Down-regulation of surface major histocompatibility complex class I by guinea pig cytomegalovirus

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Live attenuated strains of human cytomegalovirus are under development as vaccines to prevent birth defects resulting from congenital infections. These strains encode four proteins that inhibit surface expression of MHC class I, presumably to evade cytotoxic T-cell recognition and, perhaps, attenuate induction of immunity. To initiate studies of the role of class I down-regulation on congenital infection and vaccine efficacy, the ability of guinea pig cytomegalovirus to down-regulate class I was examined. Surface class I was specifically down-regulated on infected cells up to 8-fold. Sensitivity to UV irradiation and insensitivity to a viral DNA synthesis inhibitor revealed that immediate early or early viral gene(s) are responsible. Identification of these genes will permit future experiments to evaluate the role of class I down-regulation in congenital cytomegalovirus disease and its impact on vaccine efficacy. These findings should be pertinent to understanding human cytomegalovirus disease and may help guide the design of candidate vaccines.

Human cytomegalovirus (HCMV) is a herpesvirus and a major opportunistic pathogen in immunocompromised individuals, including transplant recipients, AIDS patients and developing foetuses. The most severe foetal infections occur primarily when the mother acquires a primary HCMV infection during pregnancy and transmits the virus congenitally. Over 8000 cases of congenital HCMV infections occur annually in the United States, producing sequelae that range from hearing loss and mental impairment to deafness and/or severe mental retardation (Fowler et al., 1992).

Several candidate vaccines utilizing live attenuated HCMV strains are in development (Balfour et al., 1984; Kemble et al., 1996; Plotkin et al., 1991, 1994). These strains express a number of proteins designed to modify host immune responses, including four that down-regulate surface expression of MHC class I (Ahn et al., 1997; Jones et al., 1995, 1996; Jones & Sun, 1997; Wiertz et al., 1996). As antigen presentation by class I is critical for induction of both cell- and antibody-mediated host immunity, down-regulation of class I by vaccine strains is counter-intuitive to induction of robust immune responses. Removal of the class I down-regulation genes should increase presentation of viral peptides on infected cell surfaces and may potentiate vaccine immunogenicity. However, replication in the host is also an important factor in live virus vaccines. Although reduced replication might improve vaccine safety, a class I down-regulation-deficient virus might also be rendered so vulnerable to immune clearance that replication and persistence in the host would be inadequate to establish enduring immunity. Due to the difficulties of conducting human trials with recombinant HCMV viruses, the impact of class I down-regulation on HCMV vaccine efficacy will be difficult to determine.

Several animal cytomegaloviruses have been studied as models for HCMV disease. However, only guinea pig cytomegalovirus (GPCMV) provides a small animal model for congenital infection (Griffith & Aquino-de Jesus, 1991; Griffith et al., 1981, 1990; Liu & Biegalke, 2001; Schleiss et al., 2000). Congenital transmission of GPCMV can be induced experimentally following intraperitoneal, subcutaneous or intranasal inoculation of pregnant dams (Griffith et al., 1990; Nankervis & Kumar, 1978), and prior maternal immunity, induced either by natural infection or vaccination, can reduce transmission and protect against congenital disease (Bia et al., 1980, 1982; Fong et al., 1983; Harrison et al., 1995; Johnson & Connor, 1979; Nankervis & Kumar, 1978).

In order to develop GPCMV as a model to study the significance of class I down-regulation, we evaluated the ability of GPCMV to down-regulate class I. Guinea pig embryo fibroblast (GEF) cells were cultivated in culture medium consisting of Dulbecco’s Modified Essential Medium, 10 % foetal bovine serum (FBS), 50 µg streptomycin ml⁻¹ and 50 U penicillin (BioWhittaker) ml⁻¹ as previously described (McVoy et al., 1997). Virus-infected
cells were distinguished from uninfected cells by the use of a recombinant virus, GPCMV/EGFP, which expresses enhanced green fluorescent protein (EGFP) fused to puromycin N-acetyltransferase (Abbate et al., 2001). Cells were mock-infected or infected with GPCMV/EGFP at different m.o.i. values, trypsinized at various time points and frozen at −70 °C in 90 % FBS/10 % DMSO. After thawing, cells were washed twice with staining buffer (PBS/10 % FBS) by centrifugation (500 g, 5 min, 4 °C) and incubated for 30 min on ice in 100 μL staining buffer containing monoclonal antibodies (mAbs) in ascites (0.5 μg total protein). Murine mAb HUSM-41, specific for guinea pig class I (Sato et al., 1997), was used to detect class I, while mAb HSUM-49, specific for guinea pig class II (Sato et al., 1997), served as an isotype-matched negative control. Cells were washed and then incubated for 30 min on ice in 100 μl staining buffer containing 0.1 μM biotin-conjugated rat mAb R8-140 (α-mouse Ig κ) ml−1 (PharMingen). The cells were washed again, incubated 30 min on ice in 100 μl staining buffer containing 10 μg streptavidin–R-phycocerythrin (Life Technologies) ml−1, washed and finally resuspended in PBS/1 % paraformaldehyde (Sigma). The cells were then analysed using a Beckman FACScan 2000 at wavelengths of 525 nm for EGFP and 575 nm for phycoerythrin.

As expected, the class II-specific mAb HUSM-49 failed to react with either infected or uninfected cells (Fig. 1A). Infected cells expressed EGFP whereas uninfected cells did not (Fig. 1A). Virus-infected (EGFP+) cells underwent a decrease in class I surface expression and reached a maximal 4.5-fold reduction by 72 h post-infection (p.i.). The proportion of uninfected (EGFP−) cells decreased with increasing m.o.i. but the cells maintained normal levels of class I (Fig. 1B). Similar results were obtained using a second class I-specific mAb, HUSM-20 (Sato et al., 1997) (data not shown). Thus, GPCMV specifically down-regulated surface class I expression on infected cells but not on uninfected cells in the same culture.

Herpesvirus late genes require viral DNA synthesis in order to be expressed and can be distinguished from immediate early (IE) and early genes by their failure to be expressed in the presence of viral DNA polymerase inhibitors such as 9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA). To determine the kinetic class of the viral genes responsible for class I down-regulation, cells were infected with GPCMV/EGFP at an m.o.i. of one in the presence of 10 μg HPMPA ml−1 (a gift from N. Bischofberger, Gilead Sciences Inc. California, U.S.A.), a dose previously determined to fully inhibit GPCMV DNA synthesis (not shown). The cells were analysed at different time points for surface class I by flow cytometry as described above. Even in the presence of HPMPA, class I down-regulation was observed as early as 12 h p.i. and reached a maximal 4.5-fold reduction by 72 h p.i. (Fig. 1C). Interestingly, class I on uninfected cells increased 2-fold (Fig. 1C), whereas HPMPA-treated mock-infected cells exhibited only a minor increase (<10 %) over a 72 h period (not shown). Similar events in HCMV-infected cultures have been attributed to the release of interferon-β by infected cells (Steinmassl & Hamprecht, 1994).

This result indicated that the viral proteins responsible for class I down-regulation must either be expressed with IE or early kinetics or be virion-associated and thereby carried into cells at the time of infection. The effects of virion-associated proteins can be distinguished from those requiring de novo expression by their lack of sensitivity to UV irradiation, which prevents viral gene expression by cross-linking the viral DNA (Jing et al., 2001). To determine if down-regulation is mediated by virion-associated proteins, replicate aliquots of viral inocula were exposed to increasing amounts of UV irradiation in a Stratalinker 1800 UV cross-linker (Stratagene) and then adjusted to 5 mM sodium pyruvate (Cellgro) (Fortunato et al., 2000). GEF cells were incubated with irradiated inocula for 3 h, washed twice with culture media, incubated for 72 h in culture media containing 10 μg HPMPA ml−1 and then analysed for EGFP and surface class I expression. Prior to irradiation, the amount of virus in the inocula was such that approximately 50 % of the cells would be infected. The percentage of cells expressing EGFP decreased in a dose-dependent manner, confirming UV inhibition of de novo viral gene expression (Fig. 2A). The percentage of class II flow cells (i.e. those retaining the ability to down-regulate class I) also decreased in a manner that exactly paralleled the decrease in EGFP expression (Fig. 2A), indicating that down-regulation required de novo gene expression and is therefore not mediated by virion-associated proteins. Thus, expression of one or more IE or early viral genes is responsible for class I down-regulation.

To compare the kinetics of virus-mediated class I down-regulation to the loss of class I from the surface following complete cessation of protein synthesis, class I levels were measured at different times after infection or after addition of 50 μg cycloheximide ml−1 to uninfected cells (Sigma). From these results, the half-life of class I on the surface of uninfected guinea pig cells was estimated at 6 h (Fig. 2B), consistent with class I half-lives on murine (3–9 h) and human (4–5 h) cells (Hallermalm et al., 2001; Rescigno et al., 1998). The kinetics of virus-mediated class I down-regulation closely paralleled that resulting from cycloheximide treatment (Fig. 2B). Thus, virus-mediated down-regulation was rapid and highly effective, producing results equivalent to an immediate block to synthesis of new class I molecules.

To determine if down-regulation is specific for class I and is not the consequence of a generalized virus-related inhibition of protein transport to the cell surface, we compared total surface protein profiles in both infected and uninfected cells. Cells were mock-infected or infected at an m.o.i. of 1 or 3 in the presence of 10 μg HPMPA ml−1 for 72 h. Cells were then trypsinized, washed three times with ice-cold PBS, counted, and incubated at room temperature for 30 min in PBS containing 20 μg Sulfo-NHS-LC-biotin.

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Fig. 1. Down-regulation of class I by GPCMV. (A) GEF cells were mock-infected or infected with GPCMV/EGFP at an m.o.i. of 3 and analysed 72 h p.i. by dual-colour flow cytometry for EGFP (x-axis) and for surface class II (y-axis) using mAb HUSM-49, an isotype-matched negative control for class I staining experiments. (B) Cells were infected with GPCMV/EGFP at the indicated m.o.i. values and analysed at the time points indicated by dual-colour flow cytometry for EGFP (x-axis) and for surface class I (y-axis) using mAb HUSM-41. (C) Cells were infected with GPCMV/EGFP at an m.o.i. of 1 in the presence of 10 μg HPMPA ml⁻¹ and analysed as in (B) at the time points indicated. Mean fluorescent intensities (MFI) are indicated for the populations of cells within the circles.
(Pierce) to biotinylate surface-exposed proteins. A small aliquot was stained with trypan blue to confirm the integrity of the cell membranes. The remaining cells were lysed in gel loading buffer and subjected to SDS-PAGE (Sambrook et al., 1989). Separated proteins were transferred to a Nytran N membrane (Schleicher and Schuell). The membrane was blocked for 2 h at 4˚C in blocking buffer (Sambrook et al., 1989) and incubated with streptavidin-conjugated horse-radish peroxidase (Roche) according to the manufacturer’s instructions. Biotinylated surface proteins were visualized by using Western Lightning chemiluminescence reagent plus (NEN) according to the manufacturer’s instructions, followed by exposure to Hyperfilm MP X-ray film (Amersham). Numerous surface proteins were observed in extracts of uninfected cells that remained unchanged in infected cells (Fig. 3A). One surface protein was clearly down-regulated by virus infection and had a molecular mass of approximately 46 kDa (Fig. 3A). Although the identity of this protein is unknown, its size is consistent with the molecular masses of class I heavy chains from other species (Campbell & Slater, 1994; Jones & Sun, 1997) and therefore it is possible that this protein is the guinea pig class I heavy chain.

We also used flow cytometry to examine the effects of virus infection on another cellular surface protein, Thy-1, a lipid-anchored adhesion molecule that is normally expressed on immune effector cells but is also expressed on guinea pig fibroblasts (Schäfer et al., 1999). Cells were mock-infected or infected with GPCMV/EGFP in the presence of HPMPA (10 µg ml⁻¹), and the class I mean fluorescent intensities of EGFP⁺ (infected) cells were measured by flow cytometry at the indicated times p.i.

Many viruses and some bacteria have evolved the ability to inhibit class I antigen presentation on infected cells (for review, see Lorenzo et al., 2001). Down-regulation of class I during lytic replication has now been reported for all eight human herpesviruses and several animal herpesviruses (Ambagala et al., 2000; Barnes & Grundy, 1992; Campbell & Slater, 1994; Cohen, 1998; Hariharan et al., 1993; Hirata et al., 2001; Hudson et al., 2001; Hunt et al., 2001; Ishido et al., 2000; Jones et al., 1995; Nataraj et al., 1997; Stevenson et al., 2000; Tomazin et al., 1998; Yamashita et al., 1993; York et al., 1994; Zeidler et al., 1997). Thus, class I down-regulation is clearly an important, perhaps essential, component in the herpesviral arsenal of immune evasion.
mechanisms. Because HCMV is unique among the human herpesviruses in causing congenital disease, the roles of class I down-regulation in congenital transmission and pathogenesis to the foetus represent important questions. Furthermore, at least two vaccine projects are under way utilizing live attenuated HCMV viruses as candidate vaccines (Gonzol & Plotkin, 2001). These strains retain all four class I down-regulation genes. How down-regulation of class I impacts on the ability of these vaccines to induce host immunity in vivo and protect against congenital disease is unknown.

Because GPCMV provides the only small animal model of congenital cytomegalovirus disease, we chose to investigate its ability to down-regulate class I. Our finding that down-regulation is mediated by viral genes that are expressed with IE or early kinetics should facilitate identification of the specific genes by eliminating a large number of late genes from initial consideration; however, our data do not rule out the possibility that, in addition to IE or early genes, late genes with down-regulation functions may also exist.

The various mechanisms by which herpesviruses down-regulate class I target virtually all stages of the class I expression pathway, including synthesis (Hirata et al., 2001), peptide transport (Ahn et al., 1997; Ambagala et al., 2000; Hill et al., 1995; Hinkley et al., 1998; Jugovic et al., 1998; Zeidler et al., 1997), transit to (Abendroth et al., 2001; Ahn et al., 1996; Campbell & Slater, 1994; del Val et al., 1992; Hudson et al., 2001; Jones & Sun, 1997; Jones et al., 1996; Reusch et al., 1999; Wiertz et al., 1996) and stability on the cell surface (Ishido et al., 2000). Although our data do not directly address mechanisms for class I down-regulation by GPCMV, our observation that the kinetics of class I loss in response to virus infection closely parallels the response to cycloheximide is consistent with a block to repopulation of surface class I; however, as multiple GPCMV genes may be involved, a detailed understanding of their mechanisms awaits identification of the viral genes and independent analyses of their effects.

Identification of the GPCMV class I down-regulation genes will permit construction of recombinant viruses with mutations in these genes, which can be used to investigate the importance of class I down-regulation in virus pathogenicity, congenital transmission and in utero disease. The impact of these genes can also be determined with regard to levels of induced maternal immunity and more importantly, prevention of congenital disease, when live GPCMVs are used as vaccines. Ultimately, the insights gained from the guinea pig model can be applied to HCMV disease and toward rational designs for improved HCMV live virus vaccines.

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**Fig. 3.** Down-regulation is class I-specific. (A) Cell surface proteins were biotinylated on mock-infected cells (U) or cells infected for 72 h with GPCMV/EGFP at m.o.i. values of 1 (I-1) or 3 (I-3). Cell lysates were separated by SDS-PAGE, transferred to a nylon membrane and probed using a streptavidin–horseradish peroxidase conjugate to specifically detect biotinylated surface proteins. The arrow indicates a 46 kDa uninfected cell protein that is down-regulated by virus infection. (B) GEF cells were mock-infected or infected with GPCMV/EGFP at an m.o.i. of 2 in the presence of 10 μg HPMPA ml⁻¹ and analysed 7 days p.i. by flow cytometry using mAb H154 specific for guinea pig Thy-1 or an isotype-matched negative control mAb 53-6.7 specific for mouse CD8.
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