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THE CYTOMEGALOVIRUS US28 PROTEIN BINDS MULTIPLE CC CHEMOKINES WITH HIGH AFFINITY

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Human cytomegalovirus encodes several proteins with high similarity to seven transmembrane domain receptors. We investigated the ability of one of these proteins, the product of the US28 open reading frame, to bind various chemoattractant ligands. When transfected into COS-7 cells, the US28 product conferred high affinity binding to the labeled chemokines monocyte chemoattractant protein-1 (MCP-1) (K_d = 6.0 x 10^{-10} M) and RANTES (K_d = 2.7 x 10^{-10} M). Binding of these labeled ligands could be competed by the unlabeled macrophage inflammatory proteins MIP-1α and MIP-1β, with K_d values in the range 1.2 x 10^{-9} to 7.5 x 10^{-9} M. Comparisons of the sequences of US28 and other receptors that bind chemokines should help to define regions responsible for receptor-ligand interactions.

Chemoattractant cytokines, or chemokines, are important mediators of leukocyte migration in inflammation, cancer, and atherosclerosis (for reviews see (1, 2)). The CC chemokine subfamily, so named because its members possess two adjacent cysteine residues, includes the monocyte chemoattractant proteins (MCP-1, 2, and 3), RANTES, and the macrophage inflammatory proteins-1 (MIP-1α & β). The chemokine receptors that have been characterized belong to the family of receptors with seven membrane spanning domains. Two receptors from neutrophils have been cloned that bind and transduce signals in response to the CXC chemokine interleukin-8 (3-5). A receptor found on monocytes binds and transduces signals in response to the CC chemokines RANTES and MIP-1α (6, 7). MCP-1 competed for MIP-1α binding to this receptor with a K_d of 122 nM, as opposed to 6.5 nM for MIP-1α self competition (6). Recently, the cloning of two receptors activated specifically by MCP-1 has been reported (8, 9). These are derived by alternative splicing of the 3' end of a common primary transcript, and differ only in their carboxyl terminal intracellular domains. MCP-1 receptor B binds MCP-1 with an affinity of

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Abbreviations used: monocyte chemoattractant protein-1, MCP-1; macrophage inflammatory proteins-1, MIP-1; interleukin-8, IL-8; cytomegalovirus, CMV; phosphate buffered saline, PBS; Dulbecco's modified Eagle medium, DMEM; fetal calf serum, FCS; HEPES binding buffer, HBB; bovine serum albumin, BSA; polymerase chain reaction, PCR.

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0.26 nM and shows a high specificity for MCP-1 over other chemokines (9). Finally, two proteins that are encoded by herpesviruses were found to have similarities to chemokine receptors, and subsequently receptor activity. The herpesvirus saimiri protein encoded by the gene ECRF3 transduces signals in response to IL-8 and gro/MGSA (10), while the product of the cytomegalovirus (CMV) unique sequence 28 open reading frame (hereafter referred to as the US28 protein) was shown to bind MIP-1α (6). Subsequently, it was shown that MCP-1 and RANTES could compete with nanomolar affinity for binding of labeled MIP-1α to US28 transfected K562 cells (11). We have cloned a novel US28 gene from the VHL/E strain of CMV that has five amino acid substitutions relative to previously reported US28 sequences. We show that this form of the US28 receptor can bind labeled MCP-1 and RANTES when transfected into COS-7 cells.

MATERIALS AND METHODS

Cell cultures. COS-7 cells were obtained from the A.T.C.C. and propagated in DMEM plus 10% FCS and antibiotic/antimycotic mixture (Gibco-BRL).

Cloning of the CMV US28 open reading frame. The fragment of CMV DNA containing the US28 open reading frame was amplified in a polymerase chain reaction. A frozen vial of CMV-infected endothelial cells was received from Dr. W. J. Waldman in the Department of Pathology, Ohio State University Medical School. The strain of CMV that was used, VHL/E, is a clinical isolate that has been propagated in human umbilical vein endothelial cells (12). A small sample of the frozen material was scraped directly into the reaction tube to act as template. The primers used had the sequences CTCGCTCGCCCATGACACCGACGACG and CTGGTTCGGCCCTTAC-GGTATAATTTGTGA. Reactions were done with standard conditions, purified with Ultrafree-MC filtration units (Millipore), and cloned using the pDIRECT T4 polymerase cloning system (Clontech). One entire clone was sequenced, and the portions that differed from the reported coding sequence were confirmed by sequencing a second clone. The insert was excised from the pDIRECT vector with EcoR I and Xba I and inserted into the expression vector pcDNA3.

Transfection of COS-7 cells. Exponentially growing COS-7 cells were harvested, washed twice with PBS (pH 7.4) and resuspended in PBS at 1 x 10^7 cells/ml. The expression plasmid (20μg) was electroporated into 3 x 10^6 cells. Electroporation settings were 0.25 kV, 400 ohms and 960 μF, giving rise to a pulse duration of approximately 60 msec. Transfected cells were suspended in 10 ml of DMEM (containing 10% FCS and antibiotics) and plated in 90 mm plates. Cells were allowed to recover for 3 days before use in binding experiments.

Iodination of RANTES and MCP-1. RANTES and MCP-1 were iodinated by a modification of the chloramine-T method described by Hunter and Greenwood (13). Chloramine T (26.4 μg) was added to 1.5 μg of recombinant protein with 250 μCi of [^{125}I]-NaI (Dupont-NEN) in 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.2), and the reaction was carried out at room temperature for 30 seconds. Sodium metabisulfite (100 μg) was then added to stop the reaction. Iodinated protein was separated from free ^{125}I by passing the mixture through a 1.5 ml QAE-Sepharose column using an elution solution of 5% sucrose and 0.25% BSA. Estimated specific activities for RANTES and MCP-1 were 100 μCi/μg and 90 μCi/μg, respectively. The labeled protein fraction contained less than 3% free ^{125}I as assessed by TLC on Silica G plates using normal saline as the developing solvent.

Binding experiments. Three days post-transfection, COS-7 cells were washed once with ice cold PBS and once with ice cold HEPES binding buffer (HBB; consisting of 50 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% BSA). Ice cold HBB (2ml) containing different amounts of labeled and unlabeled liquids was then added to each plate. Following incubation for 2 hours at 4°C, the binding solution was removed and each plate rinsed once with 10 ml ice cold HBB and twice with 10 ml of ice cold PBS. Cells were detached with trypsin and the plates rinsed twice with PBS. The combined eluted material was counted in a Packard Cobra gamma counter. Non-specific binding in the saturation binding experiments was determined using a 100-fold excess of unlabeled ligand. The binding data was analyzed with the LIGAND program (14).

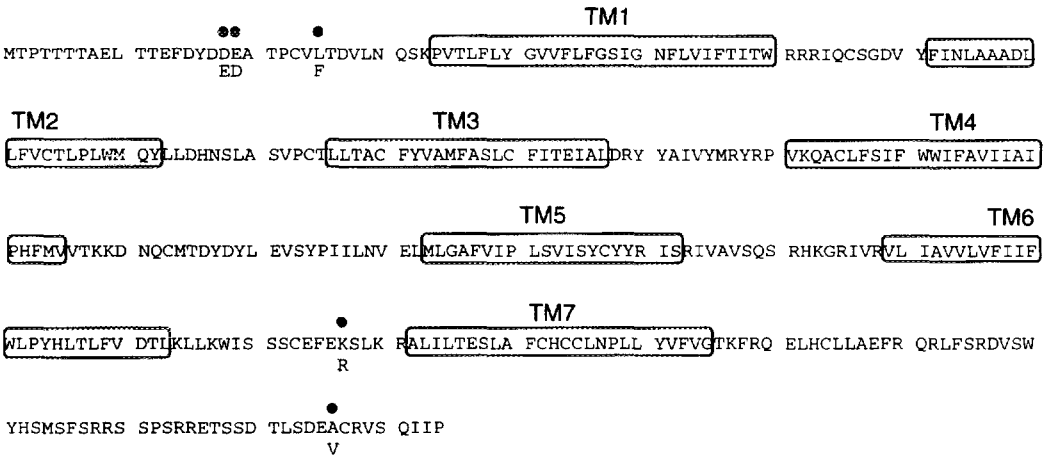

Figure 1. The deduced amino acid sequences of the US28 protein from the VHL/E strain of cytomegalovirus. The positions of amino acid substitutions are shown by black dots above the sequence with the corresponding residue from the sequence of the Towne strain (6) shown below. The positions of predicted transmembrane regions are shown with boxes. The DNA sequence is deposited in GenBank under accession number L20501.

RESULTS

Cloning of the CMV US28 gene. A clone of the US28 region was isolated by PCR from the VHL/E CMV strain. The sequence of this clone differed from clones derived from the Towne (6) and AD169 (11) strains of CMV that were reported previously, probably due to the differences in origin of the viruses. Altogether, the VHL/E sequence showed 5 amino acid substitutions from the protein sequence reported for the other strains and had 19 silent nucleotide substitutions. The amino acid changes were all rather conservative replacements, and are diagrammed in figure 1.

Transfection of the US28 gene and binding studies. We cloned the US28 cDNA into the expression vector pcDNA 3, and used the resulting plasmid to transiently transfect COS cells. Control cells were transfected with the expression vector alone. We then bound radiolabeled MCP-1 to both types of transfected cells. As shown in figure 2, the US 28 transfected cells bound high levels of radiolabeled MCP-1 while the control cells did not. We found that US28 transfected cells did not bind radiolabeled interleukin-8 but did bind labeled RANTES in similar autoradiography experiments (data not shown).

Measurements of dissociation constants of US28 binding to MCP-1 and RANTES. We measured the binding constants for the two ligands with a saturation binding approach, adding increasing amounts of labeled ligand with non-specific binding estimated by adding excess unlabeled material. The results of representative experiments are shown in figure 3. The mean K_d value for RANTES determined from multiple experiments was 1.7×10^{-10} M, while the mean K_d for MCP-1 was 4.6×10^{-10} M.

Cross competition studies of chemokine binding. We also performed competition binding studies with constant quantities of the labeled ligands RANTES or MCP-1, and increasing

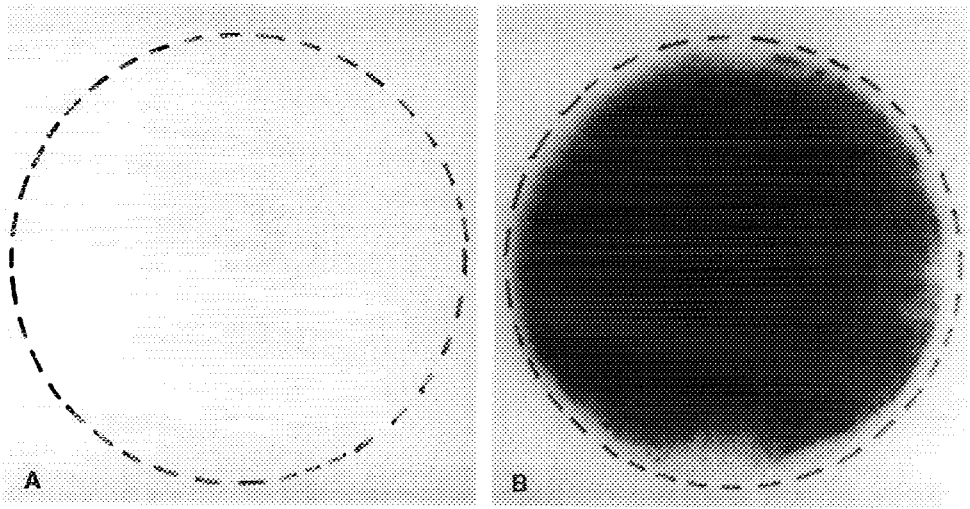

Figure 2. Autoradiograph of radiolabeled MCP-1 binding. Dishes of COS cells transfected with A) pcDNA3 vector alone or B) the US28 expression construct were used to bind [^{125}I] MCP-1, washed, dried, and exposed directly to x-ray film.

concentrations of unlabeled competitors, either the same two ligands or the CC chemokines MIP-1α and MIP-1β. By fitting the displacement curves, we calculated dissociation constants which are tabulated in table 1. RANTES and MCP-1 competed with themselves and with each other with K_d values that were slightly greater than the ones determined by saturation binding studies, Fig. 4, in the range 3.2 x 10^{-10}—7 x 10^{-10} M. MIP-1α and MIP-1β competed with either labeled ligand with higher K_d values, in the range 1.2 x 10^{-9}—7.5 x 10^{-9} M.

DISCUSSION

We find that the CMV US28 receptor expressed in COS cells binds a broad range of CC chemokines with high affinity. Although these results are in general agreement with the recent results obtained with K562 cells (11), there are some differences between the two studies. We

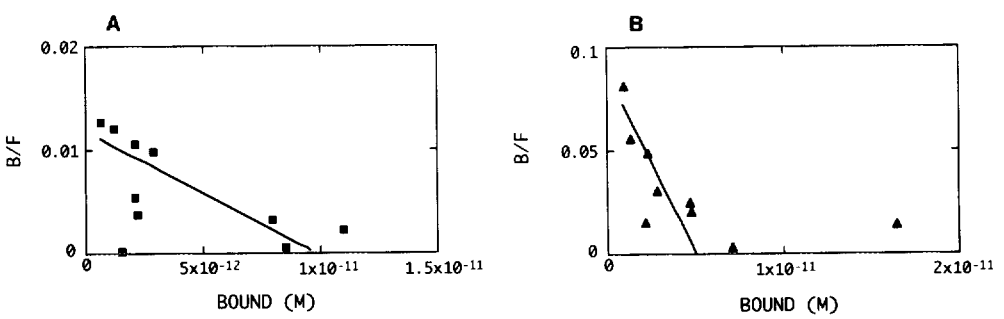

Figure 3. Scatchard plots of representative saturation ligand binding experiments with COS cells transfected with the US28 receptor expression clone. The fitted line determined by the LIGAND computer program is shown. A) Radiolabeled MCP-1 binding. B) Radiolabeled RANTES binding.

Table 1 Summary of competition binding studies with US28 transfected COS cells

Labeled Ligand	Unlabeled Ligand	Kd (x 10^{-10} M) Mean	Kd (x 10^{-10} M) Range	Number of experiments
RANTES	RANTES	3.8	1.6 - 5.5	5
RANTES	MCP-1	7.7	7.7 - 7.8	2
RANTES	MIP-1α	70	50 - 97	3
RANTES	MIP-1β	75	54 - 96	2
MCP-1	RANTES	3.2	3.1 - 3.2	2
MCP-1	MCP-1	7.4	3.7 - 12.3	6
MCP-1	MIP-1α	30	19 - 39	3
MCP-1	MIP-1β	12	9 - 14	4

find that the CMV US28 receptor binds the ligands with rank order RANTES>MCP-1>MIP-1α≈MIP-1β, while the other study found more closely equal affinities for all the ligands. This could be due to differences in the approaches used, including the use of COS cells instead of K562 cells, the use of labeled MCP-1 and RANTES instead of MIP-1α, and the presence of 5 amino acid substitutions in the US28 proteins.

The ability of the US28 receptor to bind a broad range of CC chemokines is especially interesting in view of its limited sequence similarity to the other known CC chemokine receptors, the MCP-1 receptors (8) and the CC-CKR (MIP-1α/RANTES) receptor (6, 7). Most of the

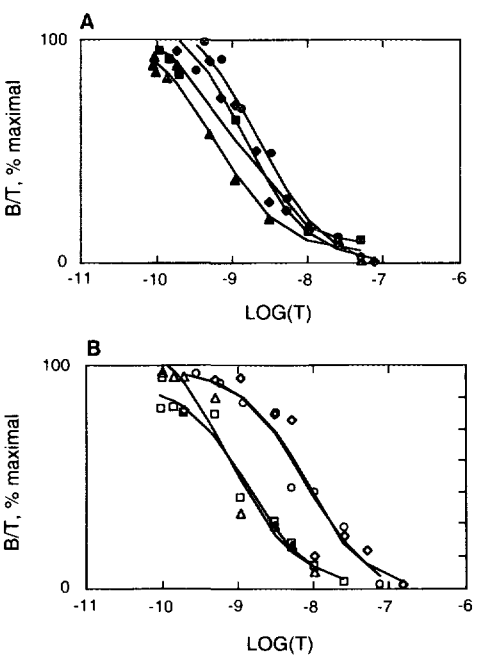

Figure 4. Representative competition binding experiments on US28 transfected COS cells. Identical quantities of labeled MCP-1 (A) or RANTES (B) were bound in the presence of increasing concentrations of unlabeled competitors, as follows: MCP-1: ■, □; RANTES: ▲, △; MIP-1α: ●, ○; MIP-1β: ◆, ◇.

similarities and identities that do exist are located either in the transmembrane domains or intracellular regions. The only conserved region in an extracellular domain is the N-terminal hexapeptide Thr-Thr-(Glu,Phe)-Phe-Asp-Tyr that begins at residue 11 of the US28 sequence. This sequence is not found in the two IL-8 receptors, but residues in the corresponding region have been shown to be important in IL-8 binding (15). Given these facts, it is tempting to speculate that this hexapeptide could form part of a CC chemokine specific recognition sequence.

ACKNOWLEDGMENTS

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