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

Uwe Reusch, Walter Muranyi, Pero Lucin¹, Hans-Gerhard Burgert, Hartmut Hengel and Ulrich H.Koszinowski²,³

Max von Pettenkofer-Institut and Genzentrum, Ludwig-Maximilians-Universität München, 81377 München, ³Max von Pettenkofer-Institut, Lehrstuhl Virologie, Pettenkofer Straße 9a, D-80336 München, Germany and ³Department of Physiology and Immunology, University of Rijeka, 51000 Rijeka, Croatia

¹Corresponding author
e-mail: koszinowski@m3401.mpk.med.uni-muenchen.de

Mouse cytomegalovirus (MCMV) early gene expression interferes with the major histocompatibility 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 β₂-microglobulin (β₂m)-associated MHC class I molecules in the endoplasmic reticulum (ER). This association is mediated by the luminal/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, β₂-microglobulin (β₂m) 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).
Here we report on the identification and the function of the MCMV-encoded glycoprotein gp48. This protein forms a tight complex with β2m-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 endosomally/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 HindIII 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 proteins directly complexing with MHC class I molecules. By monitoring cells after injection of HindIII fragment pools, single fragments and subfragments we found that the 3.4 kbp XbaI–ClaI fragment (nucleotides 4162–7528 in the MCMV genome) in the HindIII region was sufficient for gp48 expression. The XbaI–ClaI 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 Lα 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 Kd, Ld, Dd, Kb, Db and Kk 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-2d), gp48 inhibited the antigen presentation to pp89-specific, Ld-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 Ld 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 Lα 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...
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...
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 degradation of MHC class I 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 passage through 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. Immunoprecipitation 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 NH4Cl and chloroquine as well as the vacuolar type H+-ATPase inhibitors batifolimycin A1 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, middle and right panels) and MHC class I (Figure 6C, middle and right panels) was obtained. This confirmed the co-localization of gp48 with the lysosomal marker Lamp-1 (Figure 6B, middle and right panels) and MHC class I (Figure 6C, middle and right panels) 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
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 Lq 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 m06ΔCT transfectants migrate in a broad band, indicative of coprecipitating 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 m06ΔCT 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 (m06LL307/308AA) or the terminal leucines (m06LL333/334AA) were substituted by alanines. Staining of saponin permeabilized transfectants with mAb CROMA231 revealed comparable mutant gp48 expression levels in m06, m06LL307/308AA and m06LL333/334AA transfectants (Figure 7A, solid gray histograms). Since CROMA231 recognizes an epitope in the cytoplasmic tail of gp48, only a background staining was achieved in m06ΔCT 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, 14C-labeled molecular weight markers; hc, MHC class I heavy chains; β2M, β2-microglobulin; r, Endo H-resistant; s, Endo H-sensitive; dig, Endo H-digested. Please note, that the increased Lq 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), NH4Cl (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 A1 (2 µM) or Concanaamycin 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 Lq and gp48 molecules of mock-treated cells was arbitrarily set to 1. The ratio of Lq (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...
Lysosomal degradation of MHC class I complexes

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 CROMA23 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 endocyted 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 \textit{m04} product and MHC class I molecules migrate to the cell surface. Remarkably, the cytosolic tail of \textit{m04} lacks a LL motif found in \textit{m06}, \textit{m08}, \textit{m09} and \textit{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 \textit{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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{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 \textit{m06} or the mutants \textit{m06ΔCT}, \textit{m06LL307/308AA} and \textit{m06LL333/334AA} were stained with FITC-labeled goat anti-mouse IgG (dotted lines) or with mAb 28–14–8s recognizing H-2Lq molecules (bold lines) and analyzed by flow cytometry. To detect \textit{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, \textit{m06} transfectants and cells expressing gp48ΔCT were pulse-labeled for 30 min and chased for 4 h before lysis in digitonin buffer. H-2Lq 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, \textsuperscript{14}C-labeled molecular weight markers; hc, MHC class I heavy chain; β2M, β2-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%, whereas this was not the case for \textit{m06ΔCT}. This may explain the increased signals for Lq and gp48 in these lanes.}
\end{figure}
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 β2m 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 Ld 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/endocytic pathway in association with the invariant chain I molecules in endolysosomal compartments and thus possibility that gp48 molecules associate with empty class I heavy chains are apparently re-loaded with peptides (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 β2m 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 Ld 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/endocytic pathway in association with the invariant chain I molecules in endolysosomal compartments and thus possibility that gp48 molecules associate with empty class I heavy chains are apparently re-loaded with peptides (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 β2m 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 Ld 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) 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 and BALB 3T3 cells (ATCC CRL 1658) transfected with the B45-Neo vector (Ohe et al., 1995) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). NIH 3T3 cells were infected with wild-type MCMV (m.o.i. 5–10).

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 5×10^6 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 MgCl₂, 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 of ascitic fluid containing a mixture of monoclonal antibodies to K^d (MA-215, Hasenkugl 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 adjuvants, re-immunized with the suspension in incomplete Freund’s adjuvants 4 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 [35S]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 αα domain-specific mAb 28–14–8s (ATCC HB27), recognizing free and β2m-associated L^d, L^e and D^o molecules was used. Hybridoma supernatant from SF1.1.1 (ATCC HB 159) was used in an anti-sense surface H-2 K^b molecules. For immunofluorescence staining of MHC class I molecules, the rat antibody R1-21.2 (Momburg et al., 1986), kindly provided by G.Hammerling, 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 (Bionor, 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 leupentin was purchased from Boehringer Mannheim (Germany) and used at a final concentration of 200 µM. The lysosomotropic amines NH4Cl (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 A1 (Sigma) and Concarnycin 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 (Thale 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 HindIII restriction of genomic MCMV DNA destroys 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 immunodegradation 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 GTC GAG GCC GAG ACC ATG GAC ACA ACT GCG AGA GGA-3’; (m06 backward) 5’-CGC GTC GAG TTC ATT TGG TAA GCA AGG GAG AAG TG-3’ (XhoI sites underlined). The resulting PCR fragment was cloned under the control of the mouse metallothionein promoter into the Sph site of the expression 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 µg/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 GTC GAG
TTT ATT AGT AGG TGT AGA ACA ACA TAG C-3′ (m06GCT backward), and for the m06LL333/334AA mutant the backward primer 5′-GGT AGC CTT GAG TTT TagG CGG AAG T-3′ (m06LL333/334AA backward; codons for alanines instead of leucines in italics) was used. The construct for the mutant m06LL337/308AA was prepared by the two-step PCR procedure. Briefly, the first PCRs were performed to amplify the m06 fragment upstream of the LL337/308 codons with the m06 forward primer and the LL337/308AA backward primer (5′-GCC GTA TCG CTT GGC AGC TCT AGC TAG CGG 3′-CCG CTA GCT AGA 5′) containing the codons for alanine instead of leucine (italics), and the fragment downstream of the LL337/308AAs codons with the LL337/308AA forward primer (5′-CCG CTA GCT AGA GCC CAG GA TAC GCC-3′, complementary to the LL337/308AA 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 m06LL337/308AAs 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% NaN3, 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 NH4Cl and 20 mM glycine in PBS. Cells were rinsed with PBS and fixed with 3% (w/v) paraformaldehyde, and quenched with 50 mM NH4Cl 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 5×103 or 1×104 cells were analyzed for each fluorescent profile on a FACS Calibur (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 cultured in [35S]methionine and [3H]lysine (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 MgCl2, 20 mM Tris pH 7.6, 1 mM PMSF) containing 1% (w/v) digitonin (Sigma), respectively. After removal of nuclei by centrifugation, lysates were pre-cleared with the appropriate preimmune serum and protein A-Sepharose (Pharmacia). Immunoprecipitations were performed with ascites fluid or antisera 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. 14C-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 precipitation 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 NH4Cl 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 permeabilized with 0.2% Triton X-100 in PBS. To block non-specific binding of antibodies, coverslips were incubated in 0.2% (v/v) tel...
Lysosomal degradation of MHC class I complexes


Klein,M., Huppa,J.B., Lucin,P., Mukherjee,S., Farrell,H., Campbell,A.E., Koszinowski,U.H., Hill,A.B. and Ploegh,H.L. (1997) A mouse cytomegalovirus glycoprotein, gp34, forms a complex with a folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. EMBO J., 16, 685–694.


