A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface

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Murine cytomegalovirus (MCMV) interferes with antigen presentation by means of retaining major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (ER). Here we identify and characterize an MCMV-encoded glycoprotein, gp34, which tightly associates with properly conformed MHC class I molecules in the ER. Gp34 is synthesized in large quantities during MCMV infection and it leaves the ER only in association with MHC class I complexes. Many but not all class I molecules are retained in the ER during the early phase of MCMV infection, and we observe an inverse correlation between amounts of gp34 synthesized during the course of infection and class I retention. An MCMV deletion mutant lacking several genes, including the gene encoding gp34, shows increased class I retention. Thus, MCMV gp34 may counteract class I retention, perhaps to decrease susceptibility of infected cells to recognition by natural killer cells.

Keywords: antigen presentation/ER retention/gp34/MHC class I/murine cytomegalovirus

Introduction

Herpesviruses are capable of persisting in the host for extensive periods of time, in many cases in a state of latency, and can do so in the face of a fully primed immune system. In fact, all members of the Herpesviridae studied thus far modulate the immune system, often by interfering with proper antigen presentation to cytotoxic T lymphocytes (CTL). HSV-1 and HSV-2 produce a small protein, ICP47, which binds to the major histocompatibility complex (MHC)-encoded peptide transporter, thus thwarting presentation of peptide by MHC class I molecules (York et al., 1994; Früh et al., 1995; Hill et al., 1995). Epstein–Barr virus requires EBNA-1 gene expression to maintain the viral genome during latency, yet this antigen is poorly recognized, if at all, by CTL, a property attributed to the presence of a Gly–Ala repeat in EBNA-1 (Levitskaya et al., 1995). Human cytomegalovirus (HCMV) infection results in rapid destruction of newly synthesized class I molecules. Either of two gene products (US2 or US11) mediates a process that involves dislocation of the class I heavy chains from the endoplasmic reticulum (ER) to the cytosol (Jones et al., 1995; Wiertz et al., 1996). In addition, the HCMV US3 gene encodes a function capable of delaying intracellular transport of newly synthesized class I molecules (Ahn et al., 1996; Jones et al., 1996). All of these strategies have been interpreted as a means for the virus to escape immune destruction during a crucial phase of its life cycle, particularly in the production of new infectious virus upon reactivation from the latent state.

Understandably, viral functions that attenuate MHC class I expression have received much attention. This phenomenon is straightforward to establish, and such regulation has obvious implications for recognition of infected cells by CTL. However, it is becoming increasingly clear that class I molecules protect cells from lysis by natural killer (NK) cells and, consequently, viral evasive strategies that result in the elimination of class I expression render infected cells susceptible to attack by NK cells. Therefore, the relationship between virus infection, class I expression and protective immunity is likely to be complex.

Mouse cytomegalovirus (MCMV) is a herpesvirus well characterized for its interference with antigen presentation. MCMV, a member of the betaherpesvirinae, is a natural pathogen of mice. Acute infection of mice with MCMV elicits an immune response which controls the virus infection. However, the virus persists, and recurrent infection ensues if the animal becomes immunocompromised. MCMV is considered to be an NK-sensitive virus, and the NK response is important in controlling the degree of virus replication early in infection (Scalzo et al., 1992; Orange et al., 1995, 1996). Indeed, an important susceptibility locus for MCMV maps to the murine NK locus on chromosome 6 (Scalzo et al., 1992, 1995). CTL are elicited by MCMV as well, and can confer protective immunity (Koszinowski et al., 1987; Reddehase et al., 1987, 1988; del Val et al., 1988). However, MCMV interferes with antigen presentation to CTL (Campbell et al., 1992; del Val et al., 1992). In contrast to the pattern seen in HCMV infection, the MHC class I molecules are not degraded rapidly, and are apparently loaded with antigenic peptides normally yet fail to leave the ER (del Val et al., 1992), a mechanism superficially reminiscent of that employed by the adenovirus E3/19K protein. This
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Fig. 1. Gp34, a 34 kDa protein, co-immunoprecipitates with MHC class I complexes in MCMV-infected cells. FT1<sup>+</sup> cells were treated with γ-interferon and infected with MCMV at an m.o.i. of 10. One hour post-infection (p.i.), the cells were labeled continuously with [35S]methionine/cysteine. The cells were lysed 12 h p.i. and the lysates were pre-cleared with either normal rabbit serum (nrs) or normal mouse serum (nms). Immunoprecipitations were performed as indicated.

Effect is seen only with expression of MCMV early genes, and maps to the Hin<sub>IIIE</sub> region of the MCMV genome (Thäle et al., 1995).

We report here the identification of an MCMV-encoded 34 kDa glycoprotein, gp34, that forms a tight complex only with properly conformed class I molecules. Class I molecules that associate with gp34 escape from MCMV-imposed ER retention and are displayed at the cell surface. This novel class I–gp34 complex may prevent the destruction of the infected cell by immune effector cells or render it less immunogenic.

Results

A glycoprotein of 34 kDa is tightly bound to β<sub>2m</sub>-associated MHC class I molecules in MCMV-infected cells

To identify proteins involved in modulating the function of class I molecules in MCMV-infected cells, MHC class I products were immunoprecipitated from MCMV-infected primary mouse embryo fibroblasts using a number of anti-class I reagents. A protein of apparent mol. wt 34 kDa (referred to as gp34 because of its glycoprotein nature: see below) was observed in class I immunoprecipitates from H-2<sup>b</sup> fibroblasts (Figure 1). The association between gp34 and class I persisted through four washes of the immune complex with lysis buffer containing 0.1% SDS. Gp34 could be co-precipitated with K<sup>b</sup> using the antibodies Y3 [recognizing β<sub>2</sub>-microglobulin (β<sub>2m</sub>)-associated K<sup>b</sup>], and an antiserum raised against the cytoplasmic tail of K<sup>b</sup> (anti-p8); and with D<sup>b</sup> using the antibodies B22.249 and 28.14.8s. Gp34 was not immunoprecipitated by normal mouse or rabbit serum, and thus is not an Fc receptor. It was absent from uninfected cells, regardless of interferon (IFN) treatment, and thus is not likely to be a cellular protein induced indirectly by IFN as a consequence of virus infection. Class I synthesis was stimulated greatly in mouse embryo fibroblasts by MCMV infection. The level of class I synthesis and interaction with gp34 in MCMV-infected cells which had been treated previously with IFN-γ and those which had not was indistinguishable (M.F.Kleijnen and J.B.Huppa, unpublished observations).

We routinely pre-treated cells with IFN-γ in experiments where uninfected cells were used as controls, as without this treatment very little class I synthesis could be observed in uninfected cells. Both slight variations in labeling and slight differences in the conditions of viral infection, contributed to some variability in the amount of labeled gp34 co-immunoprecipitated with class I molecules.

To determine the conformational states of class I molecules that allowed co-precipitation of gp34, fibroblast lines derived from mice with targeted deletions of β<sub>2m</sub>, TAP-1 or of TAP-1 in human β<sub>2m</sub> transgenic mice (Koller et al., 1990; Van Kaer et al., 1992; Machold et al., 1995; Van Santen et al., 1995) were used for infection with MCMV (Figure 2). Each of these cell lines was infected to an equivalent extent, as determined by immunofluorescent staining of cells for MCMV-encoded early protein pp89. K<sup>b</sup> was immunoprecipitated with the anti-p8 antiserum, and D<sup>b</sup> with the monoclonal antibody 28.14.8s. Co-precipitation of class I molecules and gp34 was observed in wild-type fibroblasts (wt H-2<sup>b</sup>), but not in cells lacking β<sub>2m</sub>. Only a very faint gp34 band was seen in TAP<sup>−</sup> cells, and none at all in β<sub>2m</sub><sup>−/−</sup>/TAP<sup>−</sup> cells. Co-precipitation of gp34 was seen in cells transgenic for human β<sub>2m</sub>, even

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in the presence of TAP (hβ2m; Tap+), presumably reflecting the increased stability of 'empty' murine class I complexes in the presence of human β2m. Parenthetically, we noted a decrease in recovery of Dβ with 28.14.8s in some of these mutant cell lines, a finding that presumably reflects some degree of conformational specificity of this antibody, although 28.14.8s has been reported to interact with both conformed and free heavy chains. The presence of Dβ free heavy chains in the cell lysates from β2m−/− and Tap−/− was confirmed by sequential immunoprecipitation with a polyclonal antiserum reactive with all free murine class I heavy chains (Machold et al., 1995) (data not shown).

When H-2 b fibroblasts were infected with MCMV, gp34 co-immunoprecipitated with Dβ and Ld. However, gp34 was not observed to co-precipitate with Kβ, even though the antibody used was the same antibody (anti-p8; raised against the carboxy-terminal residues of the cytoplasmic tail encoded by exon 8 in K locus alleles) used to precipitate Kβ (data not shown).

We thus conclude that gp34 co-precipitates with a number of class I alleles, is found only in MCMV-infected cells and is observed only together with conformed, β2m-associated class I MHC complexes.

**Gp34 associates with class I molecules in the ER and contains three N-linked glycans, one of which can acquire EndoH resistance**

Since MCMV retains MHC class I in the ER, we asked whether gp34 was an ER-resident glycoprotein and could thus be responsible for this retention. Class I–gp34 complexes were recovered from MCMV-infected cells with anti-p8 and treated with a range of concentrations of endoglycosidase-H (EndoH) to determine the number of N-linked attachment sites used. EndoH cleaves the high mannose N-linked glycans found on ER-resident proteins; modification of glycans in the medial Golgi usually results in resistance to EndoH. Increasing concentrations of EndoH progressively cleaved two glycan moieties from gp34; a third glycan was removed from a small proportion of the protein. In this experiment, ~50% of the class I heavy chains remained EndoH sensitive, reflecting their retention in the ER.

The drug Brefeldin A (BFA) impedes anterograde membrane transport from the ER to the Golgi compartments and causes a reordering of intracellular membrane structures. In general, newly synthesized glycoproteins remain EndoH sensitive in the presence of BFA when treatment with the drug is for brief periods, but post-translational protein modifications may differ subtly from those found in the ER environment in untreated cells due to redistribution of Golgi enzymes including glycosyl transferases. In BFA-treated cells, all gp34 collapsed to the lower mol. wt band upon digestion with EndoH (Figure 3B). The fact that class I–gp34 complexes could be recovered in immunoprecipitates of class I molecules from BFA-treated cells indicates that complex formation can occur in the ER. Proteins on the cell surface of MCMV-infected cells were labeled with 125I and lactoperoxidase. Immunoprecipitation of cell surface-labeled class I molecules with the anti-cytoplasmic tail antibody (anti-p8) resulted in the recovery of radiolabeled gp34. The immunoprecipitates were analyzed by digestion with EndoH and, as expected for cell surface class I molecules, all of the Kβ was EndoH resistant. Two of the three glycans of the cell surface gp34 remained EndoH sensitive (Figure 3C). Combined, these results show that class I–gp34 complexes are expressed at the cell surface, and that the majority of surface gp34 recovered from MCMV-infected cells contains one EndoH-resistant and two EndoH-sensitive glycans. The apparent mol. wt of the fully deglycosylated protein was 30.5 kDa. The decreased intensity of the band corresponding to β2m in MCMV-infected cells (Figure...
The MCMV deletion mutant ΔMS94.7 fails to express gp34, but maintains ER retention of class I molecules. FT1+ cells were either mock infected or infected with the MCMV Vancouver strain or the MCMV deletion mutant ΔMS94.7 and continuously labeled with [35S]methionine/cysteine. Cells were lysed 12 h p.i. and immunoprecipitations were performed with either the anti-p38 or the anti-pp89 antiserum. (Thäle et al., 1995).

ΔMS94.5 contains a deletion in the HinDIIIE region of the genome. This virus strain lacks the ability to retain MHC class I molecules in the ER, and has been used to identify the gene responsible for ER retention (U.H. Koszinowski, in preparation). Kβ immunoprecipitated from cells infected with ΔMS94.5 was all of the mature, EndoH-resistant form. However, gp34 still co-precipitates with class I in cells infected with this virus (data not shown). A second mutant, ΔMS94.7, contains a deletion in the HinDIIIA region. Figure 5 shows that anti-pp8 immunoprecipitates from cells infected with this virus lack gp34; in contrast, gp34 co-precipitates with H2-Kβ from cells infected with the MCMV Vancouver strain, whose HinDIIIA region is intact. H2-Kβ in the ΔMS94.7-infected cells is almost entirely of the lower molecular weight, reflecting its efficient retention in the ER. The lack of gp34 co-precipitation in the ΔMS94.7 mutant is correlated with increased MHC class I retention, as compared with the Vancouver strain (Figure 5).

We conclude that (i) the functions of ER retention and co-precipitation of gp34 can be dissociated and (ii) that the co-precipitation of gp34 maps to the HinDIIIA region of the MCMV genome.

**Cloning of the candidate gene that specifies gp34**

The entire genome of MCMV has been sequenced (Rawlinson et al., 1996). Four open reading frames (ORFs) within the HinDIIIA region encode polypeptides between 28 and 40 kDa that contain at least three potential N-linked glycosylation sites. Only one, m04 (ORF 3270:4076) was considered a serious candidate to encode gp34, based on its predicted molecular weight (30243 Da), the presence

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**Fig. 4.** Gp34 is a basic protein. FT1+ cells, infected with MCMV or mock uninfected, were continuously labeled with [35S] and lysed 12 h p.i. Class I–gp34 complexes, recovered with anti-p38 antiserum, were analyzed on 2D gels. The first dimension was non-equilibrium IEF, run from acidic to basic as marked, and the second dimension was conventional 12.5% SDS–PAGE. Gp34 is indicated here as p34; due to sialylation, the Golgi-modified form runs as a more acidic spot than the ER-resident form.

3C) may be due to a decreased accessibility of β2m to surface iodination in the presence of gp34. Alternatively, exchange of murine β2m for bovine β2m—a species more readily labeled by iodination—is less efficient when class I molecules are in a complex with gp34.

**Gp34 is a basic protein**

Analysis of Kβ immunoprecipitates on one-dimensional isoelectric focusing (IEF) gels, and on 2D gels with an equilibrium IEF gel as the first dimension failed to reveal gp34, even though it was detected readily by SDS–PAGE (data not shown). 2D IEF was performed using a non-equilibrium IEF gel as the first dimension. With short IEF runs (5 h), two 34 kDa spots were apparent (Figure 4); with longer runs, these spots migrated further towards the cathode or disappeared. We conclude that gp34 has too basic a pI to be resolved adequately on equilibrium IEF gels. The pI of gp34 is therefore likely to be in excess of 7.0.

**A deletion within the HindIII region of the MCMV genome impedes expression of gp34 and decreases the degree of class I retention**

Two mutant MCMV virus strains with large deletions in their genomes were generated and have been described

(Thäle et al., 1995). ΔMS94.5 contains a deletion in the HindIII region of the genome. This virus strain lacks the ability to retain MHC class I molecules in the ER, and has been used to identify the gene responsible for ER retention (U.H. Koszinowski, in preparation). Kβ immunoprecipitated from cells infected with ΔMS94.5 was all of the mature, EndoH-resistant form. However, gp34 still co-precipitates with class I in cells infected with this virus (data not shown). A second mutant, ΔMS94.7, contains a deletion in the HindIII region. Figure 5 shows that anti-p8 immunoprecipitates from cells infected with this virus lack gp34; in contrast, gp34 co-precipitates with H2-Kβ from cells infected with the MCMV Vancouver strain, whose HindIII region is intact. H2-Kβ in the ΔMS94.7-infected cells is almost entirely of the lower molecular weight, reflecting its efficient retention in the ER. The lack of gp34 co-precipitation in the ΔMS94.7 mutant is correlated with increased MHC class I retention, as compared with the Vancouver strain (Figure 5).

We conclude that (i) the functions of ER retention and co-precipitation of gp34 can be dissociated and (ii) that the co-precipitation of gp34 maps to the HindIII region of the MCMV genome.
gp34 complexes with MHC class I molecules

1. \text{N}^{\text{GLWCLLVLITYLQSELETPVYQERARGNCCRQG
2. \text{N}^{\text{KMERKYRHRGCYT
3. \text{N}^{\text{K}^{\beta}\text{b}}
4. \text{gp34}
5. \text{0}^{\beta_{2}m}

Fig. 6. The R123 antiserum immunoprecipitates gp34. Anti-p8 immune complexes obtained from \text{35S}-labeled, MCMV-infected FT1\text{I}^{+} cells were denatured and subjected to a second round of immunoprecipitation using the anti-p8 serum or the R123 antiserum. Samples were analyzed by SDS–PAGE.

of five consensus sites for \text{N}-linked glycosylation and its predicted pI (basic). Amplification of this ORF by PCR yielded a DNA fragment of the predicted size (0.8 kb) which was cloned into pSP72 and pBluescript and subsequently sequenced to confirm its identity with m04.

\text{In vitro} transcription/translation of the cloned segment in rabbit reticulocyte lysate supplemented with dog pancreas microsomes yielded a membrane-associated polysaccharide of an apparent molecular weight identical to that of gp34 recovered from MCMV-infected cells. The number of \text{N}-linked glycans, as determined by EndoH treatment, was found to be three and consequently in agreement with the number determined for gp34 from MCMV-infected cells.

The cleavage of the signal sequence was confirmed by comparison of the gel mobility of EndoH-digested \text{in vitro} translation product translated either in the presence or absence of microsomes (data not shown). Only three of the five possible \text{N}-linked glycosylation sites are used, but we have not yet determined which sites are glycosylated in MCMV-infected cells.

**Antibody against gp34**

We raised a rabbit antiserum (R123) against the cytoplasmic tail of the candidate gene encoding gp34. This antiserum immunoprecipitates a protein with a mobility identical to that of gp34 recovered in a complex with class I molecules from MCMV-infected cells (data not shown). We performed a re-immunoprecipitation experiment to establish the identity of the candidate gene product with the class I-associated material, as follows. An immunoprecipitate obtained with anti-p8 from MCMV-infected cells was dissociated in SDS and was then used for a second round of immunoprecipitation with either anti-p8 or R123 (Figure 6). The R123 serum efficiently recognized the polypeptide associated with H2-\text{K}^{\beta} and thereby confirmed the identity of the cloned ORF with the gene encoding gp34. The cleavage site of the N-terminal signal sequence as indicated in Figure 6 was determined by the occurrence of \text{[35S]}methionine in a radiochemical sequencing of \text{[35S]}methionine/cysteine-labeled gp34 (data not shown).

Finally, a recombinant vaccinia virus encoding this ORF led to expression of a protein with characteristics indistinguishable from those of the MCMV-encoded gp34 (data not shown), indicating that gp34 can associate with class I complexes in the absence of other MCMV-encoded proteins. Hence, we conclude that MCMV m04 (ORF 3270:4076) encodes gp34 (Figure 7).

**Upon MCMV infection, gp34 is synthesized in large quantities but leaves the ER only in association with MHC class I molecules**

Our attempts to co-immunoprecipitate MHC class I molecules with the R123 serum were unsuccessful. To characterize the R123 antiserum further, we performed the following experiment. Three hours post-infection, MCMV-infected cells were metabolically labeled for 8 h and then lysed. The lysates were subjected to sequential immunoprecipitations with either anti-p8 (anti-\text{K}^{\beta}) or R123 (anti-gp34) in order to deplete for molecules carrying the corresponding epitope. Subsequently, one round of immunoprecipitation with R123 was performed on the K\text{\beta}-depleted lysate (Figure 8). To assess the maturation status of the \text{N}-linked glycans, all immunoprecipitates
Upon MCMV infection ER retention of class I molecules is not complete. At 9 h p.i., MCMV FT1/H11001 cells were pulse-labeled for 15 min and subsequently chased for different periods of time as indicated. Immunoprecipitates obtained with the anti-p8 or the R123 antiserum from cell lysates from the indicated chase time points were digested with EndoH.

Fig. 9. Upon MCMV infection ER retention of class I molecules is not complete. At 9 h p.i., MCMV FT1/H11001 cells were pulse-labeled for 15 min and subsequently chased for different periods of time as indicated. Immunoprecipitates obtained with the anti-p8 or the R123 antiserum from cell lysates from the indicated chase time points were digested with EndoH.

ER retention of H2-Kb caused by MCMV is not complete

To resolve the kinetics of intracellular transport of both class I molecules and gp34 after insertion into the ER membrane, we performed a pulse–chase experiment followed by the recovery of gp34 either complexed with (Figure 9, lanes 1–5) or devoid of (Figure 9, lanes 6–10) class I molecules.

Nine hours post-infection with MCMV, at a time point where gp34 is in great excess over class I molecules in the ER, cells were pulse-labeled for 15 min and then chased for up to 8 h. Cell lysates were subjected to immunoprecipitations using either anti-p8 or R123 serum. To monitor egress of H2-Kb molecules and gp34 from the ER, immunoprecipitates were treated with EndoH (Figure 9). The material recovered with anti-p8 (i.e. both class I molecules and gp34 in complex with class I molecules, gp34 serving as an internal control for infection) gradually acquired EndoH resistance in the course of the chase, though at a significantly slower rate compared with class I molecules from uninfected cells (data not shown). At least for the H-2b haplotype, we can conclude that even transient retention of class I molecules in the course of an MCMV infection is not complete.

The kinetics of class I–gp34 complex formation could not be addressed directly in this experiment. The accumulation of a large excess of unlabeled gp34 during the first 9 h after infection explains the low recovery of labeled class I–gp34 complexes during the first 2 h of chase. At this time point, class I molecules recovered with anti-p8 serum are in all likelihood associated with unlabeled gp34. This situation is strictly analogous to the formation of complexes of labeled class I heavy chains with pre-
with wild-type MCMV where gp34 is expressed. As the deletion in ΔMS94.7 includes other ORFs not further characterized, a mutant virus with a targeted deletion of the gp34 gene is required to examine this possibility more rigorously.

Combined, these data are consistent with the suggestion that gp34 counteracts the class I MHC retention mechanism and allows escape of class I molecules to the cell surface.

**Discussion**

The herpesviruses offer a bewildering array of functions that help the virus establish itself in an immunocompetent host. Because CTL and NK cells are required for the host to eliminate virus-infected cells, it should not come as a surprise that the expression of class I molecules is one of the functions targeted by the virus. Specifically, elimination of class I molecules, their retention in the ER or withholding peptide from class I molecules have been described as tacks taken by the virus to avoid detection. Here we describe a novel MCMV-encoded product that forms a tight complex with MHC class I products. The formation of these complexes requires the presence of an active TAP complex, as well as the light chain, β2m. In the absence of a functional TAP complex, provision of heterologous β2m can stabilize the class I molecules sufficiently to allow an interaction with gp34 (Van Santen et al., 1995). Combined, these data strongly suggest that gp34 senses the conformation of peptide loaded, properly conformed class I molecules. Although our survey was limited, products of both H-2K and -D loci are capable of forming a complex with gp34 with little allelic preference, as for both haplotypes tested, the H-2β and H-2d haplotypes, such complexes can be detected.

The identification of the gene encoding gp34 was based on the biochemical characteristics of the gp34 product itself. Importantly, cells infected with ΔMS94.7, which lacks the gene we identified as encoding gp34, fail to express the class I-associated glycoprotein. Its expression early during the infectious cycle of the virus, its molecular weight, the number of N-linked glycans and its relatively high isoelectric point narrowed the choice to a small number of ORFs. In view of its association with class I molecules, and its presence at the cell surface, gp34 was likely to be a membrane glycoprotein. Based on these parameters, our further efforts were concentrated on the synthesis of a peptide corresponding to the predicted C-terminal cytoplasmic domain of the MCMV open reading frame ORF 3270:4076. This led to the production of a recombinant vaccinia virus specifying the synthesis of gp34 likewise encoded a product reactive with this antiserum and of biochemical characteristics indistinguishable from that recovered in MCMV-infected cells, including the association with class I complexes. A mutant MCMV harboring a deletion of the segment encompassing the putative gp34 gene failed to produce class I-associated gp34, in further support of the identification of the correct gene.

Using the anti-peptide serum, we established that synthesis of gp34 is detectable some 2 h after infection, with peak levels of expression being reached at ~4 h post-infection. Based on its methionine content, we conclude
that there is a large molar excess of gp34 synthesized compared with class I heavy chains. This excess is likely to drive a sizeable portion, possibly the entire pool, of existing class I molecules into a complex with gp34. The ability of gp34 to form a complex with class I molecules in BFA-treated cells establishes that the environment of the ER is compatible with complex formation, and is consistent with the notion that complex formation occurs early in biosynthesis.

Of interest is the observation that class I-associated gp34 acquires at least one complex type N-linked glycan, indicating its passage through the Golgi when associated with class I molecules. This is striking because it shows that early during the course of MCMV infection, when the MHC class I retention mechanism is operative, a significant portion of the newly synthesized class I complexes in infected cells can escape retention. The extent to which escape of retention occurs is correlated with the amount of synthesized viral gp34. Early in infection (2 h p.i.) MHC class I retention is efficient while gp34 can hardly be detected. Progressing along in the viral life cycle results in the accumulation of massive amounts of ER-resident, mainly unassociated, gp34, and an increase in escape of retention. These results are consistent with the idea that the association of class I molecules with gp34 allows them to escape ER retention. Indeed, in a virus harboring a deletion that encodes the gp34 gene, virtually all of the newly synthesized class I molecules retain full EndoH sensitivity, whereas in cells that are infected with wild-type MCMV, gp34 is observed in association with class I molecules that carry complex type oligosaccharides. At the cell surface, the only form of gp34 that is detected carries a single complex type and two high mannose type N-linked glycans.

MHC class I molecules play two major roles in host immunity. First, they present peptides derived mostly from cytosolic and nuclear proteins to CD8-positive CTL (Townsend and Bodmer, 1989). Secondly, they deliver an inhibitory signal to NK cells (Kärre, 1995) if class I molecules disappear from the cell surface (as happens, for

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Fig. 11. Schematic model of the gp34 interaction with MHC class I complexes during infection with MCMV.
instance, when they are retained in the ER or destroyed), the inhibitory signal is lost and the cell becomes susceptible to NK lysis (Koszinowski et al., 1987; Brutkiewicz and Welsh, 1995; Yokomaya, 1995). Both CTL and NK cell responses are involved in immunity to MCMV. Induction of a CTL response against a single major antigenic determinant can protect mice from subsequent lethal challenge with MCMV, indicating that CTL can mediate protection (Jonjoc et al., 1988). Similarly, NK-deficient mice are much more susceptible to MCMV than wild-type mice, and a major susceptibility locus to MCMV maps to the murine NK locus on chromosome 6 and determines the efficacy of the NK response (Scalzo et al., 1992). Thus the ability to modulate either the NK or CTL response is likely to be advantageous to viral persistence in the immune host. Gp34 could be responsible for allowing class I molecules to travel to the cell surface and, via its association with those molecules, let them serve as non-functional, decoy class I complexes preventing effective NK activity (Figure 11). An investigation of the effect of gp34 on CTL and NK function is currently underway. The diversity of mechanisms of interference with immunity by viruses is becoming increasingly apparent, and not only allows a deeper understanding of cell biology and the immune system but also provides novel tools for investigating these processes.

Materials and methods

Cells

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin. The wild-type fibroblasts used for most experiments were FT1+ cells (gift from Dr. L. van Kaer), a primary cell line of the H-2b haplotye. In addition, several other mouse embryonic fibroblast primary cell lines were generated as follows. Twelve-day-old embryos were obtained and head, limbs, tail and intestines were removed. The remaining tissue was minced and incubated for 30 min in trypsin/EDTA (428 U/ml) at room temperature. Single cell suspensions were pelleted out to establish the primary cell lines. For generating H-2b fibroblasts, BALB/c mice were used. Furthermore, mutant mice of the b-haplotype were used to generate cell lines deficient or transgenic in components of the MHC class I-restricted antigen presentation pathway. The TAP1-, β2m- mutant H-2b mice, the β2m-/-TAP1-/- double mutant mice the H-2b mice transgenic for HLA-A2 and human β2m, and A2-β2m transgenic mice backcrossed to TAP1−/− background animals have been described elsewhere (Michold et al., 1995; Van Santen et al., 1995). The β2m−/−, H-2b mice were a gift from Dr. B. Koller (University of North Carolina, Durham, NC).

Antibodies

The following antibodies and antisera directed against MHC class I molecules have been used in this study: 28.14.8s (recognizing α3 of D0) (Ozato and Sachs, 1980), B22-249 (recognizing α3 of D0) (Hammerling et al., 1979), Y3 (recognizing α1 and α2 of K0) (Hammerling et al., 1982), rabbit anti-p8 (recognizing the cytoplasmic tail of Kp and Kq) and rabbit anti-CD8 (recognizing free class I heavy chains) (Michold et al., 1995). The anti-pp89 monoclonal antibody recognizes the MCMV IE protein pp89 (del Val et al., 1988). The R123 polyclonal antisemur was raised against a synthetic peptide corresponding to the cytosolic C-terminus of gp34 (sequence FFSNDCSNNFDPLKSN, generated by F-moc chemistry). The peptide was cross-linked to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) with 0.03% glutaraldehyde (Calbiochem, La Jolla, CA). gp34 complexes with MHC class I molecules

Cells were incubated for 45 min in methionine- and cysteine-free DMEM and labeled with [35S]methionine/cysteine (up to 250 Ci/ml, 1 Ci = 37 GBq) (protein labeling mix, NEN/Dupont, Boston, MA) for the times indicated in the Results. If indicated, cells were chased in regular DMEM supplemented with non-radioactive methionine and cysteine to a final concentration of 1 mM. Cells were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl pH 7.6, 5 mM MgCl2) containing 1 mM phenylmethylsulfonyl fluoride. Nuclei and insoluble debris were removed by centrifugation (Eppendorf, 5415C centrifuge, 14 000 r.p.m., 10 min). Supernatants were pre-cleaned twice with either normal rabbit or mouse serum and formalin-fixed Staphylococcus aureus (Staph. A) and then immunoprecipitated with the indicated antibodies. Digestions with EndoH (New England Biolabs, Beverly, MA) were performed on immune complexes bound to Staph. A according to the vendor's instruction. Re-immunoprecipitations were performed as follows: after boiling the immune complexes for 10 min in 150 μl of phosphate-buffered saline containing 1% SDS, 1 ml of NP-40 lysis buffer supplemented with 0.1% bovine serum albumin was added. A second immunoprecipitation was performed as described above. BFA (Sigma, St Louis, MO) was added to the fibroblast culture at a concentration of 10 μg/ml. Cell surface iodination with Na125I (NEN/Dupont, Boston, MA) was performed as described elsewhere (Philips and Morrison, 1970).

Gel electrophoresis

SDS–PAGE and two-dimensional (one, non-equilibrium isoelectric focusing; two, SDS–PAGE) gel electrophoresis were performed as described elsewhere (Coligan et al., 1996). The first dimension of 2D gels used the same gel composition as has been described for 1D IEF of class I MHC molecules (Neefjes et al., 1986), with the polarity of the buffers reversed. The gels were run in ** nm diameter tubes at ** V for 6 h. Cloning of the MCMV gp34 gene

PCR was performed on MCMV genomic DNA with Pfu DNA polymerase (Stratagene, La Jolla, CA) using the following primers matching the 5’ and 3’ end of ORF 3270:4076 and including restriction sites for cloning: GGA ATT CAA TCT TCG TAT GTC GG (5’ forward primer, EcoRI site underlined) and GCT GTA CAG TAG TTA AGC GGT TTG (3’ backward primer, XbaI site underlined). The resulting PCR fragment showed the appropriate size of 0.8 kb and was cloned into EcoRI–XbaI sites of pSP72 (Promega, Madison, WI) under the control of the T7 promoter. Sequence identity of the PCR product with ORF 3270:4076 was confirmed by Sanger dyeoxy DNA sequencing using the Sequenase™ Sequencing kit (USB™, Cleveland, OH). The DNA was used (i) as template for in vitro transcription and (ii) for the purpose of further subcloning.

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References


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