Identification of the Mouse Cytomegalovirus Genomic Region Affecting Major Histocompatibility Complex Class I Molecule Transport

REGINE THÄLE,¹ UWE SZEPAK,¹ HARTMUT HENGEL,¹ GERNOT GEGINAT,¹
PERO LUCIN,² AND ULRICH H. KOSZINOWSKI^{1*}

Department of Virology, Institute for Hygiene, University of Heidelberg, 69120 Heidelberg, Germany,¹ and Department of Physiology and Immunology, Faculty of Medicine, University of Rijeka, 51000 Rijeka, Croatia²

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Mouse cytomegalovirus (MCMV) functions expressed at the beginning of the early phase of the viral replication cycle interfere with the major histocompatibility complex (MHC) class I-restricted pathway of antigen presentation (M. J. Reddehase, M. R. Fibi, G. M. Keil, and U. H. Koszinowski, *J. Virol.* 60:1125–1129, 1986; M. Del Val, K. Münch, M. J. Reddehase, and U. H. Koszinowski, *Cell* 58:305–315, 1989). Nascent MHC class I heavy chains associate with β_2 -microglobulin and peptide, but the assembled trimolecular complex is retained in the endoplasmic reticulum/*cis*-Golgi compartment (M. Del Val, H. Hengel, H. Häcker, U. Hartlaub, T. Ruppert, P. Lucin, and U. H. Koszinowski, *J. Exp. Med.* 176:729–738, 1992). To locate the responsible genomic region, the cytoplasmic retention of MHC class I molecules after injection of MCMV DNA was tested. The function was mapped to the *Hind*III E fragment. A recombinant MCMV deletion mutant Δ MS94.5 lacking 15.8 kb in *Hind*III-E was constructed. Restoration of MHC class I molecule maturation and recognition of antigenic peptides by cytolytic T lymphocytes during the first hours of the early phase in mutant virus-infected cells proved the correct location to a 6.8-kb region in the *Hind*III E fragment. At later stages of the early phase, membrane-resident MHC class I molecules and cytolytic T lymphocyte recognition disappeared in Δ MS94.5 mutant virus-infected cells. These results demonstrate that more than one early-gene function of MCMV affects the MHC class I pathway of antigen presentation. The redundant MHC class I-reactive functions target the transport of MHC class I molecules at different steps.

Herpesviruses constitute a large family of host-specific viruses. A common property is the establishment of lifelong infection which is characterized by alternate stages of virus productivity and latency. Virus infection can recur despite a state of antiviral immunity. Cytomegaloviruses (CMVs) form the *Betaherpesvirus* subfamily. Usually, in humans, primary infection with human CMV (HCMV) is asymptomatic, whereas protean disease manifestations are the hallmark of infection in the immunologically immature or immunodeficient host. Infection of the mouse with mouse CMV (MCMV) is used as a model for HCMV infection and biology (18).

Previous studies on the immunobiology of MCMV infection have shown that certain genes of MCMV actively interfere with antigen presentation in the major histocompatibility complex (MHC) class I pathway. CMV gene expression is regulated in a cascade fashion typical for herpesviruses. Viral proteins encoded by immediate-early (IE) genes control the transcription of early (E) genes (24). E proteins regulate viral DNA synthesis, which is followed by the synthesis of most of the structural proteins during the late phase of infection. In BALB/c mice, the phosphoprotein pp89, a product of the *ie1* gene, is a target for MHC class I-restricted cytolytic T lymphocytes (CTL). Antigen presentation of pp89 is detected only when the expression of viral genes is restricted to the expression of IE genes despite the sustained synthesis and stability of the protein (37). Inhibition of antigen presentation for CTL is associated with the expression of MCMV E genes (13). In the

MHC class I pathway of antigen processing and presentation, endogenously synthesized proteins are degraded in the cytosol and peptides are transported into the endoplasmic reticulum (ER), where they assemble with the MHC class I heavy chain and β_2 -microglobulin to form the trimolecular MHC class I complex. This complex is exported to the plasma membrane to be recognized by peptide-specific CTL. Biochemical analysis revealed that during the E phase of MCMV gene expression, peptide-loaded MHC class I molecules are arrested in their export to the cell membrane at the stage of the ER/*cis*-Golgi compartment (12). The block of MHC class I molecule export is not absolute, and in cells upregulated for MHC class I expression by gamma interferon, the excess of MHC class I molecules can escape the transport block and present antigen at the plasma membrane (19).

As a first step toward the molecular characterization of the MCMV genes which encode MHC class I-interactive proteins, we report now on the genomic locations of viral functions involved in MHC class I retention. A recombinant MCMV deletion mutant that differs from wild-type (wt) virus in its effect on antigen presentation was constructed. In this mutant, the block of MHC class I molecule transport is lifted and antigen is presented during the E phase of MCMV gene expression. At later stages, however, MHC class I expression is lost from the plasma membrane and antigen presentation is inhibited by this mutant as well. These data show that more than one MCMV gene is involved in the regulation of MHC class I molecule expression.

* Corresponding author. Mailing address: Department of Virology, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany. Phone: 49 6221 563000. Fax: 49 6221 563953. Electronic mail address: Koszino@novsrv1.pio1.uni-heidelberg.de.

MATERIALS AND METHODS

Cells and virus. Mouse embryo fibroblasts prepared from BALB/cJ (*H-2^d*) mice were used after three passages for virus infections. For microinjection, an

immortalized cell line of BALB/c fibroblasts (14) was grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum. Mouse NIH 3T3 cells were used for preparation and titration of viruses. MCMV of strain Smith (ATCC VR-194) was used as tissue culture-grown virus.

Antibodies. The following monoclonal antibodies (MAbs) were used: SF1-1.1.1 (anti-H-2K^d) (ATCC HB 159); MA-215 (anti-K^d) (21); 34-5-8s (anti-D^d) (ATCC HB 102), 20-8-4s (anti-K^d) (ATCC HB11), 20-234-28 (anti-E1) (7), maD (anti- β -COP) (35), 1D3 (anti-protein disulfide isomerase) (42), and IM7-8.1E4 (anti-CD44) (6).

Infection conditions. Selective expression of wt or mutant MCMV IE-gene products was achieved by infection of cells with MCMV in the presence of cycloheximide (CH; 50 μ g/ml), which was replaced 3 h later by actinomycin D (ActD; 5 μ g/ml). Limited expression of E genes was achieved by incubation of cells in the absence of drugs for varied periods of time after removal of CH. IE proteins translated in this period activate the transcription of E genes, until ActD blocks further transcription. Cells were infected at a multiplicity of infection of 0.5, and the infection was performed by centrifugation at $800 \times g$ for 30 min, to enhance the efficiency by a factor of 10 to 40.

Preparation of viral DNA. The MCMV DNA was prepared from infected cells and purified by CsCl gradient centrifugation as described previously (15).

Microinjection and immunofluorescence. Cells were seeded on Cellocates (Eppendorf, Hamburg, Germany) and injected with genomic or plasmid DNA by an automatic injection system from Eppendorf as described previously (41). After injection, the cells were incubated for 24 h at 37°C and 5% CO₂ and stained by indirect immunofluorescence (31). Cells were fixed in 3% paraformaldehyde for 20 min at room temperature and permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Between antibody incubations, the cells were washed three times in PBS. For double immunofluorescence, the primary antibody specific for K^d was detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) F(ab)₂ fragments (Dianova, Hamburg, Germany), and the secondary antibodies were visualized by Lissamine rhodamine B-labelled goat anti-mouse IgG (Dianova).

Recombinant virus construction. Plasmid cloning was done by standard methods (29). The *lacZ* cassette was cloned into the *Pst*I-digested *Hind*III-E plasmid (32) after being blunt ended by using Klenow DNA polymerase or T7 DNA polymerase. To construct virus recombinants, linearized plasmid (30 fmol) and intact viral DNA (1.5 μ g) were cotransfected into NIH 3T3 cells by calcium phosphate precipitation. The mutant virus Δ MS94.5 was isolated from the resulting heterogeneous virus stock by using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) color screening and limiting dilution.

DNA analysis. One microgram of viral DNA was digested with *Hind*III and separated by gel electrophoresis. After blotting and cross-linking to a nylon membrane (NEN, Bad Homburg, Germany), hybridization with the digoxigenin-labeled *Hind*III-E plasmid and with the FITC-labeled *lacZ* cassette was performed with a multicolor detection set as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Cytolytic assays. Target cells were trypsinized and labeled for 90 min with Na₂⁵¹CrO₄, and a standard 4-h cytolytic assay was performed with 10^3 target cells and graded numbers of effector cells in fivefold dilution steps. Data represent the mean percentage of specific lysis from three replicate cultures.

Metabolic labeling, immunoprecipitation, and endo H treatment. Cells were labeled with [³⁵S]methionine (1,200 Ci/mmol; Amersham, Braunschweig, Germany) at a concentration of 500 μ Ci/ml at 37°C for 60 min and chased in the presence of 10 mM nonlabeled methionine for the times indicated as described previously (12, 19). After being washed with ice-cold PBS, cells were lysed in 1 ml of lysis buffer (1% Nonidet P-40 [NP-40], 5 mM MgCl₂, 140 mM NaCl, 20 mM Tris [pH 7.6], 0.2 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at $13,000 \times g$ for 30 min and precleared by adding 1 μ g of antiactin Mab C4 (Boehringer Mannheim) and rabbit anti-mouse IgG (Dianova). Immune complexes were retrieved with protein A-Sepharose (75 μ l of a 1:1 buffer-Sepharose slurry). Quantitative precipitations of MHC molecules were performed sequentially with ascitic fluids of Mab 34-5-8s followed by a mixture of MAbs 20-8-4s and MA-215. To ensure quantitative retrieval of immune complexes, the lysates were incubated two more times with protein A-Sepharose before addition of the next Mab. The Sepharose beads were washed twice with the first washing buffer (0.2% NP-40, 10 mM Tris-HCl [pH 7.6], 140 mM NaCl, 2 mM EDTA) and then subjected to two additional washing steps with the second (0.2% NP-40, 10 mM Tris-HCl [pH 7.6], 500 mM NaCl, 2 mM EDTA) and third (10 mM Tris-HCl [pH 7.6], 500 mM NaCl, 2 mM EDTA) washing buffers. For endoglycosidase H (endo H; Boehringer Mannheim) digestion, the immune complexes bound to protein A-Sepharose beads were resuspended in 50 mM phosphate buffer (pH 5.5) with 0.02% sodium dodecyl sulfate (SDS), 0.1% NP-40, and 0.2 mM phenylmethylsulfonyl fluoride and heated to 95°C for 4 min. Thereafter, 100 mM 2-mercaptoethanol and 2 mU of endo H were added, and digests were incubated for 18 h at 37°C and analyzed by SDS-polyacrylamide gel electrophoresis. Immune complexes were eluted by incubation with sample buffer and analyzed by 10 to 15% polyacrylamide gradient gel electrophoresis.

Flow cytometry. Trypsinized mouse embryo fibroblasts were washed with PBS (2% fetal calf serum, 0.3% NaN₃) and stained with purified antibody. Bound antibodies were visualized by addition of fluoresceinated goat anti-mouse or anti-rat IgG antibody (Dianova). Cells incubated with the second antibody alone served as negative controls. A total of 10^4 cells were analyzed for each fluores-

FIG. 1. Visualization of MHC class I retention of MCMV by immunofluorescence microscopy. Fifteen hours after infection with MCMV, BALB.SV cells were stained with Mab SF1-1.1.1 specific for K^d after saturation of the viral Fc receptor with an irrelevant immunoglobulin and subjected to indirect immunofluorescence. (a) Antibody binding visualized by staining with FITC-conjugated goat anti-mouse immunoglobulin. (b) Microinjection of the *Hind*III E fragments and the *ie1* and *ie3* genes. (c) Hoechst dye 33258 staining of cell nuclei; injected cells are marked with arrows.

cent profile by fluorescence-activated cell sorting (FACS) on a FACScan IV (Becton Dickinson & Co., San Jose, Calif.).

RESULTS

Locations of gene functions involved in MHC class I retention by microinjection of MCMV DNA. Unknown MCMV E proteins prevent nascent trimeric peptide-loaded MHC class I molecules from migrating through the Golgi complex. The

FIG. 2. Immunofluorescent colocalization of the MHC class I with β -COP and protein disulfide isomerase. Cells were injected with the *Hind*III E fragment of MCMV. After 24 h, cells were simultaneously double stained for K^d (a), K^d (b), β -COP (c), and the ER-resident protein disulfide isomerase (d).

molecules maintain the endo H-sensitive form of oligosaccharide side chains typical for the ER/*cis*-Golgi location of a glycoprotein. Pulse-chase experiments and the extraction of a normal amount of correctly processed antigenic peptides indicated that the stability of nascent molecules was not reduced (13). The export block should therefore result in an arrest of MHC class I molecules in the ER/*cis*-Golgi compartment detectable by immunomicroscopy.

By using a MAb, we studied the redistribution of the MHC class I molecule K^d after MCMV infection during expression of E genes. The appearance of a faint punctate cytoplasmic perinuclear fluorescence in infected cells was indicative of retention of MHC class I molecules (Fig. 1a). Isotype-matched control antibodies of irrelevant specificity did not react. Although the permeabilized cells were saturated with the Fc fragment of IgG to block the Fc receptor function of the MCMV *fer1* gene (41) prior to incubation with the K^d -specific antibody, a contribution of this protein could not be formally excluded at this stage of experimentation.

This redistribution of MHC class I molecules was used to locate viral gene functions involved in the block of MHC class I maturation. We tested whether the injection of MCMV DNA also resulted in the same redistribution of MHC class I molecules. First, total MCMV DNA was injected into cells, and the MHC class I distribution was studied. A similar redistribution of MHC class I molecules, restricted only to the injected cells, confirmed that virion proteins were not required for the phenomenon and that the factors which altered the MHC class I distribution were not transferred to other cells. MCMV DNA was cleaved to test which restriction enzyme would not affect the MHC class I-interactive genes. For example, the injection of cells with total MCMV DNA after cleavage with *Hind*III did not show the phenotype. *Hind*III also cleaves the coding regions for *ie1* and *ie3*, the major regulatory IE genes (27). Coinjection of the uncleaved plasmid pIE111, containing the *ie1* and *ie3* genes (33), however, restored the generation of the

FIG. 3. Construction of Δ MS94.5 lacking 15.8 kb of the *Hind*III E fragment. (a) *Hind*III cleavage map of the MCMV genome. The *Hind*III-E region is expanded. The fragment with a size of 15.8 kb was deleted by digestion with *Pst*I and then replaced by the 4.5-kb *lacZ* cassette. The resulting *Hind*III fragment has a size of 11.9 kb. (b) Ethidium bromide staining of *Hind*III-digested and agarose gel-separated MCMV DNA of MCMV and recombinant virus Δ MS94.5 (left). The gel was blotted and hybridized with the digoxigenin-labeled *Hind*III E fragment (middle) or the FITC-labeled *lacZ* cassette (right).

MHC class I redistribution phenotype of the *Hind*III fragments. Coinjection of pIE111 with *Xba*I-cleaved MCMV DNA did not result in a signal, whereas cleavage with *Eco*RI did. Thus, the requirement for IE genes confirmed the E-gene characteristics of the regulatory function and suggested that at least one E gene involved should be fully contained in a *Hind*-III or an *Eco*RI fragment. Injection of cloned *Hind*III fragments covering the entire genome (15, 32) located the function selectively to gene products encoded in the *Hind*III E fragment at the right-hand end of the MCMV genome (Fig. 1b). The *fer1* gene is located in the *Hind*III J fragment. The fact that the MHC class I redistribution function was located to a different genomic region proved that the detected phenotype was independent of the Fc receptor function.

Colocalization studies were carried out to define the intracellular redistribution of MHC class I molecules caused by

FIG. 4. Normal MHC class I molecule export in Δ MS94.5-infected cells during the E phase of gene expression. (a) Experimental protocol. Cells were infected in the presence of CH (shaded box) for 3 h, and after removal of CH, ActD (open box) was added as indicated. Cells were pulse-labelled with [³⁵S]methionine for 60 min (black box) and then chased (arrows) for the times indicated by the length of the arrow. (b) Immunoprecipitation was performed with MAb MA-215 specific for the MHC class I molecule K^d. Before gel electrophoresis, the samples marked + were incubated with endo H. The bands resulting from enzymatic digestion are indicated on the right with r (for endo H resistant) or s (for endo H sensitive). The two marginal lanes of the gel contained size markers.

gene functions encoded by the injected *HindIII* fragment. The biochemical analysis had suggested that the nascent molecules should be arrested in the ER or *cis*-Golgi (12). The punctate redistribution in the cytoplasm close to the nucleus suggested an ER location. Double labelling of the same cells with MAbs to K^d and to β -COP (Fig. 2a and c) or with anti-K^d and an antibody to the ER-resident protein disulfide isomerase (Fig. 2b and d) confirmed the ER localization; however, the association with β -COP was more pronounced. β -COP is a non-clathrin-coated vesicle-associated coat protein involved in transport in and out of the Golgi complex (35). There was no colocalization with Golgi and *trans*-Golgi network proteins (data not shown). Because MHC class I molecules are not completely retained in the ER, the function encoded by the *HindIII* E fragment apparently blocks transport of the MHC complex through the Golgi organelles.

Construction of a *HindIII*-E deletion mutant of MCMV. The next goal was to link the MHC class I redistribution phenotype in DNA-injected cells with an altered phenotype of a recombinant virus deleted for the respective genes. The comparison between herpesvirus genomes has revealed conserved gene blocks shared between alpha-, beta-, and gammaherpesviruses. In HCMV, the conserved gene blocks lie approximately between kb 50 and 170 of the 230-kb genome (10). The sequence of the MCMV genome has not yet been published, but the organization of the MCMV genome appears to be essentially similar to that of HCMV (16). In HCMV, the unique regions that flank the conserved gene blocks contain gene families comprising up to 14 members. The gene families are tandemly arranged and tend to cluster as blocks of homologous reading frames. Thus, the effect on antigen presentation could be the result of a cooperation between several genes of a gene family or a property of several genes of a gene family rather than the function of a single gene. Therefore, it was reasoned that a deletion of a single gene from the MCMV genome might not result in a different phenotype with regard to antigen presentation. Consequently, the first step was the construction of a recombinant virus mutant with a large deletion in the genomic area represented by the *HindIII* E fragment. The mutant Δ MS94.5 was prepared by homologous recombination as described by Manning et al. (30). The internal 15.8-kb *PstI* fragment of *HindIII*-E was replaced by the *lacZ* cassette (Fig. 3a), and a viable mutant was isolated. The DNA of the deletion mutant had lost, as expected, the authentic *HindIII* E fragment, and a new fragment that migrated in agarose gels at the calculated size of 11.9 kb appeared. This new fragment contained the *lacZ* gene used for selection (Fig. 3b).

Maturation and transport of MHC class I molecules in MCMV mutant Δ MS94.5-infected cells. The MCMV gene effect on the transport of newly synthesized MHC class I molecules is caused by products of genes that are transcribed during the first 30 min of the E phase (13). Biochemically, the effect is detected by the altered glycosylation pattern of MHC class I molecules (12). Infection of cells in the presence of the protein synthesis inhibitor CH results in enhanced transcription of IE genes, including gene *ie1* encoding pp89. After replacement of CH by the inhibitor of transcription ActD, IE mRNA is translated, leading to enhanced and selective IE-protein synthesis. In the absence of E-gene expression, the situation is identical to that in noninfected cells (12). Nascent MHC class I molecules mature steadily and enter the medial Golgi compartment, where they acquire a glycan structure which is resistant to cleavage by endo H. After a 90-min pulse under IE conditions, some of the MHC class I K^d molecules in mutant-infected cells had already acquired the endo H-resistant form, and the proportion of endo H-resistant molecules increased throughout the chase period, resulting in complete endo H resistance after 90 min (Fig. 4b, left). If ActD is not added immediately after removal of CH but rather is added 1.5 h later, the synthesized IE proteins can activate the transcription of E genes. In wt MCMV-infected cells, products of E-gene transcripts expressed during this period alter the transport of MHC molecules. After expression of E genes, MHC class I molecules maintain the high mannose core of N-linked oligosaccharides throughout the chase phase and are susceptible to endo H cleavage, typical for ER/*cis*-Golgi glycoproteins (Fig. 4b, right). In contrast to the situation in wt MCMV-infected cells, a glycosylation kinetics of nascent MHC class I molecules identical to that of noninfected cells became apparent after infection with mutant Δ MS94.5 under the same conditions (Fig. 4b, middle). This result suggested that viral genes causing the maturation block were deleted in mutant Δ MS94.5.

Mutant viruses may differ from wt virus by the kinetics of viral gene expression. The MHC class I molecule transport block is one of the first detectable E-gene functions. If the mutant virus was delayed in the cascade of viral E-gene expression, then the apparently normal MHC class I molecule maturation could simply reflect an altered kinetics of viral gene expression. However, the replication kinetics turned out to be indistinguishable from that of MCMV (Fig. 5a and b). The *el* gene is expressed simultaneously with the inhibitory function in wt MCMV (7). The *el* gene products of 36 and 38 kDa are expressed during the first 6 h of the E phase. Figure 5c shows that in mutant-infected cells, the *el* gene products of 38 and 36

FIG. 5. Growth kinetics of Δ MS94.5 NIH 3T3 cells were infected with wt MCMV (open circles) or mutant Δ MS94.5 (filled circles) at a multiplicity of infection of 0.1. Supernatant (a) and cells (b) were harvested at different times postinfection (p.i.) and titrated on NIH 3T3 cells in triplicate. Synthesis of the viral protein E1 was determined by [³⁵S]methionine metabolic labelling of wt MCMV- or Δ MS94.5-infected cells and immunoprecipitation of the cell lysates with MAb 234-10-28 (c). Lanes: IE, restriction of MCMV gene expression to the IE phase; IE+E, conditions of E-gene expression limited to 90 min.

kDa were expressed at the correct time. Thus, it was concluded that the difference in fate of the MHC class I molecules in Δ MS94.5-infected cells was due to the loss of those gene functions that were located to the deleted region by DNA injection and MHC class I molecule redistribution analysis.

Antigen presentation in MCMV mutant Δ MS94.5 at early times of infection. Presentation of pp89 to CTL was tested in cells infected with MCMV, Δ MS94.5, and the Vancouver strain of MCMV under the conditions described above (Fig. 4; schematically represented in Fig. 6). The Vancouver strain represents a deletion mutant that lacks 9.4 kb of *HindIII*-E (5). This deletion overlaps the deletion in Δ MS94.5. Infection of cells under conditions of selective IE expression resulted in pp89 presentation by all three virus strains (Fig. 6). Conditions permissive for 90 min of E-gene expression prevented pp89 presentation in cells infected with wt MCMV or the Vancouver strain. This result was different from that for cells infected with Δ MS94.5. As expected from the biochemical analysis of MHC

class I molecules, the Δ MS94.5-infected cells presented the L^d-restricted pp89 peptide to CTL. In the Vancouver strain, 9.4 kb of the *HindIII* E fragment are deleted. The restoration of MHC class I molecule export and antigen presentation in mutant Δ MS94.5 indicates that one essential gene function must be located in the 6.8 kb of the *HindIII* E fragment missing only in Δ MS94.5. Extending the period permissive for E-gene expression to 6 and 9 h, however, was associated with a gradual decline of antigen presentation also in cells infected with mutant Δ MS94.5.

Loss of cell surface and MHC class I in Δ MS94.5-infected cells during later stages of the E phase. The IE protein pp89 is expressed throughout the E phase (13). Therefore, the decline of CTL recognition in mutant-infected cells was suggestive of a new and unknown viral interference mechanism with antigen presentation that is distinct from the transport block of newly synthesized MHC class I molecules, because these molecules are correctly glycosylated in Δ MS94.5-infected cells also during the later stages of the E phase (data not shown). If antigen presentation is prevented despite the correct formation and glycosylation of MHC complexes, then the plasma membrane residence of MHC class I molecules should be affected. Flow cytometry analysis was carried out to study the fate of surface MHC class I molecules in infected cells. Under conditions of 3 h of E-gene expression, both wt MCMV- and mutant-infected cells showed a pattern of MHC class I expression that was almost indistinguishable from that of mock-infected cells (Fig. 7b). This result is not surprising, since a block of MHC class I molecule export for a short period cannot dramatically affect the concentration of preformed surface-resident molecules. Remarkably, the situation was different under conditions permissive for 6 and 9 h of E-gene expression (Fig. 7c and d). Virus functions expressed under these conditions had a strong effect on the surface concentration of resident MHC class I molecules. The majority of MCMV-infected cells showed reduced levels of surface-expressed MHC class I molecules, whereas a minor fraction of cells was not affected, perhaps because of a lack of infection. Cells infected with mutant Δ MS94.5 showed a similar pattern. Mutant virus-infected cells also lost MHC class I surface expression progressively, although with a slower kinetics. Thus, the deletion of *HindIII*-E sequences lifted the block of MHC class I molecule export, whereas during later stages of the E phase, the surface MHC class I molecule expression was still affected by mutant Δ MS94.5. MCMV, therefore, interferes with the MHC class I pathway of antigen presentation by more than one gene and in a fashion that is temporally controlled by sequential viral gene expression.

We tested whether the delayed effect on surface MHC class I molecule expression can be experimentally modulated in wt MCMV- and Δ MS94.5-infected cells. Figure 8 shows that preincubation with the L^d-binding peptide of MCMV pp89 did not prevent the loss of L^d molecules from the plasma membrane (compare Fig. 8a and b). Infection of cells in the presence of brefeldin blocked the surface effect on MHC class I molecules (Fig. 8c). Brefeldin disorganizes endocellular membranes and strongly affects intracellular transport (25). This result indicates that the viral protein affecting surface expression of MHC complexes is dependent on transport for function, and we expect to find eventually a glycoprotein. The surface residence of another glycoprotein, CD44, was affected by neither wt MCMV nor the mutant (Fig. 8d). Although the surface effect sets in later in the mutant because it lacks the gene affecting export, there was no difference in the reactivities of wt MCMV and the mutant in these assays. We therefore conclude that the effect on plasma membrane-resident MHC class I

FIG. 6. Restoration of antigen presentation during the E phase of gene expression in Δ MS94.5-infected cells. (a) Infection conditions for BALB/c fibroblast target cells (see Fig. 3). (b) Antigen presentation of pp89 was tested by pp89-specific BALB/c CTL infected with wt MCMV (open circles), Δ MS94.5 (filled circles), and MCMV Vancouver strain (squares) or not infected cells (diamonds). E:T, effector/target. (c) *Hind*III E fragments of the three viruses used. Dotted regions are deleted in Δ MS94.5 and the Vancouver (Vanc.) strain. The hatched region depicts the 6.8-kb region missing only in Δ MS94.5.

molecules is regulated independently from the function located in the 6.8-kb region of the *Hind*III fragment.

DISCUSSION

MCMV lacks host cell protein shutoff functions, and cellular protein synthesis in cells continues in parallel with the expression of viral functions. Nevertheless, during MCMV infection, the expression of MHC class I molecules is altered and antigen presentation is lost. This immunoevasive potential is under the control of MCMV E genes (12, 13). The first target of intervention is the transition of MHC class I molecules from ER/*cis*-Golgi to Golgi. In this paper, we report the definition of a genomic region involved in this activity. After deletion of this region in a virus mutant, the E-gene effect on MHC class I molecule glycosylation and plasma membrane transport is ablated. It became apparent, however, that the viral inhibitory functions act progressively and that later-expressed E-gene functions also affect the plasma membrane residence of MHC class I molecules.

Viruses may escape MHC class I-restricted immune control by regulating cellular proteins involved in antigen presentation at the transcriptional level (2, 17, 39). Adenovirus type 2 provided the first example of a posttranscriptional control mechanism affecting MHC class I molecule expression. In adenovirus type 2, the ER-resident virus glycoprotein E19, which is synthesized during the E phase of viral gene expression, associates with nascent MHC class I molecules and retains them in the ER (1, 21, 34, 36). Our observation that MCMV E-gene expression is incompatible with presentation of the viral IE antigen pp89 (13) demonstrated that CMVs also block this important effector arm of the immune response. This E-gene function also acts at the posttranslational stage and is associated with an arrest of intracellular MHC class I molecule transport (12). A posttranslational effect on MHC class I molecules could be confirmed for HCMV (43). In HCMV, E-gene

functions of the virus appear to destabilize nascent MHC class I molecules by an accelerated degradation of class I heavy chains (3). Recently, the effect of herpes simplex virus type 1 on MHC class I maturation could be associated with the function of the IE-gene product ICP47 (44), which appears to interfere with the function of peptide transporters (20). Thus, herpesviruses modulate the MHC class I antigen presentation pathway by different mechanisms.

Adenoviruses regulate MHC class I expression at both the transcriptional and posttranscriptional levels (39), and not only MHC class I molecules but also the expression of transporter proteins can be affected (38). Multiple targets and mechanisms of immune evasion may obscure the approach to the molecular analysis of individual functions. There is evidence for more than one effect of MCMV on MHC class I expression. The rapidity of MHC class I elimination from the plasma membrane was difficult to reconcile with the physiological half-life ($t_{1/2}$) of MHC class I molecules at the cell surface (26). Campbell and Slater (8) made additional observations, namely, a shutdown of MHC class I synthesis and a decrease of heavy-chain mRNA. These studies were carried out after infection in presence of phosphonoacetic acid (PAA) and of gamma interferon (9). Gamma interferon, however, lifts the MHC class I molecule transport block, and PAA blocks the transition from the 10- to 12-h-long E phase to the late phase of MCMV gene expression. PAA treatment, therefore, results in the accumulation of heterogeneous effects that may occur simultaneously or sequentially under the control of E genes (8, 9). This alter-

FIG. 7. Loss of MHC class I molecule expression at the cell membrane. Cells were infected with wt MCMV and Δ MS94.5. Expression of the H-2 allele K^d was assessed by FACS analysis. (a) Noninfected cells; (b) to (d) cells infected under conditions described in the legends to Fig. 3 and 5. The dotted line indicates the FITC control in the absence of the primary antibody.

FIG. 8. Comparable effects on surface MHC class I expression in wt MCMV- and Δ MS94.5-infected cells. Expression of the $H-2$ allele molecule K^d (a to c), L^d (a and b; dashed lines), and CD44 (d) was assessed by FACS analysis 8 h postinfection. The dotted line indicates the FITC control in the absence of the primary antibody. (b) Infection after preincubation and in the continuous presence of peptide YPHFPMTNL at a concentration of 10^{-4} M; (c) infection in the presence of brefeldin A at $5 \mu\text{g/ml}$.

ation of E-phase phenomena by PAA is an obstacle to the molecular analysis of individual functions.

In our first observation, we identified the virus effect on antigen presentation as an E-phase function by addition of PAA (37). Our subsequent studies showed, however, that the E-gene effect on antigen presentation required only 30 min of E-gene transcription (13). The respective gene products block the transport of nascent MHC class I molecules (12, 19). The experimental requirements to detect the intracellular redistribution of nascent MHC class I heavy chains after DNA injection, namely, the requirement of IE genes, confirmed the E-gene nature of the effect. The gene(s) causing MHC redistribution belongs to the set defined by *e1* (7) and also *fcr1*, the gene coding for the Fc receptor of MCMV that was identified by us recently by the same method (41). After deletion of the respective genome segment, the resulting mutant Δ MS94.5 had lost the capacity to block MHC class I molecule maturation. Although the *HindIII* E fragment was the only region which changed the MHC distribution, this result cannot exclude other genes with similar functions because the detection system monitored the fate of only the K^d allele. The posttranscriptional effect of adenovirus on MHC expression shows a certain degree of allele preference (4). Therefore, it is not excluded that there are additional MHC-reactive genes that do not react with $H-2^d$ alleles.

The analysis of MHC class I expression and peptide presentation during later stages of the E phase in Δ MS94.5-infected cells provided the first evidence for the existence of additional MHC class I-reactive MCMV gene functions that also act posttranslationally and that are dependent on intracellular transport. MHC class I molecules retained by the transport block remain endo H sensitive, but their $t_{1/2}$ is similar to that of correctly transported molecules (12). Cell membrane-expressed MHC class I molecules in general have a $t_{1/2}$ of several hours. The pp89 peptide has a high affinity and an exquisite specificity for the L^d molecule, but the rate of ligand dissociation is high in comparison with those of other peptides, and the $t_{1/2}$ of the complex is not longer than 2 h (40). The MHC expression on the surface of MCMV-infected cells is lost

within 3 to 6 h. Yet, the short $t_{1/2}$ of the L^d MCMV peptide interaction cannot explain this phenomenon, because D^d and K^d , more stable molecules with other peptide ligands, are lost with the same kinetics. When the export block is lifted in Δ MS94.5, MHC class I molecules mature properly and the complex is recognized by CTL. Nevertheless, these unknown viral functions destabilize MHC class I molecules also in mutant virus-infected cells. The function is expressed later because Δ MS94.5 restored antigen presentation at the beginning of the E phase. The function acts on endo H resistant MHC class I molecules because in Δ MS94.5-infected cells, the MHC class I molecules continue to mature during later stages of the E phase. Fewer than 10^3 peptide-loaded MHC class I complexes suffice for CTL recognition (11). Therefore, once expressed, the second function attacks the nascent molecules rapidly. As judged from the brefeldin block, this protein apparently needs transport to the cell membrane. If this function reflects a direct interaction between MHC class I molecules and a viral protein, such complexes might coprecipitate. A 34-kDa viral E protein that resides at the plasma membrane and coprecipitates with MHC class I molecules and represents a potential candidate was detected (28).

The effects of MCMV and HCMV on antigen presentation in the MHC class I pathway appear to differ in their mechanisms. In MCMV, an E-gene function inhibits MHC molecule transport, whereas in HCMV, E-gene function affects the stability of nascent molecules. In MCMV, the genes are transcribed during the first 2 h of the E phase, whereas in HCMV, the function has not been precisely timed. Since we now find a second MCMV E-gene function that destabilizes also membrane-resident MHC class I molecules, we wonder whether this later function is an HCMV counterpart. In this context, it is of interest that by testing a panel of HCMV recombinant deletion mutants, Jones and coworkers (22) were able to locate MHC class I-reactive functions to an 8-kb region within the U_s region of the HCMV genome (23). Testing of those mutants also provided evidence for the involvement of more than one gene.

CTL provide one of the major effector arms to limit CMV

spread. It is therefore not surprising that the virus has accumulated more than one function to evade this control in order to be effectively transmitted in face of an active immune control. MHC class I export can soon be studied at the level of the isolated MCMV gene. This mechanism and the new function indicated by the mutant virus will shed light on the intricacies on this virus-host interaction. The elucidation of the mechanisms will further our understanding of the MHC class I pathway of antigen presentation. The redundancy of mechanistically differed MHC class I-reactive genes is a remarkable trait of CMV biology.

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REFERENCES

- Andersson, M., A. McMichael, and P. A. Peterson. 1987. Reduced allorecognition of adenovirus-2 infected cells. *J. Immunol.* **138**:3960-3966.
- Beck, S., and B. G. Barrell. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature (London)* **331**:269-272.
- Beersma, M. F., M. J. Bijlmakers, and H. L. Ploegh. 1993. Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains. *J. Immunol.* **151**:4455-4464.
- Beier, D. C., J. H. Cox, D. R. Vining, P. Cresswell, and V. H. Engelhard. 1994. Association of human class I MHC alleles with the adenovirus E3/19K protein. *J. Immunol.* **152**:3862-3872.
- Boname, J. M., and J. K. Chantler. 1992. Characterization of a strain of murine cytomegalovirus which fails to grow in the salivary glands of mice. *J. Gen. Virol.* **73**:2021-2029.
- Budd, R. C., J. C. Cerottini, and H. R. McDonald. 1987. Selectively increased production of interferon-gamma by subsets of Lyt-2⁺ and L3T4⁺ T cells identified by expression of Pgp-1. *J. Immunol.* **138**:3583-3586.
- Bühler, B., G. M. Keil, F. Weiland, and U. H. Koszinowski. 1990. Characterization of the murine cytomegalovirus early transcription unit e1 that is induced by immediate-early proteins. *J. Virol.* **64**:1907-1919.
- Campbell, A. E., and J. S. Slater. 1994. Down-regulation of major histocompatibility complex class I synthesis by murine cytomegalovirus early gene expression. *J. Virol.* **68**:1805-1811.
- Campbell, A. E., J. S. Slater, V. J. Cavanaugh, and R. M. Stenberg. 1992. An early event in murine cytomegalovirus replication inhibits presentation of cellular antigens to cytotoxic T lymphocytes. *J. Virol.* **66**:3011-3017.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell. 1990. Analysis of the protein-coding content of the sequence of the human cytomegalovirus strain AD 169. *Curr. Top. Microbiol. Immunol.* **154**:125-169.
- Christinck, E. R., M. A. Luscher, B. H. Barber, and D. B. Williams. 1991. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature (London)* **352**:67-70.
- Del Val, M., H. Hengel, H. Häcker, U. Hartlaub, T. Ruppert, P. Lucin, and U. H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex I molecules into the medial-Golgi compartment. *J. Exp. Med.* **176**:729-738.
- Del Val, M., K. Münch, M. J. Reddehase, and U. H. Koszinowski. 1989. Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. *Cell* **58**:305-315.
- Del Val, M., H.-J. Schlicht, T. Ruppert, M. J. Reddehase, and U. H. Koszinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell* **66**:1145-1153.
- Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski. 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. *J. Virol.* **47**:421-433.
- Farrell, H. (University of Perth). 1994. Personal communication.
- Ge, R., A. Kralli, R. Weinmann, and R. P. Ricciardi. 1992. Down-regulation of the major histocompatibility complex class I enhancer in adenovirus type 12-transformed cells is accompanied by an increase in factor binding. *J. Virol.* **66**:6969-6978.
- Hamilton, J. D. 1982. Cytomegaloviruses and immunity. *Monogr. Virol.* **12**:3-26.
- Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U. H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. *J. Virol.* **68**:289-297.
- Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature (London)* **375**:411-415.
- Jefferies, M. A., and H. G. Burgert. 1990. E3/19K from adenovirus 2 is an immunosubversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. *J. Exp. Med.* **172**:1653-1664.
- Jones, T. R., L. A. Hanson, L. Sun, J. S. Slater, R. M. Stenberg, and A. E. Campbell. 1994. Identification of a human cytomegalovirus gene region involved in down-regulation of MHC class I heavy chain expression, abstr. 411. *In* Nineteenth Program and Abstracts of the International Herpesvirus Workshop.
- Jones, T. R., and V. P. Muzithras. 1992. A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66**:2541-2546.
- Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski. 1984. Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate early times after infection. *J. Virol.* **50**:784-795.
- Klausner, C. A., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**:1071-1080.
- Koszinowski, U. H., S. Jonjic, and P. Lucin. 1994. Cytomegalovirus persistence by evasion from immune control. *Semin. Virol.* **5**:297-305.
- Koszinowski, U. H., G. M. Keil, H. Volkmer, M. R. Fibi, A. Ebeling-Keil, and K. Münch. 1986. The 89,000-M_r murine cytomegalovirus immediate-early protein activates gene transcription. *J. Virol.* **58**:59-66.
- Lucin, P. (University of Rijeka). 1993. Unpublished data.
- Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Manning, W. C., C. A. Stoddart, L. A. Langenaur, G. B. Abenes, and E. S. Mocarski. 1992. Cytomegalovirus determinant of replication in salivary glands. *J. Virol.* **66**:3794-3802.
- Melan, M. A., and G. Sluder. 1992. Redistribution and differential extraction of soluble proteins in permeabilized cells. Implications for immunofluorescence microscopy. *J. Cell Sci.* **101**:731-743.
- Mercer, J. A., J. R. Marks, and D. H. Spector. 1983. Molecular cloning and restriction endonuclease mapping of murine cytomegalovirus genome (Smith strain). *Virology* **129**:94-106.
- Messerle, M., B. Bühler, G. M. Keil, and U. H. Koszinowski. 1992. Structural organization, expression, and functional characterization of the murine cytomegalovirus immediate-early gene 3. *J. Virol.* **66**:27-36.
- Pääbo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson. 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50**:311-317.
- Peppercok, R., J. Scheel, H. Horstmann, H. P. Hauri, G. Griffiths, and T. E. Kreis. 1993. β -COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. *Cell* **74**:71-82.
- Rawle, F. C., A. E. Tellefson, M. S. M. Wold, and L. R. Gooding. 1990. Mouse anti-adenovirus cytotoxic T lymphocytes. *J. Immunol.* **143**:2031-2037.
- Reddehase, M. J., M. R. Fibi, G. M. Keil, and U. H. Koszinowski. 1986. Late-phase expression of a murine cytomegalovirus immediate-early antigen recognized by cytolytic T lymphocytes. *J. Virol.* **60**:1125-1129.
- Rotem-Yehudar, R., S. Winograd, S. Sela, J. E. Coligan, and R. Ehrlich. 1994. Downregulation of peptide transporter genes in cell lines transformed with highly oncogenic adenovirus 12. *J. Exp. Med.* **180**:477-488.
- Schrier, P. L., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Eb. 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature (London)* **305**:771-775.
- Smith, J. D., W.-R. Lie, J. Gorka, N. B. Myers, and T. H. Hansen. 1992. Extensive peptide ligand exchange by surface class I major histocompatibility complex molecules independent of exogenous β_2 -microglobulin. *Proc. Natl. Acad. Sci. USA* **89**:7767-7771.
- Thäle, R., P. Lucin, K. Schneider, M. Eggers, and U. H. Koszinowski. 1994. Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. *J. Virol.* **68**:7757-7765.
- Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotypic antibodies of an intracellular membrane protein that recognize a mammalian endoplasmic reticulum retention signal. *Nature (London)* **345**:495-502.
- Warren, A. P., D. H. Ducroq, P. J. Lehner, and L. K. Borysiewicz. 1994. Human cytomegalovirus-infected cells have unstable assembly of major histocompatibility complex class I complexes and are resistant to lysis by cytotoxic T lymphocytes. *J. Virol.* **68**:2822-2829.
- York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8⁺ T lymphocytes. *Cell* **77**:525-535.