

# Major Histocompatibility Complex Class I Allele-specific Cooperative and Competitive Interactions between Immune Evasion Proteins of Cytomegalovirus

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## Abstract

Cytomegaloviruses (CMVs) deploy a set of genes for interference with antigen presentation in the major histocompatibility complex (MHC) class I pathway. In murine CMV (MCMV), three genes were identified so far: *m04/gp34*, *m06/gp48*, and *m152/gp40*. While their function as immunoevasins was originally defined after their selective expression, this may not necessarily reflect their biological role during infection. The three immunoevasins might act synergistically, but they might also compete for their common substrate, the MHC class I complexes. To approach this question in a systematic manner, we have generated a complete set of mutant viruses with deletions of the three genes in all seven possible combinations. Surface expression of a set of MHC class I molecules specified by haplotypes H-2<sup>d</sup> (K<sup>d</sup>, D<sup>d</sup>, and L<sup>d</sup>) and H-2<sup>b</sup> (K<sup>b</sup> and D<sup>b</sup>) was the parameter for evaluation of the interference with class I trafficking. The data show the following: first, there exists no additional MCMV gene of major influence on MHC class I surface expression; second, the strength of the inhibitory effect of immunoevasins shows an allele-specific hierarchy; and third, the immunoevasins act not only synergistically but can, in certain combinations, interact antagonistically. In essence, this work highlights the importance of studying the immunosubversive mechanisms of cytomegaloviruses in the context of gene expression during the viral replicative cycle in infected cells.

Key words: murine cytomegalovirus • BAC • immune evasion • MHC class II • allele

## Introduction

The capacity of a virus to actively modulate the host immune response defines the biological balance between a persistent virus and its host. A number of viral functions are instrumental for maintaining and tuning this balance. The highly species-specific CMVs are a representative example. Human CMV (HCMV), a herpesvirus that defines the  $\beta$ -herpesvirus subfamily, is an important pathogen only in the immunologically immature or immunocompromised host, whereas in the normal population the infection occurs without clinical symptoms. CMVs interfere with innate and adaptive cellular and humoral immune effector mechanisms by affecting cytokine networks, by activation and silencing of natural killer (NK)\* cells, by downmodulating

antigen presentation in the MHC class I and II pathways, and by the regulation of apoptosis. Some viral genes that affect host immune functions are homologues pirated from the vertebrate genome, whereas others show no apparent homology to host genes (1, 2).

Several HCMV and murine CMV (MCMV) gene products, usually glycoproteins, have been shown to interfere with the MHC class I pathway of antigen processing and presentation. With one exception, all these proteins interfere with antigen presentation by downmodulation of MHC class I cell surface expression. Individually, none of those is essential for virus growth in cell culture. They lack significant homologies to other herpesviral or cellular genes and are dispersed over the left and right genome termini (3, 4). The HCMV proteins US2 and US11 cause proteasomal degradation of MHC class I molecules by retrograde transport from the ER to the cytosol (5, 6). The US3-encoded protein binds to MHC class I molecules and arrests them in the ER (7, 8). Finally, US6 binds to the peptide transporter

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\*Abbreviations used in this paper: BAC, bacterial artificial chromosome; MEF, mouse embryonic fibroblast; NK, natural killer; wt, wild-type.

associated with processing to prevent peptide loading of MHC class I molecules (9–11).

MCMV is comparable to HCMV with regard to a variety of functions by which it can modulate the host response. The infection of the mouse with MCMV offers the advantage that defined mutants can be tested in the natural host. In MCMV, three genes encoding glycoproteins that interact with MHC class I molecules, here referred to as immunoevasins, have been identified: the immunoevasin *m04/gp34* binds to MHC class I molecules without affecting cell surface expression, which is exceptional for MHC class I interfering CMV proteins (12). Nevertheless, *m04/gp34* is involved in the prevention of antigen presentation to selected CTL clones by a so far unknown mechanism (13). The second immunoevasin *m06/gp48* reduces cell surface expression of MHC class I molecules by binding them and by retargeting the complexes to lysosomes for degradation (14). The third immunoevasin *m152/gp40* also reduces cell surface expression of MHC class I molecules, but achieves this by a different mechanism, namely by retention of the peptide-loaded complexes in the ER-cis-Golgi intermediate compartment (15). The *m152/gp40* immunoevasin was the first and is still the only MHC class I interacting CMV glycoprotein shown to be relevant for CMV replication *in vivo* in the face of functional immunity (16).

Previous studies on CMV-encoded MHC class I reactive proteins have had the aim to elucidate the specific mode of function of defined viral proteins. With the exception of *m152/gp40* of MCMV, there exist no data on the relevance of the individual immunoevasins in the course of CMV infection *in vitro* and *in vivo*. It is unknown whether, in the context of the CMV genome, the effects of these genes are additive, cooperative, redundant, or even competitive. Likewise, little is known about possible MHC class I allele-specific differences in the action of the different immunoevasins. This limited knowledge is because of a lack of appropriate virus mutants that would allow to perform a systematic analysis of immunoevasin interactions in the context of virus infection. The generation of CMVs with targeted gene deletions by recombination in cells is laborious (17) and the generation of mutants with multiple targeted mutations dispersed over the genome is almost not achievable. Cloning of CMVs as infectious bacterial artificial chromosomes (BACs; reference 18) has opened perspectives for novel site-directed and random mutagenesis techniques in *Escherichia coli* (for a review, see reference 19). Further work from several laboratories on the generation and mutagenesis of BAC-cloned CMV genomes (20–24) has confirmed the attractivity and general applicability of CMV mutagenesis in bacteria. Until now, targeted mutagenesis usually involved subcloning of viral DNA and usage of available restriction sites, which is unfavorable for the introduction of independent mutations in close proximity (a distance of <1.5 kbp; reference 18), as it is the case for genes *m04* and *m06*.

Here, we systematically investigated the influence on MHC class I molecule surface expression of the immuno-

evasins *m04/gp34*, *m06/gp48*, and *m152/gp40* during MCMV infection. We generated a complete set of MCMV mutants which lack or express these three genes in all possible combinations. To this end, we applied to the viral MCMV-BAC a fast mutagenesis procedure for targeted mutation via a double crossing-over event between PCR-generated DNA fragments and the viral DNA. We tested the effect of this set of seven MCMV immunoevasin deletion mutants on cell surface expression of H-2<sup>d</sup> and H-2<sup>b</sup> MHC class I alleles and conclude from the data the following: (i) there exists no additional MCMV gene product that would significantly interfere with MHC class I molecule export; (ii) individual MHC class I alleles are differentially affected; (iii) the isolated deletion of gene *m06* has the strongest effect on MHC class I, indicating a dominant role of the immunoevasin *m06/gp48*; and (iv) the immunoevasins *m06/gp48* and *m152/gp40* cooperate in their function. In contrast, *m04/gp34* and *m152/gp40* compete for MHC class I molecules, whereby *m04/gp34* antagonizes the function of *m152/gp40*. In summary, the viral MHC class I-modulating proteins interact in their effects on MHC class I molecules, and the outcome of this interaction is allele specific.

## Materials and Methods

**Cells, Viruses, and Plaque Assay.** BALB/c mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with 10% (vol/vol) FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. These cells were used for virus reconstitution from recombinant BAC plasmids as described previously (25). NIH3T3 cells (CRL1658; American Type Culture Collection) were cultured in DMEM supplemented with 10% (vol/vol) newborn calf serum and antibiotics (see above). These cells were used for propagation of recombinant viruses, for determination of viral growth, and for Western blot analysis. For the detection of MHC class I molecules on the cell surface after infection with the respective viruses, the following fibroblast cell lines were used: B12, which is a BALB/c (haplotype H-2<sup>d</sup>)-derived SV40-transformed fibroblast cell line (26); and C57SV, which is a C57BL/6 (haplotype H-2<sup>b</sup>)-derived SV40-transformed fibroblast cell line (27). The MCMV wild-type (wt) and wt-like strains used in these studies were strain Smith (VR-194; American Type Culture Collection) and the BAC-derived recombinant MW97.01 (20). Virus stocks were prepared following published protocols (28). *In vitro* growth kinetics of the MCMV mutants and of parental wt MCMV were determined on NIH3T3 cells as follows: NIH3T3 cells were infected with a multiplicity of infection of 0.1 PFU per cell (without centrifugal enhancement) and supernatants of infected cells were harvested every 24 h from day 0 (input virus titer) through day 5 after infection. Virus titers in supernatants were measured in triplicate by a plaque assay performed on second-passage MEF monolayers in 48-well plates as described previously (29).

**Plasmid Construction.** Plasmid cloning was performed by standard methods (30). The parental shuttle plasmid pST76A-SacB is a derivative of plasmid pST76A (31) into which the *Bacillus amyloliquefaciens sacB* gene (32) was introduced. The *sacB* gene was used for selection of clones with resolved cointegrates and lost shuttle plasmids by adding 5% (wt/vol) sucrose to the agar plates as described previously (21). The shuttle plasmid pSTm06, used

for generation of the BAC plasmid p $\Delta$ m06, was cloned as follows: the MCMV sequence from nt positions 2701–9760 was excised from plasmid pHindIII A (33) by HindIII and KpnI digestion, and was inserted into plasmid pBluescriptII (KS<sup>+</sup>) (Stratagene). Within this plasmid, the MCMV sequence from nt positions 5392–6235, containing open reading frame *m06*, was deleted by NheI treatment and replaced by the kanamycin resistance gene (*kn*) from plasmid pCP15 (34). The MCMV sequence containing the *kn* instead of *m06* was then excised by BstEII and EcoRI treatment and inserted into the SmaI site of plasmid pST76A-SacB, hereby generating shuttle plasmid pSTm06. Plasmid pSTm06 now contains the *kn* flanked by MCMV sequences upstream (nt 2701–5392) and downstream (nt 6235–9760) of *m06*.

**Mutagenesis of Viral BAC Plasmids Using Linear PCR Fragments.** A PCR-based mutagenesis procedure was used for the generation of the recombinant MCMV BAC plasmids p $\Delta$ m04, p $\Delta$ m152, p $\Delta$ m04+m06, p $\Delta$ m04+m152, p $\Delta$ m06+m152, and p $\Delta$ m04+m06+m152 that contain exact single-, double- or triple-deletions of the MCMV open reading frames *m04*, *m06*, and *m152*, respectively. The technique was based on homologous recombination mediated by the recombination functions red $\alpha$  (*exo*) and red $\beta$  (*bet*), and the exonuclease inhibitor red $\gamma$  (*gam*) of bacteriophage  $\lambda$  expressed from the plasmid pBAD $\alpha\beta\gamma$  (35) (for the strategy, see Fig. 1 A). First, linear DNA recombination fragments were generated. These fragments contained the prokaryotic *kn* from plasmid pACYC177 (New England BioLabs) or the resistance gene *zeo* (*zeo*) from plasmid pZero1 (Invitrogen), flanked by sequences of 40 to 60 nts in length, homologous to the up and downstream regions of the target gene. For the generation of these linear fragments by PCR, contiguous pairs of synthetic primers were used, which all bind to the resistance gene with their 3'-ends and which carry sequences homologous to the MCMV genomic sequence at their 5'-ends. For deletion of gene *m152* (nt 210244–211377), the PCR fragment m152-PCR, containing *zeo* flanked by 34-bp minimal FRT sites (5'-GAAGTTCCTATTCTCTAGAAAGTATAGGAAGTTC-3'), was generated by using plasmid pZero1 as template DNA and the contiguous primers m152-FRT-*zeo*-5 (5'-GCTCGAGCGAGAGCACCCGACGATCTGACATTGTCCAGTGTGCCGGTTCGCACGAACATCAGAAGTTCCTATTCTCTAGAAAGTATAGGAAGTTCACCGTTTACAATTTTCGCTGATGCG-3') and m152-FRT-*zeo*-3 (5'-TCACAAGCCGTGTCACCGCTCCACGTTT CACCGTCGTCCGCTCTCCGATCGCTAGCCTGAACAGAAGTTCCTATACTTTCTAGAGAATAGGAAGTTCGAAGTTTTAGCACGTGTCTAGTCCCT-3'). For deletion of gene *m04* (nt 3270 to 4067), the PCR fragment m04-PCR, containing *kn*, was generated using plasmid pACYC177 as template DNA and contiguous primers m04-5 (5'-TAA-TGATCTAGACGGCAATTTCTGTCTCATTTCGTTGTTCCAGAGCGACGGATGGTACAAG-3') and m04-3 (5'-TACTCAGAACACCGGAAAATGGTT TACTCAAGGGGATTTT-TATTTAGGGGGTTAGTTACT-3'). With the exception of the single *m06* deletion BAC plasmid p $\Delta$ m06 (see below), deletion of the *m06* gene (nt 5300–6334) in the MCMV BAC plasmids was performed by using PCR fragment m06-PCR, which was generated using plasmid pZero1 as template DNA and the contiguous primers m06-*zeo*-5 (5'-CACGCCAAAATCACGCAATCATATATAAATGGACAATGAAGCCAATCTAACGTTTACAATTTTCGCTGATGCG-3') and m06-*zeo*-3 (5'-GCAGTCTAGCTAGCGGCTCGCGCCGTAGGTGTA-GAACAACATAGCACCTGAAGTTTTAGCACGTGTCTAGTCCCT-3'). The PCR products were purified using the QIAGEN

PCR purification kit (QIAGEN). For removal of residual methylated template plasmid DNA, products were digested with 20 U of restriction enzyme DpnI for 1.5 h. The PCR products were then immediately precipitated with 100% ethanol, washed with 70% ethanol, and resuspended in 10  $\mu$ l of sterile water. 5  $\mu$ l of each linear fragment was electroporated into electrocompetent *E. coli* (strain DH10B), containing the respective MCMV BAC plasmid and the recombination proteins (see below), using a Bio-Rad Gene Pulser (2.5 kV, 200  $\Omega$ , and 25  $\mu$ F). After addition of 1 ml of LB medium followed by shaking for 2 h at 37°C, the bacteria were grown on LB agar plates containing 25  $\mu$ g/ml chloramphenicol and 25  $\mu$ g/ml kanamycin or zeocin and incubated overnight at 37°C.

Electrocompetent DH10B were prepared as follows: 2 ng DNA of plasmid pBAD $\alpha\beta\gamma$  (45) was introduced into DH10B, which contain the parental MCMV BAC plasmid, by chemical transformation using standard protocols (30). 2 ml of an overnight culture of these transformed DH10B were then added to 200 ml of LB growth medium containing 25  $\mu$ g/ml chloramphenicol and 25  $\mu$ g/ml kanamycin. 2 ml of 10% (wt/vol) L-arabinose in LB medium was added at an OD<sub>600</sub> of 0.2 in order to induce the expression of the *exo* protein, which is controlled by the inducible BAD-promoter (Invitrogen). After 30 min of incubation at 37°C, bacteria were put on ice for 15 min. After three washing steps with ice-cold 10% (vol/vol) glycerol, bacteria were resuspended in a total volume of 800  $\mu$ l of 10% glycerol, subdivided into 60  $\mu$ l aliquots, and frozen at -80°C.

Excision of the *zeo* from the MCMV BAC plasmid p $\Delta$ m152-*zeo* via the flanking FRT sites by FLP-mediated site specific recombination was performed by using plasmid pCP20 as described previously (34).

**Mutagenesis of BAC Plasmids Using Shuttle Plasmids.** For deletion of 82% of the *m06* gene sequence (nt 5392 to 6235) within the wt MCMV BAC plasmid pSM3fr, a two-step recombination strategy based on RecA-mediated homologous recombination in the *E. coli* strain CBTS (36) was applied as described previously (18). Shuttle plasmid pSTm06 was used for the generation of the recombinant MCMV BAC plasmid p $\Delta$ m06 (Fig.1).

**Reconstitution of Recombinant Viruses by Transfection.** For reconstitution of viral progeny, 80% confluent MEF in a 6-cm culture dish were transfected with 1.5  $\mu$ g of MCMV BAC plasmid DNA by the calcium phosphate precipitation technique as described previously (30). 5 h after transfection, the MEF were treated with 15% glycerol (vol/vol) in Hepes-buffered saline for 2.5 min as described previously (30). After 3–5 d of cultivation, virus plaques appeared. The recombinant viruses  $\Delta$ m04-MCMV,  $\Delta$ m04+152-MCMV, and  $\Delta$ m152-MCMV were already used in a previous study and published under the names  $\Delta$ m4-MW99.03,  $\Delta$ m4+m152-MW99.04, and  $\Delta$ m152-MW99.05, respectively (13).

**Isolation and Analysis of DNA.** Isolation of plasmid DNA from *E. coli* cultures was performed using an alkaline lysis procedure as described previously (30). Isolation of MCMV BAC DNA from *E. coli* was performed using the Nucleobond kit from Machery & Nagel. Viral DNA from infected cells was isolated as published previously (33).

**Western Blot Analysis.** For protein detection in infected cells, Western blot analysis was performed at 16 h after infection. For the detection of *m04*/gp34, the specific peptide antiserum m04-3 (13) was used. The *m152*/gp40 protein was detected with mAb gpM3D10 (37) and protein *m06*/gp48 was detected with polyclonal antiserum m06-2 (38). The lysates of virus-infected cells were resuspended in Laemmli sample buffer containing 5%  $\beta$ -mer-

captoethanol, sonified for 10 s, and incubated at 95°C for 5 min. After electrophoresis on a 10% SDS-PAGE, the proteins were transferred to Hybond-P membranes (Amersham Biosciences) in the presence of blotting buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol, pH 8.3). After blocking of the membranes for 30 min at room temperature with TBS-T (Tris-buffered saline/0.05% Tween-20) containing 5% (wt/vol) dry milk, the primary antibody was added in a dilution (in TBS-T) of 1:5,000. After washing with TBS-T, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the ECL system (Amersham Biosciences).

**Flow Cytometry.** For the detection of MHC class I molecules on the surface of infected cells, the following antibodies were used (see also Table I in Results): mAb 28-14-8s (HB27; American Type Culture Collection), specific for the  $\alpha 3$  domain of H-2L<sup>d</sup> and L<sup>q</sup> molecules, served for the detection of the L<sup>d</sup> molecule on cells of H-2<sup>d</sup> haplotype. mAb SF1.1.1 (HB-159; American Type Culture Collection) served for the detection of H-2K<sup>d</sup>, mAb 34-2-12s (HB-87; American Type Culture Collection) for H-2D<sup>d</sup>, mAb Y-3 (39) for H-2K<sup>b</sup>, and mAb B22.249 (40) for H-2D<sup>b</sup>. Subconfluent cell-layers in a 6-cm culture dish were infected with recombinant or wt MCMVs with a virus dose of 0.3 PFU per cell using centrifugal enhancement of infectivity, which results in an effective MOI of 6 (41). At 4, 7, 12 and 16 h after infection, the cells were washed with PBS, trypsinized, and rinsed with FACS<sup>®</sup>-buffer (PBS supplemented with 2% FCS and 0.03% [wt/vol] NaN<sub>3</sub>), followed by labeling for 60 min at 37°C with the indicated primary antibodies. After washing with FACS<sup>®</sup>-buffer, antibodies bound to MHC class I molecules were stained by adding fluorescein-conjugated goat anti-mouse IgG (Sigma-Aldrich).

A comparable infection rate for all wt and mutant viruses (>90% of the cells infected) was confirmed for a separate aliquot of the infected cells by labeling of the intranuclear viral IE1 protein pp89 with mouse mAb (IgG1) CROMA101 (provided by S. Jonjic, University of Rijeka, Rijeka, Croatia). IE1 is expressed during all kinetic phases of viral gene expression (42). For permeabilization of the cells, which is required for detection of intracellular pp89, saponin was added to the buffers in a final concentration of 0.1% (vol/vol). Fluorescein-staining was performed as specified above. Labeling of the cell surface marker CD44 with rat mAb IM7.8.1 (TIB235; American Type Culture Collection) was used to control for the expression of cell surface molecules not affected by MCMV gene products in order to exclude down-regulation of cell surface molecules due to a general cytopathogenic effect of the infection. For detection of antibodies bound to CD44, fluorescein-conjugated goat anti-rat IgG (Sigma-Aldrich) was used.

Throughout, cells incubated with the second antibody alone were used as negative controls and the resulting fluorescence signals were subtracted from the values gained with the first and second antibody for the same probes. For each expression profile,  $3 \times 10^6$  or  $5 \times 10^6$  cells were labeled and analyzed on a FACS-Calibur<sup>™</sup> (Becton Dickinson) using CELLQuest<sup>™</sup> software (Becton Dickinson) for data processing.

**Assay for Competitive Virus Fitness In Vivo.** Tissue tropism and in vivo growth characteristics of wt MCMV and of the immune evasion gene triple deletion mutant  $\Delta m04+06+152$ -MCMV were compared by coinfection of immunocompromised mice followed by two-color in situ DNA hybridization (2C-ISH) of viral genomes in infected tissues. In infected tissue cells, viral DNA is accumulated during the late phase in an intranuclear in-

clusion body, which is the site of DNA packaging and nucleocapsid assembly.

**Conditions of Infection.** Female BALB/c mice (8-wk-old) were immunocompromised by hematocytotoxic total-body  $\gamma$ -irradiation with a single dose of 7 Gy delivered by a <sup>137</sup>Cs source. At 24 h after the irradiation, recipients were coinfecting intravenously in the tail vein with a 1:1 mixture ( $5 \times 10^4$  PFU each) of the two MEF-propagated, sucrose-gradient purified viruses (41) contained in 0.5 ml of physiological saline. Infection of host tissues was determined by 2C-ISH on day 8 after infection, that is, at a prefinal stage of multiple-organ CMV disease.

**Hybridization Probes for 2C-ISH.** The hybridization probe *M55-P* spans 1541 bps from nucleotide positions 84250–85790 of the MCMV strain Smith genome (4) (GenBank/EMBL/DBJ accession no. MCMV68299[complete sequence]) within the gB-coding gene *M55*. It was synthesized by PCR using plasmid pACYC-gB (43) as the template, and oligonucleotides 5'-GCACGTCGTAGGTAATTGC-3' and 5'-TCGCTCCTC-GAGCTGGTACG-3' as primers. *M55-P* was tagged during the PCR by incorporation of fluorescein-conjugated dUTP (fluorescein-12-dUTP) (no. 1373242; Roche Laboratories). Probe *m152-P* spans 1115 bps from nucleotide positions 210254 to 211368 within gene *m152*. It was synthesized by PCR using plasmid pGEM-4Z-*m152* as the template, and oligonucleotides 5'-CAGTTGATGTAGACCAGCGATAC-3' and 5'-GCTAT-CACCTACTTGCTCCTCTCG-3' as primers. For the construction of pGEM-4Z-*m152*, the desired sequence within gene *m152* was amplified from virion DNA by using the primers mentioned above with their 5' ends extended by HindIII and BamHI cleavage sites, respectively. The amplicon was then ligated into cloning vector pGEM-4Z (GenBank/EMBL/DBJ accession no. X65305) (no. P2161; Promega). Probe *m152-P* was tagged during the PCR by incorporation of digoxigenin-11-dUTP (no. 1093088; Roche Laboratories).

**2C-ISH.** A red label is used to visualize gene *M55*, which is shared by the two viruses, whereas a black label is assigned to gene *m152* contained in the genome of wt MCMV only (for the design, see Fig. 4 A). The hybridization of viral DNA in deparaffinized 2- $\mu$ m tissue sections was performed with procedures described in greater detail by Grzimek et al. (44). In a first step, gene *M55* was stained red by using alkaline phosphatase-conjugated anti-fluorescein antibody (no. 1426338; Roche Laboratories) with fuchsin as the chromogenic substrate. In the second step, gene *m152* was stained black by using peroxidase-conjugated antidigoxigenin antibody (no. 1207733; Roche Laboratories) with diaminobenzidine tetrahydrochloride as the substrate, followed by color enhancement with ammonium nickel sulfate hexahydrate. Accordingly, cells infected by mutant  $\Delta m04+06+152$ -MCMV are marked by a clean red color, whereas in wt MCMV the red staining of gene *M55* is superimposed by the black staining of gene *m152*. Quantitative microscopic analysis as well as the photodocumentation of the tissue sections were performed as described previously (44).

## Results

**Construction of Recombinant MCMV Genomes by PCR-based BAC Mutagenesis in E. coli.** To revisit the roles of the known MHC class I interacting genes *m04/gp34*, *m06/gp48*, and *m152/gp40* in the course of infection, we intended to generate a complete set of seven mutants with single-, double-, and triple-deletions of these three genes in all possible combinations. To this end, we applied to the

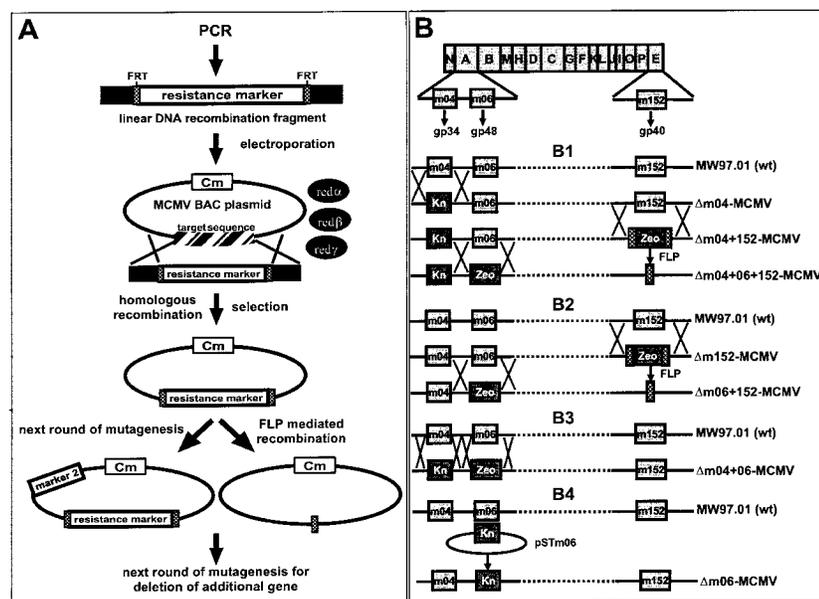
MCMV-BAC a PCR-based one-step recombination procedure (45) using conditional expression of the proteins  $\text{red}\alpha$ ,  $-\beta$ , and  $-\gamma$  from bacteriophage  $\lambda$  (Materials and Methods and Fig. 1 for the mutagenesis strategy).

Using this procedure, six recombinant MCMV BACs with deletions of the known immunoevasin genes  $m04$ ,  $m06$ , and  $m152$  in all combinations were generated:  $\text{p}\Delta m04$ ;  $\text{p}\Delta m152$ ;  $\text{p}\Delta m04+06$ ;  $\text{p}\Delta m04+m152$ ;  $\text{p}\Delta m06+m152$ ; and  $\text{p}\Delta m04+m06+m152$  (Fig. 1, B1–B3). BAC plasmid  $\text{p}\Delta m06$  was constructed by using the established two-step recombination method (18) (Fig. 1 B4). Correct deletions of the respective genes and the integrity of the whole genomes were confirmed by restriction pattern analysis using appropriate restriction enzymes followed by Southern blot analysis (unpublished data).

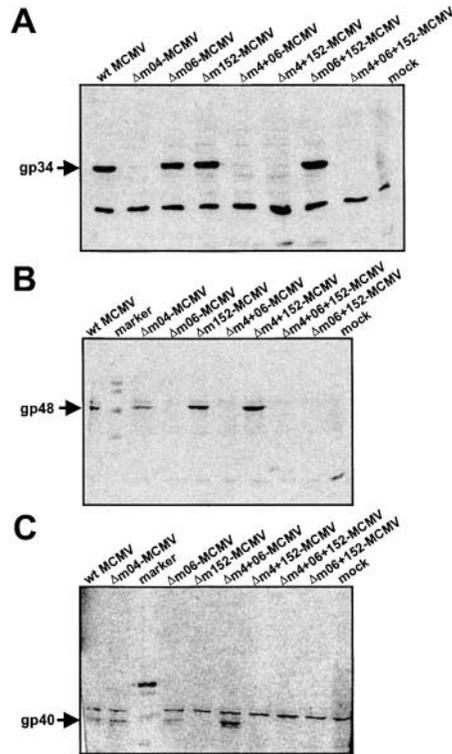
**Expression of Immunoevasins  $m04/\text{gp}34$ ,  $m06/\text{gp}48$ , and  $m152/\text{gp}40$  by MCMV Recombinants.** The MCMV BAC plasmids  $\text{p}\Delta m04$ ,  $\text{p}\Delta m06$ ,  $\text{p}\Delta m152$ ,  $\text{p}\Delta m04+m06$ ,  $\text{p}\Delta m04+m152$ ,  $\text{p}\Delta m06+m152$ , and  $\text{p}\Delta m04+m06+m152$  were transfected into permissive MEF for the reconstitution of viruses. The correct expression of  $\text{gp}34$ ,  $\text{gp}48$ , and  $\text{gp}40$  by the MCMV mutants  $\Delta m04$ -MCMV,  $\Delta m06$ -MCMV,  $\Delta m152$ -MCMV,  $\Delta m04+06$ -MCMV,  $\Delta m04+152$ -MCMV,  $\Delta m06+152$ -MCMV, and  $\Delta m04+06+152$ -MCMV, respectively, was analyzed by Western blot analysis of lysates

from NIH3T3 cells infected for 16 h. All mutants showed the expected pattern of glycoprotein expression (Fig. 2).

**Immunoevasins  $m04/\text{gp}34$ ,  $m06/\text{gp}48$ , and  $m152/\text{gp}40$  Are Collectively Dispensable for Virus Replication in Fibroblasts.** Previous work has already shown that each of the immune evasion genes  $m04$ ,  $m06$ , and  $m152$  can be deleted without affecting virus growth in fibroblast cell cultures. However, since these genes could possibly substitute each other in an essential function, deletion of all three or of combinations of two might still abolish virus growth. In addition, an impaired viral replication could result from undesired spontaneous mutations that may have accumulated elsewhere in the viral genomes during the repeated mutagenesis procedures. To explore these possibilities, we have taken multistep growth curves for wt MCMV and the complete set of mutants (Fig. 3). Specifically, NIH3T3 cells were infected at an MOI of 0.1 PFU per cell (without centrifugal enhancement), and virus titers in the supernatants were determined daily until day 5. All mutants, including the triple-deletion mutant  $\Delta m04+06+152$ -MCMV, were found to grow like wt MCMV. This finding excluded adverse effects of undesired mutations and confirmed that the three immune evasion genes are not essential for virus replication in fibroblasts in cell culture.

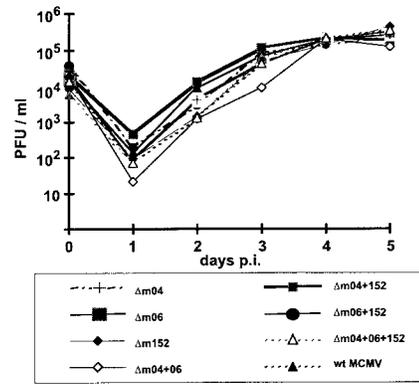


HindIII fragments of the MCMV wt (strain Smith) genome. Genes  $m04$  and  $m06$ , coding for the glycoproteins  $\text{gp}34$  and  $\text{gp}48$ , respectively, are located on fragment HindIII A. Gene  $m152$ , coding for  $\text{gp}40$ , is located on fragment HindIII E. (B1) In the first round of mutagenesis, the  $\text{kn}$  was inserted into the BAC-derived wt MCMV genome MW97.01, thereby generating the mutant genome  $\Delta m04$ -MCMV. Into this recombinant genome, the  $\text{zeo}$  gene was additionally introduced, thereby deleting gene  $m152$  and generating mutant  $\Delta m04+152$ -MCMV. Since  $\text{zeo}$  was flanked by 34-bp FRT sites (cross-hatched boxes), it could be excised by FLP-mediated recombination. This allowed again insertion of the  $\text{zeo}$  gene, this time for deletion of gene  $m06$ , generating mutant  $\Delta m04+06+152$ -MCMV. (B2) For the generation of mutant  $\Delta m152$ -MCMV, gene  $m152$  was replaced by a  $\text{zeo}$  gene that was flanked by FRT sites. After FLP-mediated excision of  $\text{zeo}$  in position of  $m152$ ,  $\text{zeo}$  was inserted once again for deletion of  $m06$ , generating mutant  $\Delta m06+152$ -MCMV. (B3) Mutant  $\Delta m04+06$ -MCMV was generated by two rounds of recombination inserting the  $\text{zeo}$  in the place of gene  $m06$  and the  $\text{kn}$  in the place of gene  $m04$ . (B4) Mutant  $\Delta m06$ -MCMV was generated by the two step replacement procedure using the shuttle plasmid  $\text{pSTm}06$ .



**Figure 2.** Expression of gp34, gp40, and gp48. NIH3T3 cells were infected with the indicated viruses for 16 h or were left uninfected (mock). The immunoevasin molecules were detected by Western blot analysis. (A) Detection of gp48 with a polyclonal antiserum confirms lack of expression in all mutants with *m06* gene deletion. (B) Detection of gp40 with a mAb confirms lack of expression in all mutants with *m152* gene deletion, and (C) detection of gp34 with a polyclonal antibody confirms lack of expression in all mutants with *m04* gene deletion. In mutants with no deletion of *m04*, *m06*, and *m152*, respectively, expression of the corresponding glycoprotein was confirmed.

*The Triple Immunoevasin Deletion Mutant  $\Delta m04+06+152$ -MCMV Has No Growth Deficiency Phenotype in Immunocompromised Mice.* So far, the data have shown that the three immune evasion genes, individually or in any possible combination, are dispensable for virus replication in fibroblast cell cultures. Yet, in vivo, MCMV replicates in a wide variety of cell types, including macrophages (46, 47), dendritic cells (48), endothelial cells, and different types of epithelial cells such as hepatocytes, pneumocytes, enterocytes, and glandular epithelial cells (49). Therefore, normal virus growth in fibroblast cell cultures did not exclude an altered cell type tropism and/or a modulated replication efficacy in host organs. If interference with antigen presentation is the only biological function of the immunoevasins, their deletion in mutant  $\Delta m04+06+152$ -MCMV should not affect in vivo replication in absence of host immune functions. For testing this prediction, we used here coinfection of severely immunocompromised mice followed by quantitative 2C-ISH analysis of virus infection of host organs to directly compare the in vivo fitnesses of wt MCMV and mutant  $\Delta m04+06+152$ -MCMV (Fig. 4). In essence, the mutant



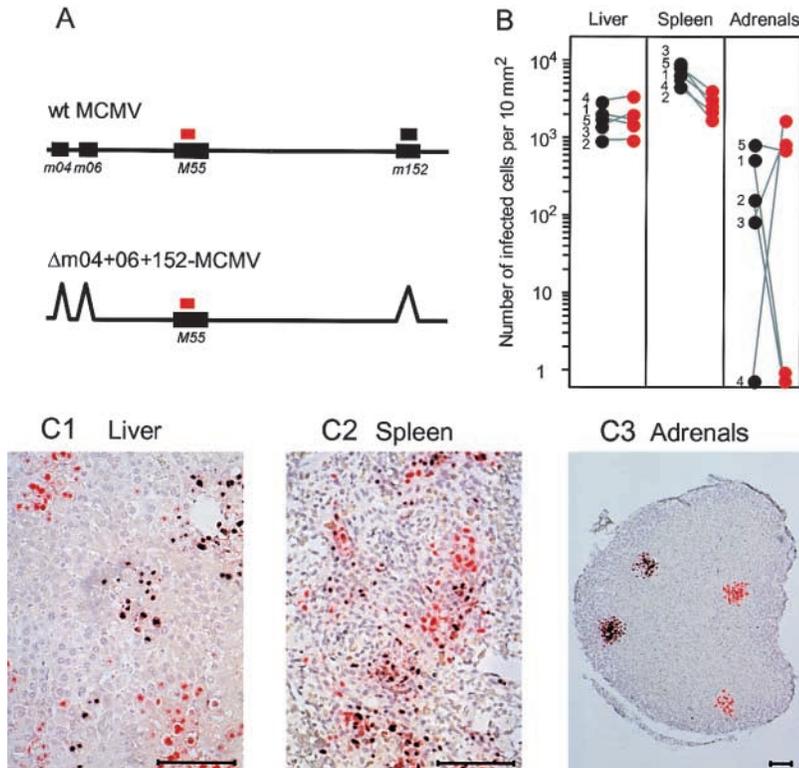
**Figure 3.** Multistep virus growth curves in cell culture. NIH3T3 cells were infected with the indicated wt and mutant viruses at an MOI of 0.1 PFU per cell and virus titers in the supernatants were detected every 24 h after infection until day 5. All seven mutants show a virus productivity comparable to wt MCMV (MW97.01).

was found to replicate at organ sites relevant to CMV disease, including liver (Fig. 4, B and C1), spleen (Fig. 4, B and C2), adrenal glands (Fig. 4, B and C3), and lungs (unpublished data). Foci of infection in tissues, each representing the clonal progeny of a single hit infection event, were either mixed black and red (representing wt MCMV) or clean-red (representing the mutant). The existence of clean-red foci proves that the mutant can replicate in the respective tissues independent of wt MCMV. The histomorphometric quantitation of infected tissue cells indicated an identical efficacy of wt and mutant virus spread in the liver and the adrenal glands, whereas a minor disadvantage of the mutant in the spleen may be attributed to some residual immunity in this lymphoid organ after the 7-Gy  $\gamma$ -irradiation. In conclusion, the data gave no indication for a nonimmunological role of the immunoevasin genes.

*Evidence Against the Expression of a Further MHC Class I Downmodulating Immunoevasin during the E Phase.* The three currently known immune evasion proteins of MCMV were identified by their interaction with MHC class I molecules. In principle, there could have existed further viral proteins performing such a function. The triple mutant  $\Delta m04+06+152$ -MCMV gave us the opportunity to address that question for the first time.

Cell surface expression of a set of MHC class I molecules specified by haplotypes H-2<sup>d</sup> and H-2<sup>b</sup> was tested by cytofluorometric analysis using the cell lines and MHC allele-specific antibodies compiled in Table I. As time point for the analysis, we chose 12 h after infection, because this is an E phase time at which all known immune evasion genes are simultaneously expressed and at which expression of other cell surface molecules, as verified for CD44 as a marker, is not yet affected by cytopathic effects of the infection (tested were 4, 7, 12, and 16 h after infection, unpublished data). An infection rate of >90% of the cells was assured by the detection of the intranuclear IE1 protein.

As it is shown in Fig. 5 A, cell surface expression of the set of MHC class I molecules of haplotypes H-2<sup>d</sup> and H-2<sup>b</sup>



**Figure 4.** Equivalent tissue tropism and comparable *in vivo* replication efficacy of wt MCMV and mutant  $\Delta m04+06+152$ -MCMV in the absence of immune control. (A) Maps (not drawn to scale) illustrating the design of probes for two-color *in situ* hybridization (2C-ISH), discriminating between wt (BAC-derived MW97.01) MCMV (mixed black & red staining) and mutant  $\Delta m04+06+152$ -MCMV (clean-red staining). (B) Comparative morphometric quantitation of infection in liver, spleen, and adrenal/suprarenal glands (per mouse, only one of the paired glands was included in the analysis) of immunodepleted (7Gy  $\gamma$ -irradiated) BALB/c recipients on day 8 after intravenous coinfection with wt MCMV and adrenal MCMV. Note that some adrenal glands were colonized clonally by either wt or mutant MCMV. Shown are linked data pairs for individual mice no. 1–5. Infected cells were counted for representative 10-mm<sup>2</sup> tissue areas, except for the adrenal glands for which the results from 2-mm<sup>2</sup> areas were extrapolated to 10 mm<sup>2</sup>. (C1–C3) 2C-IHC-stained sections of the indicated organs (derived from individual mouse no. 5), showing distinct black-red or clean-red foci of infection by wt and mutant MCMV, respectively. Counterstaining was performed with hematoxylin. Bar marker, 100  $\mu$ m.

was essentially identical for uninfected cells and cells infected with  $\Delta m04+06+152$ -MCMV. This finding allows the conclusion that there exists no further viral E gene that would significantly contribute to the downmodulation of MHC class I cell surface expression in the two MHC haplotypes tested herein. Yet, it should be emphasized that the existence of unknown functional analogs of *m04/gp34*, which interact with MHC class I molecules but do not downmodulate their cell surface expression, remains to be considered.

*The Efficacy of MHC Class I Downmodulation by the Concerted Action of All Three Immuno-evasins Shows Allele-specific Differences.* With the rationale in mind that presence and absence of all three immuno-evasins would reveal the maximal possible difference, we compared MHC class I cell surface expression after infection with wt MCMV and mutant  $\Delta m04+06+152$ -MCMV (Fig. 5 B). With the notable ex-

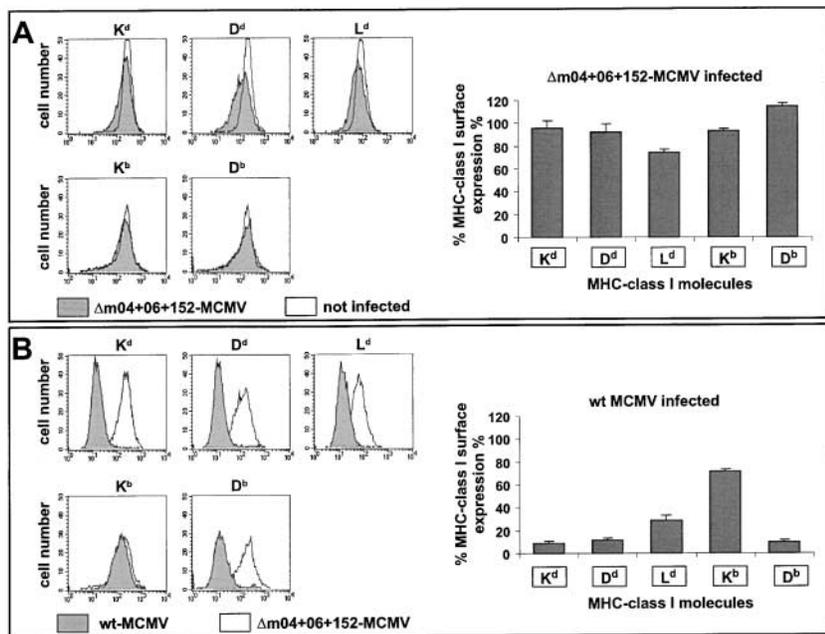
ception of  $K^b$ , the tested MHC class I molecules were significantly downregulated by the three immuno-evasins in wt MCMV. The degree of downregulation was similar for  $K^d$ ,  $D^d$ , and  $D^b$ , and a bit less for  $L^d$ . The finding that certain MHC class I molecules,  $K^b$  in the specific case, are apparently not efficiently targeted by the immuno-evasins, predicts functional, allele-specific differences in the immune control of infection.

*Combinatorial Gene Deletions Reveal Synergistic As Well As Antagonistic Interactions between the Immuno-evasins.* It is the beauty of our immuno-evasin gene deletion library of virus mutants that immune evasion proteins can now be expressed in the context of infection in any possible combination (Table II).

Fig. 6 gives a comprehensive view on the effects of immuno-evasins on the cell surface expression of all MHC class I molecules specified by haplotypes H-2<sup>d</sup> (Fig. 6 A) and H-2<sup>b</sup> (Fig. 6 B). Cytofluorometric analysis was again performed at 12 h after infection, since, at that time point, it was guaranteed that cells infected with the different viruses all expressed the corresponding immuno-evasins. The level of MHC class I surface expression observed was normalized to the expression obtained after infection with mutant  $\Delta m04+06+152$ -MCMV. As shown above (Fig. 5 A), the expression was not significantly different in uninfected cells and cells infected with  $\Delta m04+06+152$ -MCMV, but we preferred the mutant as the standard for the data normalization to account for putative general effects of the infection on MHC class I gene expression, trafficking, and metabolism.

**Table I.** Compilation of Cell Lines and Antibodies Used for the Cytofluorometric Analysis of MHC Class I Cell Surface Expression

Cell line	MHC haplotype	Class I allele	Antibody
B12	H-2 <sup>d</sup>	$K^d$	SF1.1.1
B12	H-2 <sup>d</sup>	$D^d$	34-2-12s
B12	H-2 <sup>d</sup>	$L^d$	28-14-8s
C57SV	H-2 <sup>d</sup>	$K^b$	Y-3
C57SV	H-2 <sup>d</sup>	$D^b$	B22.249



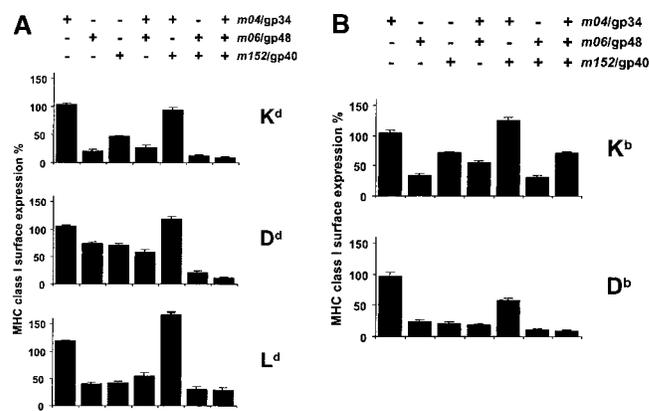
**Figure 5.** Cell surface expression of MHC class I molecules. (A) Comparison between cells infected with mutant  $\Delta m04+06+152$ -MCMV and uninfected cells. (B) Comparison between cells infected with wt MCMV (MW97.01) and cells infected with mutant  $\Delta m04+06+152$ -MCMV. The expression of MHC class I molecules of haplotypes H-2<sup>d</sup> (K<sup>d</sup>, D<sup>d</sup>, and L<sup>d</sup>) and H-2<sup>b</sup> (K<sup>b</sup> and D<sup>b</sup>) was measured by cytofluorometry at 12 h after infection (multiplicity of infection of 6) of B12 and C57SV cells, respectively. Left panels show original fluorescence histograms for one selected experiment. Right panels show data compiled from 3 to 5 independent experiments. The columns represent the mean values (standard deviations indicated by bars) of the percentage of expression relative to uninfected cells (in A) and relative to cells infected with  $\Delta m04+06+152$ -MCMV (in B).

Let us view the data in Fig. 6 from the left to the right. At a glance, isolated expression of immunoevasin *m04/gp34* by mutant  $\Delta m06+152$ -MCMV had no downmodulating effect on the cell surface expression of any of the tested MHC class I molecules. Rather, there appears to be some upregulation in the case of L<sup>d</sup>. Immunoevasins *m06/gp48* and *m152/gp40*, expressed by mutants  $\Delta m04+152$ -MCMV and  $\Delta m04+06$ -MCMV, respectively, showed comparable efficacy regarding the downmodulation of D<sup>d</sup>, L<sup>d</sup>, and D<sup>b</sup>, whereas *m06/gp48* was superior in downmodulating K locus alleles, namely K<sup>d</sup> and K<sup>b</sup>. In addition, allele-specific differences became apparent in that, for instance, D<sup>d</sup> was less affected than D<sup>b</sup>. Immunoevasin *m04/gp34*, when coexpressed with *m06/gp48* in mutant  $\Delta m152$ -MCMV, showed an indifferent role. It had no significant influence on the expression of K<sup>d</sup>, D<sup>d</sup> and D<sup>b</sup>, but

instead of supporting downmodulation it rather appeared to slightly antagonize the effect of *m06/gp48* on L<sup>d</sup> and K<sup>b</sup>. The most striking result was obtained after the coexpression of *m04/gp34* and *m152/gp40* in mutant  $\Delta m06$ -MCMV: *m04/gp34* clearly antagonized the downmodulating effect of *m152/gp40*. Remarkably, this was true for all five MHC class I molecules tested, including K<sup>d</sup>, even though *gp34* was found not to form complexes with K<sup>d</sup> at the cell surface (12). In contrast, *m06/gp48* and *m152/gp40*

**Table II.** Overview on the Expression of Immunoevasins by wt MCMV and the Set of Deletion Mutants

Virus	Immunoevasin molecule expressed		
	<i>m04/gp34</i>	<i>m06/gp48</i>	<i>m152/gp40</i>
$\Delta m04+06+152$ -MCMV	–	–	–
$\Delta m06+152$ -MCMV	+	–	–
$\Delta m04+152$ -MCMV	–	+	+
$\Delta m04+06$ -MCMV	–	–	+
$\Delta m152$ -MCMV	+	+	–
$\Delta m06$ -MCMV	+	–	+
$\Delta m04$ -MCMV	–	+	+
wt MCMV	+	+	+



**Figure 6.** Comprehensive patterns of MHC class I surface expression for all possible combinations of immunoevasins expressed by the set of immunoevasin gene deletion mutants. (A) Pattern for haplotype H-2<sup>d</sup>. (B) Pattern for haplotype H-2<sup>b</sup>. B12 and C57SV cells, respectively, were infected at an MOI of 6, and the expression of MHC class I molecules at the cell surface was determined by cytofluorometry at 12 h after infection. The columns represent the mean values (SDs indicated by bars) of the percentage of expression relative to the expression in cells infected with mutant  $\Delta m04+06+152$ -MCMV (set as 100%) from 3 to 5 independent experiments. The head legends indicate the combinations of immunoevasins expressed by the various mutants (see Table II) and (farthest right column) by BAC-cloned wt MCMV MW97.01.

gp40, coexpressed in mutant  $\Delta m04$ -MCMV, showed a cooperative function. This cooperation was particularly significant when single expression of either one was inefficient, as it was the case for D<sup>d</sup>. Finally, expression of all three molecules in wt MCMV did not further enhance the cooperative downmodulation effected by *m06/gp48* and *m152/gp40*. Instead, in the case of K<sup>b</sup>, the antagonizing function of *m04/gp34* was so strong that it partially rescued the surface expression. Thus, our previous assumption that the maximal downmodulation should be seen for wt MCMV has found an exception in K<sup>b</sup>.

In conclusion, this study has unraveled the effects of the three MCMV-encoded immunoevasins on MHC class I cell surface expression for two important, frequently studied haplotypes. The most important new finding is that the immunoevasins interact not only in a cooperative but also in a competitive fashion.

## Discussion

Both, MCMV and HCMV deploy a set of genes to alter stability and export of MHC class I molecules. These immune evasion genes are unique for each virus and have different functional properties (50). Studies on the function of individually expressed proteins have identified for each of the seven MHC reactive CMV immunoevasins the principle of the interaction with the MHC class I molecule, the intracellular compartment of activity, and the relevance of viral protein domains (for a review, see reference 51).

The fact that each of the two viruses possesses multiple genes for similar functions forces us to explain this apparent redundancy. Rapid MHC molecule degradation is the dominant phenotype of HCMV infection, whereas destabilization of nascent MHC class I complexes in the ER is definitely not the hallmark of the MCMV gene functions. The MCMV genes all affect the MHC complex export to the plasma membrane. Therefore, in MCMV-infected cells, but less so in HCMV-infected cells, the nascent MHC class I complexes could interact with more than one viral protein simultaneously or subsequently. Since the viral proteins may have allelic preferences, this could result in a complex pattern of allele-specific cooperative and competitive effects. This might lead to results completely different from what is seen when a viral protein is expressed and tested in isolation.

Experimentally, this question can only be addressed by construction and direct comparison of appropriate mutants as a set. Until recently, the ability to mutate virus genomes depended for most viruses on recombination techniques in mammalian cells, a procedure which is difficult to control. The advent of functional genomics has led to the development of cloning vectors, suitable for the maintenance of large inserts. These F-factor derived BACs fit perfectly to the insert size requirements of herpesvirus genomes (120–240 kb), and after pioneering MCMV BAC cloning by our group (18), several herpesvirus genomes have been successfully cloned as BACs. Since the large DNA inserts

in BACs preclude conventional enzymatic cleavage and ligation techniques, targeted BAC mutation in *E. coli* requires sophisticated recombination procedures (for a review, see reference 52). Here we adapted a recently developed mutagenesis approach for manipulation of conventional bacterial plasmids (45) to the viral BAC. This method uses double crossing over of a linear DNA fragment with the viral BAC mediated by the recombinases  $\text{red}\alpha$  and  $-\beta$  derived from bacteriophage  $\lambda$  (53). Compared with the conventional two-step shuttle mutagenesis, the homologies required for efficient recombination are reduced to only 25–50 nts. These short sequences can be supplied by synthetic primers. This new strategy allows repeated mutagenesis of herpesvirus genomes without any cloning and thus provided the basis for the construction of the first systematic and comprehensive set of mutants for multiple immunoevasins in the genomic context, the major intention of this study.

By testing the growth of the MCMV mutants in cell culture and in immunodepleted mice, we confirmed the immunological nature of the attenuation phenotype and the fidelity of the mutagenesis method. The *in vivo* replication is an important control, as it can reveal even subtle differences in virus fitness. According to our results, mutants that have undergone repeated mutagenesis procedures are not unspecifically attenuated and therefore can reliably be used in future *in vivo* studies on immune control and immune evasion.

Here, we focused on the hallmark of MHC class I–interfering functions, namely the surface expression of MHC class I molecules. We used cytofluorometric analysis, because this method is sufficiently standardized and reproducible to identify consistent patterns between large scale experiments performed with different cell lines. Three major findings are emerging: first, only the previously identified immunoevasins *m06/gp48* and *m152/gp40* downmodulate surface expression of MHC class I molecules after infection of cells with MCMV. Our data exclude the existence of additional unknown MCMV genes that would indirectly affect MHC molecule stability, as it is the case in HCMV for gene *US6* which inhibits transporter associated with processing-dependent peptide translocation into the ER and loading of MHC molecules (9–11).

Second, the third known immunoevasin, namely *m04/gp34*, operates in an essentially different fashion in that it does not downmodulate MHC class I cell surface expression, although it binds to MHC class I molecules in the ER as well as at the cell surface (54). This is consistent with our original hypothesis that *m04* contributes only indirectly to immune evasion, for instance through NK control (12). Since cell surface expression was the parameter tested in our study, the data cannot rule out the existence of further immunoevasins that function like *m04/gp34*. Data presented here indicate that gp34 destines MHC complexes for export to the plasma membrane. This does not yet address the functionality of the MHC molecule complexed with *m04/gp34*. One consequence of *m04* expression in cells of H-2<sup>d</sup> haplotype is the presentation of an antigenic

peptide of *m04/gp34* itself (55). Studying the recognition of MCMV-infected cells by CTL clones in the H-2<sup>b</sup> haplotype, we observed that *m04/gp34* compromises K<sup>b</sup>- but not D<sup>b</sup>-restricted CTL clones by an as yet unknown mechanism (13). Thus, although the surface export of MHC molecules is not negatively affected, the binding site for the TCR or the cargo of peptides might be different for MHC molecules escorted by *m04/gp34*.

Third, and most striking, the immunoevasins have cooperative as well as antagonistic effects with regard to surface expression of MHC molecules. Whether or not there is a hierarchy in the strength of MHC downregulation of *m06* over *m152* must await further MHC molecule testing. Clearly, *m06/gp48* and *m152/gp40* do cooperate to reduce MHC class I expression. This becomes detectable when molecules such as D<sup>d</sup> are analyzed, which are not strongly regulated by either of these two immunoevasins when expressed in isolation. Therefore, the redundancy may be helpful to effectively downregulate MHC alleles in cases in which each individual immunoevasin has only a limited effect. The moderate effect of the isolated *m152* in the genomic context comes as a surprise, given the fact that deletion of *m152* has strong functional effects on CTL recognition of antigenic peptides presented by MHC molecules such as L<sup>d</sup> (16), K<sup>b</sup>, and D<sup>b</sup> (13). The finding that *m152* does have effects on CTL recognition which do not completely parallel with its effects on the modulation of MHC expression at the plasma membrane suggests that this gene may affect CTL function through more than one mechanism. Our recent finding that *m152/gp40* has dual effects in that it, besides MHC class I downregulation, also downmodulates H-60, thereby downregulating NK cell function and possibly also accessory CTL functions, provides an explanation (56). Therefore, we have to be careful with regard to functional predictions based on the amount of MHC molecules at the cell surface. In fact, the role of *m06* is less stringently corroborated by functional testing, and future analysis of the mutants will have to show whether *m06* can control CTL recognition to the extent predicted by its effect on MHC export (14).

Remarkably, *m04/gp34* antagonizes and overrides the downmodulating effect of *m152/gp40* for all MHC class I molecules tested. As this effect is seen only after deletion of gene *m06*, a situation which is unlikely to exist in a natural MCMV genome, one apparent role of immunoevasin *m06/gp48* is to prevent an *m04/gp34*-mediated restoration of class I surface expression.

In contrast, *m04/gp34* did not antagonize the downmodulating effect *m06/gp48*. There must exist a structural basis for this difference: in accordance with the gene expression kinetics, *m06* gets the first chance to interact with MHC class I, since even for an MHC molecule such as D<sup>d</sup>, for which the downmodulation by *m06/gp48* is incomplete, the subsequent expression of the *m04* gene cannot revert the inhibition (Fig. 6). One should consider that there is stable complex formation between viral immunoevasins and MHC class I molecules only for *m04/gp34* (12) and *m06/gp48* (14) but not for *m152/gp40*. *m152/gp40* is

the earliest immunoevasin to be expressed during the viral replication cycle (15) but its interaction with MHC class I molecules is only transient. Nevertheless, after interaction with *m152/gp40* expressed in isolation, MHC molecules lose the intrinsic capacity to migrate to the plasma membrane (37). Most probably, the nascent molecules get their irreversible destination by the interaction with either *m06/gp48* or *m04/gp34*, resulting in lysosome or plasma membrane targeting, respectively, whereas the interaction with *m152/gp40* does not determine the final fate of the MHC complexes. Therefore, it is not unreasonable to speculate that in the absence of *m06/gp48* at least some of the MHC complexes that were originally retained in the ER by *m152/gp40* are eventually escorted to the cell surface by *m04/gp34*, whereas in the presence of *m06/gp48* the MHC class I traffic is preferentially directed toward lysosomal degradation.

In summary, the testing of a set of immune evasion gene mutants has disclosed functional interactions between immunoevasins. With the new mutagenesis methods, virus constructs can be generated in the near future in which by promoter exchange the expression of immunoevasins is altered from synchronous to sequential or even conditional expression patterns in order to study the temporal regulation of immunoevasin interactions. This study has already revealed that, with respect to MHC class I surface expression, the function of one immunoevasin, namely *m04/gp34*, is strongly antagonistic to *m152/gp40*, another immunoevasin encoded in a distant region of the viral genome.

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