

Cutting Edge: A Novel Viral TNF Receptor Superfamily Member in Virulent Strains of Human Cytomegalovirus¹

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The UL144 open reading frame found in clinical isolates of human CMV (HCMV) encodes a structural homologue of the herpesvirus entry mediator, a member of the TNFR superfamily. UL144 is a type I transmembrane glycoprotein that is expressed early after infection of fibroblasts; however, it is retained intracellularly. A YXXZ motif in the highly conserved cytoplasmic tail contributes to UL144 subcellular distribution. The finding that no known ligand of the TNF family binds UL144 suggests that its mechanism of action is distinct from other known viral immune evasion genes. Specific Abs to UL144 can be detected in the serum of a subset of HCMV seropositive individuals infected with HIV. This work establishes a novel molecular link between the TNF superfamily and herpesvirus that may contribute to the ability of HCMV to escape immune clearance. *The Journal of Immunology*, 1999, 162: 6967–6970.

Recent attention has focused on viral genes that target the TNF-related cytokines and their cognate receptors, which are crucial for effective cellular and humoral immune defenses (1–3). Herpesviruses possess putative immune evasion genes, each with a distinct mode of action targeting TNF superfamily. HSV-1 (α -herpesvirus), via envelope glycoprotein D, utilizes as one of its routes of entry a member of the TNFR family, the herpesvirus entry mediator (HVEM or HveA)³ (4, 5). HVEM

is a receptor for the lymphotoxin (LT) $\alpha\beta$ -related ligand, LIGHT, that also binds the LT β R (6), a critical receptor controlling organization of lymphoid tissue (7). Other examples include LMP-1 and v-FLIP in γ -herpesviruses, proteins that act to modulate distinct TNFR signaling pathways (8, 9). We reasoned that these intimate links are not fortuitous, but rather are the result of specific evolutionary history between TNF/LT cytokine systems and different species of herpesvirus. In this paper we provide evidence for a novel molecular link between the TNF superfamily and a β -herpesvirus, human CMV (HCMV), in the form of a structural homologue of HVEM encoded by the UL144 ORF of HCMV.

Materials and Methods

Cell lines and viruses

293T cells and neonatal normal human dermal fibroblasts (NHDF) (Clonetics, Walkersville, MD) were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS. HCMV-Fiala (HCMV-F) is a low passage clinical isolate from a patient with AIDS and was provided by M. Fiala (Department of Medicine, University of California, Los Angeles). Other low passage isolates, ME, and LU, are from solid organ transplant patients from Rush Clinical Labs (Rush-Presbyterian-St. Lukes Medical Center, Chicago, IL).

UL144 constructs

HCMV-F DNA was isolated and used to amplify UL144 by PCR using oligonucleotide primers of the HCMV strain Toledo (10) that contained 5' *Hind*III and 3' *Bam*HI sites to facilitate subcloning into pCDNA3.1(+) (Invitrogen, Carlsbad, CA). pUL144-myc was constructed by PCR amplification of the coding sequence of mature UL144 using oligonucleotide primers to allow for addition of a 5' *Hind*III restriction site and a 3' *Bam*HI restriction site to facilitate ligation into pCDNA3.1(+) (Invitrogen). Sequences encoding the type I leader sequence of VCAM followed by the c-myc epitope tag were PCR amplified from pLT β R-myc (11) with addition of a 5' *Nhe*I site and a 3' *Hind*III site and ligated immediately 5' of UL144 in pCDNA3.1(+) to generate pUL144-myc. The cytoplasmic domain mutant of UL144 (pUL144YA-myc) was constructed by PCR-based mutagenesis of pUL144-myc using a primer designed to introduce the point mutation Y172A. PCR amplifications were performed using *pfu* DNA polymerase (Stratagene, La Jolla, CA), and all vector sequences were verified by dideoxynucleotide sequencing using an Applied Biosystems Prism 310 genetic analyzer (Perkin-Elmer, Foster City, CA). A fusion protein consisting of the UL144-F ectodomain (aa 20-137) and a C-terminal human IgG1 Fc coding sequence (aa 231-447) (UL144:Fc) was constructed in pVL1393 (Invitrogen) for expression in baculovirus and purification as described for similar constructs (12). UL144:Fc was also constructed in PCR3 for expression in mammalian cells and purified as described (13). The anti-UL144:Fc serum was produced in Sprague-Dawley rats by immunization with native and SDS-denatured Fc fusion protein produced by insect cells. The serum was absorbed with human IgG linked to agarose beads and showed no cross-

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³ Abbreviations: HVEM, herpesvirus entry mediator; CRD, cysteine-rich domain; HCMV, human CMV; HCMV-F, Fiala variant; LT, lymphotoxin; NHDF, neonatal normal human dermal fibroblasts; ORF, open reading frame.

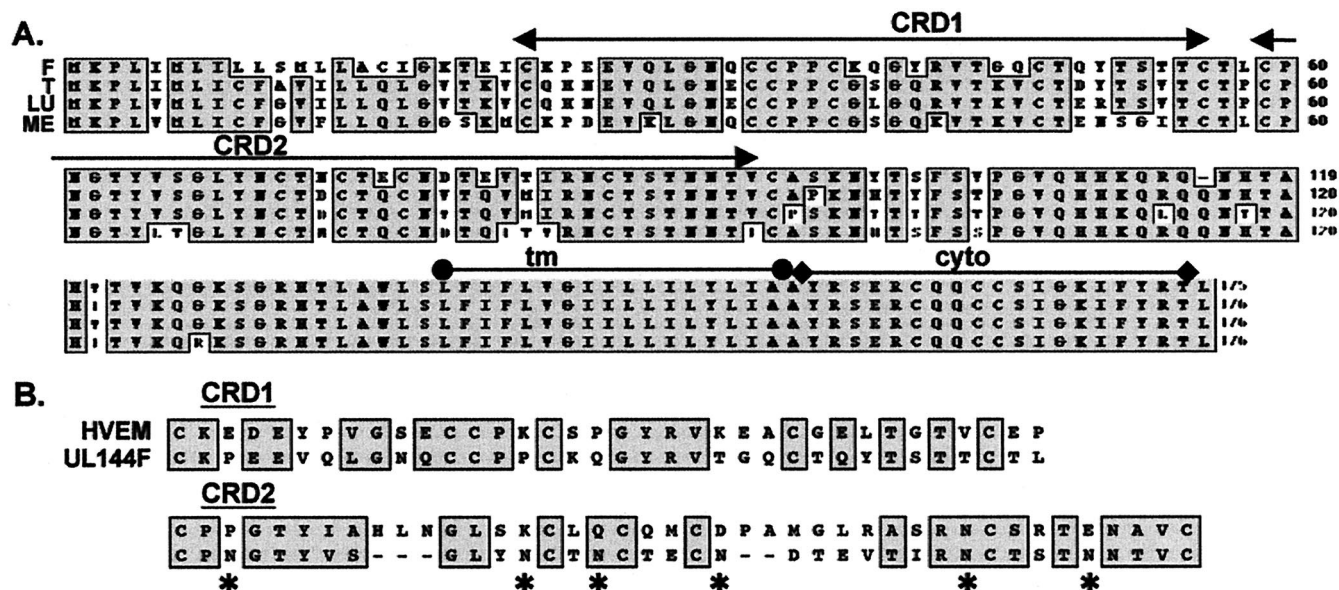


FIGURE 1. Sequence homology of UL144 with TNFR superfamily. *A*, Multiple alignment of UL144 sequences (ClustalW, PAMseries, MacVector) from Fiala (F), Toledo (T), and low passages isolates LU and ME. The outlined regions identify identical and conserved residues. The bars denote positions of CRD1 and -2, and the transmembrane (tm) and cytoplasmic (cyto) domains. *B*, Pairwise alignment of CRD1 and -2 of HVEM and UL144-F. Boxed regions show identity and conservative substitutions, and * denotes N-linked glycosylation sites. The GenBank accession numbers are AF135184 for UL144-F and U33331 for Toledo.

reaction with other TNFR:Fc fusion proteins as determined by Western blotting. Abs to IE1 (clone p63-27) and pp28 (clone 41-18) (14) were provided by William Britt (University of Alabama, Birmingham).

Analysis of UL144 by FACS, Western blot analysis, and RT-PCR

293T cells (6×10^5) were seeded in a 6-well plate, and expression vectors (5 μ g) were transfected by the CaPO₄ precipitation method as described previously (15). Transfected 293T or virus-infected NHDF cells were detached from plastic by treatment with 5 mM EDTA in PBS and resuspended in binding buffer (PBS, 2% FBS, and 0.02% sodium azide) for staining. Cells were analyzed by a FACSCalibur (Becton Dickinson, Mountain View, CA). Each histogram represents 10^4 viable cells gated on forward and side-angle light scatter (6). For analysis by Western blot, cell pellets were solubilized in SDS lysis buffer (1% SDS, 2 mM EDTA, 50 mM Tris (pH 6.8), 1 mM PMSF, and 10 μ g/ml aprotinin) and heated to 100°C for 10 min. Solubilized proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (NEN, Boston, MA). Total cellular RNA from NHDF cells (3×10^6) infected with HCMV was isolated using the RNeasy mini kit from Qiagen (Santa Clarita, CA). RNA (1 μ g) treated with one unit of DNase (Life Technologies) was reverse transcribed (1 unit Superscript II; Life Technologies). The PCR utilized UL144 forward primer, 5'-gctgagcatgctattggatgcatag-3' and reverse primer, 5'-gccgattgagcaactgttggatc-3'; and amplification at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s (Perkin-Elmer 9600).

Assay to detect UL144-specific Abs in CMV seropositive patients

Serum samples obtained from the Center for AIDS Research (University of California at San Diego) were heat-inactivated and assayed for HCMV reactivity by latex agglutination "CMVscan" assay (Becton Dickinson).

Results and Discussion

Identification of UL144 as an HVEM homologue

The second cysteine-rich domain (CRD) of HVEM containing the TNFR signature sequence CLQCQMC was used as a query in a BLAST search of the public database and identified UL144 ORF in HCMV as an HVEM homologue. The UL144 ORF is located in the unique long region of the HCMV genome, where 19 ORF were recently discovered (10). These 19 putative genes are present only in low passage, virulent strains of HCMV (originally identified in Toledo), but not in several well-characterized laboratory strains of HCMV (AD169 and Towne) (10).

The UL144 coding sequence was amplified by PCR from HCMV-Fiala (UL144-F) genomic DNA. UL144-F encodes a type I transmembrane protein with an ectodomain comprised of a leader peptide, cysteine-rich region, membrane extension region, transmembrane domain, and a short cytoplasmic tail (Fig. 1A). UL144-F differs from the sequence of UL144-Toledo in 33 of 138 ectodomain residues (82% identity at the nucleotide level). Sequence variation was also observed in two other HCMV isolates (LU and ME) and in 28 other distinct clinical HCMV isolates (N. S. Lurain et al, manuscript in preparation). However, a high level of conservation is observed in all the isolates including the number and positioning of the cysteines, as well as complete identity in the transmembrane and cytoplasmic domains. The eight putative N-linked glycosylation sites, located in CRD2 and the membrane extension region, are conserved between all of the isolates, except the Toledo variant which has seven. The UL144 ectodomain shows the highest amino acid sequence homology to HVEM (36%) (Fig. 1B), followed by other members of the TNFR superfamily (Fas, 29%; TNFR-1, 28%; LT β R, 25%; TRAIL-R2, 15%). UL144 encodes only two CRDs homologous to CRD1 and -2 of HVEM (4) and is the first identified member of the TNFR superfamily to be comprised of only these two domains.

The CRD2 of UL144, while still showing highest homology to HVEM (46% identity), displays significant homology to the ligand binding domain of TRAIL-R2 (43%) (16). Virtually all ligand-receptor contacts present in the LT α /TNFR (55 kDa) complex occur in CRD2 and -3 (17), suggesting the possibility that UL144 may not possess a complete ligand binding domain. Furthermore, in contrast to HVEM, which has a single putative glycosylation site in CRD2, UL144 has six of its eight sites located in CRD2. The UL144 ectodomain, when expressed in 293T cells as an Fc fusion protein utilizing the IgG leader sequence, is highly glycosylated (~20 kDa) as detected by digestion with endoglycosidase F (Fig. 2). The extensive glycosylation of UL144:Fc indicates that at least some of the eight predicted N-linked glycosylation sites are modified in 293T cells. There was substantially less modification of the UL144:Fc protein produced in insect cells, but this is likely

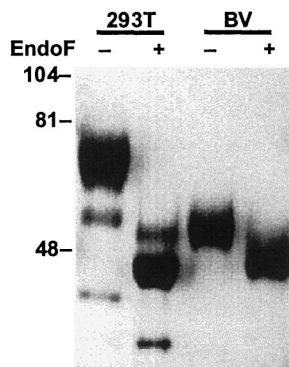


FIGURE 2. Glycosylation of UL144. UL144:Fc protein (5 μ g) purified from either 293T or insect cells infected with recombinant baculovirus (BV) was digested with endoglycosidase F (EndoF; 37°C for 60 min) for analysis of N-linked glycosylation. The gel was stained with Coomassie blue for detection of proteins.

to be due to the general restricted addition of N-linked carbohydrates seen in this cell type. The extensive homology of the UL144 ectodomain with HVEM and TNFR family members prompted us to screen for binding to TNF-related ligands. Using a sensitive ELISA method, none of the ligands, including TRAIL, LIGHT, LT α , LT α 1 β 2, TNF, FasL, CD40L, CD30L, Tweak, 41BBL, OX40L, April, RankL, or TL1 bound to UL144:Fc, whether the fusion protein was produced in insect or mammalian cells. Additionally, unlike HVEM, UL144 does not appear to serve as an entry factor for HSV-1 (C. A. Benedict, S. Borboroglu, and C. F. Ware, unpublished observations). Thus, the ability of UL144 to function as a ligand binding molecule appears unlikely, perhaps due to extensive glycosylation, although we cannot exclude interactions with a yet uncharacterized ligand. Furthermore, unlike cellular TNFR, UL144 does not appear to induce apoptosis or modulate activation of NF- κ B when over-expressed.

Expression of UL144 in HCMV infected cells

None of the putative 19 ORF in clinical HCMV isolates have been shown to be expressed during a viral infection. RT-PCR analysis of RNA from NHDF showed UL144-specific transcripts in cells infected with HCMV-F but not AD169 (Fig. 3A) (AD169 lacks the genomic locus containing UL144) (10). UL144 protein was also detected in HCMV-F-infected fibroblasts by Western blot analysis with anti-UL144:Fc serum as a major band of \sim 44 kDa and a minor 38-kDa component (Fig. 3B). In agreement with the endoglycosidase F digestion, extensive posttranslational modification of UL144 probably occurs to account for the difference in the predicted mass of 19.4 kDa. UL144 protein is expressed early (4 h) after infection, as early as the immediate early protein-1 (IE1) of HCMV, in contrast to the expression of pp28, a late protein that is not detectable until \sim 48–72 h postinfection (Fig. 3B) (18).

Surprisingly, anti-UL144 did not stain infected fibroblasts by FACS (Fig. 3C), although UL144 protein was readily detected by Western blot analysis (Fig. 3B), and this antiserum was fully capable of staining of 293T cells transfected with a UL144 expression vector (pUL144YA-myc; see below) (Fig. 3C). Furthermore, no shed or secreted UL144 was detected in the supernatant from infected cells (data not shown). Together, these results indicate that UL144 is retained in an intracellular compartment. The anti-UL144 did not block HCMV infectivity; however, this result does not preclude UL144 as a component of the virion.

The YRTL sequence in the conserved cytoplasmic tail of UL144 resembles a YXXZ motif (Z is a bulky hydrophobic amino acid),

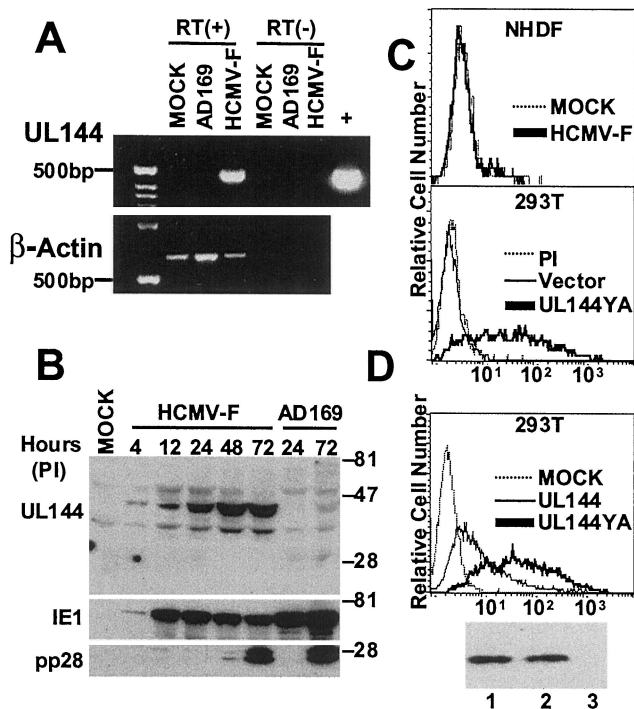


FIGURE 3. Expression of UL144 during viral infection. **A**, RT-PCR to detect UL144 expression. NHDF cells were either mock infected, infected with AD169 or HCMV-F, and cells were harvested 72 h postinfection (pi) for extraction of total cell RNA. Total cell RNA (1 μ g) was used for the reverse transcription (RT) reaction. RT negative reactions (RT-) were treated exactly the same, but enzyme was omitted from the RT reaction step. The RT reaction (1/12th) was used for PCR amplification of either UL144 or β -actin; +, positive control as UL144 cDNA (10 ng of pUL144-myc). **B**, HCMV-F or AD169-infected NHDF cells were analyzed at various times for expression of UL144 protein. UL144 protein was detected with anti-UL144:Fc (1:500) followed by incubation with sheep anti-rat IgG Ab conjugated to HRP and enhanced chemiluminescence. **C**, Detection of cell surface UL144 by FACS. NHDF cells (*top panel*), mock infected or infected with HCMV-F (moi = 5), were stained with rat anti-UL144 or preimmune (PI) serum (1:200) followed by goat F(ab')₂ anti-rat R-PE-conjugated Ab. 293T cells (*bottom panel*) transfected with empty vector or or pUL144YA-myc were stained by rat anti-UL144 as described above. **D**, 293T cells were transfected with either pUL144-myc or pUL144YA-myc, and protein levels were detected by FACS. *Inset*, Western blot comparing total cellular protein levels of cells transfected with UL144 (*lane 1*), UL144YA (*lane 2*), or vector only (*lane 3*). For FACS, cell surface protein was detected with anti-myc Ab (9E10, 25 μ g/ml) followed by incubation with goat F(ab')₂ anti-mouse R-PE-conjugated Ab. For Western blots, equivalent amounts of total protein were analyzed by SDS-PAGE, and blots were incubated with 9E10 (10 μ g/ml) followed by sheep anti-mouse IgG HRP conjugated Ab.

a motif important in sorting of some transmembrane proteins, and thus may be involved in regulating UL144 subcellular distribution. YXXZ motifs can interact directly with the adaptor complexes associated with clathrin-mediated receptor internalization at the plasma membrane and in the *trans*-Golgi network (19). A Tyr to Ala mutant of UL144 (pUL144YA-myc), when over-expressed in 293T cells, was displayed at significantly higher levels (7- to 10-fold) on the cell surface than the wild-type UL144 protein, although total cellular protein levels were equivalent (Fig. 3, C and D). Additionally, endoglycosidase H treatment of UL144YA protein immunoprecipitated from 293T cell lysates revealed a distinct banding pattern from that of UL144 (data not shown). Together, these data suggest that the sorting of UL144 is controlled in part by the YRTL sequence and is the first identification of such a motif in

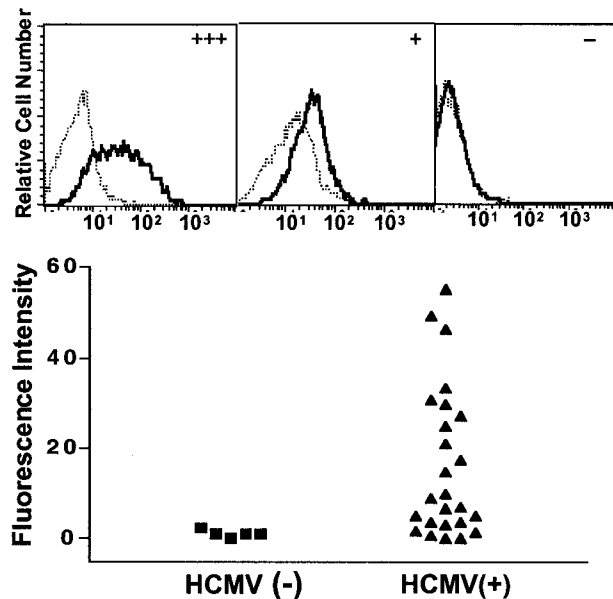


FIGURE 4. Abs to UL144 exist in human serum from HCMV seropositive individuals. Human serum from 25 HCMV seropositive patients and 3 seronegative patients (including three serial samples from one patient) were tested for Ab reactivity against the UL144 ectodomain. *Upper panel*, Representative histograms from FACS analysis of human anti-UL144 Ab reactivity. Patient serum samples were diluted 1:50 and incubated with 293T cells transfected with pUL144YA-myc or with vector. Ab binding was detected with goat anti-human IgG F(ab')₂ conjugated to R-PE. *Lower panel*, Fluorescence intensity (FI, mean peak fluorescence channel) for all tested human serum samples reactivity against UL144. FI was calculated by subtracting FI for Ab binding to vector transfected 293T cells from UL144YA-transfected 293T cells.

a TNFR superfamily member. However, preliminary evidence indicates that multiple regions, particularly the N-terminal region, may also contribute to expression and subcellular compartmentalization of UL144.

Detection of specific Abs to UL144 in HCMV seropositive patients

We looked for evidence of UL144 expression during the course of a viral infection *in vivo* by serologic detection of specific UL144 Abs. The higher cell surface levels of the UL144YA mutant expressed by 293T cells made possible a FACS-based assay for detection of UL144 Abs. As CMV can be an opportunistic pathogen in AIDS patients, sera from a HIV-positive cohort were investigated. Two groups with either high or low Abs binding levels to UL144 were identified in CMV-positive patients ($n = 25$) (Fig. 4). These sera were negative on nontransfected cells, and serum from CMV seronegative patients did not stain 293T cells expressing UL144YA protein. These results substantiate the specificity of this assay for UL144. Clinical status or antiviral therapy may contribute to waning Ab levels in some patients. Alternatively, variation in reactivity may depend on specific serologic reaction with different UL144 variants. This latter possibility is supported by the sequence diversity observed in the ectodomain of UL144 (Fig. 1B). These issues are currently being addressed.

This first identification of a TNFR family member encoded by a β -herpesvirus may be helpful in unraveling the mechanisms involved in the ability of HCMV to escape immune clearance. The inability of UL144 to bind any of the currently identified TNF family ligands or induce signaling events akin to known TNFR suggests its action will be intracellular and differ from other known immune evasion strategies. The intracellular localization of UL144

is reminiscent of the poxvirus T2 protein, a TNFR homologue, which functions biologically as a host range restriction factor to allow for productive viral replication in lymphocytes (20). Although we see no evidence for a blockade of apoptosis, it is possible that UL144 may be functioning in a similar fashion in other cell types, and this hypothesis is currently under investigation. The results presented here help establish the relevance of UL144 to the immune response and should provide additional impetus to investigate this new target, as well as the additional genes at the newly described region, with the hope to unravel this pathogen's well-guarded secrets of immune evasion.

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