Surface Expression of HLA-E, an Inhibitor of Natural Killer Cells, Enhanced by Human Cytomegalovirus gpUL40

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The nonclassical major histocompatibility complex (MHC) class I molecule HLA-E inhibits natural killer (NK) cell–mediated lysis by interacting with CD94/NKG2A receptors. Surface expression of HLA-E depends on binding of conserved peptides derived from MHC class I molecules. The same peptide is present in the leader sequence of the human cytomegalovirus (HCMV) glycoprotein UL40 (gpUL40). It is shown that, independently of the transporter associated with antigen processing, gpUL40 can up-regulate expression of HLA-E, which protects targets from NK cell lysis. While classical MHC class I molecules are down-regulated, HLA-E is up-regulated by HCMV. Induction of HLA-E surface expression by gpUL40 may represent an escape route for HCMV.

Human cytomegalovirus (HCMV) is a betaherpes virus that infects most of the population, resulting in lifelong persistent infection. The cellular immune response to CMV plays a crucial role in controlling primary infection and reactivation from latency (*1*). The virus has developed a variety of mechanisms to subvert host defenses. One of these is the ability to down-regulate expression of MHC class I molecules, which may allow evasion of recognition of cytotoxic T lymphocytes [reviewed in (*2*)]. HCMV expresses at least five proteins that interfere with the MHC class I assembly pathway. These proteins inhibit generation of HCMV-specific T cell epitopes, block the transporter associated with antigen processing (TAP), retain MHC class I molecules in the endoplasmic reticulum (ER), and recycle nascent class I heavy chains back to the cytosol (*2*). As a result, HCMV-infected cells should be more susceptible to attack by natural killer (NK) cells because MHC class I molecules, acting through various receptors, signal inhibitory messages to NK cells. The HCMV class I homolog gpUL18 may be able to substitute for this function as it binds to the inhibitory leukocyte immunoglobulin (Ig)-like receptor 1 (LIR-1) (*3–5*). Other parallel mechanisms also may contribute to evasion from NK cell lysis.

Most human NK cells express a surface C-type lectin receptor, CD94/NKG2A/B, which delivers an inhibitory signal to NK cells. It interacts specifically with a nonclassical MHC class I molecule, HLA-E, which requires binding of a nonamer peptide derived from residues 3 to 11 of the signal sequence of most classical MHC class I molecules for cell surface expression (*6–11*). Interestingly, this processed leader peptide is released in the cytosol and then transported by TAP into the lumen of the ER, where it binds to HLA-E in a fashion similar to that of classical HLA molecules (*7, 10*). Thus, HLA-E surface expression allows NK cells to monitor the expression of numerous polymorphic MHC class I molecules with a single receptor.

A database search for the conserved HLA-E binding peptide revealed that the UL40 open reading frame from HCMV strain AD169 contains a 9–amino acid sequence exactly homologous to the HLA-E binding leader peptide from HLA-C molecules (Fig. 1A). Therefore, we examined whether gpUL40 could up-regulate cell surface expression of HLA-E and inhibit NK-cell–mediated cytotoxicity and whether it could do so when transport of peptides through TAP was impaired, as occurs in HCMV infection.

The UL40 open reading frame encodes a putative type I glycoprotein of 221–amino acid residues (Fig. 1A) with an NH_2-terminal 37–amino acid signal sequence (*12*) containing the peptide homologous to the HLA-E binding peptide, a 144–amino acid domain with 3 N-glycosylation sites, a 20–amino acid transmembrane domain, and a COOH-terminus of 20 amino acids (*13*). To confirm the prediction of the NH_2-terminal signal sequence, a fusion protein that contains the UL40 protein with the transmembrane and COOH-terminal domains deleted and linked to the Fc region of human immunoglobulin G1 was expressed and the NH_2-terminus was sequenced by Edman degradation on a PE Biosystems Procise automated sequencer (Core Laboratories, New Orleans, LA). The first residues sequenced were SPAET (*14*), which correspond to residues 38 to 42 of gpUL40. These results therefore confirmed that the viral peptide homologous to the HLA-E binding peptide is located in a 37–amino acid signal sequence. DNA sequencing of the UL40 gene was done on two additional HCMV strains and five clinical isolates to determine whether the viral homolog peptide was conserved. The peptide VMAPRTLIL originally

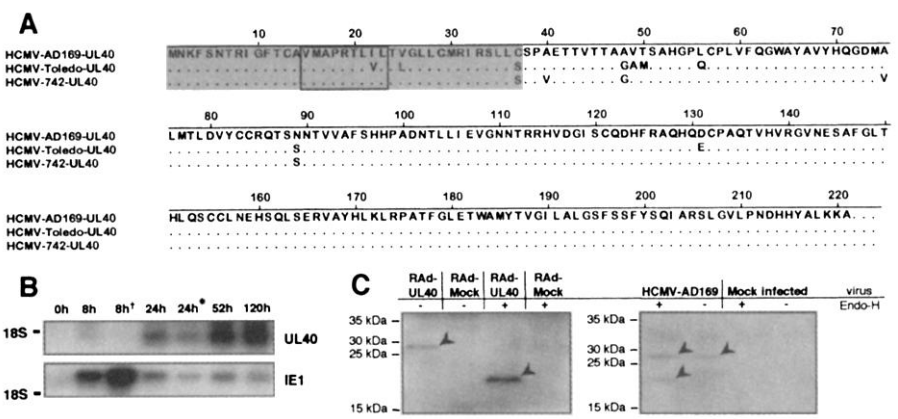

Fig. 1. HCMV UL40 gene. (**A**) Alignment of HCMV gpUL40 amino acid sequences from strains AD169, Toledo, and clinical isolate 742 (conserved residues are marked as dots). Predicted leader sequences are shaded, and homology with HLA-E binding peptides is boxed. (**B**) RNA blot illustrating the kinetics of UL40 and IE1 poly $(A)^+$ RNA synthesis during infection of HFFFs with HCMV strain AD169 [5 plaque-forming units (PFU) per cell]. Cells were treated with either cycloheximide (†) (100 μg/ml) to restrict transcription to IE genes or phosphonoformic acid (*) (200 μg/ml) to restrict expression to IE and early genes. (**C**) Immunoblot of HFFFs infected with RAd-UL40 and RAd-Mock (50 PFU/cell) and HCMV strain AD169 (10 PFU per cell), using UL40-specific polyclonal mouse serum M2083 (1/50) and guinea pig serum G2081 (1/50), respectively.

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identified in HCMV laboratory strain AD169 was conserved in the gpUL40 of seven HCMV strains and clinical isolates (Fig. 1A) (*15*). Remarkably, the variant form present in the Toledo strain sequence matched an HLA-A2 leader sequence peptide that also is known to bind to HLA-E (*6*). A change at position 1 (Val to Ala) was also found in one clinical strain and, interestingly, the resulting peptide could bind to HLA-E, although with lower affinity (*6*). The kinetics of UL40 expression during HCMV infection was determined by RNA blot analysis of polyadenylated [poly $(A)^+$] RNA from HCMV-infected human fetal foreskin fibroblasts (HFFFs). UL40 transcription was detectable in the early and late phases of infection (Fig. 1B). gpUL40 protein also was detected in HCMV-infected HFFFs by immunoblotting. gpUL40 was sensitive to Endoglycosidase H digestion, which implies retention in a pre-Golgi compartment (Fig. 1C).

To show that gpUL40 on its own was capable of up-regulating HLA-E, a replication-deficient adenovirus recombinant encoding the full-length gpUL40 (RAd-UL40) was constructed (Fig. 1C) (*16*). For detection purposes, because fibroblasts express low levels of HLA-E constitutively, a recombinant adenovirus that expressed HLA-E*0101 (RAd-HLA-E) was also engineered (*16*). HLA-E cell surface expression increased in HFFFs infected with RAd-UL40 and, as expected, more significantly in HFFFs coinfected with RAd-UL40 and RAd-HLA-E (Fig. 2, A and B). HLA-E surface expression was monitored with the monoclonal antibody (mAb) DT9, which binds to HLA-E and HLA-C (*7*). DT9 mAb consistently failed to stain uninfected fibroblasts including HFFFs. The up-regulation of DT9 mAb staining was specific for HLA-E cell surface expression because no increase in staining was detected with HLA-C–specific alloantisera (*17*). In addition, specific induction of HLA-E surface expression by gpUL40 was also observed when staining the transiently UL40-transfected cell line 721.221 (HLA-A, -B, -C, and -G negative) with DT9 mAb (*17*). Infection of HFFFs with a recombinant adenovirus expressing a mutated gpUL40 (Thr replacing Met at position 2 in the leader peptide) no longer induced HLA-E cell surface expression (*18*). This result is consistent with the inability of two-thirds of the HLA-B alleles, which possess Thr at position 2, to up-regulate HLA-E (*7*) and strongly implicates this homologous leader peptide rather than any other part of the UL40 protein in HLA-E up-regulation. By contrast, an adenovirus recombinant that expressed gpUL18 (*16*) had no effect on HLA-E expression (*17*). This result is consistent with the absence of a sequence that matches the peptides that bind to HLA-E in gpUL18.

Because gpUL40 up-regulated HLA-E surface expression, its ability to protect cells from killing by CD94/NKG2A$^+$ NK cells was examined. The interleukin-2 (IL-2)–activated NK cell line NKL expressing CD94/NKG2A and LIR-1 receptors (*19, 20*) lysed uninfected or RAd-Ctrl infected HFFFs. Blocking with an antibody to MHC class I (anti-class I) did not increase killing of these targets, which implies that the inhibitory effect of LIR-1 interaction with MHC class I molecules is not significant in the assays shown in Figs. 2C and 3B. Infection of HFFFs with RAd-UL40 alone or with RAd-UL40 and RAd-HLA-E conferred substantial protection against NK attack, which could be reversed by anti-CD94 mAb or anti-class I mAb reacting with HLA-E (Fig. 2C). Up-regulation of HLA-E by gpUL40 therefore can elicit protection from killing by CD94/NKG2A$^+$ NK cells. In contrast, a low level of protection was conferred by RAd-UL18, which may be due to the interaction between gpUL18 expressed at a low but detectable level on the surface of HFFFs (*16*) and LIR-1 on NKL (*3*). It does not result from either up-regulation of HLA-E (*17*) or a direct interaction of gpUL18 with CD94/NKG2A receptors (*4*), as anti-CD94 mAb did not reverse the protection (Fig. 2C).

Loading of HLA-E with its natural ligands, the MHC class I–derived leader peptides, is TAP dependent (*7, 10*), yet the HCMV protein US6 is an efficient inhibitor of the TAP transporter (*21–23*). Therefore, it was important to investigate whether up-regulation of HLA-E by gpUL40 could be affected when the TAP function was impaired, as in HCMV infection. An adenovirus encoding US6 (*16*) down-regulated surface MHC class I expression but did not impede the capacity of gpUL40 to up-regulate HLA-E surface expression (*17*). Two-color staining analysis could identify a population of cells with both down-regulated MHC class I molecules due to US6 expression and up-regulated HLA-E on their surface due to gpUL40 expression (*17*). Furthermore, HLA-E expression could be induced by gpUL40 on the cell surface of skin fibroblasts from a patient (N.P.) with a characterized defect in TAP expression (*24*) (Fig. 3A). Because the endogenous level of HLA-E expression in that patient was lower

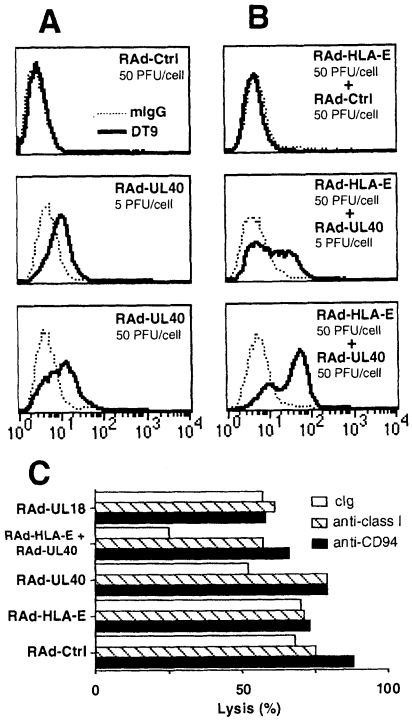

Fig. 2. gpUL40 up-regulates cell surface expression of HLA-E and protects against lysis by CD94/NKG2A$^+$ NK cells. HFFF cells were infected for 48 hours with RAd-Ctrl or RAd-UL40 as indicated (**A**) or with RAd-HLA-E and simultaneously coinfected with RAd-Ctrl or RAd-UL40 (**B**). Surface expression of HLA-E was monitored with DT9 mAb followed by fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG $(Fab')_2$. Murine IgG (mIgG) was used as control. (**C**) Susceptibility of HFFFs infected with the indicated RAd (50 pfu per cell) to lysis by the IL-2-activated NK cell line NKL (*20*). Assays (*8*) were performed at an effector-to-target-cell ratio (E:T) of 15:1 in the presence of isotype-matched IgG (clgG), anti-MHC class I (DX17), or anti-CD94 (DX22) at 5 μg/ml. NKL cells do not express CD16, which implies that the restoration seen with the mAbs cannot be explained by antibody-dependent cell-mediated cytotoxicity.

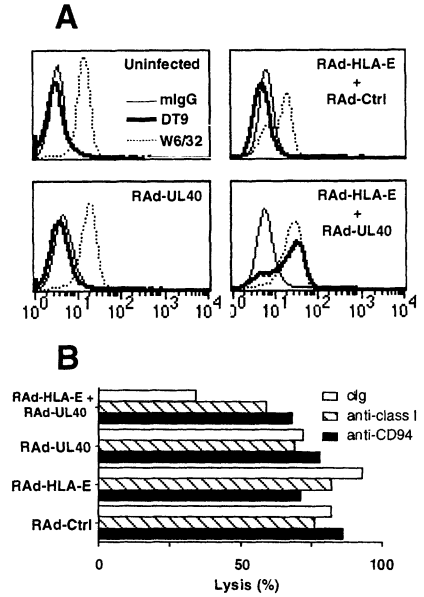

Fig. 3. gpUL40 induces surface HLA-E expression and protects against lysis by CD94/NKG2A$^+$ NK cells in a TAP-independent manner. (**A**) Fibroblasts from TAP-deficient patient N.P. were either uninfected or infected with RAd-Ctrl, RAd-HLA-E, and RAd-UL40 (50 pfu per cell), as indicated. HLA-E and MHC class I expression was monitored with DT9 and W6/32 mAb, respectively. Murine IgG (mIgG) was used as control. (**B**) Susceptibility of TAP-deficient fibroblasts from N.P. infected with the indicated RAd (50 PFU per cell) to lysis by NKL. Assays were performed at an E:T of 15:1 in the presence of isotype-matched IgG (clgG), anti-class I (DX17), or anti-CD94.

than in normal controls, up-regulation of HLA-E was observed when cells were coinfected with RAd-UL40 and RAd-HLA-E. Protection against CD94/NKG2A$^+$ NK cell–mediated lysis was conferred by infecting cells from N.P. with RAd-UL40 and RAd-HLA-E and was reversed in the presence of antibodies to CD94 and HLA class I molecules (Fig. 3B). HCMV gpUL40, therefore, is clearly capable of inducing surface expression of HLA-E and protection from NK attack in a TAP-independent manner. We previously suggested (*7*) that MHC class I signal sequences might be cleaved into two fragments with the NH_2-terminus containing the HLA-E-binding peptide being released in the cytosol (*25*). In the gpUL40 leader sequence (Fig. 1A), the HLA-E-binding peptide is not situated at the NH_2- terminus and its more distal location may direct it into the lumen of the ER, thus bypassing TAP, which is blocked by US6 during HCMV infection.

These results show that gpUL40, an early HCMV protein, can mediate up-regulation of HLA-E and therefore has the potential to counteract NK attack when HLA class I down-regulation occurs. Interestingly, while MHC classical class I molecules were down-regulated, HLA-E was up-regulated in HFFFs infected with HCMV AD169 and Toledo strains and the clinical isolate 742 (Fig. 4A). This up-regulation was enhanced when HFFFs were coinfected with RAd-HLA-E and HCMV (Fig. 4B), which implies that the limiting factor to cell surface expression was the amount of HLA-E rather than the availability of the viral peptide. Importantly, these results indicate that HLA-E is not affected by HCMV US3, US2, and US11, which are known to down-regulate some but not all MHC class I molecules [reviewed in (*2*)]. The exact role of this evasion mechanism in natural infection might be complex to unravel. In vitro studies showed that fibroblasts infected with certain HCMV strains but not others can be susceptible to NK cell–mediated lysis and that susceptibility or resistance may involve several factors that remain unidentified (*5, 26*). The isolate of HCMV, the timing, and the infected cell type used may be important. Efficient infection by HCMV in vitro is limited to primary human fibroblasts, whereas a wide range of cell types can be infected in vivo (*27*). However, the conservation of this viral HLA-E binding peptide homolog in HCMV implies that there is a selective force at work. NK cells are known to provide natural resistance to CMV infection (*28*), and a patient with an NK cell deficiency developed abnormal sensitivity to herpesviruses including HCMV (*29*). In mice, genetic susceptibility to murine CMV (MCMV) infection (CMV-1 locus) has been mapped to a region associated with NK function (*30*), depletion of NK cells enhanced virus replication, and the MCMV class I homolog m144 interferes with NK cell–mediated clearance (*31*) and confers tumor resistance to NK cell–mediated rejection (*32*). Other viruses may adopt a similar strategy, because viral homologs of the peptide binding to Qa-1 (the murine functional counterpart of HLA-E) can also be found in the databases. Development of mechanisms to escape from NK cell attack may be crucial for virus survival.

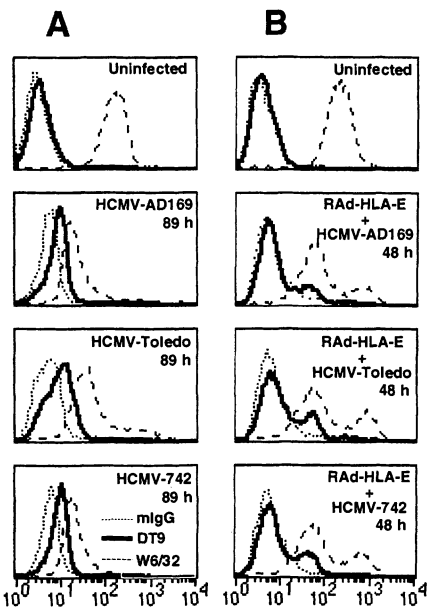

Fig. 4. HCMV infection induces cell surface expression of HLA-E. Cell surface expression of HLA-E and MHC class I molecules was monitored with DT9 and W6/32 mAb, respectively, followed by FITC-labeled anti-mouse IgG (Fab')$_2$ on HFFFs infected with HCMV strain AD169, Toledo, or clinical isolate 742 (10 pfu per cell) (**A**) and on HFFFs coinfected with RAd-HLA-E (50 pfu per cell) and HCMV strain AD169, Toledo, or 742 (10 PFU per cell) (**B**) Murine IgG (mIgG) was used as control. Staining with allosera specific for HLA-C showed that HLA-C was down-regulated after HCMV infection (*18*).

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14. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
15. Data not shown.
16. RAds were generated as described by G. W. Wilkinson and A. Akrigg [*Nucleic Acids Res.* **20**, 2233 (1992)]. Briefly, human HLA-E*0101 cDNA with a COOH-terminal His_6 tag was generated by polymerase chain reaction (PCR) with RSV5neo-HLA-E (a gift from D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) used as a template. HCMV UL40 was amplified by PCR from HCMV strain AD169 (bases 53207 to 53918) with an appropriate subgenomic DNA clone used as a template. The same PCR primers were used to amplify the UL40 gene from purified genomic DNA of strain Toledo and the clinical isolate 742. HCMV US6 and UL18 genes were amplified by PCR from HCMV AD169 bases 195396 to 195969 and bases 23632 to 27743, respectively. The PCR products were sequenced and cloned into an adenovirus transfer vector under the control of the HCMV major immediate-early (IE) promoter. Recombinant adenoviruses were generated by cotransfecting these vectors with pJM17 vector into 293T cells, thus producing RAd-HLA-E, RAd-UL40, RAd-US6, and RAd-UL18. RAd-Ctrl (also called RAd-Mock), is an equivalent adenovirus without a transgene insertion. Expression of recombinant proteins was confirmed with specific antibodies. Polyclonal mouse serum (M2083) and guinea pig serum (G2081) were raised by immunizing with RAd-UL40. The His_6 HLA-E was detected with an anti-histidine mAb (Qiagen, UK). US6 was detected by immunofluorescence with a polyclonal mouse serum raised by immunizing BALB/c mice with RAd/US6. The UL18 was detected by immunoblotting with polyclonal rabbit antibody raised against UL18/B-gal fusion protein [H. Browne, M. Churcher, T. Minson. *J. Virol.* **66**, 6784 (1992)].
17. Supplementary material on HLA-C cell surface expression on HCMV-infected HFFFs, up-regulation of HLA-E cell surface expression in gpUL40-transfected 721.221 cells, lack of HLA-E up-regulation by gpUL18, and induction of HLA-E cell surface expression by gpUL40 in Rad-US6-infected HFFF cells is available at www.sciencemag.org/feature/data/1045523.shl.
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