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Selective Down-Regulation of the NKG2D Ligand H60 by Mouse Cytomegalovirus m155 Glycoprotein

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Both human and mouse cytomegaloviruses (CMVs) encode proteins that inhibit the activation of NK cells by down-regulating cellular ligands for the activating NK cell receptor NKG2D. Up to now, three ligands for the NKG2D receptor, named RAE-1, H60, and MULT-1, have been identified in mice. The resistance of mouse strains to murine CMV (MCMV) infection is determined by their ability to generate an effective NK cell response. The MCMV gene *m152*, a member of the *m145* gene family, down-regulates the expression of RAE-1 in order to avoid NK cell control in vivo. Here we report that the *m155* gene, another member of the *m145* gene family, encodes a protein that interferes with the expression of H60 on the surfaces of infected cells. Deletion of the *m155* gene leads to an only partial restoration of H60 expression on the cell surface, suggesting the involvement of another, so far unknown, viral inhibitor. In spite of this, an *m155* deletion mutant virus shows NK cell-dependent attenuation in vivo. The acquisition of endo-β-*N*-acetylglucosaminidase H resistance and the preserved half-life of H60 in MCMV-infected cells indicate that the m155-mediated effect must take place in a compartment after H60 exits from the ERGIC–*cis*-Golgi compartment.

Natural killer (NK) cells are an important defense mechanism against pathogens, particularly against viruses belonging to the herpesvirus family (45, 48). NK cell receptor genes do not undergo somatic recombination and clonal specification (38), and their activation is tightly regulated by a balance of signaling through inhibitory receptors specific for major histocompatibility complex (MHC) class I proteins and activating NK cell receptors with diverse specificities (28). Some activating NK receptors are specific for viral proteins, such as the m157 protein of murine cytomegalovirus (MCMV) and the hemagglutinins of Sendai virus and influenza virus, which are recognized by Ly49H, NKp44, and NKp46, respectively (4, 5, 31, 46).

NKG2D is a type II C-lectin-like activating NK cell receptor that was first identified as a member of the NKG2 family (21) and is expressed on all NK cells, as well as on $CD8^+$ T cells, γδ T cells, and macrophages (6, 15, 17, 22). NKG2D is a promiscuous receptor that can recognize a broad spectrum of cell surface ligands that are distantly related to MHC class I molecules and are up-regulated on stressed, infected, or transformed cells (11). The known NKG2D ligands on human cells are the MHC class I chain-related molecules (MICA and MICB) (6, 47) and the UL-16 binding proteins (ULBP-1, ULBP-2, and ULBP-3) (12), whereas the mouse NKG2D ligands are H60 (15, 30), retinoic acid early inducible gene 1 (RAE-1α, -β, -γ, -δ, and -ε isoforms) (10), and the recently identified murine UL-16 binding protein-like transcript 1 (MULT-1) (9). H60 was originally described as a minor histocompatibility antigen recognized by T cells from C57BL/6 mice in response to BALB.B splenocytes (30), and RAE-1 has been shown to play a role during embryonic development (57).

Cytomegaloviruses (CMVs) possess a remarkable range of mechanisms to escape or subvert the immune response (2). Both human CMV (HCMV) and MCMV have developed mechanisms for evading the control of NK cells by interfering with the expression of NKG2D ligands (36). The HCMV protein encoded by the *UL16* gene binds ULBP-1, ULBP-2, and MICB (12), preventing these ligands from being expressed on the surfaces of HCMV-infected cells (16, 55). Based on their early susceptibility to MCMV infection, mouse strains can be either resistant or sensitive to this virus (18, 43). In resistant mouse strains, NK cells become activated via an interaction of Ly49H, an activating NK cell receptor, with the MCMV-encoded m157 protein (3, 46). In contrast, Ly49H-negative mouse strains, including most wild mice, show very low NK activities against MCMV, rendering them susceptible to this virus (42). The puzzling fact that Ly49H-negative mice, although being capable of mounting an effective NK cell response against other pathogens (54), are unable to create effective NK cell control of MCMV, has recently been explained by the MCMV-driven down-regulation of cellular ligands for the NKG2D receptor (27). MCMV gp40, a viral glycoprotein encoded by the *m152* gene, apart from down-regulating MHC class I molecules (56), also down-modulates NKG2D ligands from the cell surface (27). The deletion of the *m152* gene results in the conversion of an NK cell-resistant virus to an NK

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cell-sensitive virus strain. A further study by Lodoen et al. (29) revealed that *m152*/gp40 down-regulates the expression of RAE-1.

For escape from NK cell control, it is perhaps not sufficient to down-regulate only one of at least three different NKG2D ligands since the remaining ligands might be sufficient to trigger NK cell activation. Therefore, we postulated that in addition to *m152*/gp40, there are other MCMV proteins that control the expression of NKG2D ligands other than RAE-1. Here we demonstrate that the *m155* MCMV gene product down-modulates the expression of the H60 protein from the surfaces of infected cells and that the deletion of the *m155* gene affects virus fitness in vivo.

MATERIALS AND METHODS

Cells. NIH 3T3 cells (ATCC CRL1658), CV-1 cells (ATCC CCL70), and the bone marrow stromal cell line M2-10B4 (ATCC CRL1972) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Mouse embryonic fibroblasts (MEFs) prepared from BALB/c, BALB/c TAP1$^{-/-}$, and BALB/c β2-microglobulin$^{-/-}$ mice were cultivated in minimum essential medium (MEM) supplemented with 3% FCS or, alternatively, in DMEM supplemented with 10% FCS.

To obtain cell transfectants, we PCR amplified the hemagglutinin (HA)-tagged H60 open reading frame (ORF) from H60/p7.5k by using the forward primer 5′-ACGCGTCGACACCATGGCAAAGGGAGCCACC-3′ and the reverse primer 5′-GTGCGGTCGACGCTCACGCGTAATCTGGAACATCGT-3′ and cloned it into the SalI restriction site of pB45-Neo, which was kindly provided by E. R. Podack (35). The plasmid was transfected into NIH 3T3 fibroblasts by use of the SuperFect transfection reagent (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. H60-transfected 3T3 cells were selected and cultured in DMEM supplemented with 10% FCS and 500 μg of G418 (Invitrogen, Paisley, Scotland)/ml.

Viruses. A bacterial artificial chromosome (BAC)-derived MCMV, MW97.01, has previously been shown to be biologically equivalent to the MCMV Smith strain (ATCC VR-194 [recently reaccessioned as VR-1399]) and is here referred to as wild-type (wt) MCMV (52). For the preparation of virus stocks, MCMV recombinants were propagated on MEFs and purified as described previously (7). Titers of virus stocks were determined by a standard plaque assay on MEFs (7). Tissue culture-grown virus preparations were used for mouse inoculations.

Site-directed mutagenesis of MCMV BAC. For the generation of Δ*m155* and Δ*m155* Δ*m157* MCMV BACs, a PCR-based mutagenesis approach was used as described previously (53). The *m155* deletions were introduced into either the pSM3fr-16F17 (referred to as the wild type) or pΔm157-16F17 (8) MCMV BAC, resulting in pΔm155 and pΔm155-m157, respectively. To delete the *m155* gene, we introduced a zeocin resistance cassette into the BACs, replacing the MCMV sequence from positions 214443 to 215531 (nucleotide positions are numbered according to reference 39). The inserted zeocin resistance cassette was amplified by use of the High Fidelity Expand PCR system (Roche Diagnostics, Mannheim, Germany), with pcDNA4TO (Invitrogen, Paisley, Scotland) as a template and with the 5′-m155 (TTTTATTAATCGACGGGAGCGGGGGGACCGGGGTG ATCATTTGTA TTCGGATCTGATCAGCACGT) and 3′-m155 (TCGTCGAAAATGTCTGTA CGAGTATGTGCTCTCCTGCTCTTGATCTAGCACGTGTCAGTCCTGC) primers. The recombinant MCMV BACs were verified by restriction analysis and DNA sequencing. MEFs were used for virus reconstitution from recombinant BACs as described previously (52). After reconstitution of the recombinant viruses, the primary stocks were passaged six times on M2-10B4 cells to remove the BAC cassette.

The genomes of deletion mutants expressing green fluorescent protein (GFP), as described in Table 1, were constructed in *Escherichia coli* strain DH10B by homologous recombination between linear DNA fragments and the MCMV BAC pSM3fr-GFP (32), exploiting the bacteriophage λ recombination genes *red*α, *red*β, and *red*γ essentially as described previously (51). Briefly, linear fragments carrying a kanamycin resistance (Kn^r) gene were generated by PCR, with either pOri6K-F5 (in the case of the BAC MCMV-GFPΔ6) or pGP704-Kan (for all other BACs) as a template. pOri6K-F5 and pGP704-Kan contain the Kn^r gene from transposon Tn*903* flanked by mutant and wild-type FLP recombinase recognition target sites (FRT), respectively (details of the construction of plasmids pOri6K-F5 and pGP704-Kan will be published elsewhere). The primers used for amplification of the Kn^r gene contained 20 to 22 nucleotides (nt) at their 3′ ends that were specific for the Kn^r template and 50 to 60 nt at their 5′ ends that were homologous to the target region in the MCMV BAC. The linear fragments were inserted by homologous recombination into the viral target sequence via the flanking 50- to 60-nt homologies, thereby replacing the respective target gene(s). DNAs from kanamycin-resistant BAC clones were tested for correct insertion by restriction analysis. The ranges of the deletions are given in Table 1. Recombinant viruses were reconstituted by transfecting DNAs from the mutated MCMV BACs into M2-10B4 or MEF cells by electroporation. Briefly, 2 to 3 μg of BAC DNA was electroporated into 10^6 cells at 250 V and 1,500 μF by use of an Easyject Optima electroporator (Peqlap, Erlangen, Germany).

TABLE 1. MCMV deletion mutants

Mutant	Deletion characteristic	
	Range[a]	ORFs
MCMV-GFP		
MCMV-GFPΔ6	203002–217799	*m144–m158*
MCMV-GFPΔ6S2	207354–212803	*m149–m153*
MCMV-GFPΔ6S3	212946–216883	*m154–m157*
MCMV-GFPΔ*m152*	210246–211376	*m152*
MCMV-GFPΔ*m153*Δ*m154*	211591–214047	*m153–m154*
MCMV-GFPΔ*m155*	214440–215476	*m155*
MCMV-GFPΔ*m156*	215628–215837	*m156*
MCMV-GFPΔ*m157*	216038–216883	*m157*
MCMV-GFPΔ6*m155*Rev[b]	203002–217799	*m144–m154* plus *m156–m158*

[a] Nucleotide positions refer to the work of Rawlinson et al. (39).
[b] MCMV-GFPΔ6*m155* carries an identical deletion as that of MCMV-GFPΔ6 and has a reinsertion of ORF *m155* under control of the HCMV major immediate-early promoter.

For reinsertion of the m155 ORF into the Δ6 MCMV genome (lacking the genes from *m144* to *m158*), a DNA fragment containing the HCMV major immediate early promoter (MIEP) was first amplified by PCR with the primers SacI-MIEP.for (5′-GAGGAGCTCCGGGGTCATTAGTTCATAGCCCA-3′) and NotI-MIEP.rev (5′-GAGGCGGCCGCCGACCGGTAGCGCTAGCGGA TC-3′) and with the plasmid pEGFP (Clontech Laboratories, Palo Alto, Calif.) as a template, treated with SacI and NotI, and cloned into the shuttle plasmid pOri6k-AL, which contains a kanamycin resistance marker, an FRT site, and the bacterial origin of replication R6Kγ (25). A DNA fragment carrying the m155 ORF was then amplified by PCR with the primers m155rei.for (5′-AAGGGAT CCGACGCGATACACGTTTGGGATAG-3′) and m155rei.rev (5′-AAGGC GGCCGCGTCGAAAATGTCTGTACGAGTATG-3′), treated with BamHI and NotI, and inserted downstream of the MIEP, resulting in the plasmid pOri6Km155MIEP.

The BAC pSM3fr-GFPΔ6.2 carries a deletion comprising nt 203002 to 217799 and ORFs m144 to m158. The Kn^r gene in pSM3fr-GFPΔ6.2 was removed by FLP-mediated recombination excision, leaving a single FRT site in the resulting BAC, pSM3fr-GFPΔ6.2-Δkan. The pOri6k-based shuttle plasmid described above was inserted into pSM3fr-GFPΔ6.2-Δkan by FLP-mediated recombination as described elsewhere (33), leading to the creation of MCMV-GFPΔ6*m155*Rev.

For the generation of the *m155*Rev revertant virus, the m155 coding sequence (nt 214434 to 215567 [numbered according to reference 39]) was first amplified from pSM3fr by use of the m155Rfor (5′-GGGGACTAGTGGGTGATCATTT GTAGACG-3′) and m155Rrev (5′-GGAAGGTACCTCTTGATCGCTTGTGC CTA-3′) primers and then subcloned into the pGPS1.1 vector (New England Biolabs) by the use of SpeI and KpnI sites, resulting in pGPS-m155. The m155 ORF, together with the adjacent kanamycin resistance cassette of pGPS-m155, was amplified by use of the 5′-m155REV (5′-GAGTGTCATAATTGTTTTAT TAATCGACGGGAGCGGGGGGACCGGGGTGACGTCAAGTCAGCGTA ATGCT-3′) and 3′-m155REV (5′-GACTTTCGTCGAAAATGTCTGTACGA GTATGTGCTCTCCTGCTCTTGATCGCTTGTGCCTA-3′) primers and the High Fidelity Expand PCR system (Roche), and this linear fragment was inserted into the Δ*m155* BAC by RecERecT (ET) recombination (53). ET recombination restored the m155 ORF and introduced a kanamycin resistance cassette between the stop codon (nt 214434 to 214436) and the predicted polyadenylation site (nt 214398 to 214404) of the *m155* gene (numbered according to reference 39). Note the difference between the MCMV-GFPΔ6*m155*Rev and *m155*Rev viruses: the first one lacks all of the genes from the *m144-m158* region, except for *m155*, whereas the latter virus has the *m155* gene orthotopically reinserted into the Δ*m155* mutant virus.

The growth kinetics in NIH 3T3 cells of all recombinant viruses used for this study were indistinguishable from those of wt MCMV.

Production of recombinant vaccinia viruses. For the generation of a recombinant vaccinia virus (VV), the H60 cDNA sequence missing its intracellular domain (GenBank accession no. AF084643) was PCR amplified without its intracellular domain from the pcDNAI H60 plasmid. Using the forward primer 5′-CGGGATCCCGAAGACCATGGCAAAGGGAGCCACC-3′ and the reverse primer 5′-GGGGTACCCCTCACGCGTAATCTGGAACATCGTATGGGTATTTTTTCTTCAGCATACACC-3′, we added the HA epitope sequence C-terminally. The PCR products were cloned into 5′ BamHI and 3′ KpnI restriction sites of plasmid p7.5K131 (44). For the creation of a recombinant vaccinia virus bearing the m155 ORF, the cDNA was HA tagged by PCR amplification from pOri6Km155MIEP with the forward primer 5′-CGTGGATCCACCATGTCTGTACGAGTATGTGCTC-3′ and the reverse primer 5′-TGAGGATCCTCAAGCGTAGTCCGGGACGTCGTACGGGTATTTGTAGACGGGCGG-3′. The m155-HA ORF was cloned into the BamHI restriction site of p7.5K131. The constructs were used for the generation of recombinant vaccinia viruses expressing H60 or m155 by homologous recombination with the vaccinia virus strain Copenhagen. Vaccinia virus recombinants were selected by infection of thymidine kinase-negative 143 cells in the presence of 100 μg of bromodeoxyuridine/ml as described previously (50).

Flow cytometry. NIH 3T3 cells were mock treated or infected with MCMV (2 PFU/cell) and then were trypsinized at 12 h postinfection. CV-1 cells were mock treated or infected with VV (multiplicity of infection of 3/cell) and then were harvested 14 h after infection by the use of 2 mM EDTA. Both cell types were washed in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin and 0.1% NaN_3 and then stained with either a phycoerythrin (PE)-NKG2D tetramer (27), the rat anti-H60 monoclonal antibody (MAb) clone 205326 (kindly provided by J. P. Houchins, R&D Systems, Minneapolis, Minn.), or the rat anti-RAE-1αβγ MAb CX1, kindly provided by L. L. Lanier (29). After a washing step, bound antibodies were visualized by the addition of PE-labeled goat anti-rat immunoglobulin G (IgG) (Caltag Laboratories, Burlingame, Calif.) or fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Sigma-Aldrich, Munich, Germany). Cells incubated with PE-streptavidin served as a negative control for cells stained with the PE-NKG2D tetramer, and a second antibody served as a negative control for cells stained with the anti-H60 and anti-RAE-1αβγ MAbs. After being stained, the cells were analyzed with a Becton Dickinson FACScan instrument and gated for propidium iodide-negative cells. The infection of H60-3T3 transfectant cells was controlled by intracellular staining with the MAb CROMA 229, which recognizes the MCMV gp48 antigen (41), and after washing, bound antibodies were visualized by the addition of FITC-labeled goat anti-mouse Ig (BD Pharmingen, San Diego, Calif.).

Metabolic labeling of cells and immunoprecipitation. Subconfluent layers of cells were labeled with [^{35}S]methionine (Amersham Pharmacia Biotech, Freiburg, Germany) at a concentration of 500 μCi/ml at 37°C for 30 min and then chased in the presence of 10 mM unlabeled methionine. After being washed with ice-cold PBS, the cells were lysed in 1 ml of lysis buffer (140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris [pH 7.6], 1 mM phenylmethylsulfonyl fluoride, 0.1 M leupeptin, 1 μM pepstatin A) containing 1% (wt/vol) digitonin (Calbiochem-Novabiochem, La Jolla, Calif.) or 1% (vol/vol) IGEPAL (Sigma-Aldrich) for 20 min and then centrifuged at 1,300 × *g* for 30 min.

The lysates were incubated for 1 h at 4°C with 0.5 μg of anti-HA (Sigma-Aldrich) or anti-L^q (28-14-8s) or 1 μg of the anti-H60 MAb. Immunoprecipitation was performed as described previously (14, 20). In brief, immune complexes were retrieved with protein A– or protein B–CL-4B Sepharose (Amersham Pharmacia Biotech) (60 μl of buffer-Sepharose slurry [1:1] for 1 h at 4°C). The Sepharose beads were washed three times with a buffer containing 0.2% (vol/vol) IGEPAL, 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, and 2 mM EDTA, twice with a buffer containing 0.2% (vol/vol) IGEPAL, 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 2 mM EDTA, and once with 10 mM Tris-HCl (pH 7.6). The immune complexes bound to Sepharose beads were resuspended in 50 mM phosphate buffer (pH 5.5) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 0.1% (vol/vol) IGEPAL, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 M 2-mercaptoethanol. For selective immunoprecipitation of cell surface proteins, cells were metabolically labeled for 120 min before being transferred to 4°C, and antibodies were added to the cell layer for 30 min. Unbound antibodies were removed by two rounds of washing with PBS. After cell lysis, the precipitation of immune complexes was performed as described above. Sepharose-bound immune complexes were mock treated or incubated with 2 mU of endoglycosidase H (endo H; Roche Diagnostics, Mannheim, Germany) at 37°C overnight. Digestion was stopped by the addition of sample buffer, and the immune complexes were eluted from Sepharose by heating at 94°C for 5 min. The precipitates were analyzed by SDS–11.5% polyacrylamide gel electrophoresis (SDS–11.5% PAGE). Dried gels were exposed to Kodak BioMax MR films for 1 to 3 days.

Animals, infection conditions, detection of infectious MCMV in tissues, and statistical evaluation. The BALB/c (*H-2^d*) and congenic BALB.B6-*Cmv1^r* (*H-2^d*) mice used for this study were housed and bred under specific-pathogen-free conditions at the Central Animal Facility of the Medical Faculty, University of Rijeka, in accordance with the guidelines contained in the *International Guiding Principles for Biomedical Research Involving Animals*. The ethical committee at the University of Rijeka approved all animal experiments described here. Six- to 8-week-old female mice were used for experiments.

Mice were injected intravenously with 4×10^5 PFU (BALB/c mice) or 5×10^5 PFU (BALB.B6-*Cmv1^r* mice) of wt MCMV or a recombinant virus in 500 μl of diluent. Organs were collected 4 days after infection, and viral titers were determined by a standard viral plaque-forming assay performed on MEFs (40). The statistical significance of differences between experimental groups was determined by the Mann-Whitney exact rank test. Viral titers (from groups *x* and *y*) were considered significantly different for *P* values (*x* versus *y*) of <0.05 (one-sided).

Depletion of NK cell subsets in vivo. The depletion of NK1.1$^-$ cells (from BALB/c mice) was performed by intraperitoneal injections of a rabbit antiserum to asialo-GM1 (Wako Chemicals, Osaka, Japan) at a dose of 25 μl 24 to 2 h before infection. The depletion of NK1.1$^+$ cells (BALB.B6-*Cmv1^r*) was done with the MAb PK136 (26) at a concentration of 1 mg/mouse inoculated intraperitoneally 24 to 2 h before infection. The efficacy of depletion was assessed by cytofluorometric analyses of spleen cells by use of a PE-conjugated MAb directed against mouse NK1.1 molecules (BD Pharmingen) and the biotin-labeled anti-mouse pan-NK cell MAb DX5 (BD Pharmingen).

RESULTS

MCMV genes in addition to *m152* regulate the expression of NKG2D ligands. MCMV gp40, a viral glycoprotein encoded by the *m152* gene, down-modulates the expression of RAE-1 on MCMV-infected cells and thus modulates recognition and virus control by NK cells (27, 29). Considering the facts that MCMV-infected cells show a complete absence of NKG2D ligands on the plasma membrane, as shown by staining with an NKG2D tetramer, and that gp40 down-regulates RAE-1 but not H60 (29), we surmised that there must exist an MCMV gene(s) which down-regulates H60. To study the effect of MCMV on H60, we chose a cell line that constitutively expresses H60 on the cell surface and which is permissive for MCMV. Using H60-specific monoclonal antibodies, we screened several cell lines and selected NIH 3T3 cells for further studies (data not shown). To discriminate infected from uninfected cells, we took advantage of a recombinant MCMV expressing GFP (32) by gating GFP-positive cells in flow cytometry analyses. The infection of NIH 3T3 cells with the MCMV-GFP virus resulted in a strong down-modulation of NKG2D ligands from the surfaces of infected cells compared to those on uninfected NIH 3T3 cells (Fig. 1A). Identical results were also observed with wt MCMV, which does not express GFP (data not shown). Cells infected with MCMV-GFPΔ6, a mutant that lacks the genes *m144* to *m158*, however, remained positive for NKG2D staining. Because MCMV-GFPΔ6 also lacks the *m152* gene, the lack of down-modulation of NKG2D ligands could be assigned to the expression of RAE-1 molecules on the surfaces of infected cells. To separate the role of *m152* and to narrow down the genomic region encoding new NKG2D ligand regulators, we tested the mutants MCMV-GFPΔ6S2 and MCMV-GFPΔ6S3, characterized by deletions of *m149* to *m153* and of *m154* to *m157*, respectively (Table 1). The results showed that neither of these two mutants was able to down-regulate NKG2D ligands to the level of MCMV-GFP, although the intensity of NKG2D staining of

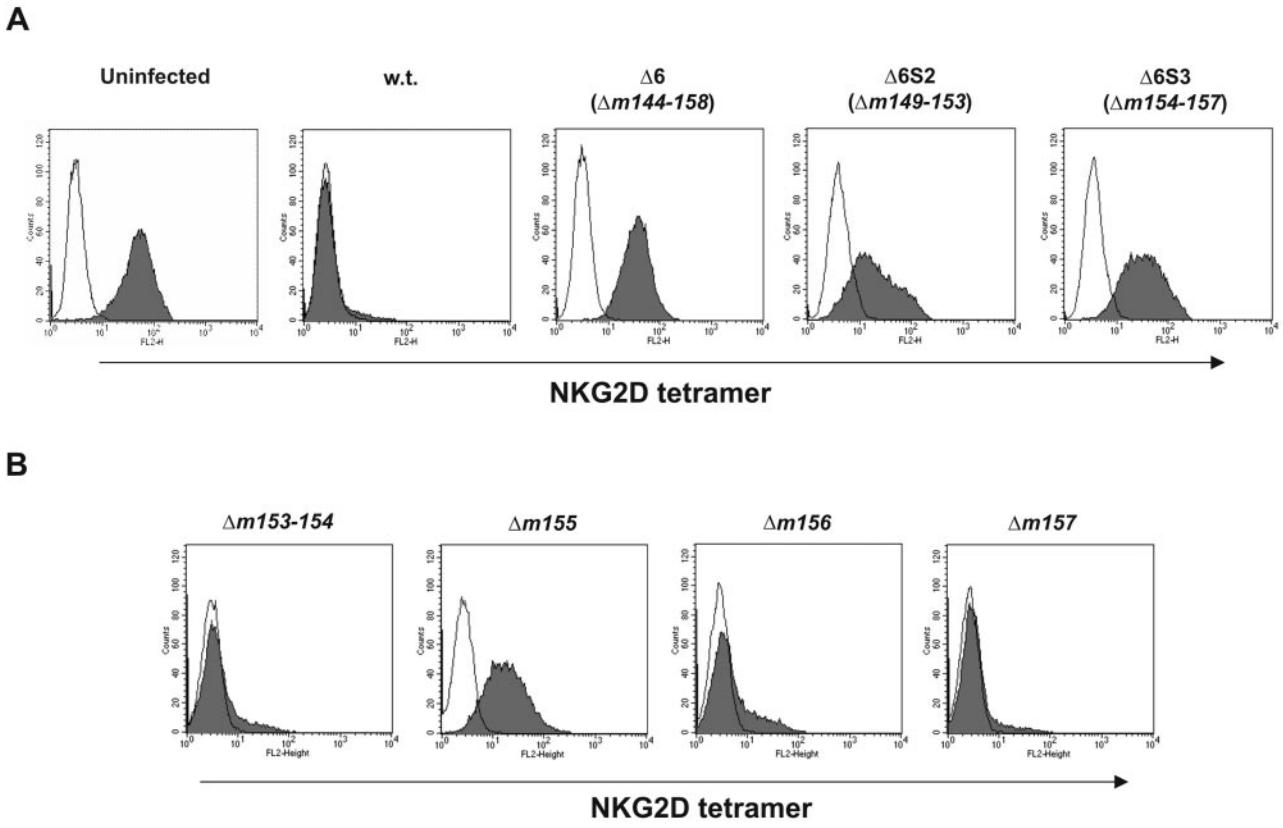

FIG. 1. The m155 protein is involved in down-regulation of NKG2D ligands. NIH 3T3 cells were infected with 2 PFU of the indicated GFP-positive viruses per cell or were left uninfected. Twelve hours after infection, the cells were collected and analyzed for the expression of surface NKG2D ligands by staining with the PE-NKG2D tetramer. Cells incubated with streptavidin-PE were used as a negative control (thin line). Each histogram represents 10,000 gated propidium iodide-negative, GFP-negative (uninfected), or GFP-positive (infected) cells. (A) Regional mutants. (B) Single mutants.

cells infected with these viruses was not the same as that observed for cells infected with the MCMV-GFPΔ6 virus (Fig. 1A). We concluded that *m152* cannot be the only MCMV gene in the region defined by the mutant MCMV-GFPΔ6 which is involved in the down-regulation of NKG2D ligands and that at least one additional gene involved in down-modulation of these ligands should be located within the *m154-m157* region.

The *m155* gene product down-regulates H60. Mutants lacking single ORFs in the *m154-m157* region were generated (Table 1) and tested for the capacity to down-modulate NKG2D ligands. As shown in Fig. 1B, all of the tested mutants still down-modulated the expression of NKG2D ligands, with the exception of MCMV-GFPΔ*m155*, which showed an NKG2D staining pattern similar to that of MCMV-GFPΔ6S3. To characterize the NKG2D ligand(s) regulated by the *m155* gene product, we directly compared the cell surface expression levels of RAE-1α, -β, and -γ and of H60 in cells infected with wild-type and mutant virus strains (Fig. 2). In accordance with previously published results (29), MCMV-GFPΔ6 and MCMV-GFPΔ*m152* did not affect the surface expression of RAE-1 in these cells (Fig. 2). In contrast, MCMV-GFPΔ6S3 and MCMV-GFPΔ*m155* still down-modulated RAE-1 expression, indicating that the *m155* product does not affect this ligand. Staining with an anti-H60 MAb revealed that the down-modulation of the expression of H60 by MCMV (Fig. 2 and 3) is strong, although not complete, as one would have predicted based on the staining with the NKG2D tetramer (Fig. 1). Infection with either MCMV-GFPΔ6 or MCMV-GFPΔ6S3 partially reconstituted the surface expression of H60. Importantly, a single deletion mutant demonstrated that *m155* gene expression is required for the down-modulation of H60 from the cell surface (Fig. 3). The reintroduction of the *m155* gene into the genome of a mutant with a larger deletion (MCMV-GFPΔ6) yielded MCMV-GFPΔ6*m155*Rev and served to confirm that the m155 protein interacts with H60 cell expression. MCMV-GFPΔ6*m155*Rev lacks all of the genes from the *m144-m158* region, with the exception of *m155*. In cells infected with this mutant, H60 was again down-modulated to the level observed for MCMV-GFP-infected cells. However, the results repeatedly showed that the expression level of H60 on cells infected with either the MCMV-GFPΔ6 or MCMV-GFPΔ*m155* virus was significantly lower than that in uninfected cells. This indicates that another viral function is also involved in the down-regulation of H60.

H60 is an MHC class I-like protein which matures independent of TAP and β2m. The H60 sequence predicts a type I transmembrane glycoprotein of 335 amino acids comprising a signal sequence, a luminal domain, a transmembrane domain, and a cytosolic domain (30). The ectodomain includes seven potential N-linked glycosylation sites. To date, studies on the

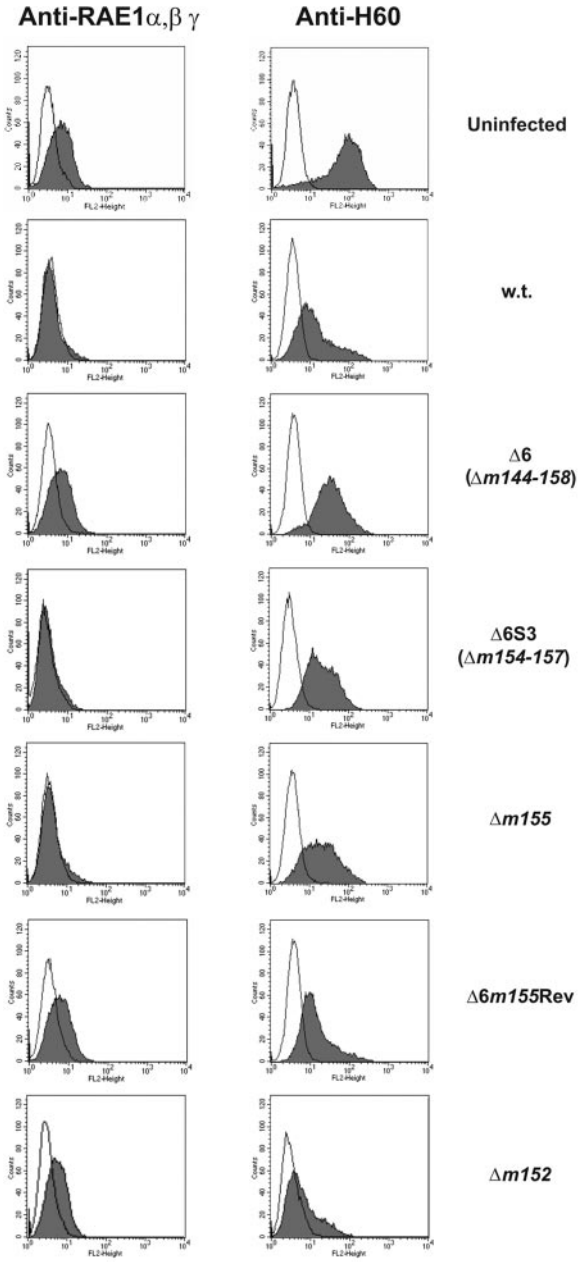

FIG. 2. The m155 protein is responsible for down-regulation of H60. NIH 3T3 cells were infected with 2 PFU of GFP-positive viruses per cell or were left uninfected. Twelve hours after infection, the cells were collected and stained with an anti-RAE-1αβγ or anti-H60 MAb, followed by PE-conjugated goat anti-rat IgG. Cells incubated with the secondary antibody in the absence of the primary antibody were used as a negative control (thin line). Each histogram represents 10,000 gated propidium iodide-negative, GFP-negative (uninfected), or GFP-positive (infected) cells.

maturation and posttranslational modifications of H60 are still lacking (30). In order to monitor the maturation of the protein, we constructed a recombinant vaccinia virus expressing HA epitope-tagged H60 (H60-VV). After 8 h of infection with H60-VV, NIH 3T3 cells were metabolically labeled for 30 min with [^{35}S]methionine, and the label was then chased for 30 min, 1 h, and 8 h. H60 was immunoprecipitated from lysates by the use of anti-HA antibodies coupled to protein A-Sepharose. The immunoprecipitated molecules were mock treated or incubated with endo H and then separated by SDS-PAGE. Wild-type vaccinia virus (wt VV)-infected cells were used as a negative control. As shown in Fig. 3A, 46- and 70-kDa bands were detected in pulse-labeled samples. The treatment of immune complexes with endo H, which cleaves high-mannose N-linked glycans that have not been processed into complex glycans by enzymes resident in the medial Golgi, resulted in a shift of the 46-kDa protein to a band of approximately 27 kDa. The size of the deglycosylated H60 protein was in accordance with its published sequence (30). Most of the newly synthesized H60 molecules were readily processed into the endo H-resistant form of about 70 kDa within 30 min of the chase. A substantial loss of the 70-kDa band occurred within 8 h of the chase. A half-life of approximately 4 to 8 h was calculated by densitometry of the bands obtained in three independent experiments, including the one shown in Fig. 3A. N-linked glycosylation of all seven predicted sites in the H60 glycoprotein was observed by partial digestion of the immunoprecipitated H60 with endo H (data not shown).

Considering that H60 is a nonpolymorphic MHC class I-like glycoprotein, we studied H60 biogenesis, processing, and transport in transporter associated with antigen processing 1 (TAP-1)- and β2-microglobulin (β2m)-deficient MEFs after its expression by H60-VV. The data showed a maturation pattern identical to the one obtained for NIH 3T3 cells, indicating that H60 matures in a TAP- and β2m-independent manner (data not shown).

Maturation of H60 in MCMV-infected cells. To study the maturation of H60 upon MCMV infection, we generated a stable NIH 3T3 H60 transfectant. H60-3T3 cells were mock treated or infected with either wt MCMV or the Δ*m155* deletion mutant and analyzed by immunoprecipitation 14 h after infection (Fig. 3B). Immunoprecipitation of pulse-labeled L^q molecules was performed as a control (Fig. 3C). The maturation pattern of H60 in transfectants (data not shown) was comparable to that in H60-VV-infected cells (Fig. 3A). A comparison between wt MCMV-infected, Δ*m155* MCMV-infected, and uninfected H60-3T3 cells revealed no difference regarding the stability of the H60 protein or its ability to reach the endo H-resistant form. In contrast, the virus caused a retention of MHC class I L^q molecules in an endo H-sensitive form upon both wt MCMV and Δ*m155* MCMV infection (Fig. 3C). Thus, the *m155* product alters neither the maturation of H60 prior to its transit through the ER-Golgi intermediate compartment (ERGIC)–*cis*-Golgi compartment nor the half-life of the endo H-resistant form of H60.

To exclude the possibility that m155 has no effect on H60 expressed by H60-3T3 cells, we performed a fluorescence-activated cell sorting analysis of the MCMV-infected H60-3T3 cells. In agreement with the results presented in Fig. 2, the surface expression of H60 was significantly reduced in MCMV-infected cells (Fig. 3D). Intracellular staining with anti-HA antibodies, however, revealed that HA-tagged H60 was detectable in MCMV-infected H60-3T3 cells, supporting the data obtained by immunoprecipitation.

Although we cannot completely rule out the possibility that H60 molecules derived from a few residual uninfected cells contribute to the background for immunoprecipitation, we as-

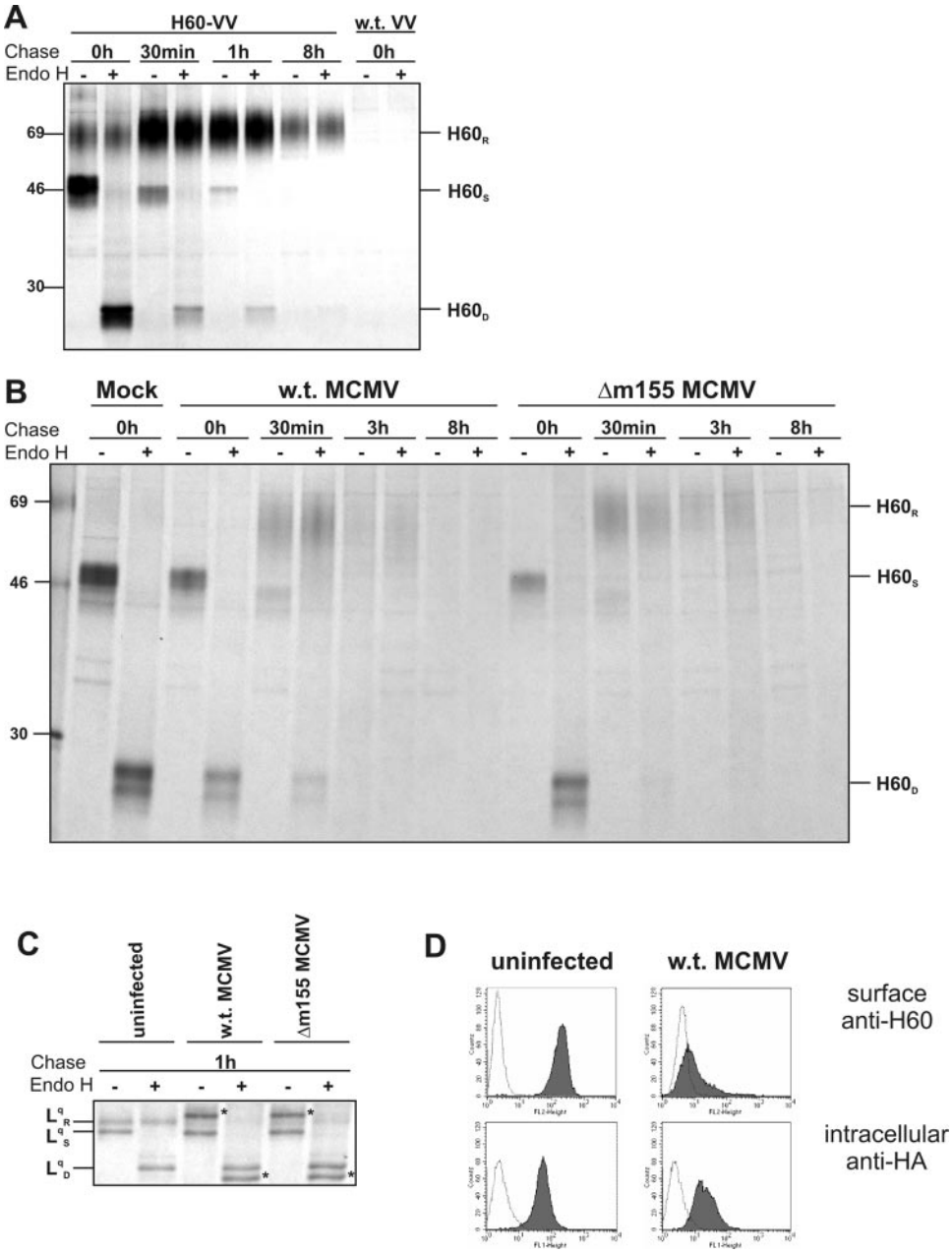

FIG. 3. MCMV does not affect maturation of the H60 protein. (A) NIH 3T3 cells were infected with H60-VV and metabolically labeled at 8 h p.i. with [^{35}S]methionine. The samples were chased in the presence of unlabeled methionine as indicated, lysed in 1% IGEPAL buffer, and immunoprecipitated with anti-HA antibodies coupled to protein A-Sepharose. Prior to separation by SDS–11.5% PAGE, the lysates were mock treated (−) or digested with endo H (+). wt VV-infected cells were used as a negative control. (B and C) H60–3T3 cells were mock treated or infected with 2 PFU per cell of either wt or Δ*m155* MCMV. At 14 h p.i., the samples were subjected to pulse-chase labeling with [^{35}S]methionine as indicated. Immunoprecipitation was performed with anti-HA (B) or anti-L^q (C) antibodies bound to protein A-Sepharose. Prior to SDS-PAGE, the lysates were mock treated (−) or digested with endo H (+). R, resistant to endo H; S, sensitive to endo H; D, digested with endo H; *, Gp48$_S$ and gp48$_D$ MCMV *m06*/gp48 protein coprecipitating with L^q (41). (D) H60-3T3 cells were infected with wt MCMV or not infected and then analyzed at 18 h p.i. for the expression of surface and intracellular H60 by the use of anti-H60 and anti-HA MAbs, respectively. Each histogram represents 10,000 GFP-negative (uninfected cells) or GFP-positive (infected cells) cells. Cells incubated with the secondary antibody in the absence of the primary antibody were used as a negative control (thin line).

sume that if m155 caused a degradation of the molecule, the quantity of the remained band would be significantly lower than that in uninfected cells. It is worth noting that the radioactivity was quantified and equalized in all of the samples prior to their being loaded into gels. Altogether, the intracellular staining of HA-tagged H60 and its immunoprecipitation strongly suggest against a significant influence of m155 on the stability of the H60 protein.

In an additional attempt to monitor the fate of the H60 protein in infected cells, we performed a selective immunopre-

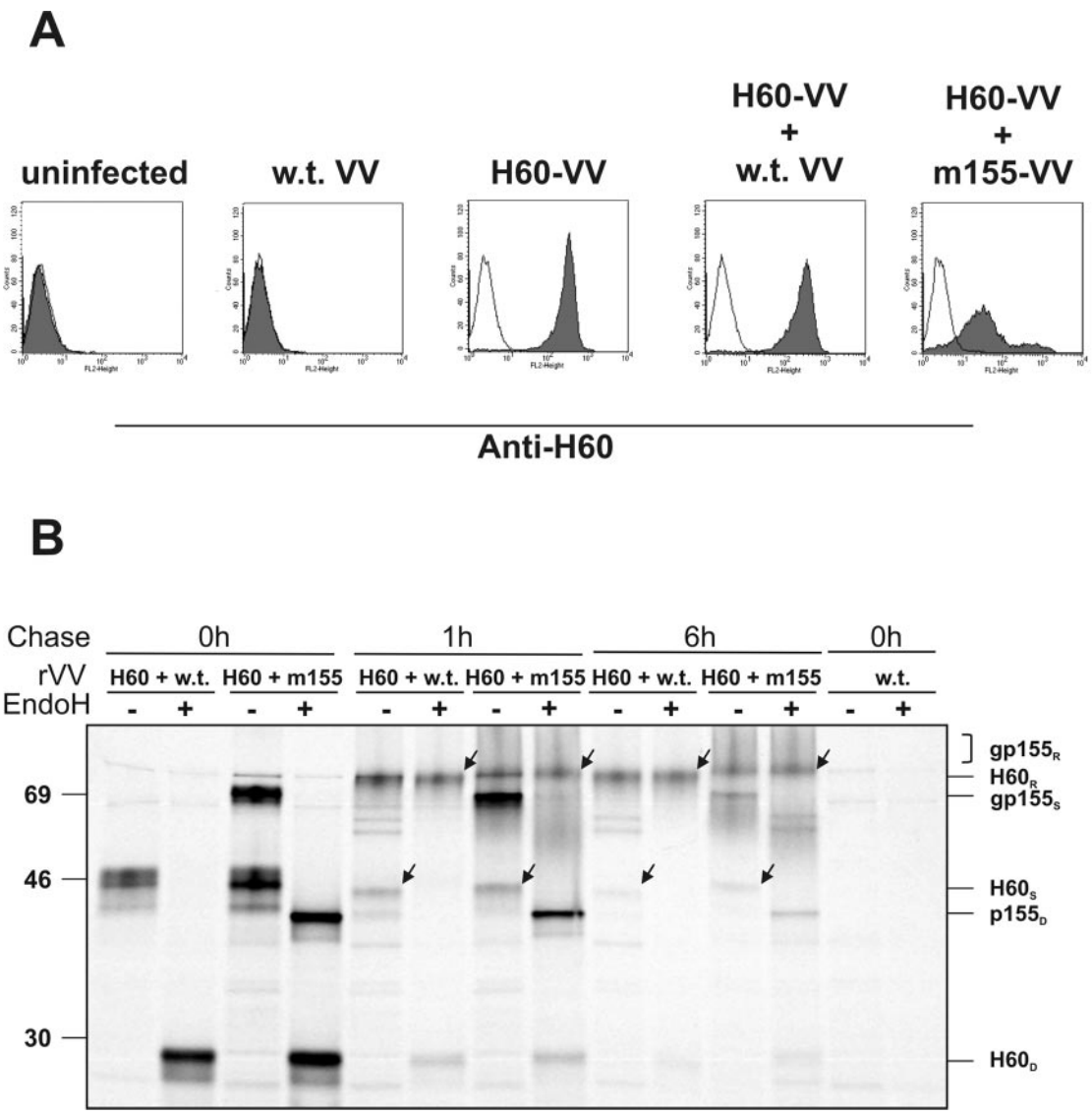

FIG. 4. Down-regulation of H60 by coinfecting CV-1 cells with m155-VV and H60-VV. CV-1 cells were infected with H60-VV or wt VV or coinfected with H60-VV and wt VV or with H60-VV and m155-VV and then were analyzed at 12 h p.i. (A) Cells were collected and stained with an anti-H60 MAb followed by FITC-conjugated anti-rat IgG. Cells incubated with the secondary antibody in the absence of the primary antibody were used as a negative control (thin line). Each histogram represents 10,000 gated propidium iodide-negative, GFP-positive cells. (B) Cells were metabolically labeled for 30 min and chased for the indicated times, and the lysates were immunoprecipitated with anti-HA antibodies coupled to protein A-Sepharose. Prior to separation by SDS–11.5% PAGE, the samples were mock treated (−) or digested with endo H (+). R, resistant to endo H; S, sensitive to endo H; D, digested with endo H. Arrows indicate H60 shifts in the presence of m155.

cipitation of surface-resident H60 molecules from H60-3T3 cells that were metabolically labeled for 120 min starting at 12 h postinfection (p.i.). The complete absence of membrane-exposed H60 molecules in wt MCMV-infected H60-3T3 cells, but not in uninfected and Δ*m155* MCMV-infected cells, suggests a rapid effect of m155 on newly synthesized surface-destined H60 molecules that do not reach the plasma membrane (data not shown). Immunoprecipitation of H60 from the supernatants of MCMV-infected cells did not detect any H60 protein (data not shown). Taken together, these results reveal that the loss of H60 from the surfaces of infected cells is not due to shedding of the H60 glycoprotein from the cell surfaces of MCMV-infected cells but to an altered intracellular distribution of H60.

To assess the effect of the isolated *m155* gene on H60 expression, we constructed a recombinant vaccinia virus expressing HA epitope-tagged m155 (m155-VV) and coinfected CV-1 cells either with H60-VV and wt VV or with H60-VV and m155-VV. Infection with H60-VV resulted in a high level of H60 expression on the cell surface (Fig. 4A). The surface expression was not altered upon coinfection with wt VV but was significantly lower upon coinfection with m155-VV. Neither uninfected nor wt VV-infected CV-1 cells showed any membrane H60 staining. These data clearly show that *m155* gene expression is sufficient for the down-regulation of H60 from the cell surface. It is worth noting that the down-modulation of H60 was not complete, suggesting that there is a threshold limit for the cellular mechanism involved.

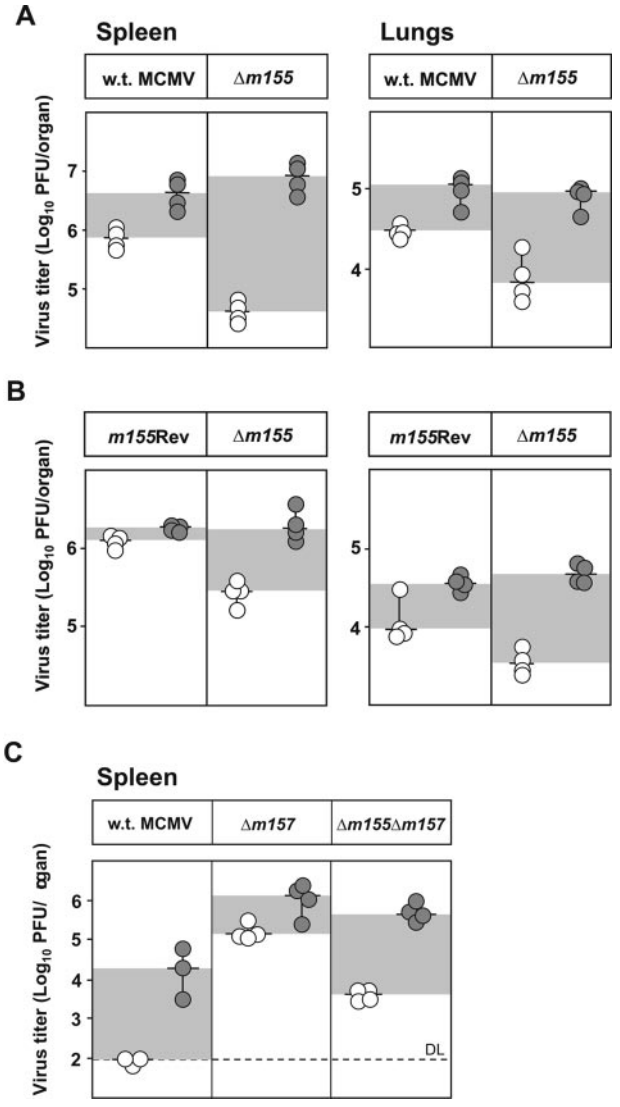

FIG. 5. Deletion of the *m155* gene sensitizes MCMV to NK cell-mediated control. NK cell-depleted or undepleted BALB/c (A and B) and BALB.B6-*Cmv1*^{r} (C) mice were injected intravenously with 4×10^5 or 5×10^5 PFU, respectively, of the indicated viruses. The mice were sacrificed 4 days after infection, and the viral titers in the organs were determined. Titers in organs of individual mice are shown; horizontal bars indicate the median values. The differences in viral titers between NK-depleted and undepleted groups of mice are indicated by shaded areas. Open circles, no depletion; gray circles, NK cell depletion; DL, detection limit.

In order to biochemically monitor the maturation pathways of both the m155 and H60 proteins, we coinfected CV-1 cells with H60-VV and wt VV or with H60-VV and m155-VV. Both proteins were immunoprecipitated from cell lysates by the use of anti-HA antibodies coupled to protein A-Sepharose 14 h after infection (Fig. 4B). Infection with wt VV was used as a negative control. The data showed the simultaneous conversion of endo H-sensitive to -resistant forms of H60 (70 and 46 kDa, respectively) and the m155 glycoprotein (85 to 100 and 70 kDa, respectively). In accordance with the results obtained upon infection of H60-3T3 cells with wt MCMV (Fig. 3B), m155-VV neither caused any change in the half-life of the H60 glycoprotein nor affected its ability to reach the endo H-resistant form. Remarkably, there was a slight shift in the migration properties of the glycosylated H60 protein in the presence of m155 (Fig. 4B, arrows), consistent with an altered posttranslational modification of H60.

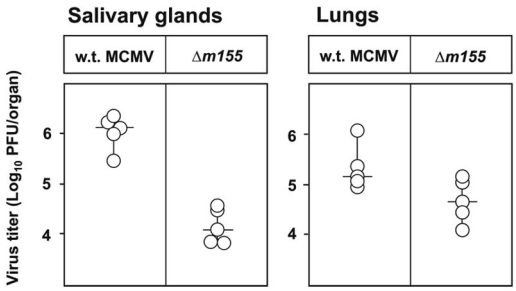

FIG. 6. The Δ*m155* virus is attenuated in salivary glands. BALB/c mice were injected intravenously with 4×10^5 PFU of the indicated viruses. The mice were sacrificed 12 days after infection, and the viral titers in salivary glands and lungs were determined. Titers in organs of individual mice are shown; horizontal bars indicate the median values.

***m155* contributes to MCMV virulence by compromising NK cell-mediated control.** We next studied whether the absence of *m155* affects virus control by NK cells in vivo. We expected the deletion of the *m155* gene to result in viral sensitivity to NK cells in vivo. In order to avoid an effect of GFP expression on the immune response to MCMV, we constructed a single *m155* deletion mutant and a double *m155 m157* deletion mutant without GFP. BALB/c mice were depleted of NK cells or left untreated, and virus titers were determined by a plaque assay 4 days after infection. The Δ*m155* virus was indeed attenuated compared to wt MCMV, and this attenuation was reversed by the depletion of NK cells (Fig. 5A). A confirmation of the specificity of the effect of m155 came from an experiment in which the *m155* gene was reintroduced into the Δ*m155* genome (*m155*Rev). Reinsertion of the *m155* gene resulted in *m155*Rev virus resistance to NK cell control in vivo (Fig. 5B). Next, we tested the Δ*m155* virus in BALB.B6-*Cmv1*^{r} mice, which express the natural killer gene complex of C57BL/6 mice in the BALB/c genetic background (Fig. 5C). Since these mice are positive for Ly49H, the role of *m155* could only be tested in a virus lacking *m157* to avoid natural killer gene complex activation via Ly49H. In accordance with our recent results (8), the deletion of the *m157* gene resulted in a gain of virulence and in resistance to NK cell control. Furthermore, the results revealed that NK cell activation via the NKG2D receptor is blocked as well. As expected, the deletion of the *m155* gene led to sensitivity of the Δ*m155* Δ*m157* mutant to NK cell-mediated control. Like the case for BALB/c mice, this attenuation could be reversed by the depletion of NK cells. Interestingly, 2 weeks after infection, the virus titers in the salivary glands of BALB/c mice infected with the Δ*m155* virus were significantly lower than the titers after infection with the control virus (Fig. 6). Given that virus control in the salivary glands is $CD8^+$ T cell independent (23), one possibility for these results is that the attenuation of virus growth in salivary glands is related to the enhanced virus sensitivity to NK cells.

DISCUSSION

In this article, we have reported data for *m155*, a member of the MCMV *m145* gene family which encodes a protein that modulates the expression of H60, one of the three cellular ligands for murine NKG2D that have been described so far. NKG2D is an activating receptor expressed on all NK cells, $CD8^+$ T lymphocytes, and γδ T cells (6, 15, 17, 22). Disruption of the *m155* gene leads to an enhanced antiviral NK cell response and an attenuation of virus growth in vivo. Thus, similar to *m152*/gp40, which regulates MHC class I alleles and RAE-1 family members, the role of the m155 protein seems to be to prevent NK cell activation during infection. Since the expression of neither RAE-1 nor MULT-1 (S. Jonjic, unpublished data) is affected by m155, it appears that MCMV devotes specialized proteins to the control of each of the NKG2D ligands. By using recombinant vaccinia viruses that express H60 or m155, we were able to demonstrate that m155 does not require the presence of other MCMV proteins to down-modulate surface H60. However, our results showed that m155 is not the only gene responsible for the down-regulation of surface H60: the fact that the down-modulation of this protein was observed in cells infected with the Δ*m155* mutant points to the existence of another viral function that regulates H60. Nevertheless, the functional significance of this second viral function in vivo is questionable since it cannot override the successful NK cell control of the Δ*m155* virus. The in vivo results are consistent with the recent finding that an MCMV possessing a transposon insertion at the *m155* ORF showed a growth deficit and attenuation in SCID mice (1).

Our data show that neither the ability of H60 to reach the endo H-resistant form nor its stability is altered upon MCMV infection. Therefore, the failure of H60 to reach the cell surface is due to a mechanism that operates beyond the ERGIC–*cis*-Golgi compartment rather than to an alteration in the kinetics of internalization after it reaches the cell surface. Although it is unlikely, it even remains possible that H60 accumulates at the cell surface in an altered conformation or in association with another protein that masks both its function and its detection by specific MAbs and NKG2D tetramer. This is not without precedent and reflects the example we described for *m04*/gp34 binding to MHC class I molecules (24). Clearly, the remarkable reduction of membrane expression in spite of the apparent maturation of the glycoprotein requires further experimentation aiming at the identification of the compartment to which H60 is targeted in the presence of m155.

The interaction of NKG2D with its cognate ligands on infected cells elicits a potent NK cell response (37). Despite possessing a functional NKG2D receptor, Ly49H-negative mice, including most wild mice, elicit only a weak NK cell response toward MCMV (13, 42, 49) and are therefore quite susceptible to this virus compared to resistant mouse strains possessing Ly49H, an activating NK cell receptor that recognizes the MCMV-encoded protein m157 (4, 46). We have recently shown that Ly49H-negative mice are constitutively able to mount an effective NK cell response to MCMV but that this activity is prevented by MCMV interference with the expression of NKG2D ligands (27). The gp40 protein encoded by the *m152* MCMV gene down-modulates the expression of RAE-1 (29). Consequently, the deletion of the *m152* gene results in the expression of RAE-1 and in viral sensitivity to NK cells in vivo. Both the *m152* and *m155* genes belong to the MCMV *m145* gene family, which has 11 members, some of which have immunomodulatory functions (e.g., *m152* and *m157)* (39, 46). Notably, we also described the *m152* product as a protein that prevents the presentation of viral peptides to $CD8^+$ T cells by retaining MHC class I molecules in an unknown compartment with ERGIC–*cis*-Golgi properties (56).

NKG2D ligand expression is restricted in normal cells and tissues to avoid becoming a target for NK cells. Transformation, infection, and stress lead to the induction of these proteins on the cell surface and thereby induce NK cell activation (11). It is not surprising that viruses have evolved mechanisms to evade NK cells by down-modulating ligands for the NKG2D receptor. Although MCMV induces the transcription of *RAE-1* genes, the translocation of the protein to the cell surface is prevented by gp40 (29). One would expect MCMV to have evolved strategies to down-modulate those immune receptor ligands whose genes are induced by infection. MCMV does not induce *H60* gene expression (29), but nevertheless, the protein is down-modulated from the surfaces of infected cells. Perhaps MCMV infection does induce H60 expression in vivo in other cell types that have not been tested so far. Note that H60 mRNA is expressed in resting splenic cells and dendritic cells as well as in activated splenocytes (30), which are potential targets for infection by MCMV. Another possibility is that the basal level of H60 expression is sufficient and perhaps necessary for the fine-tuning of NK cell activation. MCMV down-modulates MHC class I molecules on infected cells to escape cytotoxic CD8-T-cell lysis. This may already suffice as an NK activating signal. Therefore, if the down-modulation of MHC class I molecules skews the balance of NK-interacting proteins displayed on the surface of an infected cell towards the activation of NK cells, an obvious viral countermeasure would be to compromise the activation of NK cell receptors by down-modulating their ligands. This scenario is in fact supported by the findings presented here. Because the NKG2D receptor is also expressed on T cells and macrophages, it is also possible that interference with the function of NKG2D ligands represents an efficient and perhaps essential requirement for virus-mediated attenuation of both innate and specific immune responses relevant to the establishment of life-long infections and/or successful virus transmission.

What is the reason for the existence of more than one ligand for the NKG2D receptor? One possibility to account for this observation is the differential activation of these ligands between cells and tissues (11). Hamerman et al. have recently shown that signaling through Toll-like receptors in macrophages induces the transcription of RAE-1 but not of H60 and MULT-1 genes (19). Furthermore, different ligands may be differentially expressed during tissue and cell development and may have distinguishable biological functions. Although the effects of MCMV immune evasion mechanisms on NK cell activity have been well documented in vitro and in vivo, the importance of these virus-encoded functions in tissue-specific pathology is still unknown. The virus-encoded immune evasion functions that promote persistence may represent a viral virulence factor, particularly in an organ with a limited capacity for self-renewal. One observation that is relevant to this point is the recently described in vivo phenotype of the Ly49H NK

receptor and its ligand MCMV m157, which manifest in the spleen and the lungs but not in the liver (8). Furthermore, the role of viral evasion genes in modulating the expression of NK cell ligands such as RAE-1, H60, and MULT-1 in developing tissues like the central nervous system (CNS) is unknown. Ligands for NK cells such as RAE-1 can be detected in the embryonic CNS but appear to be down-regulated in cells in the adult CNS and other adult organs (10, 15, 34). It remains to be determined whether cytomegaloviruses use immunoevasion genes to explicitly limit immunopathology in vital tissues in order to preserve the vital functions of their specific hosts.

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ADDENDUM IN PROOF

Another paper presenting the evidence for m155-mediated regulation of H60 was recently published (M B. Lodoen, G. Abenes, S. Umamoto, J. P. Houchins, F. Liu, and L. L. Lanier, J. Exp. Med. **200:**1075–1081). Our work showing the effect of the *m145* gene on the expression of the third known mouse NKG2D ligand, MULT-1, was recently published (A. Krmpotic, M. Hasan, A. Loewendorf, T. Saulig, A Halenius, T. Lenac, B. Polic, I. Bubic, A. Kriegeskorte, E. Pernjak-Pugel, M. Messerle, H. Hengel, D. H. Busch, U. H. Koszinowski, and S. Jonjic, J. Exp. Med. **201:**211–220).

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