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A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation

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Mouse cytomegalovirus (MCMV) early gene expression interferes with the major histocompatibility complex class I (MHC class I) pathway of antigen presentation. Here we identify a 48 kDa type I transmembrane glycoprotein encoded by the MCMV early gene *m06*, which tightly binds to properly folded β_2-microglobulin (β_2m)-associated MHC class I molecules in the endoplasmic reticulum (ER). This association is mediated by the lumenal/transmembrane part of the protein. gp48–MHC class I complexes are transported out of the ER, pass the Golgi, but instead of being expressed on the cell surface, they are redirected to the endocytic route and rapidly degraded in a Lamp-1$^+$ compartment. As a result, *m06*-expressing cells are impaired in presenting antigenic peptides to CD8$^+$ T cells. The cytoplasmic tail of gp48 contains two di-leucine motifs. Mutation of the membrane-proximal di-leucine motif of gp48 restored surface expression of MHC class I, while mutation of the distal one had no effect. The results establish a novel viral mechanism for down-regulation of MHC class I molecules by directly binding surface-destined MHC complexes and exploiting the cellular di-leucine sorting machinery for lysosomal degradation.

Keywords: antigen presentation/cytomegalovirus/di-leucine/lysosomes/MHC class I

Introduction

Even in the face of a fully competent immune system, herpesviruses avoid elimination, establish life-long infections in their host and produce up to 200 potentially antigenic viral proteins. Several features contribute to persistence, such as virus replication at immunoprivileged sites that are poorly controlled by immune cells, the reduced transcription program during the state of viral latency and the modulation of specific immune functions (reviewed by Hengel *et al*., 1998). Interference with presentation of viral antigens in the major histocompatibility complex (MHC) class I pathway is a mechanism used by all members of the herpesvirus family studied so far (reviewed by Johnson and Hill, 1998). Viral antigens are displayed on the cell surface as peptides by polymorphic products of the MHC to T lymphocytes. MHC class I complexes comprise three protein subunits; the MHC-encoded glycoprotein heavy chain, β_2-microglobulin (β_2m) and a small peptide. The assembly of these components into a trimeric MHC class I complex is assisted by molecular chaperones in the endoplasmic reticulum (ER). Antigenic peptides are usually generated by cytosolic degradation of endogenously synthesized proteins. To reach the peptide-binding groove of MHC class I molecules, peptides are translocated across the ER membrane by a specific transporter, TAP1/2 (Momburg and Hämmerling, 1998). After binding of high-affinity peptides, class I molecules exit from the ER and are transported via the constitutive secretory pathway to the cell surface (Heemels and Ploegh, 1995). MHC class I heavy chains that fail to assemble properly are removed from the ER, translocated to the cytosol and degraded by the proteasome (Hughes *et al*., 1997).

Cytomegaloviruses (CMVs) represent the β-subgroup of herpesviruses and have evolved the most complex strategies to subvert recognition of immune effector cells. Human CMV (HCMV) expresses four genes, all encoding transmembrane glycoproteins that interrupt the MHC class I pathway of antigen presentation. Very early in HCMV replication, the *US3*-encoded glycoprotein retains MHC class I complexes in the ER (Ahn *et al*., 1996; Jones *et al*., 1996), which is followed by the subsequent expression of the US2 and US11 glycoproteins. Both proteins mediate the rapid export of newly synthesized MHC class I heavy chains back to the cytosol for degradation (Wiertz *et al*., 1996a,b). During the early and late phase of HCMV replication, the US6 glycoprotein takes control over the peptide transporter TAP1/2, thereby limiting the assembly of MHC class I complexes (Ahn *et al*., 1997; Hengel *et al*., 1997; Lehner *et al*., 1997).

Mouse cytomegalovirus (MCMV) was the first herpesvirus for which interference with MHC class I antigen presentation was demonstrated (Del Val *et al*., 1989, 1992). MCMV infection of fibroblasts causes a loss of cell surface MHC class I molecules in the early phase of the viral replication cycle (Del Val *et al*., 1992). We showed previously that a glycoprotein encoded by the gene *m152* contributes to this effect by retaining class I complexes in the ER-Golgi intermediate compartment/*cis*-Golgi compartment (Ziegler *et al*., 1997). However, downregulation of MHC class I molecules was still observed when cells are infected with a MCMV mutant missing the genes *m151* to *m165* (Thäle *et al*., 1995). Therefore, apart from the *m152* function, MCMV must contain additional gene products which affect MHC class I expression. Another glycoprotein, gp34, associates with MHC class I complexes and reaches the cell surface (Kleijnen *et al*., 1997).

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Here we report on the identification and the function of the MCMV-encoded glycoprotein gp48. This protein forms a tight complex with β_2m-associated MHC class I molecules. After passing the Golgi, the complex enters the endocytic route and reaches the lysosome where both the viral protein and MHC class I molecule undergo rapid proteolysis. The targeted transport of the complex to the endosomal/lysosomal compartment is mediated by the cytosolic domain of gp48, since removal of the cytosolic tail or mutation of a di-leucine (LL) motif restores cell-surface expression of MHC class I molecules.

Results

Identification of the MCMV protein gp48 affecting MHC class I complexes

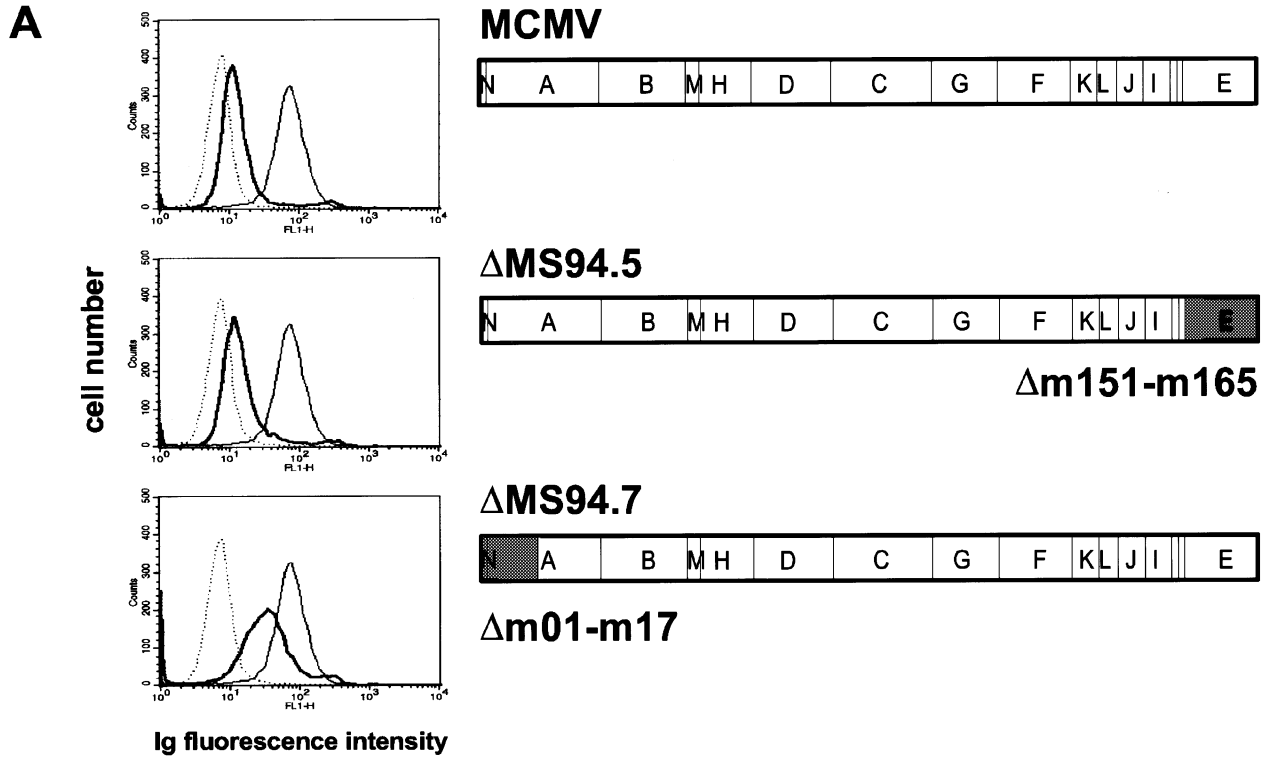
Herpesviral proteins reacting with MHC class I molecules are neither homologous to cellular proteins nor to other herpesviral proteins. To identify unknown additional genes within the MCMV genome, two approaches were used: first, recombinant MCMVs with large deletions were constructed. Mutants were screened for an impaired MHC class I downregulation phenotype in comparison with wild-type MCMV. CMV genes encoding proteins which affect MHC class I antigen presentation are members of gene families that are not included in the conserved central gene block shared by α-, β- and γ-herpesviruses (Hengel *et al*., 1998). We therefore constructed MCMV mutants with large deletions in the genome termini, the regions of the least homology to other herpesviruses. When fibroblasts were infected with the recombinant MCMV ΔMS94.7 (Kleijnen *et al*., 1997) lacking the genes *m01* to *m17* at the left-hand terminus of the viral genome, an intermediate MHC class I downregulation phenotype was observed (Figure 1A). The partial increase of surface MHC class I molecules 24 h post infection (p.i.) compared with infection with wild-type virus, suggested that mutant ΔMS94.7 had lost an MHC-reactive function. This MHC class I-reactive function located in the *Hin*dIIIA region seemed to be stronger than that defined by deletion of *m151* to *m165*, which did not significantly lift the downregulation of cell surface MHC class I molecules.

The rationale for the second strategy to identify MCMV proteins affecting MHC class I function was based on the expectation that certain viral MHC class I-regulating proteins directly complex with MHC class I molecules. For this reason, we immunized mice with MHC class I immunoprecipitates from infected cells, generated hybridoma clones and screened their supernatants for specific MCMV reactivity. Two clones, CROMA229 and CROMA231, produced antibodies that recognize a MCMV glycoprotein with an apparent molecular mass of 48 kDa, which was reduced to 34 kDa after deglycosylation. In productively infected fibroblasts the 48 kDa glycoprotein, gp48, is synthesized within the first 3 h p.i., reaches a maximum 3–6 h p.i., and is produced at high levels throughout the replication cycle (data not shown). Immunofluorescence staining of MCMV-infected cells with these antibodies revealed a distinct vesicular distribution of gp48, which was reproduced upon microinjection of total viral DNA. Therefore, we used microinjection of viral DNA fragments in combination with immunofluorescence staining for gp48 to identify the viral gene encoding gp48. This approach had previously led to the identification of the MCMV proteins that interact with cellular proteins (Thäle *et al*., 1994; Ziegler *et al*., 1997). By monitoring cells after injection of *Hin*dIII fragment pools, single fragments and subfragments we found that the 3.4 kbp *Xba*I–*Cla*I fragment (nucleotides 4162–7528 in the MCMV genome) in the *Hin*dIIIA region was sufficient for gp48 expression. The *Xba*I–*Cla*I fragment contains the open reading frames (ORFs) *m05*, *m06* and *m07* (Rawlinson *et al*., 1996). Based on the predicted molecular weight (34.5 kDa) and the presence of potential *N*-glycosylation sites, ORF *m06* (ORF 5327:6337) was considered a likely candidate to encode gp48 (Figure 2). Therefore, we established cell lines constitutively expressing gp48.

To test whether the identified gp48 had indeed the expected effect on cell surface MHC class I molecules, we analyzed the NIH 3T3 transfectants by flow cytometry. *m06*$^+$ cells are characterized by an almost complete loss of cell surface H-2 L^q molecules in comparison with vector control transfected cells (Figure 1B). Downregulation was selective since the expression of other surface molecules, such as the integrin β1 chain, remained unaltered. Other MHC class I alleles, such as H-2 K^d, L^d, D^d, K^b, D^b and K^k were also affected. Moreover, when the MCMV protein pp89 was expressed by recombinant *vaccinia* virus in *m06* transfectants of the C12 fibroblast cell line (H-2^d), gp48 inhibited the antigen presentation to pp89-specific, L^d-restricted CTL. A 10-fold greater number of effector cells were needed to achieve a similar degree of lysis compared with control cells, even though L^d expression on the cell surface was only reduced by 50% (data not shown). Thus, the MCMV *m06*-encoded protein selectively interferes with cell surface expression of MHC class I molecules and inhibits antigen presentation.

Short half life of MHC class I molecules in the presence of MCMV gp48

To understand the mechanism of the *m06* function we studied the fate of newly synthesized class I molecules in the presence of *m06*. Pulse–chase labeling of transfectants and of control cells transfected with the vector alone was performed. After precipitation of H-2 L^q molecules (Figure 3A) and gp48 (Figure 3B), half of each precipitate was subjected to endoglycosidase H (Endo H) digestion. As Endo H only cleaves high mannose *N*-linked glycans that have not been processed by *medial*-Golgi enzymes to complex glycans, the transport and maturation status of the glycoproteins can be assessed. Figure 3A shows that in *m06* transfectants the amount of newly synthesized Endo H-sensitive MHC class I molecules is not altered compared with that in control cells. However, while the MHC class I complexes in control cells acquired Endo H-resistant glycan structures during the chase period, the amount of recovered Endo H-resistant class I molecules in *m06* expressing cells was significantly decreased. During the chase period the majority of gp48 remained Endo H-sensitive (Figure 3B). This fraction seems not to be assembled with MHC class I molecules and is degraded by the proteasome (data not shown), while a proportion of molecules migrating at a position between 50 and 60 kDa acquired Endo H-resistance. The profound loss of gp48 in the course of the chase paralleled that seen for



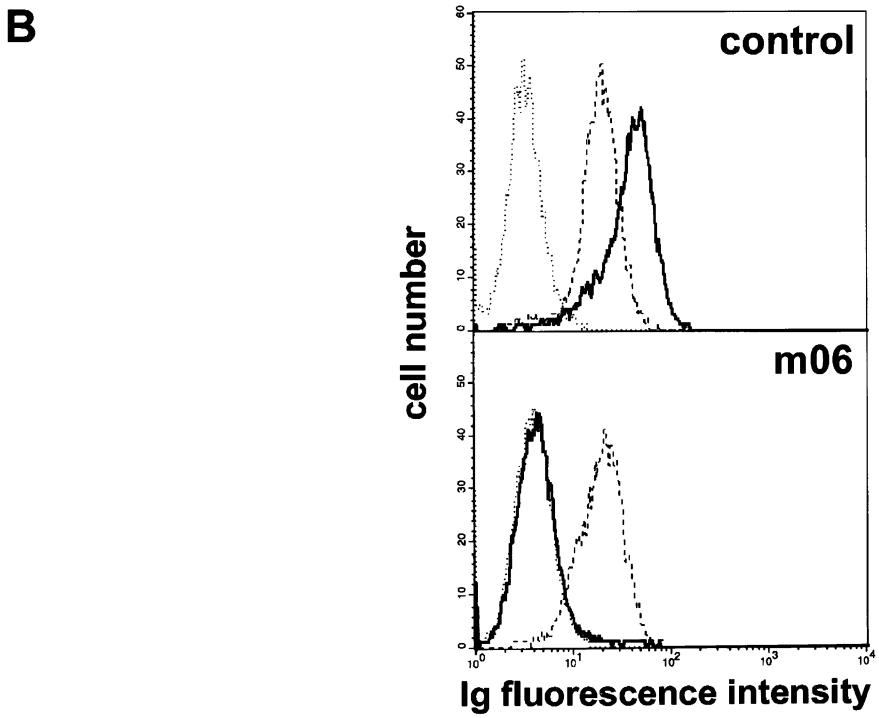


Fig. 1. Multiple early genes of MCMV affect MHC class I surface expression. (**A**) BALB.SV fibroblasts were infected with either wild-type MCMV, the mutant ΔMS94.5 or ΔMS94.7 in the presence of phosphonoacetic acid (250 μg/ml) and analyzed for their MHC class I surface expression 24 h p.i. by flow cytometry. H-2 K^d molecules of infected cells (bold lines) were stained with mAb SF1.1.1. The H-2 K^d surface expression of mock-infected cells is shown in each histogram by a fine line. The dotted lines represent staining of the cells with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) alone. (**B**) Cytofluorometric analysis of NIH 3T3 fibroblasts stably transfected with the B45-Neo vector alone (control) or the vector expressing MCMV *m06*. Cells were stained with mAb 28–14–8s (bold lines) or anti-integrin β1 chain (dashed lines) followed by goat anti-mouse IgG-FITC. Dotted lines represent control stainings with the second antibody only.

MHC class I molecules; in contrast to the rather slow turnover of MHC class I molecules in control cells, in *m06* transfectants, the half life of both MHC class I and gp48 molecules was <2 h.

Association of gp48 with MHC class I complexes

Immunoprecipitation of gp48 revealed additional co-precipitating proteins in the range of 46 and 90 kDa. To identify these proteins, gp48 was immunoprecipitated

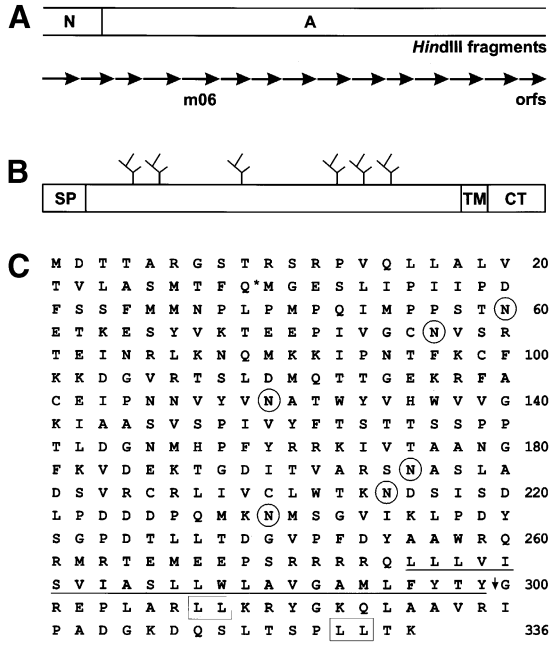


Fig. 2. The *m06* gene of MCMV. (**A**) *Hin*dIII cleavage map of the left hand genomic region of MCMV including the location of the *m06* ORF (ORF 5327:6337) within the *m02* gene family. (**B**) Proportional representation of the type I glycoprotein, deduced from the *m06* nucleotide sequence, enclosing signal peptide (SP), luminal domain with consensus sites for *N*-linked glycosylation (branched symbols), transmembrane region (TM) and cytoplasmic tail (CT). (**C**) Putative amino acid sequence of the MCMV *m06* ORF. The potential signal peptide cleavage site is shown by an asterisk. Circles indicate sites for *N*-linked glycosylation. The hydrophobic region that represents the transmembrane domain is underlined. The two di-leucine sequence motifs in the cytoplasmic tail with a potential function in intracellular targeting are boxed. The arrow indicates the C-terminal amino acid of gp48ΔCT.

with gp48-specific antibodies from digitonin lysates of metabolically labeled transfectants. After dissociation of immunoprecipitated material in SDS, a second round of immunoprecipitation was carried out in Nonidet P-40 (NP-40) lysis buffer with antibodies specific for MHC class I molecules, gp48 or calnexin, respectively. Samples were either untreated or digested with Endo H to enhance the difference in gel mobility between class I heavy chain and gp48 (Figure 4). Re-precipitation of MHC class I molecules from gp48 precipitates revealed the association of Endo H-sensitive class I heavy chains, which migrate slightly faster in the non-digested form and slower in the Endo H-treated form compared with gp48. In agreement with the results of the pulse–chase kinetics, almost exclusively Endo H-sensitive class I heavy chains could be recovered. The Endo H-resistant 90 kDa protein co-immunoprecipitating with gp48 was identified as calnexin by re-precipitation with a calnexin-specific antiserum. The reciprocal experiment using MHC-specific antibodies confirmed the co-precipitation of gp48 with Endo H-sensitive MHC class I molecules (data not shown). Thus, gp48 tightly associates with MHC class I complexes before passing the *medial*-Golgi. In addition, gp48 associates with the ER chaperone calnexin.

Lysosomal inhibitors block degradation of both MHC class I and gp48 molecules

The acquisition of Endo H-resistance of a proportion of both MHC class I and gp48 molecules together with a decreased stability indicates that these complexes are degraded after passage through the Golgi. A post-Golgi site with proteolytic properties is the endosomal/lysosomal compartment. If the gp48/MHC class I complexes were degraded in the endosomal/lysosomal compartment, inhibitors of endosomal/lysosomal transport and of lysosomal proteases should prevent destruction of the complexes. First, we tested the effect of leupeptin, a strong inhibitor of cysteine and serine proteases. Immmunoprecipitation of MHC class I molecules (Figure 5A) and gp48 (Figure 5B) after pulse–chase labeling in the absence or presence of leupeptin demonstrated that leupeptin inhibited the destruction of both MHC class I and gp48. *N*-linked glycans of the rescued glycoproteins exhibited an Endo H-resistant phenotype. Since leupeptin does not exclusively block lysosomal proteases, we tested the highly lysosome-specific epoxide E64, which binds strongly and irreversibly to most cysteine proteases and, unlike leupeptin, has no effect on non-cysteine proteases (Seglen, 1983). Treatment of the transfectants with this protease inhibitor prevented degradation to a similar extent (Figure 5C). Moreover, lysosomotropic agents such as the weak bases NH_4Cl and chloroquine as well as the vacuolar type H^+-ATPase inhibitors bafilomycin A_1 and concanamycin A, which raise the pH of endosomes and lysosomes to suboptimal working conditions for proteases, and might affect sorting in the *trans*-Golgi network (TGN) and reduce endosome–lysosome fusion, thereby preventing lysosomal degradation (Seglen, 1983; Clague *et al*., 1994), lead to an accumulation of MHC class I and gp48 molecules (Figure 5C). Taken together, these biochemical data suggests that the MHC class I/gp48 complexes undergo proteolytic destruction in an endosomal/lysosomal compartment.

MHC class I molecules and MCMV gp48 co-localize in lysosomal compartments

To localize the site of MHC class I/gp48 complex degradation more precisely, confocal laser scanning microscopy was performed after double staining of gp48 and cellular marker proteins. To increase the amount of detectable gp48 and MHC class I, cells were also treated with leupeptin. Staining of gp48 with CROMA231 (Figure 6A–C, left panels) revealed a characteristic vesicular distribution of the viral glycoprotein that resembles the gp48 distribution after MCMV infection (data not shown). This staining was clearly different from that obtained for the luminal ER protein calreticulin (Figure 6A, middle and right panels) and the *medial*-Golgi marker α-mannosidase II (data not shown). The latter overlaps only in a few spots with the gp48 distribution. Staining with an antibody specific for the TGN/late endosomal marker, CI-MPR (cation-independent mannose 6-phosphate receptor), did not overlap with that of gp48 either (data not shown). However, a strong co-localization of gp48 with the lysosomal associated membrane protein Lamp-1 (Figure 6B, middle and right panels) and MHC class I (Figure 6C, middle and right panels) was obtained. This confirmed that the gp48/MHC complexes are directed to lysosomes.

The membrane proximal LL motif of the gp48 cytoplasmic tail is crucial for downregulation of MHC class I molecules

The amino acid sequence of *m06* revealed two LL motifs at amino acid position 307/308 and 333/334 in the putative

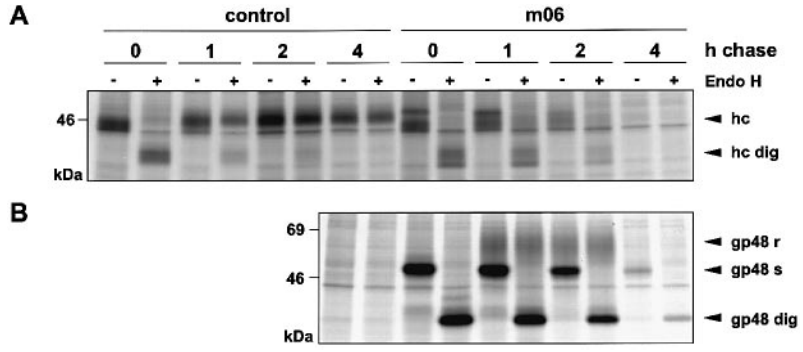


Fig. 3. Short half-life of MHC class I molecules and gp48 in *m06* transfectants. NIH 3T3 cells stably expressing *m06* and control cells transfected with the vector alone were pulse labeled for 30 min and chased for the indicated periods of time. H-2 L^q molecules were immunoprecipitated with mAb 28–14–8s (**A**) and gp48 with mAb CROMA229 (**B**) from aliquots of NP-40-lysates, and were either Endo H-digested or mock treated prior to 11–14% SDS–PAGE. hc, MHC class I heavy chains; r, Endo H-resistant; s, Endo H-sensitive; dig, Endo H-digested.

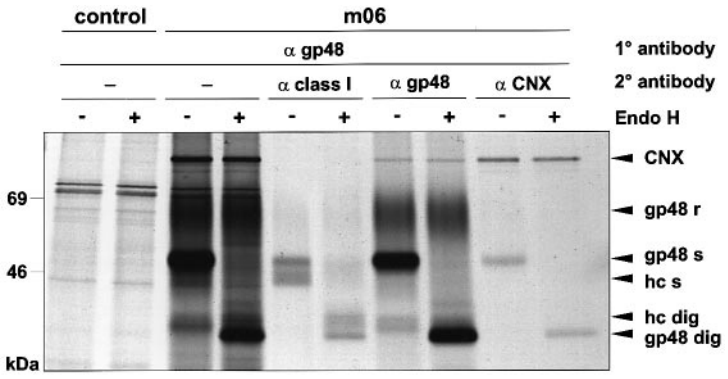


Fig. 4. Association of gp48 with MHC class I molecules and calnexin. NIH 3T3 cells stably expressing *m06* and control cells transfected with the vector alone were continuously labeled for 4 h and lysed in digitonin buffer. After precipitation of MCMV gp48 with mAb CROMA229, aliquots of the immune complexes were either directly analyzed or dissociated in SDS and subjected to a second round of precipitation with the antibodies 28–14–8s, CROMA229 and a polyclonal rabbit serum against calnexin (CNX), respectively. Half of the samples were treated with Endo H prior to separation by 11–14% SDS-PAGE. hc, MHC class I heavy chains; r, Endo H-resistant; s, Endo H-sensitive; dig, Endo H-digested.

cytoplasmic tail (Figure 2). LL motifs are known for their endosomal/lysosomal targeting capacity (reviewed by Sandoval and Bakke, 1994). To examine whether the gp48 cytoplasmic tail containing these LL motifs acts as a lysosomal targeting module, we constructed an *m06* deletion mutant lacking the 37 C-terminal amino acid residues (gp48ΔCT). The truncated protein was stably expressed in NIH 3T3 fibroblasts, and the transfectants were tested in parallel with cells expressing full-length *m06* and control cells for their MHC class I phenotype. Flow cytometry analysis (Figure 7A) revealed a substantial restoration of plasma membrane transport of class I molecules in gp48ΔCT expressing cells. The immunoprecipitation and Endo H-analysis of H-2 L^q molecules after pulse–chase labeling showed that class I molecules and gp48ΔCT achieve Endo H-resistant glycan structures and are stable for several hours (data not shown; Figure 7B). These Endo H-resistant class I molecules in the *m06*ΔCT transfectants migrate in a broad band, indicative of co-precipitating proteins. Digestion of the class I precipitates with peptide-*N*-glycosidase F (PNGase F), which cleaves Golgi-modified complex *N*-linked oligosaccharides, revealed, in addition to the class I heavy chain, a protein with a molecular weight of ~32 kDa, representing gp48ΔCT (Figure 7B). Thus, the class I molecules in *m06*ΔCT transfectants have a different fate than in *m06* cells; they are still bound to the viral protein, but instead of being targeted to the lysosomes they are exported to be expressed at the cell surface. We concluded that the gp48 cytoplasmic tail is essential for directing MHC class I/gp48 complexes to lysosomes. As the association with MHC class I molecules is apparently not affected by deletion of the gp48 cytoplasmic tail, the luminal domain and/or the transmembrane domain is required for binding to class I molecules.

To test whether one of the LL motifs is required for the gp48 mediated targeting function, we established cell transfectants stably expressing gp48 tail mutants in which either the membrane proximal (*m06*LL307/308AA) or the terminal leucines (*m06*LL333/334AA) were substituted by alanines. Staining of saponin permeabilized transfectants with mAb CROMA231 revealed comparable mutant gp48 expression levels in *m06*, *m06*LL307/308AA and *m06*LL333/334AA transfectants (Figure 7A, solid gray histograms). Since CROMA231 recognizes an epitope in the cytoplasmic tail of gp48, only a background staining was achieved in *m06*ΔCT transfectants. Flow cytometry analysis of surface MHC class I molecules (Figure 7A, bold lines) demonstrated that either the deletion of the cytoplasmic tail or the substitution of alanines for the membrane proximal LL motif LL307/308 restored cell surface expression of MHC class I molecules. In contrast, mutation of the terminal di-leucine LL333/334 did not significantly alter class I surface expression. Thus, the proximal LL motif is essential for the gp48-mediated class I complex targeting to the lysosome.

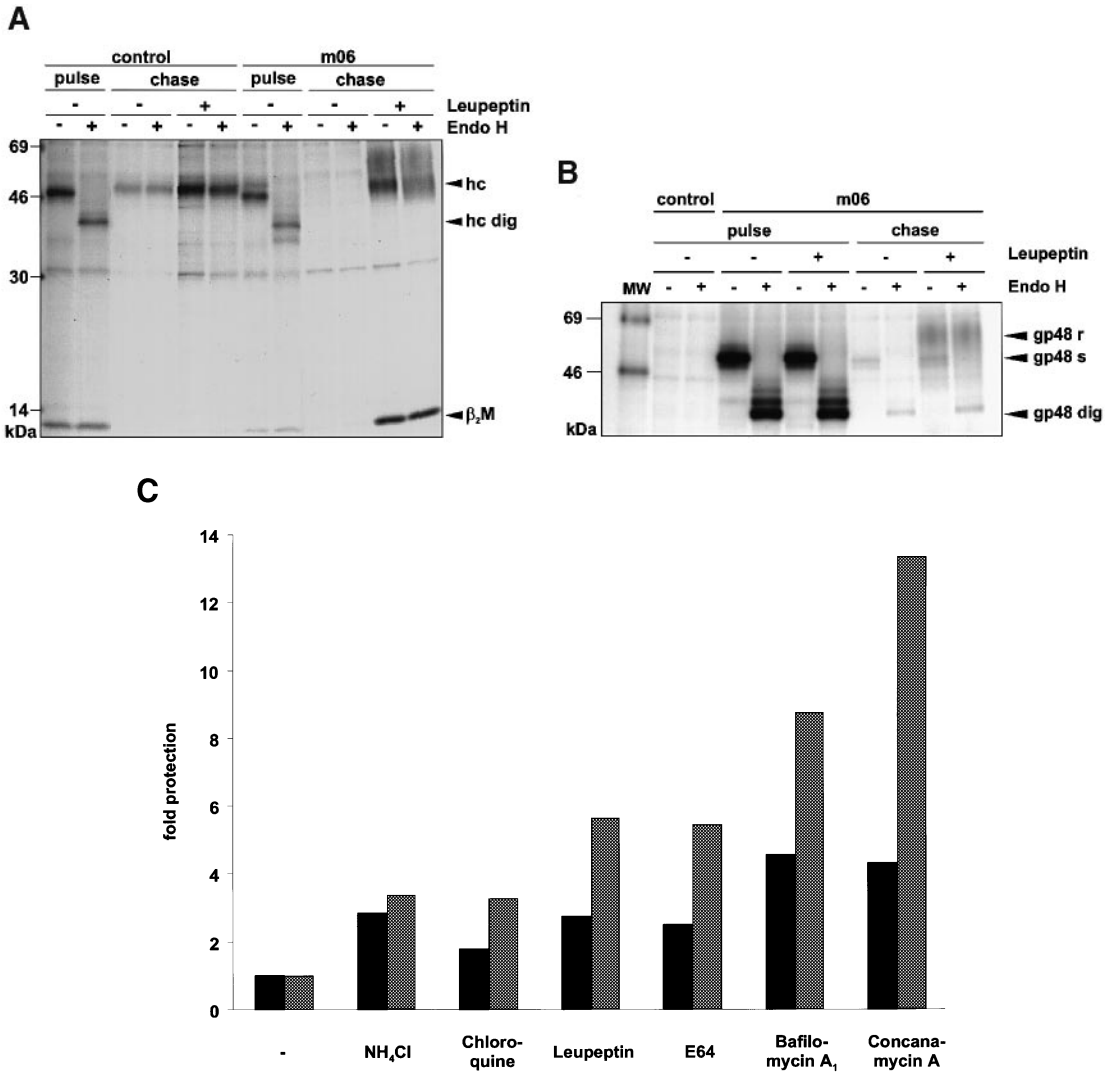


Fig. 5. Lysosomal inhibitors block degradation of both MHC class I molecules and gp48. NIH 3T3 *m06* transfectants were pulse labeled for 30 min and chased for 6 h in the presence of leupeptin as indicated. Cells were lysed in buffer containing NP-40, and aliquots of the lysates were precipitated with mAb 28–14–8s (**A**) or mAb CROMA229 (**B**). Half of each sample was digested with Endo H prior to 11.5–13.5% SDS–PAGE analysis. MW, ^{14}C-labeled molecular weight markers; hc, MHC class I heavy chains; β_2M, β_2-microglobulin; r, Endo H-resistant; s, Endo H-sensitive; dig, Endo H-digested. Please note, that the increased L^q precipitation observed in control cells upon leupeptin treatment is not significant but rather reflects the use of lysates containing more radioactivity. In other experiments this was not observed. (**C**) NIH 3T3 *m06* transfectants were treated with leupeptin (200 μM), NH_4Cl (50 mM) or chloroquine (100 μM) during 1 h pulse and 6 h chase, or 1 h pre-pulse and chase with E64 (L-*trans*-epoxysuccinylleucylagmatine, 100 μM), Bafilomycin A_1 (2 μM) or Concanamycin A (50 nM), and aliquots of lysates were precipitated with mAb 28–14–8s or mAb CROMA229 followed by separation of the samples by SDS–PAGE. Bands were quantified using a PhosphorImager (Molecular Dynamics), and the amount of recovered H-2 L^q and gp48 molecules of mock-treated cells was arbitrarily set to 1. The ratio of L^q (black) and gp48 (gray) in treated cells versus mock-treated cells is shown as fold protection.

Discussion

Here, we report on the isolation and functional characterization of a viral protein reacting with MHC class I complexes, demonstrating a new principle of function. MCMV affects MHC class I cell surface expression apart from the *m152* function by an additional transmembrane glycoprotein. This effect is mediated, unless other as yet unidentified functions contribute, by the MCMV *m06* gene product gp48. The gene was identified by an approach which combined searching for MCMV proteins that associate with MHC class I molecules, analysis of mutant viruses for the lack of the MHC class I complex reducing phenotype, and by transfection of genome fragments.

gp48 dominantly affects cell surface expression of MHC class I molecules. Soon after biosynthesis, gp48 associates with MHC class I molecules in the ER. Pulse–chase analysis in *m06* transfectants revealed that both MHC class I and gp48 molecules obtain Endo H-resistant carbohydrates but are subsequently degraded in lysosomes. In support of the latter, treatment of cells with lysosomotropic agents inhibited degradation of gp48/MHC class I complexes.

Interestingly, the cytoplasmic tail of gp48 contains two LL motifs, which may serve as sorting modules. LL-related signals have been implicated in the internalization and lysosomal targeting of a variety of cellular proteins (reviewed by Sandoval and Bakke, 1994). Therefore, it was tempting to speculate that one or both of these LL motifs are important for lysosomal delivery of gp48 and associated MHC molecules. Consistent with this idea, we found that in cells expressing a mutant gp48 molecule

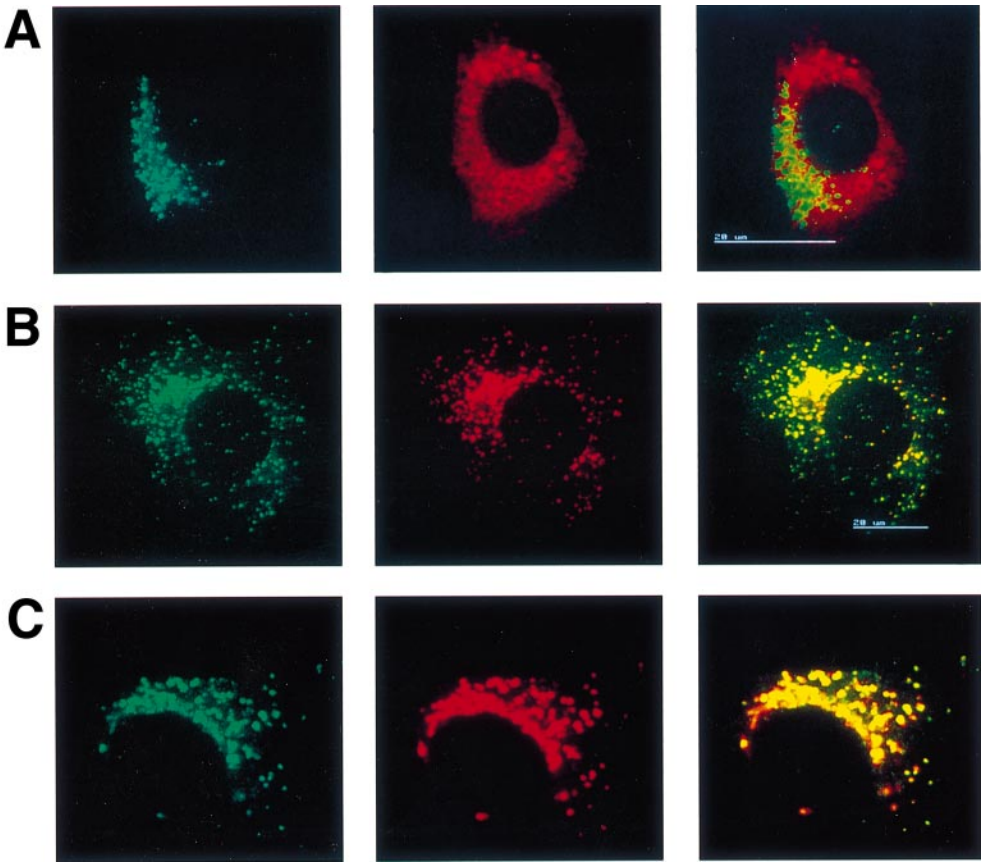


Fig. 6. MHC class I molecules and MCMV gp48 colocalize in lysosomal compartments. NIH 3T3 cells stably expressing MCMV *m06* were treated with leupeptin (200 μM) for 2 h prior to fixation and permeabilization and subsequent double staining with following antibodies. Mouse mAb CROMA231 anti MCMV gp48 (**A**–**C**, left panels) together with rabbit anti-calreticulin [(A), middle panel], rat anti-Lamp-1 [(B), middle panel] and rat anti-mouse MHC class I [(C), middle panel]. First, antibodies were visualized with fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit or rat IgG, respectively. The stained cells were analyzed with a laser scanning confocal microscope. The right panel represents an overlay of the corresponding double staining.

lacking the cytoplasmic tail, gp48ΔCT/MHC class I complexes obtain complex-type carbohydrates and are not degraded. In addition, mutation of the membrane proximal LL motif was sufficient to restore class I surface expression comparable to that observed in gp48ΔCT expressing cells, whereas mutation of the terminal LL motif did not release the gp48 mediated class I downregulation. Thus, the cytoplasmic tail of gp48 containing LL motifs is required for delivery and degradation of MHC molecules in lysosomes.

The molecular mechanism that mediates di-leucine-based sorting is not completely understood. There is evidence that heterotetrameric clathrin-associated protein (AP)-complexes (AP-1, AP-2 and AP-3) concentrate transmembrane proteins to clathrin-coated pits by direct interaction with tyrosine- and di-leucine-based sorting signals in the cytoplasmic domains of the target proteins (Le Borgne and Hoflack, 1998). It was proposed that acidic clusters in the vicinity of di-leucine based motifs (in positions –4 and –5 relative to the LL motif, e.g. in Iip31, Pond *et al*., 1995; in CD3-γ chain, Dietrich *et al*., 1997; and in LIMP-II and tyrosinase, Höning *et al*., 1998) are critical for AP-binding and sorting. In line with this hypothesis we find a glutamic acid residue in position –5 relative to the membrane proximal di-leucine while the nonfunctional distal LL motif lacks an acidic acid residue within the same distance.

At present, it is unclear whether the MHC class I/gp48 complexes reach first the cell surface and are subsequently endocytosed via the AP-2 pathway, or whether they are directly targeted from the TGN to endosomes/lysosomes by the AP-1 or AP-3 (Le Borgne and Hoflack, 1998). So far, we have been unable to detect gp48 on the cell surface. Therefore, it is likely that gp48/MHC class I complexes are directly sorted from the TGN to lysosomes, unless we assume that the residence time at the cell surface is extremely short. Studies are under way to examine the extent of co-localization with the respective AP complexes.

Interestingly, three other MCMV ORFs of the *m02* gene family, i.e. *m08*, *m09* and *m10*, all encoding type I transmembrane glycoproteins contain a membrane proximal LL-based motif but not the distal one (Rawlinson *et al*., 1996). Assuming that these gene family members have homologous functions and use this conserved sorting motif, it is tempting to speculate that binding to target proteins in the early secretory pathway and subsequent re-routing to the lysosomes for degradation is a more widely used principle of this viral gene family. Another member of the *m02* gene family, *m04*, also binds to MHC molecules, but does not re-route them to the lysosome.

Instead, the complex of the *m04* product and MHC class I molecules migrate to the cell surface. Remarkably, the cytosolic tail of *m04* lacks a LL motif found in *m06*, *m08*, *m09* and *m10*.

A number of viral proteins have been discovered that bind to MHC class I molecules and interfere in various ways with the antigen-presentation pathway. The adenovirus E3/19K protein (Burgert and Kvist, 1985), the HCMV US3 glycoprotein (Ahn *et al*., 1996; Jones *et al*., 1996), and the MCMV gp40 (Ziegler *et al*., 1997) prevent efficient export of MHC class I complexes from the ER without altering the half-life of the class I complexes significantly. In contrast, MHC class I heavy chains of cells expressing HCMV US2 and US11 proteins are dislocated from the ER into the cytosol where they are rapidly destroyed by the proteasome (Wiertz *et al*., 1996a,b). The Herpes simplex virus ICP47 protein (Früh *et al*., 1995; Hill *et al*., 1995) and the HCMV US6 protein (Ahn *et al*., 1997; Hengel *et al*., 1997; Lehner *et al*., 1997) inhibit the peptide supply into the ER by blocking the transporter associated with antigen processing (TAP). As a result, both prevent assembly of trimeric MHC class I complexes leading to ER-associated degradation of MHC class I molecules (Hughes *et al*., 1997). In contrast to the gp48-mediated lysosomal degradation of MHC class I molecules, all above-mentioned viral functions lead to a retention or degradation of newly synthesized MHC class I molecules in pre-Golgi compartments.

The MHC class I delivery phenotype to a post-Golgi compartment employed by gp48 resembles that described for the HIV-1 Nef protein. Nef induces degradation of MHC class I molecules in an ammonium chloride-sensitive compartment, most likely the lysosomes (Schwartz *et al*., 1996). Nef affects not only MHC class I molecules but exhibits multiple functions including downregulation of CD4 by enhancing its endocytosis (Mangasarian *et al*., 1997; Lu *et al*., 1998), modulation of T-cell receptor signaling (Iafrate *et al*., 1997) and induction of Fas ligand expression (Xu *et al*., 1997). Secondly, Nef is a cytosolic protein which can bind to membranes upon myristylation. Thirdly, it does not contain an LL-targeting motif, and finally, no complex formation has yet been observed between Nef and MHC class I molecules. Thus, a closer analysis reveals clear mechanistic differences between HIV-1 Nef and MCMV gp48.

In *m06* transfectants, the MHC class I molecules are targeted to an endolysosomal compartment. For certain cell types a physiological pathway for MHC class I molecules to enter the endocytic route has been described (Machy *et al*., 1987; Dasgupta *et al*., 1988; Vega and Strominger, 1989). After internalization, MHC class I

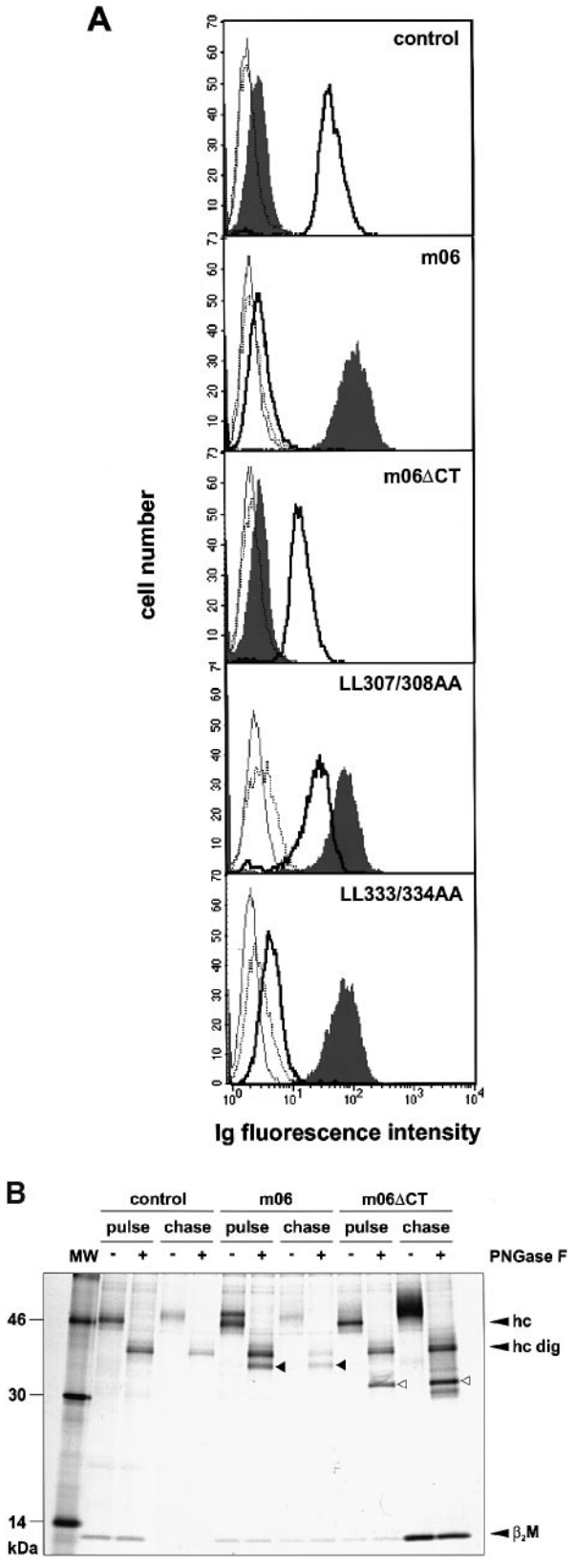


Fig. 7. The membrane proximal LL motif of the gp48 cytoplasmic tail is crucial for MHC class I downregulation. (**A**) NIH 3T3 control cells and transfectants expressing *m06* or the mutants *m06*ΔCT, *m06*LL307/308AA and *m06*LL333/334AA were stained with FITC-labeled goat anti-mouse IgG (dotted lines) or with mAb 28–14–8s recognizing H-2 L^q molecules (bold lines) and analyzed by flow cytometry. To detect *m06* expression, cells were permeabilized with saponin and stained with mAb CROMA231 specific for the cytoplasmic tail of gp48 (solid gray histogram); incubation of the permeabilized cells with secondary reagent alone served as negative control (gray line). (**B**) Control cells, *m06* transfectants and cells expressing gp48ΔCT were pulse-labeled for 30 min and chased for 4 h before lysis in digitonin buffer. H-2 L^q molecules, precipitated with mAb 28–14–8s, were left untreated or were digested with PNGase F and separated by 11,5–13,5% SDS–PAGE. MW, ^{14}C-labeled molecular weight markers; hc, MHC class I heavy chain; β_2M, β_2-microglobulin; dig, Endo H-digested; closed triangles, gp48 dig; open triangles, gp48ΔCT dig. All chased samples exhibit a reduction of radioactivity compared with that in the pulsed samples by ~40%, while this was not the case for *m06*ΔCT. This may explain the increased signals for L^q and gp48 in these lanes.

molecules are usually degraded in an acidic compartment (Machold and Ploegh, 1996). However, some empty class I heavy chains are apparently re-loaded with peptides derived from endocytosed and processed exogenous antigens, associate with β_2m and recycle to the cell surface for presentation to $CD8^+$ T cells (reviewed by Watts, 1997). The biological relevance of this pathway is still not clear. Our finding that gp48 can be co-immunoprecipitated with non-peptide-associated forms of H-2 L^d molecules which are recognized by mAb 64–3–7 (U.Reusch and U.H.Koszinowski, unpublished observation) raises the possibility that gp48 molecules associate with empty class I molecules in endolysosomal compartments and thus interfere with MHC recycling. There is also evidence that newly synthesized MHC class I molecules may reach the endocytic pathway in association with the invariant chain Ii (Sugita and Brenner, 1995). While Ii association was suggested to stabilize MHC class I/β_2m heterodimers in the ER (Vigna *et al*., 1996), the complex was also found in endocytic compartments (Sugita and Brenner, 1995) due to sorting signals present in the Ii cytoplasmic tail (Bakke and Dobberstein, 1990). Operationally, gp48 could be viewed as a dominant viral variant of Ii which fails to dissociate from the MHC complex. Thus, gp48 could have a physiological role in preventing endosomal loading and recycling of MHC class I molecules in cells using this pathway.

The discovery of multiple viral gene products in HCMV and MCMV that affect various steps of the MHC class I antigen-presentation pathway is a puzzle in itself. It raises the question of whether physiologically each of these proteins act in isolation, sequentially or in concert.

Interestingly, no cellular homologue has yet been identified for any of the herpesviral proteins reacting with MHC class I molecules. The functional dissection of the role of protein domains explains to some extent the functional diversity of the proteins. Although the products of MCMV *m04*, *m06* and the adenovirus E3/19K protein all bind to MHC class I molecules, the different fates of these complexes is defined by cellular targeting motifs adopted by the viral proteins. A more precise definition of the protein binding motif in the luminal part may also reveal certain rules shared by such proteins.

Materials and methods

Cell lines and viruses

BALB 3T3 cells (ATCC CCL 163) and BALB.SV fibroblasts (Del Val *et al*., 1991) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). NIH 3T3 fibroblasts (ATCC CRL 1658) transfected with the B45-Neo vector (Ohe *et al*., 1995) were cultured in DMEM supplemented with 10% (v/v) newborn calf serum and 1 mg/ml G418 (Gibco-BRL).

MCMV strain Smith (ATCC VR-194) and MCMV mutants ΔMS94.5 and ΔMS94.7 (Kleijnen *et al*., 1997) were used as tissue culture-grown viruses.

Preparation of MHC class I co-immunoprecipitates for immunization

BALB 3T3 cells were infected with wild-type MCMV (m.o.i. 5–10). Cytoplasmic extracts from ~3×10^7 cells were prepared at 7 h p.i. by lysis in 2 ml of buffer containing 1% NP-40, 20 mM Tris–HCl pH 7.6, 140 mM NaCl, 5 mM $MgCl_2$, 10 μM leupeptin, 1 mM PMSF. After pre-clearing with an irrelevant ascites (ascites of ACT1 hybridoma, anti-prokaryotic actin), MHC class I complexes were immunoprecipitated with 15 μl/ml of ascitic fluid containing a mixture of monoclonal antibodies to K^d (MA-215, Hasenkrug *et al*., 1987), D^d (34–5–8s, ATCC HB102) and L^d (28–14–8s, ATCC HB28), and protein A–Sepharose (Pharmacia). Immunoprecipitates were eluted in a 0.1 M citrate buffer (pH 4.0), and after elution the pH was adjusted to 7.0 by 0.1 M Tris–HCl (pH 8.0). The eluate was used for immunization of mice.

Immunization and monoclonal antibody production

BALB/c mice were immunized with a 1:1 emulsion of the immunoprecipitate in complete Freund's adjuvans, re-immunized with the suspension in incomplete Freund's adjuvans 3 weeks later, and boosted intraperitoneally with the suspension in phosphate-buffered saline (PBS) twice at 2-week intervals. Spleen cells of the immune mice were fused with myeloma cells Sp2/0 by polyethylene glycol procedure (PEG). Hybridoma supernatants were screened by ELISA on extracts prepared from cells infected with MCMV at 6 h p.i., and tested further by immunofluorescence and immunoprecipitation. For immunoprecipitation we used cytoplasmic extract of MCMV infected BALB 3T3 cells metabolically labeled with 200 μCi/ml of [^{35}S]methionine from 3 to 8 h p.i. Positive hybridoma cultures were cloned by limiting dilution following by at least one round of re-cloning. Two hybridoma clones originating from different wells (CROMA229 and CROMA231) produced a monoclonal IgG1 antibody that precipitated a protein of 48 kDa.

Antibodies and reagents

For detection of MHC class I molecules by flow cytometry and precipitation, the α_3 domain-specific mAb 28–14–8s (ATCC HB27), recognizing free and β_2m-associated L^d, L^q and D^b molecules was used. Hybridoma supernatant from SF1.1.1 (ATCC HB 159) was used to stain surface H-2 K^d molecules. For immunofluorescence staining of MHC class I molecules, the rat antibody R1-21.2 (Momburg *et al*., 1986), kindly provided by G.Hämmerling, was used. Integrin β1 chain was stained with purified rat anti-mouse CD29 (Pharmingen, CA). For precipitation of calnexin, a mixture of polyclonal rabbit antibodies raised against the C- and N-termini of canine calnexin was used (Biomol, Germany). Polyclonal rabbit antibodies to luminal ER protein calreticulin were from StressGen (Victoria, Canada). Cell culture supernatant from the rat hybridoma clone 1D4B, recognizing the lysosomal associated membrane protein Lamp-1 was purchased from Development studies hybridoma bank.

The protease inhibitor leupeptin was purchased from Boehringer Mannheim (Germany) and used at a final concentration of 200 μM. The lysosomotropic amines NH_4Cl (Merck, Germany) and chloroquine (Sigma) were added to cell cultures at final concentrations of 50 mM and 100 μM, respectively. E-64 (Fluka, Sigma-Aldrich, Germany) was used at a concentration of 100 μg/ml. Bafilomycin A_1 (Sigma) and Concanamycin A (Sigma) were used at final concentrations of 2 μM and 50 nM, respectively, to inhibit vacuolar type H^+-ATPase. Late-phase gene expression was prevented by treatment with phosphonoacetic acid (PAA, 250 μg/ml; Sigma), which arrests CMV-infected cells in the early phase.

Microinjection

Cells were seeded on Cellocates (Eppendorf, Germany) and injected with genomic or plasmid DNA as described previously (Thäle *et al*., 1994). Injected cells were incubated for 24 h at 37°C and 5% CO_2 and stained by indirect immunofluorescence with CROMA231. Since *Hin*dIII restriction of genomic MCMV DNA destroyes the major regulatory immediate-early genes *ie1* and *ie3* (Koszinowski *et al*., 1986), the plasmid pIE111, containing the ie1 and ie3 region, was co-injected to restore the gp48-specific immunofluorescence phenotype.

Cloning and expression of the MCMV gene m06

Polymerase chain reaction (PCR) was performed on genomic MCMV DNA for amplification of the *m06* gene using the following primers matching the 5′ and 3′ end, respectively, of ORF 5327:6337 and including the following restriction sites for cloning: (*m06* forward) 5′-CGC GGG CTC GAG GCC GCC ACC ATG GAC ACA ACT GCG AGA GGA-3′; (*m06* backward) 5′-CGC GGG CTC GAG TTT ATT ATT TGG TAA GCA AGG GGG AAG TG-3′ (*Xho*I sites underlined). The resulting PCR fragment was cloned under the control of the mouse metallothionein promoter into the *Sal*I site of B45-Neo vector (Ohe *et al*., 1995). NIH 3T3 cells were transfected with plasmid DNA by electroporation using a Bio-Rad gene pulser. Bulk cultures of transfectants were selected in the presence of 1 mg/ml G418 (Gibco-BRL) and tested for gp48 expression by immunoprecipitation and immunofluorescence microscopy. To generate the *m06* ORF coding for a protein with a deleted cytoplasmic tail, the backward primer 5′-CGC GGG CTC GAG

TTT ATT AGT AGG TGT AGA ACA ACA TAG C-3′ (*m06*ΔCT backward), and for the *m06*LL333/334AA mutant the backward primer 5′-CGC GGG CTC GAG TTT ATT ATT TGG T*TG CTG C*GG GGG AAG T-3′ (*m06*LL333/334AA backward; codons for alanines instead of leucines in italics) was used. The construct for the mutant *m06*LL307/308AA was prepared by the two-step PCR procedure. Briefly, the first PCRs were performed to amplify the *m06* fragment upstream of the LL307/308 codons with the *m06* forward primer and the LL307/308AA backward primer (5′-GCC GTA TCG CTT *GGC AGC* TCT AGC TAG CGG-3′) containing the codons for alanine instead of leucine (italics), and the fragment downstream of the LL307/308 codons with the LL307/308AA forward primer (5′-CCG CTA GCT AGA *GCT GCC* AAG CGA TAC GGC-3′, complementary to the LL307/308AA backward primer) and the *m06* backward primer. The PCR products were mixed and re-amplified using the *m06* forward primer and the *m06* backward primer to generate the complete *m06*LL307/308AA reading frame. The PCR fragments were cloned by the strategy described for *m06*.

Flow cytometry

Trypsinized cells were rinsed with PBS supplemented with 2% FCS and 0.03% NaN_3, and stained with the indicated antibodies. After washing, bound antibodies were visualized by addition of fluorescein-conjugated goat anti-mouse (Sigma) or goat anti-rat IgG (Dianova, Germany). For intracellular staining, cells were detached with 1 mM EDTA, fixed with 3% (w/v) paraformaldehyde, and quenched with 50 mM NH_4Cl and 20 mM glycine in PBS. Antibody incubation and washing was performed in buffer containing 0.1% Saponin. Cells incubated with the second antibody alone served as negative controls. A total of 5×10^3 or 1×10^4 cells were analyzed for each fluorescent profile on a FACSCalibur (Becton Dickinson, CA).

Metabolic labeling and immunoprecipitation

Immunoprecipitation was performed as described previously (Del Val *et al*., 1992). In brief, subconfluent layers of cells were labeled with [^{35}S]methionine and [^{35}S]cysteine (1200 Ci/mmol; Amersham, Germany) at a concentration of 350 μCi/ml and chased in the presence of 10 mM unlabeled methionine for the indicated periods of time. Cells were lysed in buffer (140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris pH 7.6, 1 mM PMSF) containing 1% (v/v) NP-40 (Sigma) or 1% (w/v) digitonin (Sigma), respectively. After removal of nuclei by centrifugation, lysates were precleared with the appropriate preimmune serum and protein A–Sepharose (Pharmacia). Immunoprecipitations were performed with ascites fluid or antiserum as indicated. To ensure quantitative retrieval of immune complexes, the lysates were incubated twice with protein A–Sepharose. Endo H- and PNGase F-(Boehringer Mannheim, Germany) digestion and SDS–PAGE were carried out as described previously (Del Val *et al*., 1992). The dried gels were exposed for different periods of time to BioMaxMR films (Kodak) at –70°C. ^{14}C-methylated proteins (Amersham, Germany) were used as molecular weight markers. In some experiments, bands were quantified using a Storm 860 Molecular Imager (Molecular Dynamics, CA).

For re-immunoprecipitation, the first precipitation was performed in 1% (w/v) digitonin lysis buffer as described above. After dissociation of immune complexes in 100 μl 1% SDS for 15 min at 37°C, 1 ml NP-40 lysis buffer was added, and first antibodies were removed by two rounds of incubation with protein A–Sepharose before re-immunoprecipitation with the indicated antibodies. When the first precipitate was analyzed directly, 5-fold fewer counts were used for immunoprecipitation analysis.

Confocal laser scanning microscopy

Subconfluent layers of NIH 3T3 *m06* fibroblasts were grown on glass coverslips. Cells were rinsed with PBS and fixed with 3% (w/v) paraformaldehyde in PBS for 20 min. After blocking unreacted aldehyde groups with 50 mM NH_4Cl and 20 mM glycine in PBS, cells were permeabilized with 0.2% Triton X-100 in PBS. To block non-specific binding of antibodies, coverslips were incubated in 0.2% (v/v) teleostean gelatin (Sigma, MO) in PBS. Double immunofluorescence was performed by incubating primary antibodies together in 0.2% gelatin in PBS for 45 min. After extensive washing in PBS, cells were incubated with fluorescein- or rhodamine-conjugated goat anti-mouse, goat anti-rabbit or goat anti-rat IgG, respectively (Dianova, Germany), in 0.2% gelatin for 45 min. After repeated washing the coverslips were mounted on glass slides with Histosafe (Camon, Germany). The mounted cells were analyzed with a laser scanning confocal microscope (Leitz DM IRB; scanner, Leica TCS NT).

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