Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10)

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We identified a viral IL-10 homolog encoded by an ORF (UL111a) within the human cytomegalovirus (CMV) genome, which we designated cmvIL-10. cmvIL-10 can bind to the human IL-10 receptor and can compete with human IL-10 for binding sites, despite the fact that these two proteins are only 27% identical. cmvIL-10 requires both subunits of the IL-10 receptor complex to induce signal transduction events and biological activities. The structure of the cmvIL-10 gene is unique by itself. The gene retained two of four introns of the IL-10 gene, but the length of the introns was reduced. We demonstrated that cmvIL-10 is expressed in CMV-infected cells. Thus, expression of cmvIL-10 extends the range of counter measures developed by CMV to circumvent detection and destruction by the host immune system.

IL-10 is a pleiotropic immunomodulatory cytokine produced by $CD4^+$ and $CD8^+$ T cells, monocytes/macrophages, keratinocytes, and activated B cells (1). In addition, its expression is elevated in patients with a variety of peripheral blood or bone marrow-derived leukemias, certain B cell and T cell lymphomas and nasal natural killer cell lymphomas and other hematopoietic and solid tumors (1–5). Two mechanisms of IL-10 action can be used by tumors. IL-10 appears to act as an autocrine growth factor for B cell lymphomas. In addition, IL-10 selectively inhibits certain aspects of the cellular immune response. It blocks proinflammatory cytokine synthesis and suppresses the ability of macrophages to serve as antigen-presenting or costimulatory cells (6–8). Thus, IL-10 is a powerful anti-inflammatory agent and a potent immunosuppressor.

Many viruses exploit the strategy of using homologs of cellular cytokines or cytokine receptors to shield virus-infected cells from immune defenses and enhance virus survival in the host. The presence of virus-encoded homologs of cellular proteins may be an indicator of the importance of these cellular components in immune mechanisms for combating this virus *in vivo*. A number of herpes viruses harbor homologs of IL-10. Epstein–Barr virus (EBV)-encoded IL-10 (ebvIL-10), the first viral homolog of IL-10 identified (9, 10), shares many but not all of the biological activities of cellular IL-10 and may play an important role in the host-virus interaction (1, 11, 12). In addition to EBV, another virus, the Orf poxvirus (OV), which can infect humans, has its own IL-10 homolog, ovIL-10 (13). Whether it is active on human cells remains to be shown. The exact *in vivo* roles of viral IL-10 homologs in the viral life cycle, in immune evasion, and/or in helping virus-infected cells to survive immune surveillance remain to be defined.

Human cytomegalovirus (CMV) is a widespread herpes virus that is able to persist for decades in its host. CMV is the major cause of a variety of life-threatening diseases in immunocompromised individuals, including transplant and AIDS patients, and is a leading cause of congenital birth defects (14). CMV is also associated with the development of atherosclerosis, restenosis after coronary angioplasty, chronic rejection in organ transplant patients (15–17), and chronic graft-versus-host disease in bone marrow transplant patients (18, 19). We discovered that CMV harbors its own IL-10 homolog, which we have designated cmvIL-10. In this report, we describe the identification and cloning of cmvIL-10 and its functional characterization.

Materials and Methods

PCR, Reverse Transcription–PCR (RT-PCR), and Plasmid Construction. Plasmid pEF-SPFL is a derivative of the pcDEF3 vector (20), in which the fragment encoding the human IFN-γ receptor 2 (IFN-γR2) signal peptide followed by the FLAG epitope (21, 22) is introduced into *Kpn*I and *Bam*HI sites of the pcDEF3 vector. Primers 5′-TAGGATCCTTCCGAGGAGGCGAAG-3′ (cmv1) and 5′-ATGAATTCGTTGTTACCTCT-3′ and C101AD135–175 cosmid DNA (from T. Shenk, Princeton University, Princeton, NJ) were used for PCR to clone the cmv_1 fragment into plasmid pEF-SPFL with *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmv_1. The same primers and total RNA isolated from COS-1 cells transfected with plasmid pEF-SPFL-cmv_1 were used for RT-PCR to clone the cmv_1-spliced (cmv_{1SP}) fragment into plasmid pEF-SPFL with *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmv_{1SP}. Primers cmv1 and 5′-AGCGGAATTCAAATCGCAACGC-3′ (cmv17) and C101AD135–175 cosmid DNA were used for PCR, to clone the cmv_2 fragment into plasmid pEF-SPFL vector with *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmv_2. The same primers and total RNA isolated from COS-1 cells transfected with plasmid pEF-SPFL-cmv_2 were used for RT-PCR to clone the cmv_2-spliced (cmv_{2SP} or cmvIL-10) fragment into plasmid pEF-SPFL with *Bam*HI and *Eco*RI restriction endonucleases, resulting in plasmid pEF-SPFL-cmvIL-10. To create cellular IL-10 protein tagged with FLAG-epitope at the N terminus, primers 5′-CGGGATCCCAGCCCAGGGCAGGGCACC-3′ and 5′-GCTCTAGATCAGTTTCGTATCTTCAT-3′ and cellular IL-10–encoded plasmid DNA (23) were used for PCR. The PCR fragment was cloned into the pEF-SPFL vector with *Bam*HI and *Xba*I restriction endonucleases. COS-1 cells were transfected with the resultant plasmid, and FL-IL-10 was purified on an anti-FLAG affinity column from conditioned media. To create the pEF-cmv_3 (or pEF-cmvIL-10) expression vector, in which the cmvIL-10 gene is cloned with its own signal peptide, primers 5′-CGGGATCCTGCGGCGATGCTG-3′ (cmv4) and cmv17 and C101AD135–175 cosmid DNA were used for PCR. The resulting PCR fragment was cloned into the pcDEF3 vector with *Bam*HI and *Eco*RI restriction endonucleases (GenBank accession no. AF182315). The nucleotide sequences of the modified regions of all the constructs were verified in their entirety by DNA sequencing.

CMV-infected or uninfected HEL 299 cells were lysed in TRIzol reagent (GIBCO/BRL), and the RNA and DNA were isolated by protocols suggested by the manufacturer. RNA samples were used for nested RT-PCR with cmvIL-10-specific primers. The first round was performed with primers cmv4 and 5′-AGTAACTGGGTGAACGACAC-3′, and the second round was performed with primers cmv1 and cmv17 with the GeneAmp (RT-PCR) kit (Perkin–Elmer). RNA samples also were used for RT-PCR with β-actin-specific primers 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACGCACGATTTCC-3′. Genomic DNA isolated from CMV-infected or uninfected cells, as well as plasmid DNA, was used for PCR with the

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Abbreviations: EBV, Epstein–Barr virus; OV Orf poxvirus; CMV, cytomegalovirus; RT, reverse transcription; IFN-γR1 or -R2, IFN-γ receptor 1 or 2; PBMC, peripheral blood mononuclear cell; EMSA, electrophoretic mobility-shift assay; FL, FLAG epitope.

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same sets of primers. The PCR products were analyzed on a 1.2% agarose gel stained with ethidium bromide.

Cells, Virus Infection, Transfection, Cytofluorographic Analysis, and IL-10 Reagents. The Chinese hamster 16–9 cell line and its derivatives were described (21). Leukocytes were obtained from a normal donor by leukapheresis as described (21). HEL 299 cells (human diploid embryonic lung fibroblast-like cell line; ATCC no. CCL-137) were grown to ≈80% confluence in a 90-mm dish and were infected with CMV strain AD-169 (ATCC VR-538) as described (24). COS-1 cells were transiently transfected with the expression vectors by the DEAE-dextran procedure with a DMSO shock step as described (21).

To detect cytokine-induced MHC class I antigen (HLA-B7) expression, cells were treated with purified recombinant IL-10 or cmvIL-10 (COS cell supernatant or purified recombinant protein), as indicated in the text, for 72 h and analyzed by flow cytometry as described (25).

Electrophoretic Mobility-Shift Assays (EMSAs), Immunoprecipitation, and Blotting. Isolated PBMCs were treated directly, and cultured cells were starved overnight in serum-free medium, then treated with IL-10 or cmvIL-10 for 15 min at 37°C, and used for EMSA experiments to detect activation of Stat1 and Stat3 as described (21, 26, 27).

Three days after transfection, conditioned medium from COS-1 cells transiently transfected with expression plasmids was collected and subjected to immunoprecipitation and Western blotting with anti-FLAG M2 mAb (Sigma).

Results

Identification of a Fragment of the CMV Genome Encoding an IL-10 Homolog. A search of the GenBank database with the TBLASTN program for possible IL-10 homologs revealed two closely positioned regions of the CMV genome with weak amino acid homology to the IL-10 sequence (Fig. 1*A*). The first reading frame had an ATG codon (Met) just upstream of the region where homology starts (Fig. 1*A*). In addition, a hydropathy plot of the first reading frame predicted that the amino terminus of the protein is highly hydrophobic (Fig. 1*B*), suggesting that this region could encode a signal peptide. To determine whether these two coding frames represent two exons of one gene and can be spliced together, we first examined the size of the protein encoded by this region of the CMV genome. The PCR-derived fragment, designated cmv_1 [nucleotides 159735–160178 of the CMV genome (Fig. 1*C*)], that encompassed the first reading frame starting from Ser-20 (the first amino acid after the hypothetical signal peptide) and the entire second predicted reading frame, was cloned into plasmid pEF-SPFL, resulting in plasmid pEF-SPFL-cmv_1. Because plasmid pEF-SPFL encodes the signal peptide derived from the human IFN-γR2 chain followed by the FLAG epitope, this cloning abutted the first reading frame to the frame of the FLAG epitope.

COS-1 cells were transiently transfected with plasmid pEF-SPFL-cmv_1. Three days after transfection, conditioned medium was collected and subjected to Western blotting (Fig. 2). The predicted molecular mass of a protein encoded by the first ORF was ≈8 kDa. However, a protein of ≈14 kDa, immunoreacting with anti-FLAG antibody, was observed in the experiment (Fig. 2*A*, lane 2), raising the possibility that the first translation frame was linked to the second one, where the second part of IL-10 homology was positioned (Fig. 1 *A* and *C*). To determine whether this occurred because of the splicing rather than a frame shift event, we cloned the corresponding mRNA by RT-PCR from COS-1 cells transfected with plasmid pEF-SPFL-cmv_1. The region including nucleotides 159858–159933 was spliced out in the cloned cmv_1 RT-PCR-derived (cmv_{1SP}) fragment (Fig. 1*C*), indicating the existence of the small intron in this region of the CMV genome. The splicing event connected the two frames with IL-10 homology. The cmv_{1SP} fragment was cloned into the pEF-SPFL vector to yield the expression vector pEF-SPFL-cmv_{1SP}. This expression vector was transfected into COS-1 cells, and the medium was collected after 3 days. On Western blotting of the medium, a protein of ≈14 kDa was detected with anti-FLAG antibody [Fig. 2*A*, lane 3 (FL-cmv_{1SP}IL-10)] and was identical to the band seen with the unspliced expression vector pEF-SPFL-cmv_1 [Fig. 2*A*, lane 2 (FL-cmv_1IL-10)]. However, the conditioned medium from COS-1 cells transfected with either pEF-SPFL-cmv_1 or pEF-SPFL-cmv_{1SP} failed to induce Stat activation in hamster cells with the reconstituted human IL-10 receptor complex or in human PBMCs. In addition, when the predicted amino acid sequence of the cmv_{1SP} fragment was aligned with the IL-10 sequence, the COOH-terminal third of the IL-10 protein was not represented in the cmv_{1SP}-encoded protein. We hypothesized that there is a third portion of the CMV genome encoding the missing COOH-terminal part of the CMV-encoded IL-10-like protein. We then cloned a bigger fragment of the CMV genome (cmv_2) from nucleotides 159735 to 160376 (Fig. 1*C*) into plasmid pEF-SPFL by PCR, resulting in plasmid pEF-SPFL-cmv_2. Western blotting demonstrated that the size of the protein recognized by an anti-FLAG antibody increased from ≈14 kDa to ≈21 kDa, a size comparable with that of cellular IL-10 [Fig. 2*A*, lane 4 (FL-cmv_2IL-10)]. The cmv_2-derived mRNA was cloned from COS-1 cells by RT-PCR as described above, and the resulting plasmid pEF-SPFL-cmvIL-10 was sequenced. In addition to the first intron determined in previous experiments, the RT-PCR fragment was missing the region including nucleotides 160135–160217 of the CMV genome, revealing a second intron within this region of the CMV genome (Fig. 1*C*). Splicing caused the second reading frame to be shifted to another frame (Fig. 1*C*). The resulting ORF encoded a protein of 175 aa (Fig. 1*C* and Fig. 3), which was designated cmvIL-10. Medium from COS-1 cells transfected with the expression vector pEF-SPFL-cmvIL-10 and analyzed by Western blotting with an anti-FLAG antibody revealed a 21-kDa band identical to cmv_2IL-10 [Fig. 2*A*, lane 5 (FL-cmvIL-10); cf. lane 4 (FL-cmv_2IL-10)]. After longer exposure, several additional bands were observed in the region of ≈30–35 kDa, suggesting possible glycosylation of cmvIL-10. Indeed, there is a site for N-linked glycosylation—Asn-151–X–Thr-153. Treatment of the conditioned medium with Peptide: *N*-glycosidase F results in the disappearance of the higher bands and enhancement of the 21-kDa band (results not shown), consistent with glycosylation of the 30- to 35-kDa proteins.

Alignment of the predicted cmvIL-10 with human IL-10 and other viral homologs that are active on human cells (Fig. 3) revealed 27–28% identity and 36–41% similarity to the IL-10 sequence of ebvIL-10, ovIL-10, or cellular IL-10.

cmvIL-10 Binds to and Signals Through the IL-10 Receptor Complex. The active IL-10 receptor complex is composed of two subunits (21), the ligand-binding IL-10R1 chain (28) and the second IL-10R2 chain, which is required for signaling (21). To determine whether cmvIL-10 can bind and signal through the human IL-10 receptor complex, we used four hamster cell lines (21) expressing different components of the modified human IL-10 receptor complex (Fig. 4*A*). The native receptor complex was modified to facilitate the detection of the IL-10-induced biological activities. The IFN-γR1 intracellular domain was substituted for the IL-10R1 intracellular domain. With this exchange, IL-10 can activate IFN-γ-like biological responses, such as MHC class I antigen induction and Stat1 activation, in hamster cells expressing the chimeric IL-10R1/γR1 chain and the intact second chain, IL-10R2 (21).

Parental hamster CHO-derived 16–9 cells and three cell lines expressing either each receptor subunit alone or both together were used in ligand binding and cellular activation experiments (Fig. 4). FLAG epitope-tagged cmvIL-10 (FL-cmvIL-10) and IL-10 (FL-IL-10) were used to detect ligand binding by flow cytometry (Fig. 4, row II). The experiments demonstrated that cmvIL-10 binds to the cell surface of hamster cells expressing IL-10R1/γR1 alone or with IL-10R2, but not to the parental 16–9 cells or cells expressing IL-10R2 alone (Fig. 4 *A–D*). Furthermore, IL-10 competes for receptor binding with FL-cmvIL-10 in a concentration-dependent manner on cells expressing both chains of the IL-10 receptor

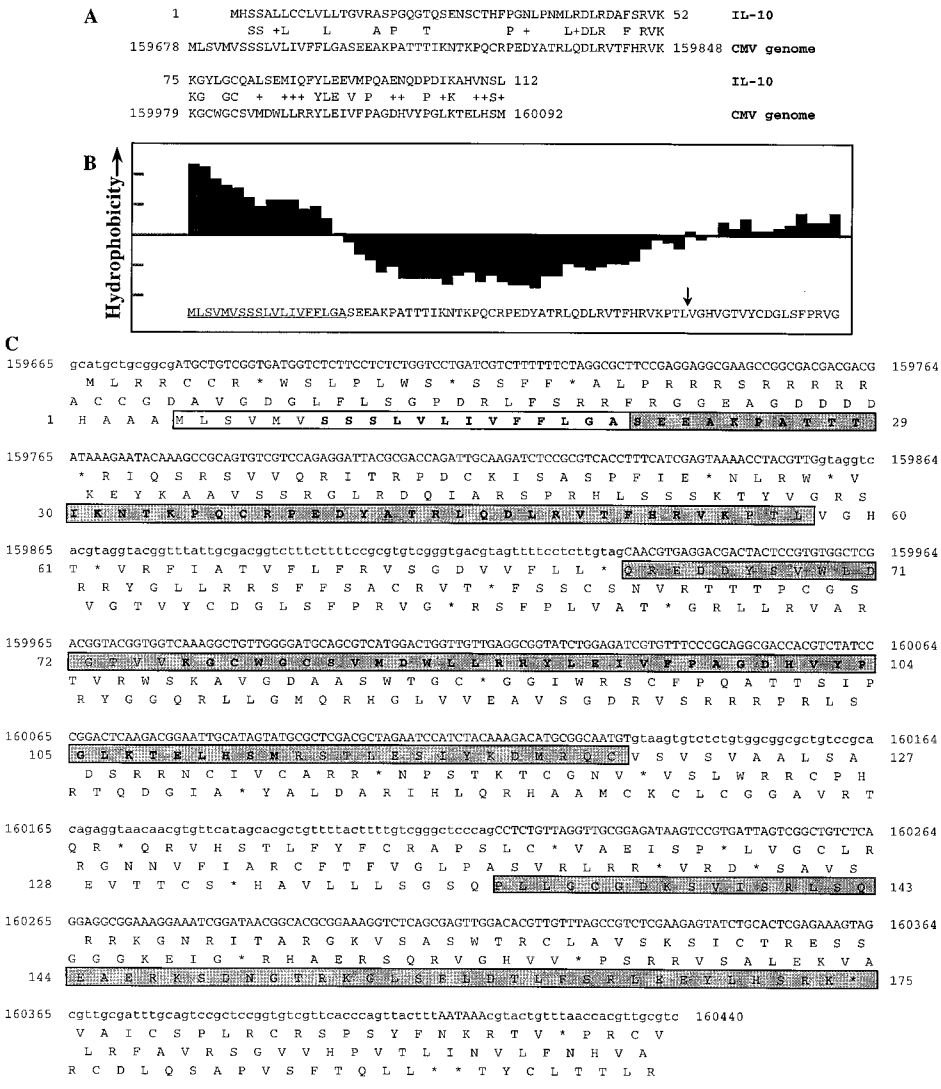

Fig. 1. Fragment of the CMV genome encoding the IL-10 homolog. (*A*) Two coding frames starting at nucleotides 159678 and 159979, respectively, of the CMV genome (GenBank accession no. X17403) and identified by the TBLASTN homology search with human IL-10 as a query sequence (top lines). Letters in the center lines indicate identical amino acids; +, similar amino acids. Numbering is as follows. Upper lines, amino acids 1–52 and 75–112 of human IL-10 starting from Met1; lower lines, nucleotide numbering starting at 159678 and 159979, respectively, and corresponding to reading frames from the CMV genome. (*B*) Hydropathy plot of the whole first reading frame of the CMV genome with homology to the N-terminal portion of IL-10. ↓, end of the first exon. The predicted signal peptide is underlined. (*C*) Fragment of the CMV genome encoding the IL-10 homolog. Fragments of the two coding frames that were identified by the TBLASTN search program to have homology to human IL-10 are shown in boldface. Nucleotides of the two introns are shown in lowercase letters. Nucleotides of the three exons are shown in uppercase letters, and exon-encoded protein sequences are in shaded boxes. The predicted signal peptide is in an open box. Nucleotide numbering corresponds to the numbers of the CMV genome (GenBank accession no. X17403). Amino acid residues in the boxed regions are numbered starting from first Met residue in the first reading frame with only exon-encoded amino acid residues (boxes) counted.

complex (Fig. 5*A*). Conversely cmvIL-10 can compete in a concentration-dependent manner with FL-IL-10 (Fig. 5*B*).

Because the chimeric IL-10 receptor complex with the intracellular domain of the IL-10R1 replaced by the IFN-γR1 intracellular domain was used, IL-10 activated IFN-γ-like responses. As previously demonstrated with cellular IL-10 (21), cmvIL-10 induced MHC class I antigen expression (Fig. 4 *E–H*) and Stat1 activation, as evaluated by EMSAs (Fig. 6), only in hamster cells expressing both subunits of the modified IL-10 receptor complex. PBMCs were used to determine that cmvIL-10 activated the same pattern of the Stat1 and Stat3 DNA-binding complexes that was characteristic of IL-10 signaling (Fig. 6).

To demonstrate that cmvIL-10 can be secreted with its own signal peptide and can bind and activate the IL-10 receptor complex, the PCR-derived fragment of the CMV genome between nucleotides 159670 and 160376 (Fig. 1*C*) was cloned into the pcDEF3 vector (20), resulting in the plasmid pEF-cmv$_3$. Conditioned medium of COS-1 cells transiently transfected with this expression vector was used for competition binding with FL-IL-10, as well as in MHC class I induction experiments and in the EMSA experiments. cmvIL-10 that was produced with its own putative signal peptide was able to compete with FL-IL-10 binding, as shown earlier (Fig. 5*B*) and demonstrated the same activities (data not shown) as FL-cmvIL-10 (Figs. 4 and 6). *Escherichia coli*-produced recombinant cmvIL-10 was active in all of the experiments described above (data not shown).

cmvIL-10 Is Expressed by CMV-Infected Cells. To demonstrate that cmvIL-10 is expressed by virus-infected cells, HEL 299 cells were infected with CMV strain AD169, and several assays were performed 48 h after infection. DNA and RNA were isolated from virus-infected and control-uninfected HEL 299 cells. Infection of the cells was confirmed by the presence of the CMV DNA, assayed by PCR with CMV-specific primers and the isolated DNA samples (Fig. 2*B*, lanes 4 and 5). The RNA samples were subjected to RT-PCR with the same sets of primers that were used for assessment of the presence of the CMV genome in infected cells (Fig. 2*B*, lanes 6 and 7). Plasmids carrying the cmvIL-10 gene [pEF-cmv$_3$ (Fig. 2*B*, lane 2)] or cmvIL-10 cDNA [spliced form, pEF-SPFL-cmvIL-10 (Fig. 2*B*, lane 5)] were used as positive controls for the PCR. The PCR product obtained with DNA from CMV-infected cells was collinear with the PCR product from control pEF-SPFL-cmvIL-10 plasmid (Fig. 2*B*). RT-PCR with RNA from CMV-infected cells resulted in two products. The size of the smaller RT-PCR product was identical to that of the PCR product from the control pEF-SPFL-cmvIL-10 plasmid, whereas the size of the other RT-PCR product corresponded to the size of the PCR product from the control genomic construct (pEF-cmv$_3$). The larger RT-PCR product was derived from unspliced cmvIL-10 mRNA, because PCR (without the RT step) with this RNA sample did not produce any products (data not shown). No PCR or RT-PCR products were obtained with samples isolated from control uninfected cells. RT-PCR also was performed with primers for β-actin

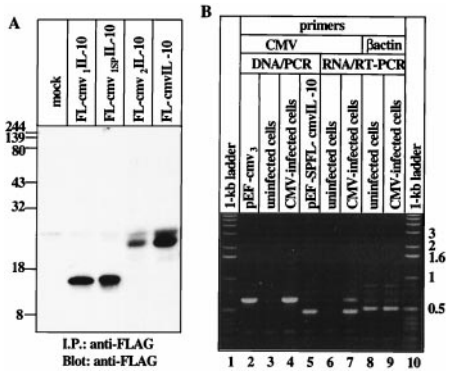

Fig. 2. cmvIL-10 expression. (*A*) Western blotting analysis of COS-1 cell-conditioned media. COS-1 cells were transiently transfected with the pEF-SPFL [lane 1 (mock)], the pEF-SPFL-cmv$_1$ [lane 2 (FL-cmv$_1$IL-10)], the pEF-SPFL-cmv$_{1SP}$ [lane 3 (FL-cmv$_{1SP}$IL-10)], the pEF-SPFL-cmv$_2$ [lane 4 (FL-cmv$_2$IL-10)], or the pEF-SPFL-cmvIL-10 [lane 5 (FL-cmvIL-10)] expression vectors. Three days later, 1 ml of the conditioned media was subjected to immunoprecipitation and Western blotting experiments with anti-FLAG antibody. The molecular weight markers are shown on the left. (*B*) CMV-infected cells express cmvIL-10. PCR (lanes 3 and 4) or RT-PCR (lanes 6 and 7) with the same sets of primers was performed with DNA or RNA isolated from virus-infected (lanes 4 and 7) or uninfected (lanes 3 and 6) cells as described in *Materials and Methods*. Plasmids pEF-cmv$_3$ (lane 2) and pEF-SPFL-cmvIL-10 (lane 5) were used for PCR as positive controls. A 1-kb ladder was run in lanes 1 and 10.

cDNA to evaluate the integrity and quantity of the isolated RNA samples. PCR and RT-PCR fragments were isolated and sequenced. The sequence of the PCR product was identical to the sequence of the cmvIL-10 gene (Fig. 1), and the sequence of the

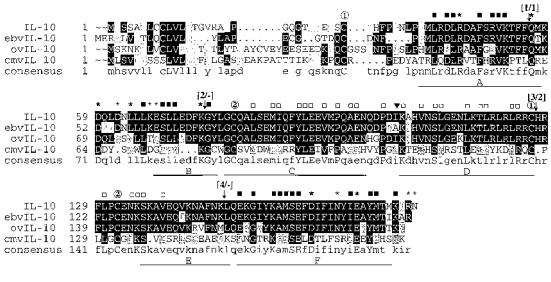

Fig. 3. Alignment of amino acid sequences of human IL-10 and its viral homologs. The alignment of the amino acid sequences of cellular IL-10 encoded by the human genome (23) and viral IL-10s encoded by EBV [ebvIL-10 (10)], OV [ovIL-10 (12)], and CMV [cmvIL-10 (this study)] are shown. A consensus sequence is shown on the bottom. Identical amino acids corresponding to the consensus sequence are shown in black outline with white lettering. Similar amino acids are shown in gray outline with white lettering. Amino acid residues are numbered starting from first Met residue (signal peptide amino acids are included). The α-helices A through F, taken from the crystal structure of IL-10 and ebvIL-10 (30, 36), are underlined. Symbols: ① and ② designate Cys residues of IL-10 that form two intramolecular disulfide bridges (30). Asterisks denote amino acids predicted to be involved in interaction with IL-10R1 (32). The bold asterisks represent those residues involved in the interaction with IL-10R1 that are conserved among all the IL-10s. ■ points to conserved amino acids within regions involved in interaction with IL-10R1. □ points to conserved amino acids in the middle of IL-10 homologs that may be involved in interaction with IL-10R2. Arrows indicate positions of introns within IL-10 and cmvIL-10 genes. Numbers in parentheses represent the number of introns in IL-10 and cmvIL-10 (intron number within IL-10/intron number within cmvIL-10); the minuses at (2/−) and (4/−) denote no intron in cmvIL-10 at these positions. The triangle represents the position of Ala-98 of ebvIL-10. The program PILEUP of the Wisconsin Package, Version 9.1, Genetics Computer Group, Madison, WI, was used with the following parameters: the gap creation penalty 10, the gap extension penalty 2. The BOXSHADE 3.21 program was used for shading of the alignment file.

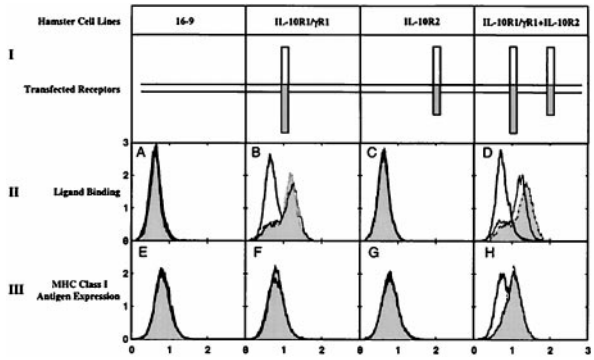

Fig. 4. Ligand binding and MHC class I antigen induction. (row I) Schematic of four cell lines used in these experiments: the parental Chinese hamster 16–9 cells and three 16–9-based cell lines expressing human IL-10R1/γR1 chimeric receptor or human IL-10R2 alone or both receptors together, which were created and described in detail (21). (Row II, *A–D*) The cells described in row I were incubated for 30 min at 4°C with conditioned medium from COS-1 cells transfected with one of the following plasmids: the control vector pEF-SPFL (open areas, thick lines); the pEF-SPFL-IL-10 (open areas, thin lines), or the pEF-SPFL-cmvIL-10 (shaded areas, thin lines). Ligand binding to the cell surface was determined by flow cytometry with anti-FLAG antibody (Sigma) as the primary antibody and FITC-conjugated goat anti-mouse IgG (Santa Cruz) as the secondary antibody. Here and in row III, the ordinate represents relative cell number, and the abscissa is relative fluorescence. (Row III, *E–H*). The ability of IL-10 and cmvIL-10 to induce MHC class I antigen expression was demonstrated by flow cytometry as described (21). The cells described in row I were left untreated (open areas, thick lines) or treated with conditioned media (100 μl) from COS-1 cells transfected with the pEF-SPFL-cmvIL-10 plasmid (shaded areas, thin lines) or with Hu-IL-10 (100 units/ml; open areas, thin lines).

RT-PCR product revealed that both introns within the cmvIL-10 gene were spliced as they were spliced in COS cells transfected with pEF-cmv$_2$ plasmid.

The conditioned media from virus-infected and uninfected HEL 299 cells were assayed for the presence of IL-10 activity (Fig. 6). The hamster cells expressing the chimeric IL-10R1/γR1 chain and the intact second chain IL-10R2, as well as PBMCs, were used to perform the EMSA. Only medium from infected cells was able to induce Stat DNA binding complexes with the same pattern as IL-10 or cmvIL-10 treatment produced.

Structural Features of cmvIL-10, Alignment with Human IL-10, and Other Viral IL-10 Homologs. The sequence of cmvIL-10 can be put in the context of known and predicted structural features of IL-10. The crystal structure of IL-10 revealed topological similarity to that of IFN-γ (29–32), and both IL-10 and IFN-γ receptors belong to the same class II cytokine receptor family (33, 34). Based on the structure of the IFN-γ–IFN-γR1 complex (35), the amino acid residues of IL-10 involved in its interaction with IL-10R1 were predicted (ref. 32; Fig. 3, asterisks). The crystal structure of ebvIL-10 also has been solved (36) and is almost identical to that of IL-10. The fact that cmvIL-10 competes with IL-10 for receptor binding indicates that these proteins interact with the same receptor components. IL-10 has two intramolecular disulfide bridges (Fig. 3). Positions of all but one Cys residue are conserved. The Cys residue in the vicinity of the N terminus, although present in all four cytokines, is at a slightly different position in each. Also, cmvIL-10 has one additional Cys residue at position 78, which is apparently unpaired. Assuming that the amino acid residues of cellular and viral IL-10s involved in receptor interaction are well conserved, the similarity between the primary sequences of cellular and viral IL-10s (ovIL-10, ebvIL-10, and cmvIL-10) allowed us to evaluate the validity of the model for the IL-10:IL-10R1 interaction and speculate about sites within IL-10 molecules which are involved in interaction with IL-10R2. Most of the residues within helices A and B and the AB loop and helix F that were predicted to participate in interaction with IL-10R1 are well conserved in all IL-10s (Fig. 3,

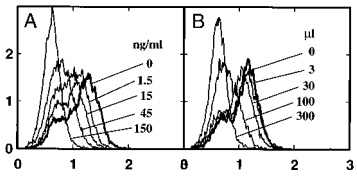

Fig. 5. Ligand-binding competition. Cells expressing both chains of the IL-10 receptor complex were incubated with FL-cmvIL-10 alone [30 μl of conditioned media from COS-1 cells expressing FL-cmvIL-10; (*A*) thick line] or with increased concentrations of IL-10 (expressed as ng/ml; *A*, thin lines) or with FL-IL-10 alone [30 μl of conditioned media from COS-1 cells expressing FL-IL-10; *B*, thick line] and with increased quantities of conditioned media from COS-1 cells expressing cmvIL-10 [expressed as μl; *B*, thin lines]. Cells were treated as described for row II in the legend to Fig. 4.

bold asterisks). However, several residues in these regions and the last two residues at the C terminus (Fig. 3, regular asterisks) that were predicted to be involved in interaction with IL-10R1 (32) are not conserved. Thus, it is likely that they are not involved in interaction with IL-10R1, particularly because there are charge differences among these residues. These differences among the IL-10s may reflect differences in signal transduction and likely represent subtle differences in their interactions with the receptor components. However, there are other conserved residues within helix A and the AB loop and helix F (Fig. 3, filled squares), suggesting their importance either for interaction with IL-10R1 or for maintaining the structural integrity of the proteins. In addition, the existence of a few very well conserved residues within helices C and D and the DE loop (Fig. 3, open squares) in the middle part of the IL-10s, the region that is apparently not involved in interaction with IL-10R1, suggests that these residues may be involved in interaction with IL-10R2, the second chain of the IL-10 receptor complex (21).

It is interesting that ebvIL-10 shares many immunosuppressive activities but lacks several immunostimulatory activities of IL-10 (23, 37–39). A single amino acid, Ala, at position 98 of ebvIL-10 and present only in ebvIL-10 (Fig. 3, filled triangle), was demonstrated to be responsible for this difference (40). This Ala also resides outside the regions predicted to be involved in interaction with IL-10R1 and, thus, is likely to be involved in interaction with IL-10R2.

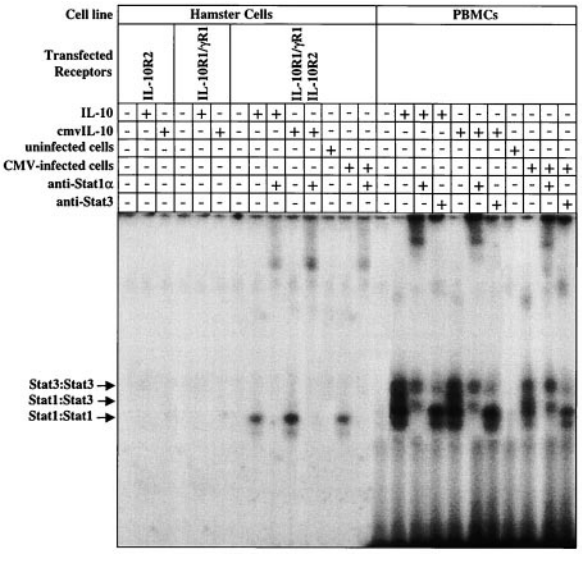

Fig. 6. Stat activation induced by cmvIL-10 in hamster cells and PBMCs. EMSAs were used to measure activation of Stat1 and Stat3. Hamster cells expressing both receptor chains (Fig. 4) and PBMCs were used. Cells were left untreated or treated with recombinant IL-10 (100 units/ml) or with conditioned media (200 μl) from COS cells transfected with the pEF-SPFL-cmvIL-10 plasmid or from uninfected or CMV-infected cells. Cellular lysates were prepared and assayed for Stat activation in the EMSA as described in *Materials and Methods*. Positions of Stat DNA-binding complexes are indicated by arrows. Antibodies against Stat1 and Stat3 were added as indicated to reduce the mobility of complexes containing these proteins.

Discussion

Viruses have developed elegant mechanisms to circumvent detection and destruction by host immune system. One of these strategies to escape immune surveillance is to express immune modulators encoded within viral genomes. Many viruses capture host genes encoding cellular cytokines and use them to their own advantage. The identification of such gene products and the elucidation of their functions not only improve our knowledge of virus-host interaction but enhance our understanding of the regulation of normal immune mechanisms, because these viral proteins have their cellular counterparts.

The complete sequence of the CMV strain AD169 genome was determined in 1990 (41), but the functions of many ORFs within the CMV genome have yet to be discovered. We identified the function of the UL111a ORF. This gene encodes a viral homolog of cellular IL-10 so that we have designated it cmvIL-10, thus extending the number of herpes viruses harboring homologs of IL-10. cmvIL-10 reveals a number of distinct features when compared with other viral IL-10 homologs encoded by herpesviruses that are able to infect humans, including EBV and OV. It demonstrates only 27% identity to human IL-10, whereas other vIL-10s are ≈85% identical to human IL-10 (Fig. 3). The cmvIL-10 gene also has a unique structure. The IL-10 gene is composed of five exons (GenBank accession no. U16720), the position of the two introns within the cmvIL-10 gene matches the position of the first and third introns of the IL-10 gene (Figs. 1 and 3), suggesting that these genes are related. The 5′ and 3′ intron/exon splice sites (Fig. 1*C*) for both introns within the cmvIL-10 gene conform well to consensus sequences (exon/GT-intron-AG/exon). In contrast, ovIL-10 and ebvIL-10 genes do not have introns. Thus, CMV might have captured the partially spliced IL-10 mRNA sequence from infected cells. Alternatively, CMV might have captured the human IL-10 gene that subsequently evolved to eliminate two introns and shorten the remaining two.

We demonstrated that the cmvIL-10 gene is transcribed and the primary transcript is spliced to generate the cmvIL-10 mRNA (Fig. 2*B*). In addition, we showed that cmvIL-10 is secreted (Fig. 6) by CMV-infected cells. Despite the limited homology between cmvIL-10 and IL-10, cmvIL-10 binds to the IL-10 receptor complex and competes with IL-10 for binding sites on the receptor (Fig. 5). cmvIL-10 is capable of inducing signal transduction events characteristic of IL-10 signaling and requires both chains of the IL-10 receptor complex to exert its biological activities (Figs. 4 and 6). Thus, CMV encodes its own unique, functionally active IL-10 homolog.

CMV uses several strategies to avoid clearing by the host immune system (42). One of these strategies is to inhibit antigen presentation through interruption of MHC class I and II synthesis and/or assembling pathways. Although down-regulation of the cell surface expression of MHC class I antigens allows cells to evade attack by cytotoxic T-lymphocytes, it makes them vulnerable to natural killer cell-mediated lysis. To circumvent natural killer attack, CMV expresses a virus-encoded MHC class I homolog on the cell surface that serves as a decoy molecule for natural killer cells. Another strategy, which is used by murine CMV and EBV infections, is to express secreted proteins that are able to suppress immune and inflammatory responses, such as cellular murine IL-10 (43) and ebvIL-10 (10). IL-10 has several immunosuppressive activities that would be favorable for CMV, such as down-regulation of MHC class I and II expression, inhibition of production of inflammatory cytokines, and interference with antigen presentation. Murine CMV infection induces transient early expression of IL-10, which plays an essential role in MHC class II down-regulation (43). EBV

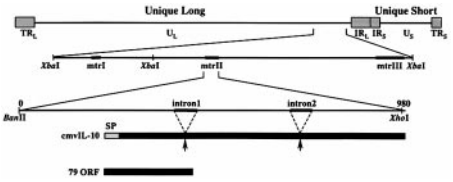

Fig. 7. Schematic map of the CMV genome. The CMV genome is organized as two regions of unique sequences, unique long (U_L) and unique short (U_S), flanked by two sets of inverted repeats (TR_L/IR_L) and (IR_S/TR_S) (light shaded boxes). mtrI, mtrII, and mtrIII represent three morphological transforming regions identified within the CMV genome (24, 48). *Xba*I, *Ban*II, and *Xho*I are sites for digestion with restriction endonucleases. The 79 ORF (dark shaded boxes) is an ORF of 79 aa whose disruption abolishes mtrII transforming ability. cmvIL-10 is encoded by three exons (see text). The position of two introns within the mtrII region is shown. The junction of the segments of the cmvIL-10 protein encoded by each exon is shown by the arrows. SP represents the signal peptide of the cmvIL-10 (light shaded box). The 79-aa ORF is slightly larger than the cmvIL-10 segment encoded by the first exon; it is the product of an mRNA that includes the first exon and intron 1 (Fig. 1).

infection causes expression not only of ebvIL-10, but also of human IL-10 (44, 45). We demonstrated here that human CMV encodes its own functional IL-10 homolog, which is expressed by CMV-infected cells. Thus, it seems likely that herpes viruses acquire many advantages by using IL-10-specific biological activities either of virus-encoded or host IL-10s.

CMV has been reported to be associated with a variety of human cancers, including cervical carcinoma, adenocarcinoma of the colon and prostate, and Kaposi's sarcoma (46, 47). Three regions of the CMV genome have been implicated in morphological transformation (Fig. 7) (47). However, the constant presence of only one of them, the morphological transforming region II (mtrII), was shown to be required in transformed cells to maintain the transformed phenotype. The mtrII subsequently was mapped to a minimal 980-bp *Ban*II/*Xho*I fragment located at the end of the unique long region (U_L) close to the invertible repeat [IR_L (Fig. 7)]. The region of the CMV genome encoding cmvIL-10 is collinear with this region. Moreover, the interruption of a 79-aa ORF, the first reading frame with homology to IL-10 (Figs. 1 and 7), abolished the transforming activity of the *Ban*II/*Xho*I fragment (24, 48). Thus, interruption of the first exon of the cmvIL-10 gene destroys transforming ability of mtrII. This 79-aa polypeptide, encoded by the first exon of the cmvIL-10 gene, could possibly be expressed by CMV-infected cells when splicing of the cmvIL-10 mRNA is interrupted. Thus, although it remains to be determined whether intact cmvIL-10 can function as an oncoprotein or the tumorigenic activity is exclusively the function of the 79-aa mtrII protein, the normal or abnormal production of cmvIL-10 may be the mechanism by which CMV supports malignant transformation.

In conclusion, we have identified, cloned, and characterized a homolog of IL-10 encoded by CMV, which has been designated cmvIL-10. Although the homology between IL-10 and cmvIL-10 is modest (27% aa identity), cmvIL-10 binds to and induces signal transduction through the same IL-10 receptor complex as IL-10. Thus, production of cmvIL-10 by CMV-infected cells increases the diverse arsenal used by CMV to combat the host immunity.

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