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Genetic Variability of the Major Histocompatibility Complex Class I Homologue Encoded by Human Cytomegalovirus Leads to Differential Binding to the Inhibitory Receptor ILT2†

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Human cytomegalovirus carries a gene, UL18, that is homologous to cellular major histocompatibility complex (MHC) class I genes. Like MHC class I molecules, the protein product of the UL18 gene associates with β2-microglobulin, and the stability of this complex depends on peptide loading. UL18 protein binds to ILT2 (CD85j), an inhibitory receptor present on B cells, monocytes, dendritic cells, T cells, and NK cells that also recognizes classical and nonclassical MHC molecules. These observations suggest that UL18 may play a role in viral immune evasion, but its real function is unclear. Since this molecule has similarity with polymorphic MHC proteins, we explored whether the UL18 gene varied between virus isolates. We report here that the UL18 gene varies significantly between virus isolates: amino acid substitutions were found in the predicted α1, α2, and α3 domains of the UL18 protein molecule. We also studied the ability of several variant UL18 proteins to bind to the ILT2 receptor. All of the variants tested bound to ILT2, but there were marked differences in the affinity of binding to this receptor. These differences were reflected in functional assays measuring inhibition of the cytotoxic capacity of NK cells via interaction with ILT2. In addition, the variants did not bind other members of the CD85 family. The implications of these data are discussed.

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that is able to establish lifelong persistent infection in immunocompetent hosts. Acute primary infection of adults is usually associated with a mild mononucleosis that disappears unnoticed. After that, HCMV remains in a latent state inside host hematopoietic cells, with occasional, mainly asymptomatic, episodes of reactivation and replication. In contrast, in immunologically immature children and immunocompromised individuals, such as transplant recipients and AIDS patients, HCMV infection can be associated with severe disease (38). These observations emphasize that while the pathogenesis of HCMV disease depends on many factors that can affect its complex life cycle, one of the most important of these factors is the interaction of the virus with the host immune system. Much of the complexity of this relationship was acquired during the coevolution of the virus with its host (34), as the latter developed strategies to eliminate the virus while the virus gained mechanisms to avoid recognition by the immune system (32). Viral immunomodulatory genes may thus be important determinants of viral pathogenesis.

Another feature of HCMV biology that likely influences the outcome of viral infection is genetic variability between viruses. Two types of variation have been described. First, large-scale deletions and rearrangements of the viral genome can occur, with the result that different HCMV isolates can have different numbers of genes (12). Second, nucleotide sequence variability of individual viral genes has also been documented. The major viral envelope glycoprotein B (gB), for example, shows a high degree of variation within the virus population. Four main genotypes of gB have been identified (36), and it has been proposed that this variation can be correlated with viral tropism in vivo (37), but it is unclear whether it influences the virulence of HCMV (see reference 44 and references therein). It seems reasonable to propose that studies of the variability of viral immune evasion genes could shed light on the importance and function of these molecules during natural infections. To date, however, immune subversion by HCMV has been investigated only with laboratory strains, and little is known about the natural variability of viral proteins and what this implies for their function. Thus, we decided to study the variability of UL18, an HCMV glycoprotein and candidate viral immunomodulatory protein that is related to major histocompatibility complex (MHC) class I antigens (7), which are encoded by a very polymorphic set of cellular genes.

Although the similarity between UL18 and MHC molecules is relatively low (about 20% in the extracellular region), UL18 forms a complex with β2-microglobulin (β2m) and binds endogenous peptides (11, 21); indeed, the stability of the complex depends on peptide binding (13). Moreover, UL18 has been reported to be resistant to the downregulation of host MHC molecules mediated by the viral gene products US2, US3, US6, and US11, which block the normal trafficking of MHC molecules to the cell surface (43). Thus, HCMV-infected cells lack host MHC class I molecules and instead express a decoy virally encoded MHC antigen. These observations led to the idea that UL18 might be involved in evading immune recognition of the HCMV-infected cell: reduced expression of MHC on the surface would be expected to help evade recognition by T cells but to enhance susceptibility to attack by NK cells (the missing-self hypothesis [31]). Thus, it was proposed that UL18 might reg-

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ulate NK cell recognition of HCMV-infected cells (21). In support of this suggestion, UL18 has been shown to have the potential to inhibit lysis by NK cells when expressed in isolation (27, 45). However, when the role of UL18 was tested in a more physiological context, comparing the susceptibility to NK lysis of fibroblasts infected with either HCMV or a UL18 knockout virus, no protective effect of UL18 expression was noted (28). Consistent with these data, NK cells do not efficiently kill HCMV-infected macrophages and endothelial cells (probable in vivo reservoirs of virus-infected cells) (25, 52) independently of the expression of UL18 (42), although it is also true that HCMV appears to have developed multiple mechanisms, including UL16 and UL40 (33), to modulate the susceptibility of the virus-infected target to NK cell lysis.

UL18, however, has been shown to bind leukocyte immunoglobulin-like receptor 1 (LIR1)/immunoglobulin-like transcript 2 (ILT2), an inhibitory receptor expressed mainly in B cells and monocytes (19, 46). LIR1/ILT2 is now known to be identical to the previously described CD85j molecule (6) and also to be expressed on the membranes of dendritic cells and subsets of T and NK cells (6, 10, 47).

ILT2 is a 110-kDa transmembrane protein with four immunoglobulin domains in the extracellular portion and four ITIMs in the cytoplasmic tail that allow it to function as an inhibitory molecule. It belongs to a multigene family of receptors which have either two or four immunoglobulin (Ig)-like domains and whose cytoplasmic tail is also variable. Some ILT receptors have two, three, or four immune receptor tyrosine-based inhibitory motifs (ITIMs), while others have no ITIMs but instead associate with Fcœ receptor γ chain and are activating receptors (9). The ligands for most of the ILT/LIR genes are unknown, but ILT2 recognizes both classical and nonclassical MHC class I molecules (10, 18, 19, 29, 40) and is able to deliver a negative signal to NK and T cells (17, 47). HCMV UL18 binds ILT2 with very high affinity (in the nanomolar range), a binding affinity much greater than that of ILT2 for HLA molecules (15), so even if only a very small number of UL18 molecules are expressed at the surface of the infected cell, the interaction between UL18 and ILT2 could result in inhibition of an effector cell. However, the identity of that effector cell remains unknown, and so the true function of UL18 during HCMV infection, although widely debated, is still not clear (see reference 22 and references therein).

The amino acid sequence of the UL18 protein used for all previous studies was that of the laboratory strain HCMV AD169. Here, we sequenced UL18 DNAs from several clinical samples of HCMV and found a significant degree of variability in the UL18 gene. We investigated the ability of these naturally occurring genetic variants of UL18 to bind ILT2 and found that all of them could be recognized by this receptor, but with markedly different affinities. These variations in binding affinities were reflected in the different abilities of the different UL18 proteins to inhibit NK cell killing in assays of redirected lysis.

MATERIALS AND METHODS

Cell lines and antibodies. 293T cells were grown and maintained in Dulbecco's modified Eagle's medium–10% fetal calf serum (FCS). The rat mastocytoma RBL and the ILT-2 transfectant were gifts of Miguel López-Botet (Universitat Pompeu Fabra, Barcelona, Spain) and were grown and maintained as previously described (18). Daudi cells were maintained in RPMI–10% FCS. YT cells transfected with ILT-2, a kind gift of Chiwen Chang (Department of Pathology, Cambridge University), were grown and maintained in RPMI–10% FCS supplemented with 2 μg of puromycin (Calbiochem) per ml. B2KA cells (BHK cells transfected with human FcγR1) were a gift from Mike Clark (3). Monoclonal antibodies (MAbs) specific for ILT-2 were GH1/75 (purchased from BD PharMingen) and HP-F1 (a gift of Miguel López-Botet). MAb anti-2B4 (clone 2-69) was also purchased from BD PharMingen.

Cloning and sequencing of UL18 variants from clinical isolates. Serum samples from patients with confirmed CMV disease were supplied by Jim Gray (Public Health Laboratory Service, Addenbrookes Hospital, Cambridge, United Kingdom), and CMV DNA was isolated from 100 μl of serum by using the DNeasy kit (Qiagen).

UL18 genes were amplified with the primers 5′CACACGGCTAAGAGGATACATC3′ (forward) and 5′GGTGGTAAAGTAGTGCAGGAACGC3′ (reverse), followed by nested PCR with the proofreading polymerase Pwo (Roche) and the primers 5′AGGGTACCCCACGTGTTGCGTTAC3′ (forward) and 5′GTCATGGTGTTATAGCGTTTGTTAGC3′ (reverse), corresponding to the predicted extracellular domain of UL18.

The PCR products were cloned into the vector pCR4Blunt-TOPO according to the instructions of the manufacturer (Invitrogen, Carlsbad, Calif.) and multiple clones from each patient were sequenced by using the oligonucleotides 5′TCACAGAGCGTGCTGACGTG3′ and 5′ACCCAACGCAATTTCCAG3′.

Sequence analysis was done with the set of tools on the web pages of the European Bioinformatics Institute (www.ebi.ac.uk); specifically, sequence alignments were prepared with the ClustalW program (53).

Expression of UL18 gene products as Ig fusion proteins. For expression of UL18 as an Ig fusion protein, the PCR fragments from the procedure described above were subcloned into the vector pCDM7 as KpnI-BamHI fragments. Recombinant proteins expressed from this vector are synthesized with the CD5 leader sequence and fused at the C terminus to the hinge, CH2, and CH3 regions of human IgG1 (4).

Expression of recombinant proteins. Recombinant proteins were expressed by transfection of 293T cells with 47 μg of plasmid DNA (80% UL18 and 20% β2m)/5×10^6 cells/175-cm^2 flask, using calcium phosphate. After removal of precipitate at 16 h, cells were incubated for 48 to 72 h in X-Vivo-10 serum-free medium (BioWhittaker, Walkersville, Md.) before the supernatant was recovered. Supernatants were concentrated in Centricon-YM10 spin concentrators, and the concentration of UL18 Ig protein was determined.

Enzyme-linked immunosorbent assay. The concentrations of the various Ig fusion proteins were measured by enzyme-linked immunosorbent assay with polyclonal antibody against human Ig (Jackson) as a capture antibody and a horseradish peroxidase-conjugated goat antibody directed against human Ig (Southern Biotechnology) for detection. The reaction was developed with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche). Dilution series of the various UL18-Igs were assayed and compared with standard curves prepared with highly purified human IgG1 protein (Sigma).

Immunoprecipitation and Western blotting. UL18-Ig proteins were immunoprecipitated with protein A-Sepharose (Amersham Pharmacia), and the immunoprecipitates were run on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blotting was performed either with MAb BBM1 (specific for human β2-microglobulin) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Dako) or with biotinylated goat F(ab′)2 antibody directed against human Ig (Caltag) followed by horseradish peroxidase-conjugated streptavidin. Bound antibody was visualized by using the ECL Plus system (Amersham Pharmacia).

Flow cytometry. Cells (10^5) were preincubated in PBA (phosphate-buffered saline containing 1% bovine serum albumin [BSA] and 0.1% sodium azide [Sigma]). After washing and careful removal of all liquid, cells were then incubated for 1 h on ice with the indicated concentrations of the various UL18-Ig proteins diluted in PBA. After being washed in PBA, cells were incubated with phycoerythrin-labeled goat $F(ab)_2$ fragment of antibody directed against human Ig (Immunotech) for 30 min on ice. Cells were analyzed with a FACScan (Becton Dickinson).

Expression, refolding, and iodination of soluble ILT2 proteins. Soluble ILT2 protein (d1d2) was expressed in bacteria as inclusion bodies and refolded and purified as described previously (50). For iodination, the protein was exchanged into borate-buffered saline (pH 8.5) and diluted to 1 mg/ml in 0.1 M borate (pH 8.5). Ten micrograms of protein was labeled with 500 μCi of Bolton-Hunter reagent (^{125}I) (Amersham). The iodinated protein was separated from the iodinated Bolton-Hunter reagent on a gel filtration column prepared with Ultrogel Aca202 (IBF Biotechnics) previously blocked with 1% BSA–phosphate-buffered saline and then equilibrated in binding buffer (128 mM NaCl, 5 mM KCl, 5 mM

$MgSO_4$, 1.3 mM $CaCl_2$, 50 mM HEPES [pH 7.6]). Fractions containing iodinated ILT2 were pooled and used immediately in scintillation proximity binding assays.

Scintillation proximity binding assay. FlashPlate (NEN, Life Sciences Products) is a 96-well polystyrene microtiter plate with scintillant-coated wells. After a target protein is bound to the wall of a FlashPlate well, radiolabeled molecules are added and allowed to bind to equilibrium. The radioactive decay associated with the bound radiolabeled molecules causes a scintillation effect that is detectable on a microplate scintillation counter. Control, purified human IgG1 (Sigma) and the UL18-Ig proteins were immobilized in the wells of a protein A-coated FlashPlate Plus plate by incubation for 2 h in phosphate saline buffer (pH 7.2). After washing with binding buffer, nonspecific binding was blocked by incubation for 2 h at room temperature with 5% BSA in binding buffer. After three washes with binding buffer containing 1% BSA, ^{125}I-labeled soluble ILT2 protein was added at the concentrations indicated and incubated for 2 h to 16 h at room temperature. The plates were counted in a microplate scintillation counter (Wallac), using a 1-min counting period. Nonspecific binding was also determined by inclusion in the assays of a 60-fold excess of unlabeled soluble ILT2 protein. Best-fit values for the K_d of each interaction were calculated by using nonlinear regression analysis to fit the model $Y = B_{max} \times X/(K_d + X)$ to the experimental data. This model describes the binding of a ligand to a receptor that follows the law of mass action; B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. These analyses were performed with Prism version 4.0 for Windows (GraphPad Software, San Diego, Calif.).

Transient transfection of Flag-tagged ILT/LIR genes. 293T cells were transiently transfected with the indicated ILT/LIR genes by using Fugene 6 (Roche). Plasmids carrying Flag-tagged ILT and LIR genes were a gift of Rachel Allen (Department of Pathology, Cambridge University) and have been described previously (1). Cells transfected with the activating ILT1 and LIR-6 ILT/LIR genes were cotransfected with a plasmid encoding the gamma chain of FcRε (a gift of Chiwen Chang). Cells were stained 48 h after transfection with either MAb M2 (anti-Flag; Sigma) or saturating amounts (as judged by staining of ILT2 transfectants) of the indicated UL18-Ig fusion protein (except UL18 variant E, for which insufficient protein was available). Negative controls included mock-transfected 293T cells, cells transfected with KIR2DL3, and the staining of ILT/LIR-transfected cells with control human IgG1 protein.

Cytotoxicity assays. The cytolytic activity of the YT-ILT2 NK cell line against B2KA target cells line was assessed in 4-h ^{51}Cr release assays. B2KA cells were plated (5,000 cells/well) in medium with $Na^{51}CrO_7$ (3μCi/well) and incubated for 16 h at 37°C with 5% CO_2 to allow cells to adhere. Cells were washed three times and then incubated with a constant amount of anti-2B4 MAb, to trigger cytotoxicity, and the indicated amounts of the various UL18-Ig proteins or controls. The target cells were then washed once, and YT-ILT2 cells were added at effector-to-target cell ratios of 50:1 and 100:1. Assays were performed in triplicate, and values differed by <10% (on average, ~5%) of the mean. In all presented cytotoxicity assays, the spontaneous release of ^{51}Cr was <25% (on average, ~10%) of the maximal release.

Nucleotide sequence accession numbers. The sequences of the various UL18 variants have been deposited in GenBank with accession numbers AJ583658 to AJ583668.

RESULTS

The UL18 gene of HCMV varies markedly between viruses from patients. The sequences of UL18 known when this study was initiated were obtained from the laboratory strains HCMV AD169 (PubMed accession number NC_001347) and Towne (PubMed accession number AY072777). Interestingly, these two UL18 sequences differ in five amino acids (Fig. 1; see Fig. S1 in the supplemental material). In order to assess whether UL18 varied between clinical specimens of the virus, the UL18 gene was amplified from HCMV DNAs isolated from sera obtained from transplant recipients suffering from HCMV disease, and the region coding for the extracellular portion of UL18 was amplified, via nested PCR, from 15 patient samples. A number of conclusions could be drawn from inspection of these sequence data. First, from only 15 samples, 12 different sequences were obtained (for three of the patients the UL18 gene amplified was identical to the UL18 gene of AD169), revealing that there exists significant variability of the UL18 gene in the virus population. Second, among these sequences, there were amino acid positions that varied from the AD169 sequence and which were found in several patients, suggesting that these mutations may influence virus biology. One striking feature found in several of the sequences (A, B, E, J, K, and Q) is a deletion of three adjacent nucleotides, i.e., one amino acid loss. This deletion occurs at position 16 of the polypeptide, at the beginning of what would be the predicted α1 domain, and, in the sequences analyzed so far, it is part of a cluster of changes from DDT to GE*, where the asterisk represents a deletion (Fig. 1). Third, although there are clusters of mutations in all three domains, most mutations, especially those unique changes defining each distinct sequence, occur in what would be predicted to be the α2 domain of the protein. Fourth, a number of silent mutations are shared by several sequences, perhaps indicating how these variants of UL18 arose.

We measured the extent of divergence between sequences by determining two parameters: the number of amino acid differences between two sequences and the proportion of different amino acids (Table 1). The most different pairs of sequences differ at 21 amino acids, and the most similar have only one amino acid change.

The UL18 variants obtained from patient samples display between 93.42 and 98.36% identity with the known sequence from the laboratory strain AD169, a range very similar to the degree of similarity found among all of them (93.09 to 99.67%). Independent confirmation of these data comes from inspection of the sequences of the UL18 genes from the genomes of a number of HCMV laboratory and clinical isolates that have been cloned in bacterial artificial chromosomes and sequenced (20, 39). The UL18 gene products of these viruses vary by up to 16 amino acids from the sequence of UL18 from AD169.

In control experiments the UL16 gene, which is located very close to UL18, was also amplified from the same samples and sequenced. In this group of patients the sequence of the UL16 gene was essentially identical in all samples (M. Valés-Gómez, unpublished observations).

The various UL18 gene products bind to ILT2, but with different affinities. The UL18 protein from AD169 binds the inhibitory receptor ILT2. Thus, we next investigated whether the naturally occurring variation of UL18 seen in patient samples affected binding to this receptor. For these experiments, several UL18 variants were expressed as Ig fusion proteins, and their ability to bind to ILT2-expressing cells was studied. Figure 2 depicts a representative result of these fluorescence-activated cell sorting (FACS) experiments. All of the genetic variants studied were able to interact with ILT2 but apparently with different affinities, as they bound differentially. This result was consistently obtained in a large number of experiments, using several batches of protein to stain several cell lines expressing ILT2 on their surfaces (RBL-ILT2, Daudi, and YT-ILT2). In all cases the results were comparable to the ones shown in Fig. 2.

In order to estimate the strength of the interaction between the UL18 variants and ILT2, the concentration of UL18 required for half-maximal binding to ILT2 was calculated from flow cytometry experiments in which a titration of the amount of UL18 binding to RBL-ILT2 cells was performed. The result of a representative experiment is shown in Fig. 3. One group of

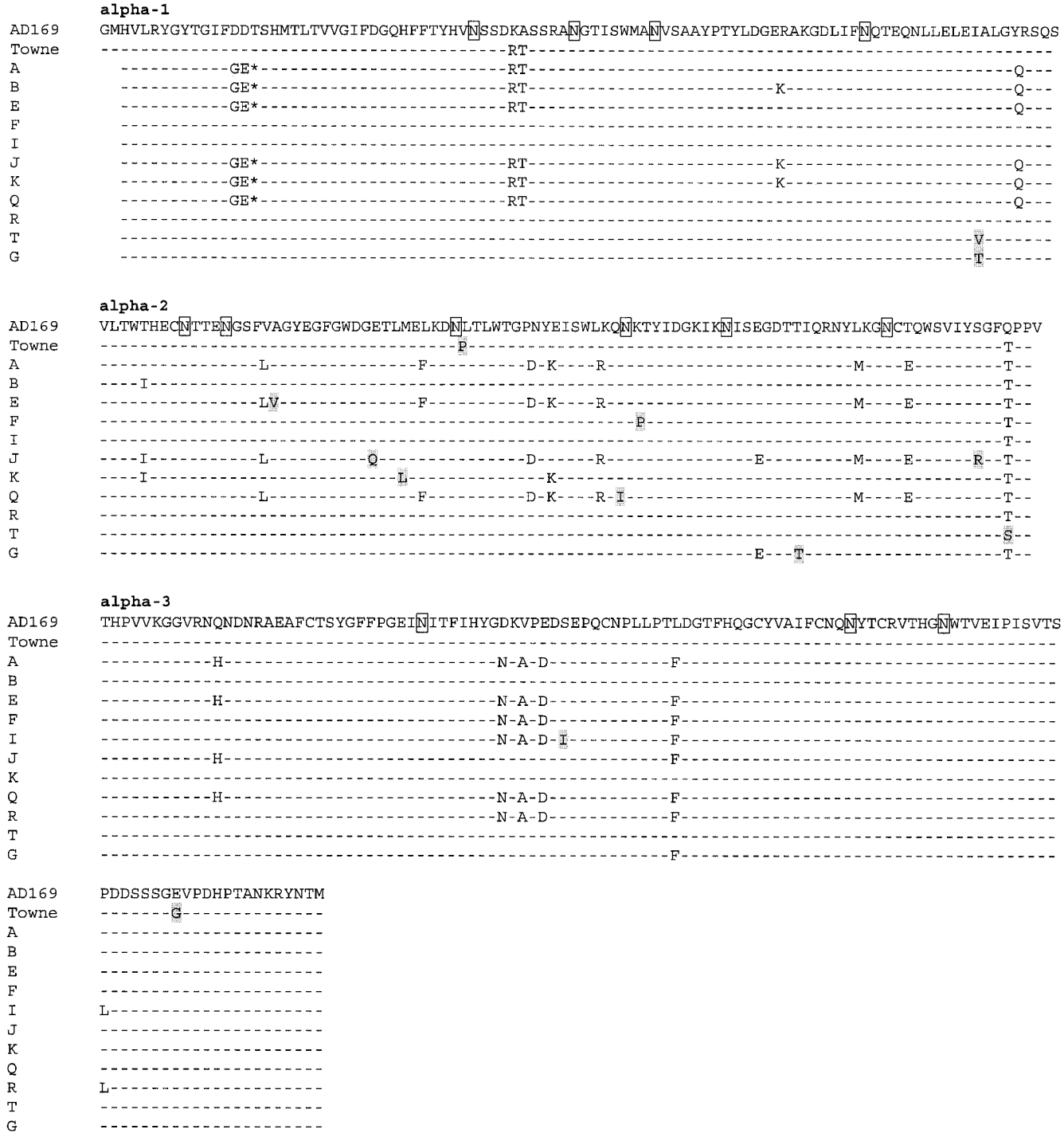

FIG. 1. Amino acid comparison of the extracellular portions of several UL18 variants. Dashes represent identity with AD169, and asterisks represent deletion of a residue. Amino acid changes that are unique to a single UL18 variant are shaded in gray. Sites of potential N glycosylation are boxed.

proteins (F and K) showed saturation curves similar to those seen with AD169 UL18 protein, while one variant, UL18J, bound with higher affinity. Another variant protein, UL18E, did not reach saturation at the amounts of protein used. This implies that there is at least an order of magnitude of difference in the affinities of the variants of UL18 studied. We have not presented calculated affinities derived from the raw data because the values estimated from these experiments are relative strengths of binding and not affinity, in strict kinetic terms, as the UL18-Ig constructs are bivalent. Further, any calculations of values for K_d are influenced by the molecular masses of the UL18-Ig proteins used for the calculation (110

TABLE 1. Amino acid differences between UL18 sequences

Strain of variant	No. of amino acid differences and proportion of different amino acids[a] with respect to:												
	AD169	Towne	A	B	E	F	I	J	K	Q	R	T	G
AD169		5	18	8	19	6	7	18	10	20	6	2	5
Towne	0.0164		17	7	18	9	10	17	8	17	9	6	8
A	0.0592	0.0559		13	1	14	14	10	14	1	13	16	19
B	0.0263	0.0230	0.0428		15	11	13	10	2	15	12	9	11
E	0.0625	0.0592	0.0033	0.0493		15	16	10	15	2	14	20	20
F	0.0197	0.0296	0.0461	0.0361	0.0493		3	20	9	15	2	7	7
I	0.0230	0.0329	0.0460	0.0428	0.0526	0.0099		21	15	16	2	8	8
J	0.0592	0.0559	0.0329	0.0329	0.0329	0.0658	0.0691		12	11	20	18	17
K	0.0329	0.0263	0.0461	0.0066	0.0493	0.0296	0.0493	0.0395		15	14	11	13
Q	0.0658	0.0559	0.0033	0.0493	0.0066	0.0493	0.0526	0.0362	0.0493		14	19	20
R	0.0197	0.0296	0.0428	0.0394	0.0461	0.0066	0.0066	0.0658	0.0461	0.0461		7	7
T	0.0066	0.0197	0.0526	0.0296	0.0658	0.0230	0.0263	0.0592	0.0362	0.0625	0.0230		5
G	0.0164	0.0263	0.0625	0.0362	0.0658	0.0230	0.0263	0.0559	0.0428	0.0658	0.0230	0.0164	

[a] The number of amino acid differences between two sequences is given above the diagonal. The proportion of different amino acids is given below the diagonal. Deletions were eliminated from the computation. The total number of amino acids used was 304, corresponding to the extracellular portion of UL18.

kDa), which are only approximations derived from analysis by SDS-polyacrylamide gel electrophoresis. Bearing in mind these limitations, the apparent affinity obtained for AD169 UL18 binding to ILT2, about 13.7 nM, is a value broadly consistent with those obtained from BIAcore analysis of the ILT2-UL18 interaction (15).

Next, scintillation proximity binding assays were done to determine precisely the affinities of the interactions between ILT2 and some of the variants of UL18 and to confirm the hierarchy of affinities seen in the FACS experiments. The UL18-Ig constructs were immobilized on a protein A-coated FlashPlate (see Materials and Methods), and the binding at equilibrium of soluble iodinated ILT2 (d1d2) protein was measured. Figure 4A depicts the results of a representative experiment showing the specific binding of the UL18 variants E, J, and AD169 to ILT2 d1d2. Figure 4B shows the Scatchard transformations of these dose curves. Nonspecific binding was determined as either the binding of labeled ILT2 to wells

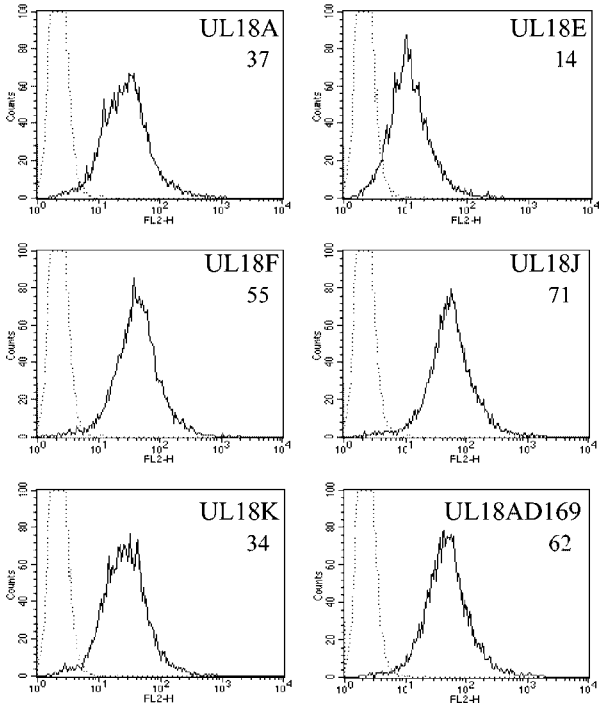

FIG. 2. All of the UL18 genetic variants can bind to ILT2-transfected cells. The indicated variants of UL18 were expressed as soluble Ig fusion proteins. These proteins were assayed, by flow cytometry, for their ability to bind RBL-ILT2 cells (solid line: UL18-Ig; dotted line: human IgG1 control). Below the name of each UL18 variant appears the observed mean fluorescence intensity. The mean fluorescence intensity of the Ig control was 2.

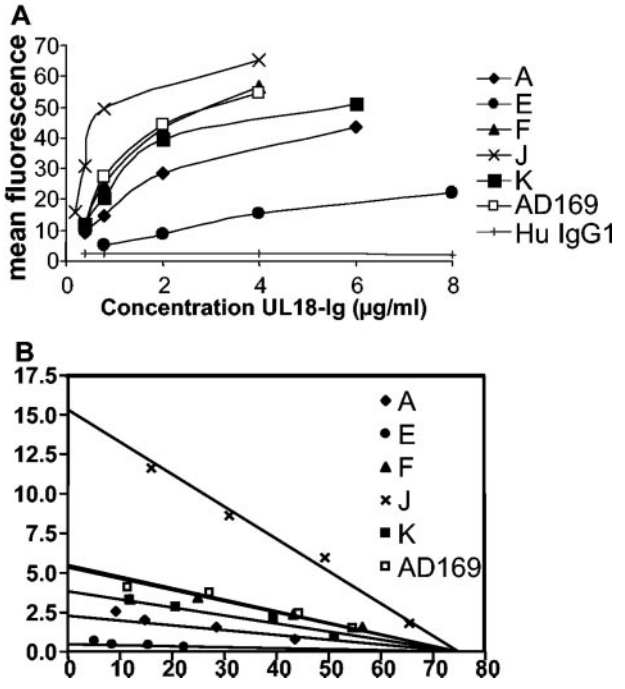

FIG. 3. Titration of the binding of UL18 genetic variants to ILT2-transfected cells. (A) RBL-ILT2 cells were stained with the indicated concentrations of the various UL18-Ig proteins and analyzed by flow cytometry. The graph shows the measured fluorescence plotted against the concentration used for staining. Data are representative of those from three experiments. (B) Scatchard transformation of the data shown in panel A.

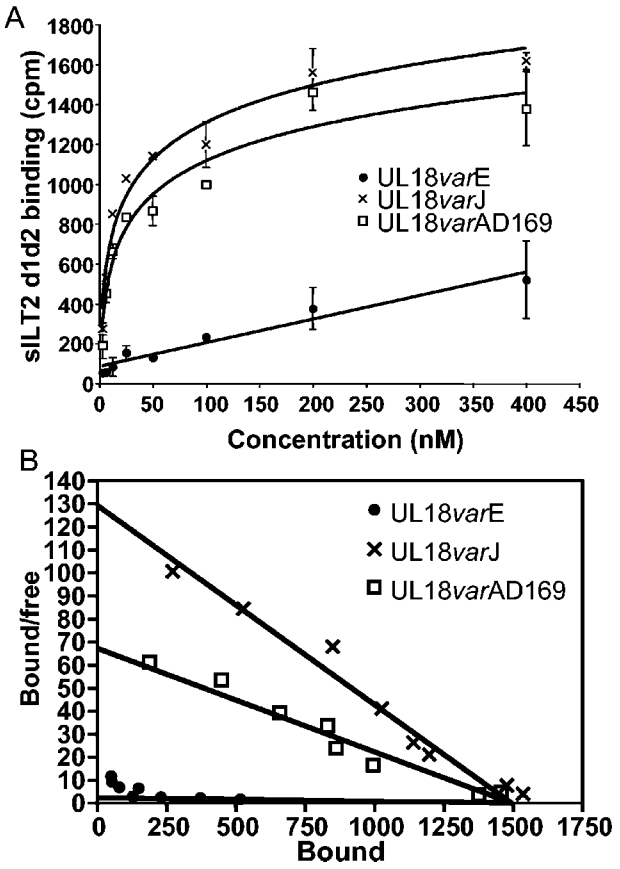

FIG. 4. Equilibrium binding of ^{125}I-labeled ILT2 d1d2 to UL18-Ig variants. (A) The indicated UL18-Ig constructs were tested with a FlashPlate binding assay in the presence of the indicated concentrations of soluble ILT2 (d1d2). Counts were read after an overnight incubation. Background counts (^{125}I-ILT2 on human IgG1-coated wells) were subtracted from each point. Results from one representative experiment of three are shown, and the data points are given as means ± standard deviations. (B) Scatchard transformation of the data shown in panel A.

coated with human IgG1 or the binding of labeled ILT2 to the various UL18 proteins in the presence of a 60-fold excess of unlabeled ILT2 and was around 30 to 40% of the total binding. The variants J and AD169 of UL18 bound specifically to ^{125}I-ILT2 with the values of K_d calculated at 11.55 and 22.2 nM, respectively. The UL18 variant E bound much more weakly, not reaching saturation at the concentrations of soluble ILT2 used (estimated K_d of ≥637 nM). Thus, the hierarchy of binding affinities measured in scintillation proximity binding assays is in good agreement with the data obtained in the FACS experiments, in which the variant E bound ILT2 with a much lower affinity than the other variants. The affinity measured for the binding of UL18 AD169 to ILT2 is consistent with those obtained from BIAcore analysis of the ILT2-UL18 interaction (15).

The different UL18 variants are able to bind β2m. Since, at least for ILT2 binding to HLA class I molecules, many of the contact residues involve the β2m molecule, we checked for differential association of the UL18-Ig proteins with β2m as a possible explanation for the differences in binding to ILT2. As the stability of UL18 depends on association with peptide and

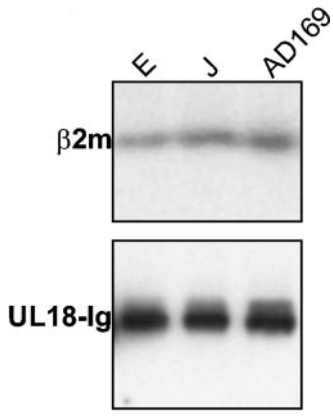

FIG. 5. The UL18 genetic variants can bind β2m. Ig fusion proteins UL18-E, -J, and -AD169 were immunoprecipitated with protein A-Sepharose, run in SDS-polyacrylamide gel electrophoresis, and then analyzed by immunoblotting with MAb BBM1, specific for human β2m. The blots were then stripped and reprobed with antiserum specific for the Fc portion of human Ig to confirm equal loading of the UL18-Ig proteins.

β2m, this could also be thought of as a test of whether the soluble UL18-Ig molecules were correctly folded. Ig fusion proteins were immunoprecipitated and then analyzed by immunoblotting with MAb BBM1, which is specific for human β2m (Fig. 5), and after stripping were reprobed with antiserum specific for the Fc portion of human Ig to confirm even loading of the UL18-Ig proteins (Fig. 5). These data indicate that UL18 variants that bind ILT2 very differently bind β2m roughly equally.

The different binding affinities of the UL18 variants for ILT2 correlate with their ability to inhibit ILT2-expressing NK cells. The experiments described above show that the different UL18 variants bind ILT2 with different affinities. However, the affinity of HLA class I molecules for ILT2 is on the order of 8 to 20 μM, which is three orders of magnitude lower than that of UL18 for ILT2 yet still sufficient to inhibit NK cell lysis. Thus, it was important to test whether the different UL18

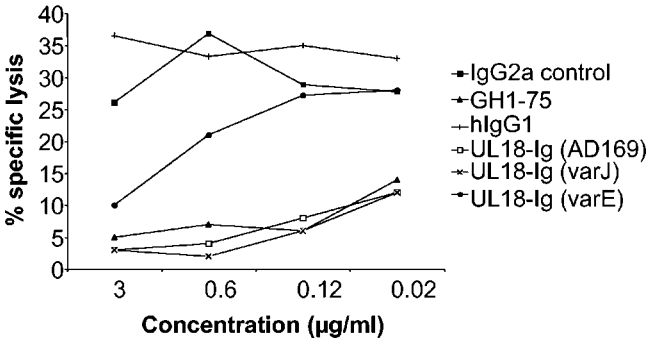

FIG. 6. Cytotoxicity by NK cells can be blocked with UL18-Ig proteins. The percent specific lysis of BHK-CD64 cells by YT-ILT2 cells at an effector-to-target cell ratio of 50:1 in the presence of different amounts of UL18-Ig soluble proteins is depicted. Target cells were preincubated with the indicated amounts of either antibody or UL18-Ig constructs. After 30 min, effector cells were added and a 4-h ^{51}Cr release assay was performed. These data are representative of those from three independent experiments with YT-ILT2 cells.

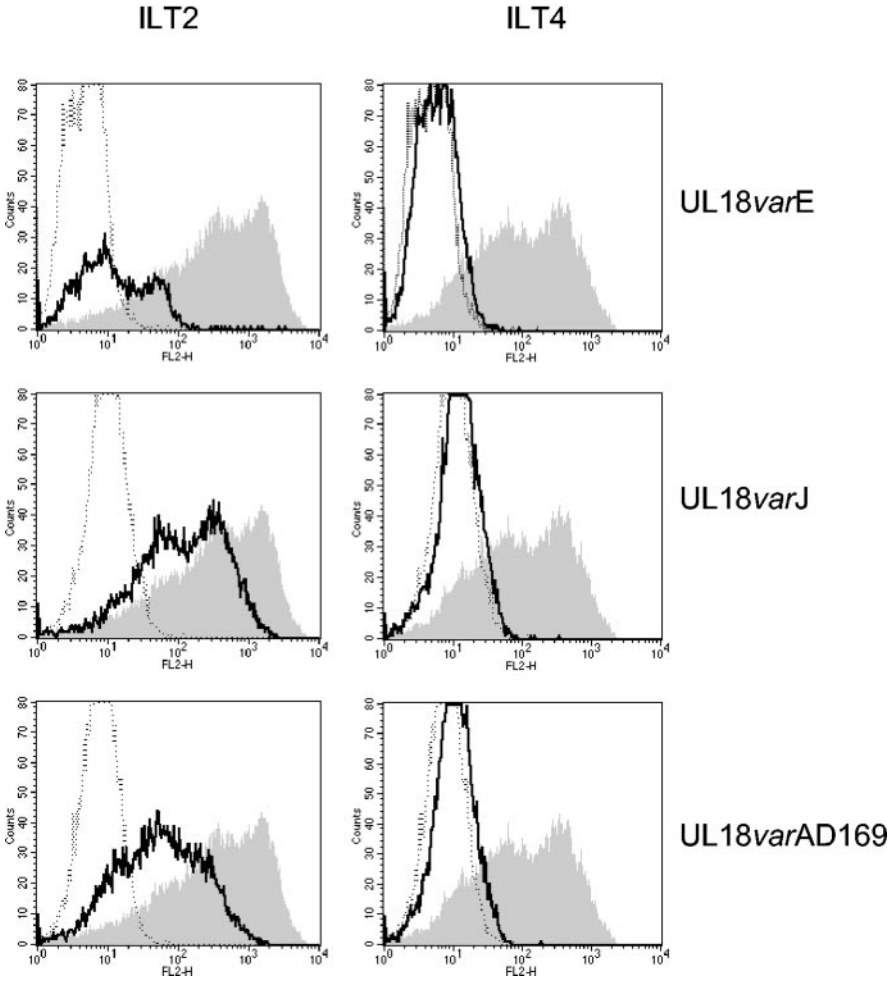

FIG. 7. None of the UL18 genetic variants bind to cells transfected with other members of the ILT family. Cells were transiently transfected with a panel of different ILTs (here, only ILT2 and ILT4 are shown) and stained with either anti-Flag antibody (transfection control, shaded), or the UL18-Ig construct (solid line) and analyzed by flow cytometry. As a negative control, the different UL18-Ig proteins were used to stain 293T cells transfected with an irrelevant (KIR) gene (dotted line). Cells were also stained with human IgG1 as negative control (not shown). Data are representative of those from three experiments.

variants differed in their ability to inhibit NK cell killing. For this purpose, redirected lysis assays were carried out with a constant amount of anti-2B4 monoclonal antibody to trigger NK cell lysis and titrating in increasing amounts of UL18-Ig, to produce differing levels of cell surface UL18. These experiments were done with, as a target, the B2KA cell line (BHK cells transfected with human CD64/FcγR1), since both murine IgG2a and human IgG1 bind efficiently to this Fc receptor. The NK tumor cell line YT transfected with ILT2 was used as the effector in the majority of the experiments, since this cell line is not known to express other inhibitory receptors that could interfere in this assay. Figure 6 shows the results of a representative experiment. UL18-Ig variant J or AD169 inhibited NK cell lysis efficiently, even at low concentrations, whereas variant E was able to inhibit lysis only at much higher concentrations. Similar data were obtained with the ILT2-expressing NK cell tumor line KHYG-1 as effector cells (not shown).

The variant UL18 genes tested do not bind to other members of the ILT/LIR gene family. ILT2 belongs to a multigene family of receptors with 2 or 4 Ig domains. Thus, one question arising from the existence of UL18 variability was whether any of these variants would gain the capacity to bind other members of the ILT/LIR family. With this idea in mind, cytometry experiments were performed on 293T cells transiently transfected with either ILT1, ILT2, ILT3, ILT4, ILT5, ILT7, or LIR-6. Of all the ILT/LIR cDNAs tested, the UL18 genetic variants bound only to ILT2. Figure 7 shows the results of a representative experiment testing for ILT2 and ILT4 binding to UL18 from AD169, variant E (the one with lowest affinity), and variant J (the one with highest affinity).

DISCUSSION

This paper describes the existence of genetic variability in UL18, the MHC class I homologue encoded by HCMV. The UL18 gene shows significant variability throughout the extracellular domain; different UL18 variants can vary by up to 21 amino acids. Two types of sequence variation are seen in the UL18 gene. First, there are regions where both coding and noncoding nucleotide changes are found and which occur in

several clones. This type of sequence variation probably arises from recombination between multiple strains of infecting or reactivating CMV (16, 35), and these putative recombination events can affect any of the α1, α2, or α3 domains. The second type of variations observed in these sequences are point mutations, which occur mainly in the predicted α2 domain of UL18 and often define the individual variants. It is not clear how these various mutations arose, but a variety of evidence suggests that the observed variation in UL18 is not just random genetic drift. First, for every sequence the majority of the nucleotide changes observed (with respect to the AD169 sequence) result in replacement of an amino acid. Further, analysis of the pattern of nucleotide substitutions by using the algorithm developed by Nei and Gojobori (41), which takes into account that not all nucleotide changes are equally likely to cause an amino acid change, also indicates that there is evidence for positive selection acting at all of the observed sites of amino acid change (Z. Yang, M. Valés-Gómez, and H. T. Reyburn, unpublished observations). Second, no mutations which might inactivate UL18 expression were observed in any of these samples; e.g., although there is a nucleotide deletion in the predicted alpha 1 domains of several of the variant viruses, the reading frame is maintained. Lastly, analysis of the full-length UL18 genes of several HCMV laboratory strains and clinical isolates, whose entire genomes have been cloned and sequenced (20, 39), reveals that amino acids which vary between the UL18 sequences of these viruses are found only in the predicted extracellular domain of the protein. Restriction of the variability in UL18 to a region that could affect interactions with other molecules again suggests that this variability is functionally significant.

These genomic sequence data also allow comparison of the variability of UL18 genes with the variability of other genes carried by HCMV (20, 39). Although a few open reading frames vary greatly between strains, the sequences of the majority of HCMV genes appear to be quite conserved. If the extent of sequence variability in other known immune evasion genes is compared to that in UL18, then it can be seen that US2, US3, US6, and US11 (all interfering with the correct surface expression of MHC molecules) and UL16 (which blocks surface expression of ligands for the NKG2D receptor) are highly conserved between strains (97 to 99.9% amino acid identity) (20, 39, 49). Further, at least for the US3 gene, this sequence variability does not appear to have any impact on function (26). The viral genes that encode Fc receptors (TRL11/IRL11 and UL119-118) (5, 30) are slightly more variable (93.5 to 100% identity between strains), but whether this affects their function is unknown. Interestingly, UL40, the HCMV protein involved in immune evasion by providing a peptide that can stabilize HLA-E molecules (94 to 100% identical between strains), is variable in the region that encodes the HLA-E binding peptide, and it is not clear whether all of these variants would be able to assemble with HLA-E for interaction with the CD94/NKG2 heterodimer. UL18 is considerably more variable than any of these other known immune evasion genes (90 to 99% identity between strains), and, importantly, this sequence variability can modulate the strength of the UL18-ILT2 interaction.

In the experiments reported here, the avidity of the binding of the different UL18 variants to the ILT2 receptor varied over orders of magnitude. Some variants (K and F) bound to ILT2 with an avidity similar to that of UL18 from AD169, but variants could be identified that bound better (J), worse (A), or much worse (E) than UL18 from AD169. The molecular basis of this differential binding is unclear. The amino acid changes that define the different variants occur throughout the predicted α1, α2, and α3 domains, and while the α3 domain of UL18 is known to be critical for ILT2 binding (15), there does not appear to be any simple correlation between the α3 domain sequences of the variants and the strengths with which they bind ILT2. This perhaps indicates that other regions of the UL18 protein can influence binding to ILT2, a suggestion supported by comparison of variants A and E, which differ by only one amino acid, an alanine-to-valine change at position 114 in the α2 domain, and yet bind ILT2 with very different affinities. No contacts are seen between ILT2 and the α2 domain of HLA-A2 in the recently solved crystal structure of this complex (55), but it is possible that this mutation could affect local elements of the UL18 structure that alter receptor binding, analogous to the effect of the mutation at position 145 in the α3 domain of HLA-Aw68 on CD8 binding (24). In any event, the fact that a single amino acid change can have such a profound effect on the affinity of the interaction between receptor and ligand emphasizes the difficulty in predicting the effect on function of any given sequence change in the absence of detailed structural information.

It is also of some interest to point out that the mutations in some of the sequences result in the loss of a potential N-glycosylation site (Fig. 1). These include the laboratory strain Towne and the UL18 variants F and Q from clinical isolates. However, UL18 variant F binds to ILT2 very similarly to UL18 from AD169. Thus, in this case the lack of one molecule of carbohydrate does not influence binding to the receptor. This observation is consistent with other data which show that the nature of the glycan moieties on UL18 does not affect binding to ILT2 (15), but it does not rule out that alterations in UL18 glycosylation could influence other aspects of the biology of this molecule; for example, it is possible that the heavy glycosylation of UL18 contributes to the ability of this molecule to resist the attack of other HCMV proteins which act to downregulate expression of host cell MHC class I molecules (43).

What might be the functional significance of the observed variation in the affinity of UL18 binding to ILT2? It has been argued that the high affinity of the interaction is significant, since only a small amount of UL18 would have to be expressed on the surface of the infected cell to bind ILT2 and mediate inhibition (15). In this context, the difference in the binding to ILT2 of these variant UL18 proteins could translate into a difference in signaling within the ILT2-expressing cell, and a low-avidity interaction might not reach the threshold necessary to provoke a cellular response. This last point is especially relevant for UL18 variant E, which binds ILT2 much more weakly than the other UL18 variants tested. Indeed, the hierarchy of binding affinities of the different UL18 variants for ILT2 correlates well with the ability to inhibit NK lysis of the different soluble UL18-Ig proteins at low concentrations (Fig. 3, 4, and 6). Given that the UL18 variants described in this paper were all cloned from patients presenting with symptoms of CMV disease, these data imply that the UL18-ILT2 interaction might be dispensable or that even low-affinity binding to

ILT2 is sufficient for function; however, it is clear that low levels of surface UL18 are able to inhibit NK cells efficiently only if the UL18 binds to ILT2 with high affinity (Fig. 6).

These data prompt the question as to what might be the selective pressure promoting the variation of the UL18 gene; specifically, what might be the advantage to a virus to express a variant UL18 with a lowered binding affinity for ILT2? A possible answer to this question is suggested by a recent report that specific recognition of the viral protein UL18 by ILT2 on $CD8^+$ T cells mediates non-MHC-restricted lysis of human cytomegalovirus-infected cells (48). In this context, the advantages of expressing a UL18 molecule with a reduced binding affinity to ILT2 are obvious. However, this hypothesis does not explain why all of the sites of amino acid change appear to be under positive selection for diversity and yet many of the variant UL18s tested have an affinity for the ILT2 receptor that is similar (within a two- to threefold range) to that of the index UL18 from AD169. Thus, another explanation for these observations would be that UL18 is able to interact with receptors other than ILT2 and that perhaps the evolution of diversity in UL18 is related to modulation of the strength of this interaction. This suggestion would be consistent with the observation that in some systems expression of UL18 can result in enhanced killing of target cells by NK cells (28). The idea that viral proteins can interact with both activating and inhibitory receptors expressed on NK cells and that attempts by the virus to modulate these interactions can lead to mutation in viral proteins is familiar from recent studies on the interactions of the murine CMV protein m157 with Ly-49 receptors (2, 23, 51, 54). Given that ILT2 belongs to a multigene family containing receptors with activating and inhibitory potentials and prompted by reports that UL18, while binding strongly to ILT2, is also able to bind weakly to the LIR2/ILT4 receptor (14), we tested whether any of the new UL18 variants described here could bind other receptors of the LIR/ILT family. No evidence was obtained for any new interaction between the genetic variants of UL18 and the other LIR/ILT molecules tested, but we cannot rule out that other genetic variants of UL18 might bind other CD85s or indeed other, unrelated molecules.

Consistent with this last possibility, it is interesting that many of the mutations found in these UL18 variants occur in the predicted $\alpha1$ and $\alpha2$ domains of the molecule, distant from the proposed site of union with ILT2. Many of these mutations occur in regions that would be predicted to form the peptide-binding groove of an MHC-like molecule, and although the low level of homology between UL18 and HLA impedes confident assignment of the locations of individual amino acids, the observed pattern of sequence variation might imply that different UL18 molecules bind different peptides. Interestingly, while it has been shown that UL18 binds peptides and that the stability of the heavy-chain/$\beta2$m complex depends on peptide binding (13, 21), the functional significance of this aspect of the biology of UL18 is unknown; "peptide-empty" soluble UL18, for example, has been shown to bind ILT2 almost as well as peptide-filled molecules (15). Perhaps UL18 can interact via its $\alpha1$ and $\alpha2$ domains with other, as-yet-unidentified, receptors.

One outstanding question posed by these data is whether there might be any correlation between UL18 sequence variants, their altered binding to ILT2, and the pathogenesis of HCMV disease. This question is particularly relevant since the early increase in ILT2/LIR-1 expression on peripheral blood lymphocytes has been correlated with the development of cytomegalovirus disease after lung transplantation (8). This, however, is a very difficult issue to address, and currently there are not sufficient data. There are two major problems to be overcome. First, the extensive sequence diversity (12 different sequences were obtained from only 15 patient samples) implies that a very large number of patient samples would have to be examined to have a reasonable possibility of determining whether there is any statistically significant relationship between UL18 sequence variation and pathogenesis. Second, the fact that variation of even one amino acid can have a profound effect on the affinity of the interaction between receptor and ligand means that prediction of the biological significance of any variation would be very difficult in the absence of a detailed structural and biochemical characterization of how ILT2 binds UL18.

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