique to this virus (Virgin et al., 1997), there are many others that are conserved among the herpesvirus subfamilies. The majority of these have identified functions, but there are a significant number of shared, non-homologous herpesvirus genes without a known function. One of these is ORF20 from MHV68, which has a predicted N-terminal domain homologous to herpesvirus UL24 gene products (Alba et al., 2001). The universal presence of UL24 in herpesviruses genomes from the three subfamilies, alpha-, beta- and gammaherpesviruses, suggests a fundamental and conserved role in the biology of herpesviruses.

Here, we have demonstrated that, as reported for its human herpesvirus homologues, ORF20 localizes in the nucleus. More importantly, expression of MHV68 ORF20 induced G2/M cell-cycle arrest followed by apoptosis of
both mouse and human cells. The mechanism of its action is to maintain Cdc2 in its phosphorylated inactive state, so that Cdc2–cyclin B complexes in cells expressing ORF20 exhibit almost no kinase activity.

**METHODS**

**Cells.** HeLa, 293T and murine NIH3T3 cells were cultured in 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C.

**Plasmids.** MHV68 ORF20 was cloned into plasmid pCDNA3 fused in frame with an N-terminal influenza haemagglutinin (HA) peptide tag. The human cytomegalovirus (CMV)–vesicular stomatitis virus G envelope protein, the packaging plasmid pCMVR8.9 and the vector pHR-CMV-eGFP constructs have been described previously (Ikeda et al., 2002).

For construction of a recombinant lentivirus vector (pHR-CMV-ORF20-eGFP), ORF20 was excised from pCDNA3, together with the HA tag, by BamHI/Xhol digestion and cloned into the vector pHR-CMV-eGFP upstream of an internal ribosome entry site-driven enhanced green fluorescent protein gene (eGFP).

**Production and measurement of lentivirus.** Lentivirus was produced by transient transfection of 293T cells with a weight ratio of 3 : 1 : 1 of vector to packaging to envelope plasmids using Fugene 6 (Roche) according to the manufacturer’s instructions. Control lentivirus was produced by co-transfection of the packaging and envelope plasmid together with the empty pHR-CMV-eGFP plasmid. For production of recombinant lentivirus expressing ORF20, the plasmid pHR-CMV-ORF20-eGFP was used. supernatants containing the lentivirus were collected at 48, 72 and 96 h post-transfection and clarified by low-speed centrifugation, and the lentivirus was collected by ultracentrifugation (25 000 r.p.m. in an SW28 rotor in a Beckman centrifuge). Virus pellets were resuspended in fresh culture medium and frozen at −80°C. Lentivirus titres were measured by infection of 293T cells with a dilution factor of 4. Analysis of lentivirus-infected cells was done by detecting eGFP-positive cells by flow cytometry at 48 h post-infection (p.i.).

**Immunofluorescence.** NIH3T3 and HeLa cells were infected with recombinant lentivirus containing the MHV68 ORF20 gene (pHR-CMV-ORF20-eGFP) or the control ‘empty’ lentivirus (pHR-CMV-eGFP) at an m.o.i. of 10. At the indicated time points, cells were trypsinized (Gibco-BRL), washed once with PBS and fixed with 90% ethanol overnight at 4°C. After fixation, cells were washed once with PBS and resuspended in PBS/0.1% Triton X-100 and incubated with 50U DNase-free RNase A (Calbiochem) (30 min, room temp). After incubation, cells were stained with propidium iodide (Sigma) (20 µg ml⁻¹ in PBS, 15 min, room temperature) before analysis. Flow cytometry analysis was performed using a FACS Calibur (Becton Dickinson) and cell-cycle analysis was performed using CELLQUEST software.

**TdT-mediated dUTP nick end labelling (TUNEL).** To determine the percentage of apoptosis, a FlowTACS kit (R&D Systems) was used as specified by the manufacturer. Briefly, at 72 h p.i., cells were trypsinized, washed with PBS and fixed in 3.7% formaldehyde solution (10 min, room temperature). After fixation, cells were washed in PBS, permeabilized in Cytonin and incubated in labelling reaction mix (1 h, 37°C). The reaction was terminated by adding ‘stop’ buffer, cells were centrifuged and visualization of the apoptotic cells was achieved by incubation with streptavidin–allophycocyanin (BD Biosciences Pharmingen) (15 min, room temperature, in the dark). Positive and negative controls were prepared using the same procedure and as provided by the supplier. Cells were washed in PBS and analysed using a FACS Calibur (Becton Dickinson) and data analysis was performed using CELLQUEST software.

**Immunoblotting.** Cells cultured in 60 mm diameter Petri dishes were infected with different lentivirus constructs at an m.o.i. of 10 and harvested at various times p.i. The dishes were placed on ice and washed in PBS before scraping off the cells.

Cells were lysed in 50 µl lysis buffer containing 0.1 M potassium phosphate, 1% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF and a mixture of protease (Sigma) and phosphatase (Roche) inhibitors (30 min, 0°C). Protein concentrations were determined by the Bradford assay (Bio-Rad). For SDS-PAGE, 50 µg protein was loaded per lane and the separated proteins were transferred to PVDF membrane (Bio-Rad) and blocked with 5% non-fat milk (1 h, room temperature). Development of the blots was performed with a monoclonal antibody for Cdc2 and polyclonal antibodies for cyclin B1 (Santa Cruz Biotechnology), anti-phospho-Tyr-15 Cdc2 (Cell Signalling Technology) and anti-α-tubulin (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies were purchased from Dako. Finally, the identified molecules were revealed with SuperSignal chemiluminescent reagents (Perbio Science) according to the manufacturer’s instructions.

**In vitro kinase assays.** A histone H1 kinase assay was performed to measure the kinase activity of the Cdc2-cyclin B complex. NIH3T3 cells infected with lentivirus constructs at an m.o.i. of 10 were lysed at 48 h p.i. in a buffer from Cell Signalling Technology containing 1 mM β-glycerophosphate, 1 mM sodium orthovanadate and a mix of protease inhibitors from Roche. Lysates containing 600 µg protein were incubated for 1 h at 4°C in 300 µl lysis buffer with 5 µg anti-cyclin B1 antibody (Santa Cruz Biotechnology) and immune complexes were collected on 30 µl of protein G–Sepharose beads. The beads were washed three times with lysis buffer and once with kinase buffer containing 20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂ and 0.25 mM dithiothreitol, before being incubated for 10 min at 30°C in 30 µl kinase buffer containing 2.5 µCi [γ-³²P]ATP (Amersham-Pharmacia), 10 µg histone H1 (Sigma) and 50 µM ATP. The reaction mixture was centrifuged and SDS sample buffer was immediately added to the supernatant prior to 12% SDS-PAGE. The gel was dried and exposed for autoradiography for 1 h at −80°C. The protein G beads were collected from the kinase reaction, washed three times in kinase buffer and denatured in 30 µl SDS sample buffer for separation by SDS-PAGE and subsequent transfer to PVDF.
membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk (1 h, room temperature), developed with a monoclonal antibody for Cdc2 (Santa Cruz Biotechnology) and a secondary horseradish peroxidase-conjugated antibody, and revealed with SuperSignal chemiluminescent reagents (Perbio Science) according to the manufacturer’s instructions.

RESULTS

Modification of cell-cycle progression in stable cell lines expressing the MHV68 ORF20 gene

In order to study the function of ORF20, the virus gene was expressed in mouse and human cells through a lentivirus expression vector encoding eGFP. Transduced cells were first tested for expression of ORF20 by Western blot analysis of the HA tag. As can be seen in Fig. 1, the expressed protein migrated with a molecular mass of 28 kDa.

High rates of infection (~90%) were demonstrated by GFP expression (FACS analysis) and most of the infected cells (80–90%) expressed the ORF20 gene, as judged by counterstaining for the HA peptide tag introduced in the N-terminal region of the ORF20 protein. In these cells clearly expressing the virus protein, however, immunofluorescence revealed a nuclear localization of the virus protein in the typically enlarged cells at 24–48 h p.i. (Fig. 2). Significantly, mouse NIH3T3 cells infected with ORF20-recombinant lentivirus failed to divide and died after 3–5 days, showing the typical fragmented nuclear morphology of apoptosis. Prior to this, the cells became enlarged and flat, suggesting that ORF20 might interfere with cell-cycle progression.

Induction of cell-cycle arrest in human and murine cell lines by MHV68 ORF20

To determine the impact of ORF20 on cell-cycle progression, murine NIH3T3 cells were infected with the lentivirus construct containing the ORF20 gene. Cells were harvested at different time points for cell-cycle analysis by flow cytometry after staining with propidium iodide. Cells expressing MHV68 ORF20 showed a significantly increased G2/M : G1 ratio from 24 to 48 h p.i. (Fig. 3, second row). As molecules involved in the cell cycle are highly conserved in mammalian cells, we repeated the same experiment with human 293T and HeLa cells and obtained a similarly increased G2/M : G1 ratio in both cell types (Fig. 3 bottom row; this only shows the experiment in the HeLa cells).

Fig. 1. The expressed ORF20 gene product migrates with a molecular mass of 28 kDa in SDS-PAGE. NIH3T3 cells were infected with recombinant lentivirus. At the indicated time points, cells were lysed for immunoblotting and the ORF20 protein was developed with anti-HA (top panel) antibody. Anti-a-tubulin antibody was used as a loading control (bottom panel). Molecular mass markers are indicated.

Fig. 2. Nuclear localization of MHV68 ORF20 in NIH3T3 cells. NIH3T3 cells infected with lentivirus expressing MHV68 ORF20 (top two rows) or eGFP (bottom two rows) were seeded onto glass coverslips (see Methods). At 24 and 48 h, cells were fixed, permeabilized and subjected to double staining with DAPI to visualize nuclei (blue) and with anti-HA monoclonal antibody (red) to visualize ORF20 expression in the GFP–lentivirus–infected cells (top row, 24 h; second row, 48 h). An identical experiment was conducted with the eGFP ‘empty’ non-recombinant lentivirus (third row, 24 h; fourth row, 48 h).
Infection with a control, non-recombinant lentivirus did not change the cell-cycle profile (Fig. 3, top and third rows).

**Induction of apoptosis by MHV68 ORF20**

Staining cells with DAPI at 72 h p.i. revealed that NIH3T3 cells expressing ORF20 did not progress in the cell cycle, but instead underwent apoptosis, showing typical apoptotic nuclear bodies (Fig. 4a, top and middle rows). To confirm this observation, the cells were also examined by FACS analysis at 72 h p.i. for levels of lentivirus infection (eGFP expression) and apoptosis by the TUNEL procedure. The level of infection was 80–90% and almost 50% of recombinant lentivirus-infected NIH3T3 (Fig. 4b) and HeLa (data not shown) cells were apoptotic. There were no significant levels of apoptosis in control cells infected with the lentivirus containing the empty vector. Identical results were obtained with human (HeLa) cells infected with control and recombinant lentivirus (data not shown).

**Cells expressing MHV68 ORF20 enter apoptosis in G2 phase**

To define whether the arrest of cells by ORF20 was in the G2 phase of the cell cycle or in mitosis, and to determine whether the cell entered mitosis prior to apoptosis, expression of the mitosis marker phosphorylated histone H3 was studied. Histone H3 phosphorylation plays an important role in several cellular functions, including chromosome condensation and cell division. Mitotic chromosome condensation is always associated with phosphorylation of histone H3 at Ser-28, making it a frequently used marker of mitosis.

Lentivirus-infected NIH3T3 cells expressing ORF20 were stained with anti-phospho-histone H3 at 48 and 72 h after infection with recombinant lentivirus. Only cells negative for the GFP marker of infection stained positively for phospho-histone H3, whereas lentivirus-infected cells did not express phospho-histone H3 (Fig. 5). This experiment demonstrated that cells infected with ORF20 arrest in G2 and enter apoptosis without entering mitosis.

**ORF20 expression results in an increase in Cdc2 phosphorylation**

The G2/M transition in mammalian cells is mediated by the Cdc2–cyclin B complex. The activity of this complex is positively regulated by the availability of the cyclin B subunit. Expression of the cyclin B subunit fluctuates through the cell cycle. In addition, its activity is negatively regulated by phosphorylation of two amino acids (Thr-14 and Tyr-15) of Cdc2.

To test whether ORF20 was arresting the cell cycle in G2 and acting at the level of the cyclin complex, NIH3T3 cells were infected with lentivirus expressing ORF20 and...
cyclin B expression was determined by Western blot analysis with anti-cyclin B antibody. A clearly increased level of cyclin B was observed in recombinant lentivirus-infected cells from 48 to 60 h p.i., consistent with arrest at the G$_2$/M phase of the cell cycle (Fig. 6). As the dephosphorylated form of Cdc2 is required for entry into mitosis, a similar experiment was performed, but the Western blots were developed with an antibody specific for the Tyr-15-phosphorylated form of Cdc2. The experiment revealed an increase in the phosphorylated (inactive) form of Cdc2 relative to the expression of total Cdc2 from 24 to 72 h after infection with recombinant lentivirus (Fig. 6). These changes were not observed when cells were transduced with the control, non-recombinant lentivirus. Similar results were obtained in human 293T cells (data not shown).

**ORF20-mediated G$_2$ arrest is caused by inhibition of the Cdc2–cyclin B complex**

The demonstration that Cdc2 is in its inactive phosphorylated form and the clear increase in levels of cyclin B indicated that ORF20 blocks the progression of cells to mitosis by acting at the level of the Cdc2–cyclin B complex. To confirm the hypothesis that ORF20 induced G$_2$ arrest by inactivation of the Cdc2–cyclin B complex, we compared the relative kinase activities of Cdc2–cyclin B in control and ORF20-expressing cells. Nocadazol-treated cells, which are blocked in mitosis, were used as a positive control. Lysates from NIH3T3 cells infected with recombinant lentivirus expressing ORF20 or with lentivirus control (eGFP) were prepared. Complexes of Cdc2–cyclin B were precipitated from cell lysates with protein G–Sepharose.

![Fig. 5. NIH3T3 cells expressing MHV68 ORF20 arrest in G$_2$. NIH3T3 cells infected with ORF20-recombinant lentivirus were seeded onto glass coverslips and, at the indicated time points, cells were fixed, permeabilized and subjected to staining with DAPI to visualize the nuclei (blue). ORF20 expression was assessed using an anti-HA monoclonal antibody (red) and lentivirus infection by eGFP expression. In the middle row, amplification of the indicated field reveals the presence of fragmented apoptotic nuclei. (b) NIH3T3 cells were infected with ORF20-recombinant lentivirus (thick line) and control eGFP lentivirus (thin line) and examined for apoptosis by the TUNEL technique at 72 h p.i.](image-url)

and an antibody to cyclin B, and their kinase activity was determined in a kinase assay using histone H1 as the substrate. The supernatants were examined for phosphorylated histone by SDS-PAGE and autoradiography (Fig. 7a) and the immunocomplexes bound to protein G were submitted to SDS-PAGE and Western blot analysis for anti-Cdc2 (Fig. 7b). Cells expressing ORF20 contained the higher-molecular-mass phosphorylated Cdc2 (inactive) complexed with cyclin B (Fig. 7b), which was almost completely inactive in the kinase assay (Fig. 7a). In contrast, in the nocadazol positive control, Cdc2 was present as the active non-phosphorylated form (Fig. 7b) and there was clear phosphorylation of the histone substrate in the kinase assay (Fig. 7a). The control, non-recombinant lentivirus (eGFP)-infected cell lysate contained both active and inactive forms of Cdc2, which catalysed a lower level of histone phosphorylation.

**DISCUSSION**

Manipulation of the host cell cycle is a frequent virus strategy for host evasion, presumably in order to achieve a cellular environment favourable for their replication. Small DNA viruses interact with the host cell-cycle control mechanism in a way that promotes entry into the S phase of the cell cycle. In contrast, herpesviruses encode their own DNA polymerase and accessory factors and do not require the environment of an S phase for virus replication. At the same time, however, herpesviruses have a more profound interaction with host cell-cycle machinery, promoting both cell-cycle arrest (Lu & Shenk, 1999; Song et al., 2000; Sunil-Chandra et al., 1992a) and cell-cycle progression (Boldogh et al., 1990; Hobbs & DeLuca, 1999).

The key element in the regulation of the eukaryotic cell cycle is the periodic synthesis and destruction of cyclins, the proteins that associate and activate cyclin-dependent kinases. The related sequential activation and inactivation of cyclin-dependent kinases, through the periodic synthesis and destruction of cyclins, provide the basis of cell-cycle regulation. In addition to the formation of cyclin complexes, the phosphorylation state of cyclin-dependent kinases controls the activity of these kinases during the cell cycle. The key downstream target of G2 arrest is the mitosis-promoting kinase complex Cdc2–cyclin B. Entry
into mitosis requires activation of Cdc2 following dephosphorylation of Tyr-15 and Thr-14. Various pathways converge at this point, shifting the balance towards entry into mitosis or arrest at G2 (Abraham, 2001; Castedo et al., 2002; Kastan & Bartek, 2004; Lukas & Bartek, 2004). In this work, we demonstrated that the non-essential ORF20 gene of the mouse herpesvirus MHV68 arrests cells in the G2 phase of the cell cycle (Fig. 3) in both human and mouse cells, followed by induction of apoptosis (Fig. 4). Our observation that the ORF20 gene of MHV68 induces cell-cycle arrest in both human and mouse cells strongly suggests that this virus gene plays a fundamental role in herpesvirus strategy for host-cell modification and adaptation, and one that might be exploited for the rational understanding and control of these important human infectious agents.

The general mechanism of ORF20 focuses on the inactivation of the Cdc2–cyclin B complex through a block in the dephosphorylation-dependent activation of the Cdc2 molecule, as indeed occurs with the human papilloma virus (HPV) E2 protein (Fournier et al., 1999) and the human immunodeficiency virus (HIV) Vpr protein (He et al., 1995). Consequently, in cells transduced with ORF20, there is an increase in Cdc2 phosphorylated at the inhibitory site (Fig. 6). The exact sequence of events in the inhibition is still to be elucidated, but the localization of ORF20 to the nucleus (Fig. 2), as for its herpes simplex virus 1 (HSV-1) homologue (Pearson & Coen, 2002), suggests a primary influence on transcription. The HSV-1 homologue of ORF20, UL24, is not required for growth in cultured cells (Pearson & Coen, 2002), but, in mice, mutation of the HSV-1 UL24 gene impairs virus replication in tissue culture and in mouse eye (Jacobson et al., 1995). In conclusion, ORF20 of the mouse gammaherpesvirus MHV68 is a virion-associated protein with nuclear localization, which has evolved for host cell-cycle arrest at G2 through inactivation of the Cdc2–cyclin B complex. A more profound understanding of its mechanism and function provides a fascinating problem for further investigation. For example, whether the observed apoptosis induced by ORF20 is a default consequence of the G2 arrest or an independent mechanism will be addressed by deletion mutant analysis. A similar functional analysis of the homologous human herpesviruses gene UL24 is clearly a priority.

ACKNOWLEDGEMENTS

This work was supported by Fundação Portuguesa para a Ciência e Tecnologia (POCI/SAU-MMO/59444/2004). R. N. was a recipient of a fellowship from Fundação Portuguesa para a Ciência e Tecnologia (SFRH/BD/4853/2001).

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