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The Murine Cytomegalovirus Immune Evasion Protein m4/ gp34 Forms Biochemically Distinct Complexes with Class I MHC at the Cell Surface and in a Pre-Golgi Compartment¹

Daniel G. Kavanagh,* Ulrich H. Koszinowski,[†] and Ann B. Hill²*

We have recently demonstrated that the murine CMV (MCMV) gene *m4* is an immune evasion gene that protects MCMV-infected targets from some virus-specific CTL clones. *m4* encodes m4/gp34, a 34-kDa glycoprotein that binds to major histocompatibility complex class I in the endoplasmic reticulum and forms a detergent-stable complex that is exported to the surface of the cell. To investigate how m4/gp34 promotes CTL evasion, we analyzed the assembly and export of m4/gp34-K^b complexes. We found that 50-70% of K^b exported over the course of MCMV infection was m4/gp34 associated. Because these complexes are present at the cell surface, it is possible that *m4* mediates CTL evasion by interfering with contact between class I and receptors on the T cell. In addition, we found that K^b retained by the MCMV immune evasion gene *m152* formed a novel type of complex with Endo H-sensitive m4/gp34; these complexes are distinguished from the exported complexes by being stable in 1% digitonin and unstable in 1% Nonidet P-40. Because this association occurs in a pre-Golgi compartment, m4/gp34 might also interfere with Ag presentation by affecting some aspect of class I assembly, such as peptide loading. Although m4/gp34 requires β_2 -microglobulin to bind class I, there was no significant binding of m4/gp34 to β_2 -microglobulin in the absence of class I H chain, demonstrating that m4/gp34 forms Nonidet P-40-stable complexes specifically with folded conformations of class I. We conclude that m4/gp34 promotes immune evasion by a novel mechanism involving altered assembly and/or T cell recognition of class I molecules. *The Journal of Immunology*, 2001, 167: 3894–3902.

ytomegaloviruses, including murine CMV (MCMV)³ (1) and human CMV (HCMV), are herpesviruses that cause lifelong infections, but are rarely associated with acute disease in immunocompetent hosts. As with other herpesviruses, CTLs play a vital role in controlling infections, and CMVs in turn encode multiple immune evasion genes that alter the normal assembly of MHC class I, and protect infected cells from CTL activity (reviewed in Refs. 1–4). MCMV (5) and HCMV (6, 7) have been completely sequenced, and have collinear genomes. MCMV contains 170 open reading frames (ORFs) of predicted length greater than 300 bp. MCMV ORFs are numbered sequentially from one end of the linear genome: m1-m170. ORFs with recognized homology to HCMV genes are labeled with a capital M.

During normal assembly, murine class I H chain (HC) is cotranslationally translocated into the endoplasmic reticulum (ER), where it associates first with the chaperone calnexin and binds to the L chain, β_2 -microglobulin (β_2 m). The HC- β_2 m heterodimer associates with an assembly complex, including the chaperones calreticulin, tapasin, and ERp57, and the peptide transporter TAP (for recent reviews, see Refs. 8 and 9). TAP transports short peptides, generated by proteasomal degradation of cytosolic proteins, into the ER, where they are loaded onto empty class I molecules. The trimolecular complex of HC, β_2 m, and peptide dissociates from the assembly complex, leaves the ER, and travels through the Golgi and out to the cell surface.

Three MCMV genes have been shown to alter this process in infected cells. m152 encodes a glycoprotein (m152/gp40), which, by an unknown mechanism, prevents normal export of class I and causes immature class I molecules to accumulate in the ER-Golgi intermediate compartment (ERGIC) (10, 11). Because normal amounts of antigenic peptide can be extracted from MCMV-infected cells that are not recognized by cognate CTL, it is believed that these retained molecules are loaded with peptide (10, 11). It has not been possible to detect a direct interaction between m152/ gp40 and class I, and the lumenal domain of m152/gp40 is sufficient to cause retention of MHC class I (12). The immune evasion gene m6 encodes a glycoprotein (m6/gp48) that binds to class I in the ER and redirects it to the lysosome for degradation (13). Expression of either m152 or m6 via recombinant vaccinia is sufficient to protect targets from CTL lysis in vitro (11, 13). The third CTL evasion gene is m4.

m4 encodes a 34-kDa type 1 glycoprotein (m4/gp34) that is abundantly expressed in the ER. m4/gp34 remains ER resident until it binds to class I, forming tight complexes that are readily observed by immunoprecipitation of class I from Nonidet P-40 lysates of infected cells. These complexes exit the ER and are expressed at the cell surface, where they can be labeled by surface iodination (14). Based on our initial observations, we hypothesized that these complexes might serve as decoy signals to prevent NK activation due to loss of class I expression in infected cells. To

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³ Abbreviations used in this paper: MCMV, murine CMV; β_2 m, β_2 -microglobulin; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; HC, H chain; HCMV, human CMV; MEF, mouse embryo fibroblast; NRS, normal rabbit serum; ORF, open reading frame; TfR, transferrin receptor.

date, however, there is no functional evidence for an effect of m4on NK activity. A recent report (15) demonstrates that peptides derived from m4/gp34 are recognized by some CTLs in the context of the class I molecule D^d. This antigenicity has no obvious connection to the function m4, and is probably a consequence of the abundance of m4/gp34 in the ER of infected cells.

We have recently demonstrated that m4 does inhibit CTL activity: mutant MCMV strains lacking m4 are recognized by some MCMV-specific CD8⁺ CTLs, whereas wild-type MCMV is not. In addition, the functions of m4 and m152 are complementary with respect to different class I molecules: in H-2^b fibroblasts, m152 retards the export of D^b more than K^b. In contrast, we observed that *m4* was required for maximal immune evasion from three K^b-, but not two D^b-restricted CTL clones.⁴ Because the mechanism by which m4 inhibits CTLs is unknown, we undertook the biochemical analysis described in this study to gain a clearer understanding of how and when m4/gp34 affects the MHC class I Ag presentation pathway. In this study, we demonstrate that at least 50% of mature (Endo H-resistant) K^b in infected fibroblasts was found in Nonidet P-40-stable complexes with m4/gp34. This is consistent with a mechanism for m4 inhibiting CTLs by direct interference with contact between class I and the TCR or CD8. However, we could not demonstrate m4/gp34 association for a significant minority of these mature K^b molecules, which, along with the fact that the vast majority of m4/gp34 is resident in a pre-Golgi compartment, prompted us to look for other effects of m4 on K^b. We found that K^b accumulated, in an *m152*-dependent manner, with m4/gp34 in a novel type of complex that is unstable in Nonidet P-40 lysate, but stable in 1% digitonin. These complexes are largely confined to a pre-Golgi compartment. This suggests a second possible mechanism by which m4 may interfere with Ag presentation: by altering aspects of class I assembly, such as peptide loading. In addition, we investigated factors required for Nonidet P-40-stable binding of class I by m4/gp34. We (14) showed that m4/gp34 and class I do not form complexes in the absence of β_2 m; in this study, we show that m4/gp34 did not bind significantly to β_2 m in the absence of classical class I molecules, demonstrating that the requirement for β_2 m is due to a specific association of m4/gp34 with a folded conformation of class I.

Materials and Methods

MCMV strains

The recombinant Δ m4-MC95.33, with an insertion of the *lacZ* gene in place of the m4 ORF, was generated using the plasmid construct pm4 and performing insertional mutagenesis in eukaryotic cells, as described previously (16). pm4 was constructed, as follows. The homologous recombining region was produced by flanking the lacZ gene with MCMV genomic sequences adjacent to the 5' (nt 2739 to 3250 left flank) and 3' (nt 4041 to 4737 right flank) ends of the m4 ORF. Plasmid DNA (pHindIIIA) (17) serving as MCMV genomic template and primer pairs for the left flanking sequence (sense, 5'-AACTCGAGCATCACGGTGAACGATACCA-3'; antisense, 5'-TTGGATCCTGGAACAACGAATGAGACAGA-3') and right flanking sequence (sense, 5'-ATGCGGCCGCTCGAACTTCAAACCGCT TAAGAG; antisense, 5'-AACCGCGGACTTATCGACGTACAATCCT GT) were used in separate PCR to produce fragments with convenient restriction sites to ligate to the *lacZ* gene (*XhoI*, *BamHI*, *NotI*, and *SacII*, respectively; restriction sites denoted in bold). These fragments were inserted into corresponding sites within the plasmid pIC4, which contains the lacZ gene under control of the Rous sarcoma virus promoter, SV40 poly(A), and flanking loxP sites (18). A total of 30 fmol of linearized pm4 plasmid DNA was cotransfected with wild-type (Smith) MCMV DNA (1.5 μ g) into NIH3T3 fibroblasts by calcium phosphate precipitation to generate the recombinant virus $\Delta m4$ -MC95.33. Recombinant virus was isolated and plaque purified, as described previously (18). Correct recombinatorial mutagenesis within the genome of Δ m4-MC95.33 was confirmed by restriction enzyme analysis (data not shown).

All other mutant MCMVs used in this study have been previously described. (Δ MC96.24 (*m*152 knockout) and Δ MC96.27 (revertant) are described in Ref. 16, and Δ MS94.5 (lacking ORFs *m151–165*) in Ref. 19.)

Experimental animals

C57BL/6 (B6) mice were purchased from Simonsen (Gilroy, CA). D^{b-/-} and $K^{b^{-/-}}D^{b^{-/-}}$ mice (20) were a gift from F. Lemmonnier (Institut Pasteur, Paris, France). C57BL/6 β_2 m⁻ mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Virus stocks and cell culture

Mouse embryo fibroblasts (MEFs) from B6 and $\beta_2 m^{-/-}$ mice were grown from trypsin-digested day 12-14 mouse embryos, and used between passage 3 and 6. MEFs from $D^{b-/-}$ and $K^{b-/-}D^{b-/-}$ mice were the gift of D. Raulet (University of California, Berkeley, CA). NIH3T3s (CRL-1658) and BALB3T3s (CCL-163) were obtained from American Type Culture Collection (ATCC, Manassas, VA). MEFs and 3T3s were maintained in DMEM supplemented with 10% fetal (for MEFs, adult fibroblast lines, and NIH3T3s) or newborn (for Balb3T3s) calf serum. Virus stocks were generated by infecting subconfluent MEFs with low passage seed stock at a multiplicity of infection of 0.001. Cells were then switched to DMEM + 10% normal calf serum until the monolayer became 100% infected. Stocks were harvested by scraping and sonication of cells. Titer of PFU was determined by serial dilution and agarose overlay on Balb3T3s.

Antibodies

Serum 8010 (anti-p8) was generated by immunizing rabbits with synthetic peptide corresponding to exon 8 of K^b. Sera 8142 and 8139 (anti-m4/gp34) were both generated as follows. Serum R123 against the cytoplasmic tail of m4/gp34 (14) was used to precipitate m4/gp34 from MCMV (Smith)-infected MEFs. After washing, the immune complex was suspended in CFA (Sigma, St. Louis, MO) and used to immunize rabbits s.c. Rabbits were boosted first with immune complex suspended in IFA (Sigma), and then by infection with recombinant vaccinia virus expressing m4/gp34, and finally with recombinant soluble m4/gp34 protein purified from baculovirus (the kind gift of P. Bjorkman, California Institute of Technology, Pasadena, CA) in IFA. Anti-D^b mAb 28.14.8S (ATCC HB-27) and anti-β₂m mAb Lym11 (a gift from A. Simmons, Adelaide University, Adelaide, Australia) were purified from hybridoma supernatants. Anti-transferrin receptor (anti-TfR)/CD71 Ab (rat IgG2a) was purchased from Leinco Technologies (Ballwin, MO).

Ab-binding reagents

Rabbit Abs were precipitated using either fixed Staphylococcus aureus or 5% w/v protein A-agarose (Sigma). Rat IgG2a Ab was precipitated using 5% w/v protein G-agarose (Sigma). Just before use, Ab-binding reagents were washed three times in the appropriate lysis buffer.

Metabolic labeling and immunoprecipitations

All immunoprecipitations used adherent MEFs, which were pretreated with mouse rIFN-y at 50 U/ml for 48 h before metabolic labeling. Unless otherwise indicated, virus infections used a multiplicity of infection of 10, and infected cells were continuously grown in the presence of 0.3 mg/ml phosphonoacetic acid (Sigma). One hour before the addition of metabolic label, cells were washed in PBS and placed in labeling medium (cysteine/methionine-free DMEM (Life Technologies, Grand Island, NY) supplemented with antibiotics and 5% FCS); at the end of 1 h, cells were labeled with [³⁵S]cysteine/methionine (NEN, Boston, MA) for the time periods indicated in the figures. For pulse-chase experiments, cells were washed with chase medium (DMEM supplemented with antibiotics, glutamate, 10% FCS, and saturating concentrations of tissue culture-grade cysteine and methionine; Sigma) at the end of the labeling period, after which they were cultured in chase medium for the chase period indicated in the figures. All lysis and precipitation procedures were conducted at 4°C. At the time of lysis, tissue culture plates were placed on ice, washed in cold PBS, lysed in the plate with lysis buffer, scraped, and transferred to tubes. Lysis buffer was either Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂) or digitonin lysis buffer (1% high purity digitonin (Calbiochem, La Jolla, CA) in PBS). Just before use, lysis buffer was supplemented with protease inhibitor: either 1 mM PMSF (Sigma) or $1 \times$ inhibitor cocktail (complete EDTA-free protease inhibitor mixture; Boehringer Mannheim, Indianapolis, IN). To remove nonspecific Ab-binding

⁴ D. G. Kavanagh, M. G. Gold, M. Wagner, U. H. Koszinowski, and A. B. Hill. The multiple immune-evasive genes of murine cytomegalovirus are not redundant: m4 and m152 inhibit antigen presentation by K^b and D^b in a complementary and cooperative fashion. Submitted for publication.

proteins, lysates were precleared with normal rabbit serum (NRS): each ml of lysate received at least 20 μ l of NRS and 500 μ l of *S. aureus*, after which lysates were mixed by slow rotation for 2 h, and centrifuged for 5 min at 15,000 × g to remove nonspecific proteins and cellular membranes. Precleared lysates were then subjected to specific immunoprecipitation, as indicated in the figures. Unless otherwise indicated, each aliquot of lysate received 10 μ l of Ab plus 150 μ l of protein A or G suspension. Lysates were mixed by slow rotation for 2 h and then centrifuged for 1 min at 12,000 × g. Lysate supernatant was removed and stored at -80° for further analysis. Immune complex pellets were washed four times in 1× NET buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, and 0.05% Nonidet P-40) + 0.1% SDS. Samples were digested with Endo H_f (NEB, Beverly, MA), according to manufacturer's protocol, and separated by SDS-PAGE on a 12.5% gel. Quantitation of labeled protein was performed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Results

Most of the K^b reaching the surface of infected MEFs is associated with m4/gp34 in Nonidet P-40-stable complexes

m4 is required to protect MCMV-infected B6 MEFs from lysis by K^{b} -restricted T cell clones, which demonstrates that *m*4 expression interferes with Ag presentation by K^b. It is possible that this interference occurs at the level of TCR-K^b contact, because, unlike any other immune evasion protein, m4/gp34 forms complexes with class I that are expressed on the surface of the cell (14). However, it was previously not known what percentage of K^b reaching the cell surface of MCMV-infected MEFs was m4/gp34 associated. We were unable to estimate this quantity because we did not precipitate significant amounts of K^b with our anti-m4/gp34 serum R123, generated against a peptide in the cytoplasmic tail (14); we did not know whether this failure was due to a steric blockage of the R123 epitope or to a stoichiometric excess of free m4/gp34 over m4/gp34-class I complexes. Therefore, we generated two new sera, 8139 and 8142, by immunizing rabbits against the entire m4/ gp34 protein (see Materials and Methods). As shown below, we have defined conditions under which it is possible to coprecipitate some class I using any of our Abs to m4/gp34. Previously, we observed the CTL evasion function of m4 using MCMV-infected MEFs, which were pretreated with IFN- γ to increase class I expression, and treated with the viral DNA synthesis inhibitor phosphonoacetic acid to limit viral cytopathic effect. Therefore, we performed all of our biochemical analyses shown here using the same cell type and infection conditions.

To estimate what percentage of K^b synthesized during the course of MCMV infection is exported in complexes with m4/gp34, we undertook the following experiments, shown in Fig. 1. Nonidet P-40 lysates of metabolically labeled, MCMV-infected cells were subjected to serial depletion with anti-m4/gp34 serum to remove all of the m4/gp34 protein from the lysates; we then compared the amount of K^b present before or after total m4/gp34 depletion. This comparison allowed us to estimate the minimal percentage of total labeled K^b that was in Nonidet P-40-stable complexes with m4/gp34. Because the only class I molecules on infected MEFs that are likely to be relevant to the antiviral CTL response are those synthesized after viral infection, we chose to begin the metabolic labeling after infection of MEFs with MCMV.

Fig. 1*A* shows a typical initial immunoprecipitation of m4/ gp34-K^b complexes from lysates of infected cells; as a control, we performed parallel immunoprecipitations from lysates of uninfected cells. B6 MEFs were infected with MCMV and metabolically labeled for 16 h, lysed in 0.5% Nonidet P-40 buffer, and subjected to immunoprecipitation, as shown. As previously demonstrated (14), m4/gp34 coprecipitates with K^b (*lanes 15* and *16*). This coprecipitated m4/gp34 runs in two positions after Endo H digestion: a lower band (m4-S), representing ER-resident m4/ gp34, has lost all three N-linked glycans; an upper band (m4-R), representing m4/gp34, which has traversed the medial Golgi, has retained one of its three glycans. We have previously demonstrated (14) that m4/gp34 is an unusual protein, in that only one of its three N-linked glycans becomes Endo H resistant as it passes through the Golgi; therefore, Endo H-resistant m4/gp34 (lane 16) is of a lower m.w. than undigested m4/gp34 (lane 15). Immunoprecipitation with either of two anti-m4/gp34 sera yields an Endo Hsensitive m4/gp34 band (lanes 7, 8, 11, and 12). At this exposure, there is no Endo H-resistant m4/gp34 visible. However, both immunoprecipitates do contain faint bands that comigrate with class I HC and β_2 m and that are absent in precipitates from uninfected cells (lanes 5, 6, 9, and 10). In this experiment, coprecipitation of class I was much more apparent with serum R123 (lanes 11 and 12) than 8142 (lanes 7 and 8). The amount of coprecipitating class I seen with different sera is somewhat variable between experiments, due largely to the vast stoichiometric excess of m4/gp34 over class I in these lysates (see Fig. 1B). However, these complexes can be shown to contain K^b by reprecipitation with specific antiserum (data not shown). Thus, these sera (R123 and 8142) can initially precipitate some small number of class I-m4/gp34 complexes.

To remove all available m4/gp34 from the lysates, we took the supernatant lysate from the immunoprecipitation shown in Fig. 1A, lanes 7 and 8, and subjected it to serial depletion with 40-µl aliquots of serum 8142. Fig. 1B shows that two rounds of serial depletion (lanes 17 and 18), using a total of 80 µl Ab, removed all remaining m4/gp34 from the lysate, because there is no further m4/gp34 band visible in *lane 19*, even on overexposure of the gel. The immunoprecipitates shown in lanes 17 and 18 also contain bands of m.w. corresponding to K^{b} , $\beta_{2}m$, and Endo H-resistant m4/gp34. Furthermore, when K^b was immunoprecipitated from the fully depleted supernatant, we found no more m4/gp34 associated with this remaining pool of K^b (lane 22), even after extensive overexposure of the gel, as shown. Thus, serial immunoprecipitation with serum 8142 was able to remove all K^b-associated and -unassociated m4/gp34 from the lysate. We conclude that our previous failure to coprecipitate class I with m4/gp34 was largely due to a stoichiometric excess of free m4/gp34 over the Kb-m4/gp34 complexes in Nonidet P-40 lysates. It is also possible that prolonged exposure to anti-m4/gp34 Abs has the effect of dissociating m4/gp34-class I complexes; for this reason, we kept the total time of incubation of lysates with Abs to a minimum for both experiments shown in Fig. 1. Based on a comparison of the amount of K^b precipitated from the original lysate (Fig. 1A, lane 16), with the amount precipitated from the same volume of lysate after total depletion of m4/gp34 (Fig. 1B, lane 22), we concluded that a substantial proportion of the total labeled K^b in the lysate was m4/ gp34 associated.

To estimate this percentage more accurately, we repeated the procedure using additional quantitative controls (Fig. 1*C*). In this case, Nonidet P-40 lysates from infected and uninfected cells were totally depleted of m4/gp34 using serum 8139, or mock depleted with NRS. The amount of labeled K^b precipitated before and after depletion was determined by PhosphorImager analysis. These quantities are shown in Table I. Mock depletion with NRS serves as a control for nonspecific loss of class I due to serial immuno-precipitations. The fact that no class I was lost from the lysates of uninfected cells depleted with serum 8139 shows that this serum is not cross-reactive with class I. After total depletion of m4/gp34, 33% of the original Endo H-resistant K^b remained in the lysate.



FIGURE 1. Over 50% of Endo H-resistant K^b is m4/gp34 associated in Nonidet P-40-stable complexes. B6 MEFs were infected with MCMV (Smith) or mock infected, labeled 16 h with [35] methionine, and lysed in Nonidet P-40 lysis buffer. A, Primary immunoprecipitation using 10 µl each of NRS (lanes 1-4), anti-m4/gp34 serum 8142 (lanes 5-8), anti-m4/gp34 cytoplasmic tail serum R123 (lanes 9-12), or anti-K^b (lanes 13-16). Samples were divided in half and digested with Endo H or mock digested. B. Supernatant from the precipitation shown in Fig. 5A, lanes 7 and 8, was subjected to serial depletion of m4/gp34 protein. For each of lanes 17-20, 40 µl of serum 8142 was used to precipitate m4/gp34, and the supernatant from that precipitation was subjected to the next round of immunoprecipitation with another 40 µl of 8142. Finally, the supernatant from the immunoprecipitation shown in *lane 20* was cleared of residual Abs with S. aureus, divided in half, and subjected to immunoprecipitation with anti-m4/gp34 serum R123 (lane 21), or anti-K^b (lane 22). All samples were treated with Endo H before analysis by SDS-PAGE. The immunoprecipitation shown in lane 22 represents an equal amount of the original lysate as that shown in A, lane 16. Overexposure of the gel in B demonstrates that there is no Kb-associated m4/gp34 detectable in lane 22. Bands of a similar weight as K^b in *lanes 19* and 20 probably represent an unidentified/nonspecific protein, which sometimes coprecipitates with anti-m4/gp34 sera. C, Cells were infected and labeled as above, and subjected to immunoprecipitation with NRS (lanes 1 and 8), anti-m4/gp34 (serum 8139, lanes 2 and 9), or anti-K^b (lanes 3 and 10). The lysates from which m4/gp34 had been precipitated were then completely depleted of m4/gp34 by four rounds of immunoprecipitation. A parallel mock depletion was performed with NRS. After depletion, these lysates were then subjected to immunoprecipitation with NRS or anti-K^b. Lanes 5 and 12, Show the amount of K^b remaining after mock depletion with NRS; lanes 7 and 14, show the amount remaining after specific depletion of m4/gp34 with serum 8139. Each lane of C shows proteins precipitated from an equal volume of the original lysate. All samples were treated with Endo H. PhosphorImager data from this gel are shown in Table I. Relevant bands are labeled -R and -S to designate Endo H-resistant and -sensitive proteins, respectively.

There is of course a large error inherent in sequential immunoprecipitations, the range of which is indicated by the fact that 0% of class I was removed by depletion of m4/gp34 from lysates of uninfected cells, whereas between 14 and 33% of class I was nonspecifically depleted by NRS from lysates of infected cells. Taking this into account, we conclude that between 50 and 70% of Endo H-resistant, metabolically labeled K^b was associated with m4/ gp34. We note that the depletion of Endo H-resistant K^b is much greater than that of Endo H-sensitive K^b. This is consistent with our previous observation that Nonidet P-40-stable complexes of K^b with m4/gp34 are exported to the cell surface.

It is possible that, of the remaining mature K^b not coprecipitated with m4/gp34, some may have been m4/gp34 associated at the time of lysis, but disassociated during the immunoprecipitation procedure. If this were the case, up to 100% of the surface-exposed labeled K^b could be m4/gp34 associated. However, the present data suggest that at least 30%, and up to 50% of K^b that matures over the course of MCMV infection may be m4/gp34 unassociated.

Table I. Over half of the exported K^b , but less than half of the retained K^b , is in Nonidet P-40-stable complexes with m4/gp34 in infected cells^a

		Amount of K ^b (arbitrary units)		
Lysate	Endo H Sensitivity of K ^b	Initial IP	After mock depletion (% remaining)	After anti-m4/gp34 depletion (% remaining)
Infected	Resistant	140	93 (67)	42 (30)
	Sensitive	296	209 (71)	174 (59)
Uninfected	Resistant	133	115 (86)	141 (106)
	Sensitive	n/a		

^{*a*} The amount of radiolabeled K^b in Fig. 1*C* was determined by PhosphorImager analysis; measurements are expressed in arbitrary volume units. We determined the initial amount of exported (Endo H-resistant) and retained (Endo H-sensitive) K^b for infected (Fig. 1*C*, *lane 3*), and uninfected (Fig. 1*C*, *lane 10*) cells; uninfected cells did not have a measurable amount of retained K^b. Lysates were subjected to serial depletion as explained in Fig. 1*C*. The amount of K^b remaining after mock (Fig. 1*C*, *lanes 5* and *12*) or anti-m4/gp34 (Fig. 1*C*, *lanes 7* and *14*) depletion is shown. For each depletion, we calculated the percentage of K^b remaining as (depleted IP/initial IP). Thus, 70% of exported K^b was removed from infected lysates by anti-m4/gp34 serum, indicating that 70% of these molecules were m4/gp34 associated. The fact that a mock preclear with normal serum yields between 14 and 33% depletion of K^b indicates the degree of error in the system.

Given this possibility, we wanted to look for other potential mechanisms for m4-mediated immune evasion besides direct interference with contact between class I and the receptors on the T cell.

K^{b} molecules whose export is blocked by MCMV infection are found in Nonidet P-40-unstable complexes with m4/gp34

Comparing the transport of different MCMV immune evasion proteins provides some interesting contrasts. Although m152 causes class I to be retained in the ERGIC, m152/gp40 itself is rapidly exported from the ER to the lysosome for degradation. m6/gp48 is also targeted to the lysosome, where it is destroyed, along with any associated class I proteins. Although m4 was originally discovered because m4/gp34 forms complexes with class I found on the cell surface, the vast majority of m4/gp34 in infected cells remains in a pre-Golgi compartment (see Fig. 1). This suggested that m4/gp34 might have a separate function in a pre-Golgi compartment; therefore, we undertook a closer examination of m4/gp34 in these compartments.

MCMV infection has a mixed effect on maturation of K^b. Some molecules are retained in a pre-Golgi compartment by the effects of m152, whereas others are exported to the cell surface. As demonstrated above, the majority of K^b on the cell surface is m4/gp34 associated, but we also wanted to know more about the disposition of K^b that is retained in the ERGIC. Therefore, we used a more sensitive assay for protein-protein interactions: lysis of infected cells in the weak detergent digitonin and identification of coprecipitating bands in immunoprecipitations. We chose digitonin because weak interactions such as those between class I, TAP, and tapasin are preserved in 1% digitonin, but disrupted by Nonidet P-40. This led to a discovery (Fig. 2): m4/gp34 and K^b were found in a novel type of complex, which, unlike the complexes described above and previously (14), were unstable in 1% Nonidet P-40 lysis buffer, but stable in 1% digitonin lysis buffer. Furthermore, these Nonidet P-40-unstable complexes were almost entirely composed of Endo H-sensitive proteins, as opposed to the previously described Nonidet P-40-stable complexes, which were mostly Endo H resistant.

Fig. 2A is a schematic representation of the method used to distinguish Nonidet P-40-stable and -unstable complexes. Infected cells are metabolically labeled and lysed in 1% digitonin buffer, and lysates are subjected to immunoprecipitation with the primary

serum (e.g., anti-m4/gp34). Precipitated complexes are then resuspended in 1% Nonidet P-40 buffer so that any Nonidet P-40-unstable associations will be disrupted. Precipitated complexes are recentrifuged. Nonidet P-40-stable complexes remain with the pellet, whereas proteins from Nonidet P-40-unstable complexes are now found in the supernatant, from which they can be specifically reimmunoprecipitated.

The results shown in Fig. 2B demonstrate that K^b and m4/gp34 are found together in Nonidet P-40-unstable complexes. Cells were metabolically labeled starting 5 h postinfection with MCMV, and 4 h later were lysed in 1% digitonin buffer. K^b from Nonidet P-40unstable complexes was isolated as described above. The results show that if the primary antiserum is anti-K^b, no K^b is recovered from the Nonidet P-40 supernatant. This is because the Abs still bind strongly to K^b in this buffer, and all of K^b remains in the primary pellet. Likewise, NRS does not precipitate any K^b in Nonidet P-40-unstable complexes. However, if anti-m4/gp34 serum is used for the primary precipitation, then K^b is found in the Nonidet P-40 supernatant, indicating that some m4/gp34 and K^b are in Nonidet P-40-unstable complexes. In cells infected with wild-type MCMV, this K^b is almost entirely Endo H sensitive, indicating that these complexes reside in a pre-Golgi compartment. In cells infected with MCMV AMS94.5, a mutant lacking ORFs 151-165 (19), the overall amount of K^b in these complexes is greatly reduced, and of the amount remaining a greater percentage is Endo H resistant. This suggests a role for some gene from the deleted region in retaining and stabilizing Nonidet P-40-unstable complexes. This observation is pursued in Fig. 3.

We wanted to confirm that these Nonidet P-40-unstable complexes represented a specific interaction between K^b and m4/gp34, and were not merely a side effect of the abundance of m4/gp34 in these lysates. Therefore, we compared the protein content of Nonidet P-40-stable and -unstable complexes with K^b, m4/gp34, and the irrelevant control protein TfR (Fig. 2C). Primary immunoprecipitations from digitonin lysate of wild-type MCMV-infected cells were performed using the following sera or Abs: NRS, anti-TfR, anti-K^b, or either of two anti-m4/gp34 sera (8139 and 8142). These pellets were dissociated, and the resulting Nonidet P-40 supernatants were subjected to secondary immunoprecipitation, as described above. Nonidet P-40-stable pellets are shown in lanes 1-10; immunoprecipitations from Nonidet P-40 supernatants are shown in lanes 11-20. As seen previously in Fig. 1, Nonidet P-40stable pellets from immunoprecipitation with anti-K^b serum contain m4/gp34 (lanes 5 and 6). In addition, the Nonidet P-40 supernatant from this immunoprecipitation also contains m4/gp34 (lane 16), but not TfR (lane 15). Thus, the Nonidet P-40-unstable interaction between K^b and m4/gp34 is specific to these two proteins and does not include an irrelevant protein. The reciprocal precipitations with anti-m4/gp34 sera for the primary immunoprecipitation (lanes 7-10) also demonstrate a specific interaction with K^b (lanes 18 and 20), but not TfR (lanes 17 and 19). Primary immunoprecipitation with NRS (lanes 1 and 2) or anti-TfR (lanes 3 and 4) yields no specific protein in the Nonidet P-40 supernatant (*lanes 11–14*), confirming the specificity of the m4/gp34-K^b interactions.

We are confident that Nonidet P-40-stable and -unstable complexes are distinct entities because they reside in different cellular compartments. All of the m4/gp34 and K^b from Nonidet P-40stable complexes eventually becomes Endo H resistant (*lane 6* and Fig. 3), whereas all of the m4/gp34 (*lane 16*) and K^b (*lanes 18* and 20) from Nonidet P-40-unstable complexes remains Endo H sensitive, and is thus confined to some pre-Golgi compartment.



FIGURE 2. Specific association of Endo H-sensitive m4/gp34 and Endo H-sensitive K^b in Nonidet P-40-unstable complexes. *A*, Method for distinguishing Nonidet P-40-stable and -unstable complexes. Metabolically labeled cells are lysed in 1% digitonin in PBS, and the primary immunoprecipitation is performed in this buffer. The primary complex is then disrupted in 1% Nonidet P-40 buffer and subjected to secondary immunoprecipitation as shown. *B*, K^b is found in Nonidet P-40-unstable complexes with m4/gp34 in cells infected with MCMV. B6 MEFs were infected with wild-type (Smith) MCMV or mutant Δ MS94.5 (lacking ORFs 151–165). MEFs were infected for 5 h, metabolically labeled for 4 h, and lysed in 1% digitonin. Lysates were then subjected to immunoprecipitation, as shown for *part A*, and digested with Endo H. *C*, Nonidet P-40-unstable complexes represent a specific interaction between m4/gp34 and K^b. MEFs were infected with MCMV and labeled as above. Primary lysates were subjected to immunoprecipitation with NRS (*lanes 1* and 2), mAb against mouse TfR (*lanes 3* and 4), anti-K^b (*lanes 5* and 6), anti-m4/gp34 8142 (*lanes 7* and 8), or 8139 (*lanes 9* and *10*). Each lane represents precipitation with roughly 10 μ g of Ab. Precipitates were resuspended in Nonidet P-40 lysis buffer to disrupt Nonidet P-40-unstable complexes, after which they were recentrifuged to precipitate the Nonidet P-40-stable complexes shown in *lanes 1–10*. Supernatant remaining from these immunoprecipitations was divided in half and subjected to sequential precipitation with two different Abs, as shown (*lanes 11–20*). All samples in *B* and *C* were digested with Endo H.

Cooperative effects of m152 and m4 on retained, immature class I

Given the discovery of m4/gp34 and K^b in novel Nonidet P-40unstable complexes, we wanted to know to what degree the specific association of m4/gp34 with Endo H-sensitive class I in these complexes was an independent function of m4, or a cooperative function of m4 with other MCMV genes. Fig. 2B demonstrates a decrease of these complexes in the absence of ORFs m151-165, and this led us to suspect that m152 might play a role. Fig. 3 shows a pulse-chase analysis of K^b from Nonidet P-40-unstable complexes with m4/gp34, isolated from cells infected with wild-type, Δ m152, or revertant (m152R) MCMV. Five hours after infection, B6 MEFs were pulsed with [³⁵S]methionine/cysteine for 1 h, and chased with medium containing excess unlabeled methionine/cysteine for 1, 2, or 3 h. K^b from Nonidet P-40-unstable complexes with m4/gp34 was isolated as described in Fig. 2, and K^b that was not associated with m4/gp34 was subsequently isolated from the supernatant that remained after depletion of m4/gp34. In cells infected with wild-type or revertant MCMV, K^b accumulated in Nonidet P-40-unstable complexes with m4/gp34 over the course of the chase period. By contrast, in cells infected with $\Delta m152$, K^b initially entered into Nonidet P-40-unstable complexes with m4/ gp34 (chase time 0) and then rapidly left the complexes, presumably to be exported to the cell surface.

Class I retained by the effects of m152 is thought to accumulate in the ERGIC, forming a distinct population from newly synthesized, ER-localized class I molecules (12). The most striking result from Fig. 3 is that in wild-type MCMV-infected cells, nascent K^b is gradually recruited into Nonidet P-40-unstable complexes with m4/gp34 over a period of hours. This accumulation is dependent on the expression of m152, although the initial formation of the complexes is not. This implies that m4/gp34 molecules are colocalized and specifically interacting with some portion of the m152retained K^b. Whether this interaction represents a significant function of m4 (for example, related to peptide loading), or an adventitious interaction with class I already on a dead-end pathway, remains to be determined.

m4/gp34 does not associate significantly with $\beta_2 m$ in the absence of class I HC

It is known that m4/gp34 specifically associates with class I HC that is bound to β_2 m rather than with free HC; this is demonstrated by the observation that Nonidet P-40-stable m4/gp34-HC complexes were absent in fibroblasts lacking β_2 m (Ref. 14 and this



FIGURE 3. Pulse-chase analysis shows that K^b accumulates in Nonidet P-40-unstable complexes with m4/gp34 in an *m152*-dependent manner. MEFs were infected as above, metabolically labeled for 1 h, and chased with excess cysteine/methionine for the indicated time period. K^b from Nonidet P-40-unstable complexes with m4/gp34 was isolated as for Fig. 2 above. As a control to demonstrate the efficacy of the chase protocol, the total remaining (m4/gp34-unassociated) K^b was isolated from the remaining supernatants. All samples were digested with Endo H.

report, Fig. 4, lanes 13-16), and reduced in fibroblasts lacking TAP (14). This led to the tentative conclusion that m4/gp34 associates specifically with peptide-loaded, fully conformed MHC class I (14). However, the data were also compatible with the possibility that m4/gp34 might bind directly to β_2 m, and that β_2 m might be the primary bridge between m4/gp34 and HC. The availability of primary fibroblasts from mice lacking both K^b and D^b made it possible for us to test whether m4/gp34 could associate directly with β_2 m in the absence of classical class I HC (Fig. 4). Immunoprecipitation of β_2 m from Nonidet P-40 lysates of wildtype or $D^{b-/-}$ cells coprecipitated m4/gp34 (*lanes 4* and 8). However, very little or no m4/gp34 coprecipitated with β_2 m from lysates of $K^{b-/-}D^{b-/-}$ cells, despite a comparable level of β_2m expression in these cells (lane 12). Likewise, there is a prominent β_2 m band in the anti-m4/gp4 precipitations from wild-type or $D^{b-/-}$ cells (*lanes 1* and 5), but little or no $\beta_2 m$ apparent in precipitations from $K^{b-/-}D^{b-/-}$ cells (*lane 9*). Thus, the association of β_2 m with m4/gp34 is almost entirely dependent on class Ia expression. We conclude that m4/gp34 does not form significant Nonidet P-40-stable associations with either free HC or free $\beta_2 m$, but only with a specific conformation of class I, which is $\beta_2 m$ associated. Whether or not the class I in these complexes is peptide loaded remains to be demonstrated.

We also note that if m4/gp34 formed abundant complexes with nonclassical class I molecules such as Qa-1 or the viral class I homologue m144, we should still have seen a prominent β_2 m band in *lane 9*. Because the amount of β_2 m in *lane 9* is extremely small, we conclude that such complexes, at least in fibroblasts, are few or absent.

Discussion

CMVs share an extensive evolutionary history with their mammalian hosts, over the course of which the viruses have developed

FIGURE 4. m4/gp34 does not significantly associate with β_2 m in the absence of class Ia expression. MEFs from wild-type H-2^b mice (B6, expressing both K^b and D^b) or knockout mice, as shown, were infected with MCMV (Smith) and metabolically labeled overnight. Cells were lysed in Nonidet P-40 lysis buffer, and lysates were subjected to immunoprecipitation with serum against m4/gp34 (8139) or K^b (8010), or purified hybridoma supernatant against D^b (28.14.8S) or β_2 m (Lym11). Immunoprecipitates were not Endo H digested. Molecular weights of class I HC, m4/gp34, and β_2 m are indicated. intimate and complex relationships with the host immune systems. The altered assembly of class I MHC in MCMV-infected cells is a prime example of this complexity. Two MCMV genes have been identified in published reports as directly interfering with CTL recognition of fibroblasts: m152 (11, 16), which causes retention of nascent class I molecules in the ERGIC, and m6 (13), which redirects class I to the lysosome, where it is degraded. In addition, we have recently demonstrated that a third gene, m4, cooperates with m152 and m6 to modulate immune responses restricted by K^b. We know that m4 is necessary for evasion of K^b-restricted, MCMV-specific CTL clones, because fibroblasts infected with $\Delta m4$ MCMV were readily recognized by these clones, whereas those infected with wild-type virus were not.⁴ However, the mechanism by which m4/gp34 interferes with Ag presentation is not known.

We undertook the experiments described in this study to define more carefully the nature of the interaction of m4/gp34 with class I. Because m4/gp34 was originally identified on the basis of its association with class I in Nonidet P-40-stable complexes expressed on the cell surface (14), we considered it possible that m4might block Ag recognition by directly interfering with contact between class I and the TCR or CD8. However, previous work was unable to demonstrate whether a significant percentage of K^b at the cell surface was associated with m4/gp34. We now show (Fig. 1) that at least half of K^b synthesized over the course of infection and exported past the medial Golgi is associated with m4/gp34 in Nonidet P-40-stable complexes. This finding seems consistent with a mechanism by which m4/gp34 directly interferes with contact between K^b and the TCR. Such inhibition could involve steric hindrance of TCR contact with MHC peptide, although the ability of Abs such as Y3 and B22.249, which recognize the $\alpha 1$ and $\alpha 2$ domains of class I molecules, to coprecipitate m4/gp34 (14) argues



against this idea. Alternatively, m4/gp34 might prevent CD8 coreceptor contact with class I, exclude class I from the immune synapse, alter the association of class I with the cytoskeleton, or prevent class I dimerization. Distinguishing these possibilities would be greatly assisted by knowing the peptide content of m4/ gp34-class I complexes. However, none of the MCMV Ags recognized by K^b-restricted CTLs are currently known, which makes determining the peptide content of these complexes difficult. The fact that m4/gp34 associates specifically with folded conformations of class I (Fig. 4) and that these associations are promoted by TAP expression (14) suggests that peptide loading is required at least for the initial formation of these complexes.

Despite the above findings, we were unable to demonstrate, over the course of several experiments, complete association of mature K^b with m4/gp34: a significant percentage (at least 30%) always failed to coprecipitate with antisera to m4/gp34. This might be due to anti-m4/gp34 sera causing dissociation of m4/gp34-class I complexes, or to a genuine population of m4/gp34-unassociated (free) K^b at the surface of infected cells. The latter possibility, along with the fact that the vast majority of m4/gp34 resides in a pre-Golgi



FIGURE 5. Hypothetical relationships among m4/gp34-containing complexes. β_2 m-associated K^b forms Nonidet P-40-unstable associations with m4/gp34 in the ER or ERGIC. The transport of K^b from these complexes to the Golgi is retarded by *m152*. It may be that K^b in this compartment undergoes peptide exchange or some other alteration, which makes the molecules nonimmunogenic. Eventually, a significant amount of K^b escapes the effects of *m152* and is transported to the cell surface. Over half of these molecules are associated with m4/gp34 in Nonidet P-40-stable complexes; up to 30% may be unassociated with m4/gp34, but *m4* nevertheless renders the target incapable of stimulating a CTL clone. Thus, *m4* might prevent Ag presentation by altering peptide loading in a pre-Golgi compartment and/or by interfering at the cell surface with contact between K^b and receptors on the T cell. Arrows indicate multiple hypothetical pathways of traffic between compartments.

compartment, prompted us to look for additional interactions between m4/gp34 and K^b aside from those at the cell surface.

Therefore, we undertook a more sensitive assay for protein-protein interactions, using the weak detergent digitonin. Fig. 2 demonstrates that K^b and m4/gp34 engage in a novel type of complex in a pre-Golgi compartment. These complexes are defined by their stability in digitonin lysis buffer and their instability in Nonidet P-40 lysis buffer. We know that these complexes are specific because neither protein is found in association with an irrelevant control protein (TfR) or with immunoprecipitates of NRS. They are distinct from the Nonidet P-40-stable complexes, because they accumulate in a pre-Golgi compartment over time, whereas the latter are exported and become Endo H resistant. The accumulation of K^{b} in these complexes is dependent on *m152*. The biochemical basis of the difference in detergent stability of the two types of complex, and the kinetic relationship between them, is unknown. The existence of these novel Nonidet P-40-unstable complexes suggests that m4/gp34 may be altering K^b assembly and function at either or both of two different points: in a pre-Golgi compartment at the level of class I assembly, and/or at the cell surface at the level of contact between class I and receptors on the MCMVspecific T cell.

 K^{b} that is retained by the effects of *m152* accumulates in Nonidet P-40-unstable complexes with m4/gp34 in a pre-Golgi compartment, most likely the ERGIC (Fig. 3). Immature class I in the ER associates with a large assembly complex, including tapasin, TAP, ERp57, and calreticulin (9), and it is possible that Nonidet P-40-unstable complexes between m4/gp34 and K^b represent an indirect interaction mediated by one or more ER chaperones. Current models of class I assembly suggest that class I in the ER repeatedly exchanges peptides in a process that favors the eventual presentation of immunodominant peptides. By associating with ER- or ERGIC-resident class I molecules, m4/gp34 might alter this process. We have found that MCMV infection also prolongs the association of K^b with tapasin (our unpublished observation). Taken together, these results provide circumstantial evidence for an effect of m4/gp34 on an early aspect of K^b assembly and export, such as peptide loading.

We have shown that m4/gp34 associates with K^b in distinct complexes in different cellular compartments. One or both of these interactions is presumably responsible for the immune evasive effects of m4. A general model of interactions between K^b and m4/ gp34 is shown in Fig. 5. As shown, m152 causes the partial retention of K^b in a pre-Golgi compartment, where it accumulates in Nonidet P-40-unstable complexes with m4/gp34. Eventually, a significant amount of K^b escapes the effects of m152 and progresses to the cell surface, where much of it is found in Nonidet P-40-stable complexes with m4/gp34. It is possible that the effects of m4/gp34 in the ER have a direct impact on the function of complexes at the cell surface. For example, if all of the Nonidet P-40-unstable complexes eventually become stable complexes, interference with peptide loading in the ER would result in a different spectrum of peptides being found on free and m4/gp34-associated class I at the cell surface. A number of other scenarios can be imagined, and it should be possible to test these hypotheses after we have identified the MCMV epitopes recognized by K^b-restricted CTL.

In any case, it is clear that m4/gp34 inhibits CTL activity by some novel mechanism, because the biochemical relationships described in this work are unlike those seen for any other immune evasion protein. Results shown in this study provide insight into the complex and dynamic biochemical relationship between a class I molecule and viral immune evasion proteins. We thank Irena Crnkovic-Mertens for generating the virus Δ MC95.33, and Markus Wagner and Michael Munks for assistance with the manuscript. We thank Pamela Bjorkman for the gift of recombinant m4/gp34, and Francois Lemmonier for the gift of K^{b-/-}D^{b-/-} mice.

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