CC and CX3C Chemokines Differentially Interact with the N Terminus of the Human Cytomegalovirus-encoded US28 Receptor*

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Human cytomegalovirus (HCMV) is the causative agent of life-threatening systemic diseases in immunocompromised patients as well as a risk factor for vascular pathologies, like atherosclerosis, in immunocompetent individuals. HCMV encodes a G-protein-coupled receptor (GPCR), referred to as US28, that displays homology to the human chemokine receptor CCR1 and binds several chemokines of the CC family as well as the CX3C chemokine fractalkine with high affinity. Most importantly, following HCMV infection, US28 activates several intracellular pathways, either constitutively or in a chemokine-dependent manner. In this study, our goal was to understand the molecular interactions between chemokines and the HCMV-encoded US28 receptor. To achieve this goal, a double approach has been used, consisting in the analysis of both receptor and ligand mutants. This approach has led us to identify several amino acids located in the N terminus of US28 that differentially contribute to the high affinity binding of CC versus CX3C chemokines. Additionally, our results highlight the importance of secondary modifications occurring at US28, such as sulfation, for ligand recognition. Finally, the effects of chemokine dimerization and interaction with glycosaminoglycans (GAGs) on chemokine binding and activation of US28 were investigated as well using CCL4 as model ligand. In line with the two-state model describing chemokine/receptor interaction, we show that an aromatic residue in the N-loop region of CCL4 promotes tight binding to US28, whereas receptor activation depends on the presence of the N terminus of CCL4, as shown previously for CCR5.

Chemokines are a group of small (8–14 kDa), mostly basic soluble proteins that have been shown through crystallographic and nuclear magnetic resonance structure determination to adopt a similar fold, even in cases of low overall sequence identity (1, 2). Chemokines are classified (CC, CXC, CX3C, and XC) based on the number and sequential relationship of the first two of four conserved cysteine residues (3).

Chemokines play key roles in many aspects of the immune and inflammatory responses, primarily by attracting and activating leukocytes (4, 5). Chemokine signaling is dependent on binding to a family of seven transmembrane-spanning, G-protein-coupled receptors (6), which triggers intracellular signal transduction events, such as calcium mobilization and phosphorylation of serine/threonine kinases (7).

Another common aspect of chemokines is their ability to interact with glycosaminoglycans (GAGs), usually heparan sulfate, and several lines of evidence point out the importance of heparan sulfate in promoting chemokine activity (8). Such interactions are of physiological relevance, as they presumably contribute to the formation of chemotactic gradients at the cell surface and in the extracellular matrices, which are essential for chemokine-mediated cell trafficking (9). It is currently debated whether GAG binding may also influence chemokine structure and activity in other ways. In support of this notion, enzymatic removal of cell surface GAGs reduces the ability of Gro-α/CXCL1 to bind and activate its cell surface receptor, CXCR2 (10). GAG-induced increase in local chemokine concentration could be critical to potentiate the interaction with its receptor, or GAGs could represent a fundamental component of the receptor-ligand complex. The understanding of chemokine action is further complicated by the fact that many chemokines have been shown to form tight dimers (11–14). The physiological role of the chemokine dimer in the interaction with the chemokine receptor is not yet fully understood.

Because chemokines are key players in orchestrating the migration and activation of leukocytes, viruses have developed several ways to affect the chemokine signaling for their own benefit (15). These strategies represent efficacious ways to evade host immune responses. For example, several viruses belonging to the β- and γ-herpesvirus family encode for GPCRs which show sequence homology to mammalian chemokine receptors and bind chemokines with high affinity (16). These vGPCRs might help the virus to subvert the host chemokine signaling system. Additionally, they might help the virus in immune evasion, acting as scavengers of endogenous chemokines through rapid endocytosis (16, 17).

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1 The abbreviations used are: GAG, glycosaminoglycan; HA, hemagglutinin; WT, wild type; ELISA, enzyme-linked immunosorbent assay; HCMV, human cytomegalovirus; GPCR, G-protein-coupled receptor; SMCs, smooth muscle cells; RANTES, regulated on activation normal T cell expressed and secreted.
The β-herpesvirus human cytomegalovirus (HCMV) HCMV is a widely spread virus that is able to establish a lifelong persisting infection in the host in a latent form (18, 19). HCMV has been implicated as a risk factor for restenosis after coronary angioplasty and for atherosclerosis (20–25). Most interestingly, HCMV encodes four vGPCRs (26).

HCMV-encoded chemokine receptor US28 is so far the best characterized and has been shown to display several interesting pharmacological features. First of all, US28 possesses a large spectrum chemokine binding profile, because it recognizes chemokines belonging to the CC family, such as RANTES/CCL5, Mip-1α/CCL3, Mip-1β/CCL4, and MCP-1/CCL2, as well as the CX3C chemokine fractalkine/CX3CL1 (27, 28). In accordance with its binding profile, US28 has been suggested to act as a chemokine scavenger. Depletion of chemokines from the medium was in fact demonstrated following HCMV infection, due to internalization of US28 (29, 30). Because chemokines play a crucial role in regulation of the immune system, chemokine sequestration by US28 could help HCMV escape immune surveillance.

Additionally, US28 can activate several intracellular pathways. Most interestingly, some of these pathways are constitutively activated by the US28 receptor. We and others have shown previously that US28 constitutively signals through a Goq pathway leading to activation of phospholipase C when transiently expressed in COS-7 cells, as well as after HCMV infection (31–34). Moreover, US28 can constitutively activate NF-κB, a transcription factor that plays an important role in inflammatory events such as atherosclerosis (31, 34). Most interestingly, chemokines belonging to the CC family do not modulate the constitutive activation of phospholipase C and NF-κB by US28 in both transfected as well as HCMV-infected cells (31, 33). In contrast, US28 activates other pathways in a ligand-dependent fashion. For example, CC chemokines, such as RANTES/CCL5 and Mip-1α/CCL3, induce calcium release in US28-transfected cells (35, 36) as well as in HCMV-infected fibroblasts (37). Additionally, Streblow and colleagues (38) demonstrated that stimulation with CC chemokines, including RANTES/CCL5 or MCP-1/CCL2, induces a significant cellular migration in US28-expressing smooth muscle cells (SMCs). In collaboration with Streblow (38), we have recently shown that this agonistic effect of CC chemokines on US28 involves activation of focal adhesion kinases (39). US28-mediated SMC migration provides an additional molecular basis for the correlation evidence that links HCMV to the acceleration of vascular disease (38).

In this study, we aimed at better understanding the molecular interactions between chemokines and the HCMV-encoded US28 receptor. To achieve this goal, a double approach has been used, consisting in the analysis of both receptor and ligand mutants. Moreover, the effect of chemokine dimerization and interaction with GAGs on chemokine binding and activation of US28 was investigated as well.

MATERIALS AND METHODS
DNA Constructs

The two N-terminal truncation mutants of US28 were generated by PCR and subsequently subcloned in the mammalian vector pcDEF3 (kindly provided by Dr. J. Langer). Single amino acid mutations in the hepad of US28 were introduced using the Altered Sites® II in vitro Mutagenesis System (Promega, Madison, WI) according to the manufacturer’s protocol, using the HA-tagged US28 receptor as template. Subsequently, all mutants were subcloned in pcDEF3. All constructs were verified by dyeoxy sequencing.

Production and Purification of CCL4 Variants

Mutations of CCL4 were produced using the Quikchange procedure (Stratagene, La Jolla, CA) in a variant of the Novagen pET32LIC vector as described previously (40). Wild type and all point mutants were expressed in the pET-32 vector and contained no N-terminal modification. The plasmids were transfected into BL21(DE3) cells (Novagen), and the protein was produced and purified as described previously (40), using a modified procedure from Kuna et al. (41) to refold each mutant.

Cell Culture and Transfection

COS-7 cells were grown as described previously (31). Transfection of the COS-7 cells was performed by DEAE-dextran, using 2 μg of DNA of each US28 construct per million cells (31). SVEC4-10 is an endothelial cell line derived by SV40 (strain 4a) transformation of endothelial cells from murine auxiliary lymph node vessels (ATCC CRL2181). SVEC4-10 cells were grown at 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. SVEC4-10 cells were transfected with the US28-YFP fusion construct inserted into the PTEJ8 expression vector with Superfect (Qiagen) according to the manufacturer’s protocol. Cells stably expressing US28 were selected in media containing 500 μg/ml neomycin G418. Clones were selected based on fluorescence, and US28 expression was further confirmed by chemokine binding analysis.

Equilibrium Binding Experiments

Labeling of CCL3, CCL4, CCL5, and the chemokine domain of CX3CL1 (PeproTech, Rocky Hill, NJ) with 125I was performed with IODO-GEN as described previously (42). Chemokine binding affinity for the different US28 mutants was determined in homogenous displacement studies, as described previously (32). Briefly, 2 days after transfection, COS-7 cells were incubated with 0.3 nM of radiolabeled chemokine (1 nM for 125I-CCL3) in binding buffer (50 mM Hepes, pH 7.4, 1 mM CaCl2, 5 mM MgCl2, and 0.5% bovine serum albumin) in the presence or absence of various concentrations of cold competitor for 3 h at 4 °C. After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 M NaCl. In some experiments, transfected COS-7 cells were incubated with sodium chloride (10 mM) in sucrose-free medium for 48 h before performing the binding experiment.

Kinetic Studies

Membrane isolation and purification was performed as described previously (43). Briefly, 2 days after transfection COS-7 cells were resuspended in buffer B (7.5 mM Tris-HCl, pH 7.5, 12.5 mM MgCl2, 0.3 mM EDTA, 1 mM EGTA, homogenized, and spun down for 30 min at 48,000 × g). The pellet was resuspended in buffer B (7.5 mM Tris-HCl, pH 7.5, 12.5 mM MgCl2, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), aliquoted, and stored at −80 °C until use. For dissociation kinetic studies, membranes (3 μg/sample) were first allowed to equilibrate with 0.4 mM 125I-CCL5 or 125I-CX3CL1 in binding buffer (50 mM Hepes, pH 7.4, 1 mM CaCl2, 5 mM MgCl2, 0.5% bovine serum albumin) at 37 °C for at least 1 h. Afterward, the cold chemokine (100-fold excess) was added, and samples were further incubated at 30 °C. At the different time points, samples were filtered through Whatman GPC filters pre-soaked in 0.3% polyethyleneimine, followed by three washes with binding buffer supplemented with 0.5 mM NaCl, using a Brandel harvester. Samples with membranes from mock-transfected cells were carried along to determine aspecific binding at each time point.

Calcium Measurements

SVEC4-10 cells stably expressing US28 were seeded in 96-well black wall clear-bottom microplates (Corning Glass) at 4 × 103 cells/well. After 24 h, cells were loaded for 30 min at 37 °C in the dark with 4 μM cell-permeant Fluo-AM (Fluo-4 acetoxyethyl ester) and 0.04% pluronacetate (both from Molecular Probes) in Hanks’ balanced salt solution supplemented with 20 mM Hepes, pH 7.4, 2.5 mM probenecid, and 0.5% bovine serum albumin. Cells were then washed twice and preincubated for 1 h at 37 °C in the dark in Hanks’ balanced salt solution supplemented with 20 mM Hepes, pH 7.4, 2.5 mM probenecid, 1% bovine serum albumin. Intracellular Ca2+ mobilization upon stimulation with tested chemokines was monitored at 37 °C as fluorescence at excitation and emission wavelengths of 485 and 520 nm, respectively, using a Novostar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany). After 60 s cells were lysed by adding 5% Triton X-100 to determine maximum fluorescence. All data shown are expressed as percentage of maximum fluorescence.

[3H] Inositol Phosphate Production

Experiments in COS-7 cells were performed as described previously (31).
ELISA Test

48 h after transfection, receptor expression in COS-7 cells was measured by ELISA as described previously (32). Mouse anti-HA monoclonal antibody (kindly provided by Dr. J. van Minnen, Vrije University, Amsterdam, The Netherlands) was used as primary antibody, and goat anti-mouse horseradish peroxidase conjugate (Bio-Rad) was used as secondary antibody. The TMB solution (Sigma) was used as substrate, and the optical density was measured in a Victor2 (PerkinElmer Life Sciences) at 450 nm.

Immunofluorescence

Feeding Experiments—To directly examine that the mutant US28 receptors were in fact intact and reached the cell surface (and were not merely stuck intracellularly or retained in the endoplasmic reticulum due to improper protein folding), we conducted antibody feeding experiments (see also Ref. 44). This experimental setup allows us to assess distributions of receptors that were accessible to the HA antibody during a 30-min incubation period. COS-7 cells expressing the receptor constructs 2 days after transfection were used for immunofluorescence. 24 h post-transfection, cells were plated to coverslips and allowed to grow for 24 h. Living cells were fed monoclonal anti-HA 11 antibody (Covance, Berkeley, CA) for 30 min to label receptors. Subsequently, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature and permeabilized with 0.1% Triton X-100, essentially as described (44).

Staining Experiments—For cells not fed with monoclonal anti-HA11 antibody, living cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature, permeabilized with 0.1% Triton X, and first incubated with monoclonal anti-HA11 antibody for 40 min at room temperature.

Subsequently, all cells were visualized with a fluorescein isothiocyanate-conjugated secondary donkey-anti-mouse antibody (Molecular Probes). Following staining, cells were mounted in Vectashield Mounting Medium (Vector Laboratories) and analyzed using a Zeiss LSM510 META Axioplan 2 confocal microscope.

RESULTS

Chemokine Interaction with US28 as Studied with MIP-1β/CCL4 Mutants—In order to better understand the molecular interactions occurring between chemokines and US28, we chose Mip-1β/CCL4 as a model ligand and analyzed the behavior of several mutants for their ability to bind and activate US28.

Functional analysis of several chemokines has led to the general conclusion that the N-terminal portion of the protein preceding the Cys motif is responsible for receptor activation (45, 46). To check whether this model applies to US28 as well, CCL4-Δ8, a truncated analogue which lacks the first 8 amino acids, was tested. Our results indicate that CCL4-Δ8 binds to US28 with high affinity, similarly to CCL4 wild type (pKᵦ = 8.8 ± 0.1 and 8.7 ± 0.1, respectively; Fig. 1A), whereas it is strongly impaired in promoting US28-mediated calcium release (Fig. 1B). These data imply that the N terminus of the chemokine does not contribute to high affinity binding, whereas it is an essential determinant for receptor activation.

Furthermore, we analyzed whether chemokine dimerization and interaction with GAGs affect the pharmacology of CCL4 at US28. For CC chemokines, it is known that basic residues in the 40s loop are involved in GAGs binding (47), whereas hy-
drophobic interactions along the N terminus are essential for dimer formation (13).

The single amino acid mutant CCL4-P8A, for example, was shown previously to be unable to dimerize even at very high concentrations, although it can bind to GAGs similarly to CCL4-WT (40). The triple mutant in the 40s loop CCL4-[K45A/R46A/K48A], on the other hand, dimerizes similarly to WT protein but is impaired in the interaction with GAGs (48). This set of mutants can therefore be used to analyze separately the protein but is impaired in the interaction with GAGs (48). Taken together, these data imply that both chemokine oligomerization and interaction with GAGs are not necessary for chemokine binding and activation of the US28 receptor in vitro.

According to the two-state model describing chemokine/receptor interaction, the N-loop region of chemokines (residues 13–20) promotes tight binding to the chemokine receptor (46, 49). An amino acid alignment of all chemokines known to bind to US28 shows that an aromatic residue present in the N-loop region after the CXC cluster is highly conserved (Fig. 2A). The data are normalized for nonspecific binding (0%) and maximal specific binding in the absence of a competitor (100%). A representative experiment out of three experiments is shown, each performed in triplicate. C, functional response curves were obtained on SVEC4-10 cells stably expressing US28. The black arrow indicates the time when chemokines (40 nm) were added to the cells. The data are normalized to the maximal fluorescence signal resulting from addition of Triton X-100 (100%). The displayed curves represent a typical experiment of three performed independently.

Fig. 2. Importance of a highly conserved aromatic residue in the N-loop of CCL4. A, alignment of chemokines known to bind US28. The highly conserved aromatic residue after the CXC cluster is shown in white on black background. B, competition binding curves were determined in US28-expressing COS-7 cells against 125I-CCL4. The data are normalized to the maximal fluorescence signal resulting from addition of Triton X-100 (100%). The displayed curves represent a typical experiment of three performed independently.
ing, is not necessary for the US28-mediated constitutive activity (32). Moreover, these results indicate that the truncated US28 mutants are correctly expressed at the plasma membrane of cells.

Thereafter, we tested the consequence of progressive truncation of US28 N terminus on chemokine binding. As shown in Fig. 3C, US28-Δ(2–10), which still contains the hexapeptide sequence, binds CX3CL1/fractalkine (pKᵢ = 9.2 ± 0.1) and CCL5/RANTES (pKᵢ = 8.8 ± 0.1) with unchanged affinity, when compared with US28-WT, and CCL4/Mip-1b with only slightly lower affinity (pKᵢ = 8.1 ± 0.1; 5-fold decrease in binding affinity compared with US28-WT). Taken together, these results indicate that the first 10 amino acids in the N terminus of US28 do not play an important role in chemokine binding. On the other hand, US28-Δ(2–16) is fully impaired in binding all chemokines tested (Fig. 3C).

**Generation and Expression of US28 Mutants Carrying Single Amino Acid Changes**—Comparison of the behavior of the two N-terminal truncation mutants clearly indicates the functional relevance of the hexapeptide sequence in chemokine binding. To understand in more detail which amino acids within this region are responsible for chemokine/receptor interaction, we replaced each residue in the hexapeptide region of US28 individually with alanine. All cDNA constructs were transiently

| US28     | MTP-TTTA-ELTEDTDEATPVCVLTDVLNSK |
|----------|---------------------------------|
| CCR1     | METPNTTEDY-DTTPEDYD-ATPCQKVNERAFGA |
| CCR2     | MLSTSSRFIRNTESGEEVTTPFDY-GAPCHKFVQIGA |

**Fig. 3. Characterization of N terminus truncation mutants of US28.** A, alignment of the N termini of US28, CCR1, and CCR2. The highly conserved heptad is shown in white on black background. The lines indicate which is the first residue following methionine in the sequence of the N-terminal truncation mutants US28-Δ(2–10) and -Δ(2–16). B, COS-7 cells expressing the various US28 mutants and the reporter gene NF-κB-luciferase were assayed for NF-κB-induced transcription. A representative experiment out of three experiments is shown, each consisting of six data points. C, competition binding curves were determined in COS-7 cells expressing US28-WT (filled circles), US28-Δ(2–10) (open circles), or US28-Δ(2–16) (open triangles) mutants. The data are normalized for nonspecific binding (0%) and maximal specific binding in the absence of a competitor (100%). A representative experiment out of two experiments is shown, each performed in triplicate.
Expression and binding profiles of US28 mutants

Two days after transfection, COS-7 cells were tested for receptor expression by using an ELISA test against the HA tag present at the US28 N terminus. Data are expressed as percentage of US28-WT expression. The average of three experiments is shown, with each data point performed in triplicate. Binding affinities for the different chemokines were determined in homologous displacement studies and are expressed in nanomolar. $B_{\text{max}}$ values are expressed in fmol/10⁶ cells. The average of at least two experiments is shown, with each data point performed in triplicate.

| US28 mutant | Expression (% US28 WT) | CCL3 $K_d$ (nM) | CCL3 $B_{\text{max}}$ (fmol/10⁶ cells) | CCL4 $K_d$ (nM) | CCL4 $B_{\text{max}}$ (fmol/10⁶ cells) | CCL5 $K_d$ (nM) | CCL5 $B_{\text{max}}$ (fmol/10⁶ cells) | CX3CL1 $K_d$ (nM) | CX3CL1 $B_{\text{max}}$ (fmol/10⁶ cells) |
|-------------|------------------------|-----------------|---------------------------------|-----------------|---------------------------------|-----------------|---------------------------------|-----------------|---------------------------------|
| WT          | 100                    | 1.3             | 23                             | 1.7             | 60                             | 1.6             | 104                            | 0.5             | 63                             |
| T11A        | 88 ± 7                 | 3.0             | 19                             | 2.7             | 49                             | 2.0             | 102                            | 0.5             | 54                             |
| T12A        | 77 ± 12                | 4.1             | 10                             | 5.7             | 55                             | 2.3             | 91                             | 0.4             | 42                             |
| E13A        | 91 ± 14                | 1.2             | 22                             | 2.2             | 81                             | 1.7             | 106                            | 0.5             | 54                             |
| F14A        | 78 ± 15                | 15.0            | 19                             | 18.4            | 53                             | 5.7             | 104                            | 0.5             | 46                             |
| D15A        | 104 ± 4                | 1.4             | 33                             | 1.9             | 71                             | 1.9             | 110                            | 0.7             | 82                             |
| Y16F        | 128 ± 6                | 12.0            | 24                             | 10.2            | 52                             | 4.6             | 101                            | 1.2             | 64                             |
| DFDF        | 115 ± 7                | 1.0             | 22                             | 1.8             | 64                             | 1.6             | 101                            | 0.7             | 66                             |

Chemokine Binding to Mutant US28 Receptors—All mutants generated were tested for their ability to bind the CC chemokines RANTES/CCL5, Mip-1α/CCL3, and Mip-1β/CCL4 as well as fractalkine/CX3CL1 after transient expression in COS-7 cells. Affinities were determined in homologous displacement studies on whole cells, because chemokines do not show a truly competitive binding to US28 (27). As described before (27, 28), all chemokines bind with (sub-)nanomolar affinity to the wild type receptor US28 (Table I). The $B_{\text{max}}$ values for the various chemokines are shown in Table I. In accordance with previously published data (27), $B_{\text{max}}$ values obtained with the different radioligands differ among each other, with RANTES/CCL5 giving consistently the highest value and Mip-1α/CCL3 the lowest value. These results possibly suggest that different chemokines recognize different receptor sub-populations, a phenomenon that has already been described for other chemokine receptors (51) and is currently under investigation.

Chemokines possess several positively charged residues, and ionic interactions are thought to be important for high affinity binding (52, 53). However, mutation of the two acidic residues Glu-13 and Asp-15 within the N terminus of US28 did not affect the binding of any of the tested chemokines (Table I). Mutation of the two hydrophilic threonine residues Thr-11 and Thr-12 also had minor effects on chemokine binding, although a small impairment of the high affinity binding of both Mip-1α/CCL3 and Mip-1β/CCL4 can be noticed (Table I). However, more dramatic changes were observed with the mutation of Phe-14 and Tyr-16 in the N terminus of US28.

Mutation of the aromatic residue phenylalanine 14 with an alanine resulted for all tested CC chemokines in the most significant loss of high affinity binding. Most interestingly, the loss of affinity for both Mip-1α/CCL3 and Mip1β/CCL4 (11.5- and 10.8-fold decrease, respectively) (Fig. 5, A and B) was significantly stronger than the loss observed for RANTES/CCL5 (3.6-fold decrease) (Fig. 5C). Moreover, mutation of F14A did not affect US28 binding of CX3CL1 at all (Fig. 5D), suggesting that considerable differences exist in the interaction of US28 with chemokines belonging to different classes. Analysis of the US28-Y16F mutant also resulted in loss of binding affinity. Unfortunately, the role of the aromaticity of the tyrosine residue could not be investigated, as US28-Y16A was impaired in its transport to the cell surface (Fig. 3B), and therefore no binding could be detected (data not shown). However, elimination of the hydroxyl group from Tyr-16 by the exchange with a phenylalanine residue (resulting in US28-Y16F) significantly reduced the binding affinity for all tested CC chemokines. As observed with US28-F14A, the loss of affinity was less dra-
matic for RANTES/CCL5 compared with Mip1α/CCL3 and Mip1β/CCL4 (Table I). In contrast to the F14A mutant, US28-Y16F also showed a small but statistically significant 2-fold decrease in affinity for fractalkine/CX3CL1 (Table I).

Radioligand dissociation studies are a very sensitive method to study receptor/ligand interaction. Because of the equation, $K_d = k_{off}/k_{on}$, the dissociation rate constant ($k_{off}$) is directly proportional to the affinity of a ligand for a given receptor. In order to investigate the importance of the small difference in affinity observed for CX3CL1 binding to US28-Y16F, dissociation studies were performed for the binding of $^{125}$I-labeled Fractalkine/CX3CL1 to US28-wild type, -F14A, and -Y16F receptors. As a control, the dissociation of $^{125}$I-labeled RANTES/CCL5 was studied at the same time. Membranes isolated from COS-7 cells transiently transfected with US28-wild type, -F14A, and -Y16F receptors were first allowed to equilibrate with the radioligand. Dissociation was started by the addition of a large excess of cold competitor, and the residual specific binding was monitored in time. As shown in Fig. 5A, the half-life of dissociation of $^{125}$I-CCL5 is 12 ± 3 min for wild type US28 and, as expected, the dissociation occurs much faster for both mutants F14A (4 ± 1 min) and Y16F (5 ± 1 min) (Fig. 6A).
In this study, we aimed at identifying key residues involved in the chemokine/US28 receptor interaction. In the effort to better understand how chemokines interact with US28, several Mip-1/β/CCL4 mutants were tested in equilibrium binding and functional assays. Structure-function studies indicate that chemokines have two major sites of interaction with their cognate receptors, comprising the flexible N-terminal portion that precipitates receptor activation (31, 34), and to induce a chemokine-mediated SMC migration (38). These US28 properties could potentially help HCMV escape immune surveillance and spread in the host (16).

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**Discussion**

US28 is a HCMV-encoded chemokine receptor that displays several interesting pharmacological features, among which the ability to act as a chemokine scavenger (30), to constitutively activate several pathways (31, 34), and to induce a chemokine-dependent SMC migration (38). These US28 properties could potentially help HCMV escape immune surveillance and spread in the host (16).

In summary, we have studied the role of aromatic residues in US28 binding to chemokines. Our findings suggest that sulfation of these residues is not important for high-affinity chemokine binding, if sulfation occurs at all. To test the hypothesis of tyrosine sulfation of Tyr-16 in more detail, US28-expressing COS-7 cells were incubated with sodium chlorate, NaClO₃ (10 mM), a competitive inhibitor of tyrosine sulfation enzymes (56–58). Indeed, upon incubation of US28-expressing cells in the presence of NaClO₃, chemokines showed a reduced affinity as shown for CCL4 (Fig. 8). The reduction in affinity determined with NaClO₃ (8-fold decrease) is comparable with the observed effect of the Y16F mutation (6-fold), suggesting that Tyr-16 of US28 is normally sulfated and that this modification is important for the high-affinity binding of chemokines to US28.

**Characterization of the Binding Site for HCMV-encoded US28**

**Fig. 7.** Role of aromatic/aromatic interactions. A and B, CCL4-WT (filled circles) and CCL4-F13A (open circles) were tested in competition binding experiments in COS-7 cells expressing either US28-WT (A) or US28-F14A (B) against 125I-CCL4. The data are normalized for nonspecific binding (0%) and maximal specific binding in the absence of a competitor (100%). A representative experiment out of three experiments is shown, each performed in triplicate.

**Fig. 8.** Role of tyrosine sulfation for chemokine binding at US28. COS-7 cells expressing US28-WT were incubated in the presence (open circles) or absence (filled circles) of sodium chlorate, an inhibitor of sulfation (10 mM), as described under “Material and Methods.” Subsequently, cells were tested in homologous competition binding experiments against 125I-CCL4. A representative experiment out of two experiments is shown, each performed in triplicate.

The dissociation of 125I-CX3CL1 from US28 wild type was ~3-fold slower (32 ± 3 min) compared with 125I-CCL5. No changes were observed for the dissociation of 125I-CX3CL1 binding to wild-type- and F14A-US28 receptor (31 ± 2 min), whereas the binding of 125I-CX3CL1 to Y16F-US28 showed a significant faster dissociation profile (16 ± 1 min; Fig. 6B). This observation implies that the small change in affinity of CX3CL1 for US28-Y16F, as determined with the equilibrium displacement studies, has important consequences in terms of dissociation profile.

**Mip-1/β/CCL4 Binding to US28 via Aromatic/Aromatic Interactions**—Following the identification of phenylalanine 14 in the N terminus of US28 as an important feature for high affinity binding of CC chemokines, we hypothesized that this aromatic residue might represent the interaction partner for the highly conserved aromatic residue after the C(X)₃C cluster in the chemokines. As mentioned before and can be seen in Fig. 2A, this aromatic residue is not present in CX3CL1, potentially explaining the observed lack of effect of the US28-F14A mutation on the affinity of CX3CL1. Moreover, in this study we show that mutation of the conserved aromatic residue in the chemokine Mip-1/β/CCL4 to alanine (CCL4-F13A) indeed resulted in a 11-fold decrease in affinity for US28-WT (Figs. 2A and 7A). Most interestingly, the shift in affinity observed for CCL4-F13A on US28-WT is in the same order of magnitude as observed for CCL4-WT on US28-F14A, suggesting that the two aromatic residues might be potential interaction partners. To test this hypothesis, we determined the affinity of CCL4-F13A for US28-F14A. As shown in Fig. 7B, no further shift in affinity is observed compared with CCL4-F13A, as determined in competition binding experiments against 125I-CCL4.

**Tyrosine Sulfation and Chemokine/US28 Interaction**—Mutation of tyrosine 16 in phenylalanine retains the aromatic character of the amino acid but eliminates the possibility of secondary modifications occurring at the hydroxy group, such as sulfation. Several studies have shown that the N terminus of chemokine receptors, such as CCR2 and CCR5, often shows sulfation of tyrosine residues and that this modification is affecting chemokine binding (50, 54). In silico prediction (“sulfinator” program available at www.expasy.org) indicates that Tyr-16 of US28 has a high probability of sulfation, and this hypothesis was also recently suggested by Lin et al. (55). Additionally, two tyrosine residues in the second extracellular loop of US28 (170DYYD177 motif) are also predicted to be sulfated (55). As shown in this study, the US28-Y16F mutant indeed loses considerable affinity for the tested chemokines. To check the role of the predicted sulfation of the second site (DYYD motif), the double mutant US28-DFDF was generated. As shown in Table 1, US28-DFDF binds all tested chemokines with high affinity and shows no difference with wild type US28, suggesting that sulfation of these residues is not important for high affinity chemokine binding, if sulfation occurs at all.
two contact regions to overall ligand affinity varies depending on the receptor examined and reflects synergy between several important contacts. Usually, the flexible N-terminal sequence of chemokines plays an important role in determining agonist activity (60). For example, N-terminal modification of RANTES/CCL5 by addition of a methionine, or truncation, results in an antagonist, whereas affinity for the cognate receptors is not affected (61). Similarly, here we show that truncating the N terminus of Mip-1α/CCL4 (resulting in CCL4-Δ8), does not influence binding at US28 (Fig. 1) but strongly impairs the agonistic properties of this chemokine. These data fit the currently accepted model describing chemokine/receptor interaction, the so-called two-site model, according to which the chemokine N terminus is an essential determinant for agonistic behavior. The N-loop of the chemokine, instead, is generally thought to play an important role for high affinity binding (46, 49). An amino acid alignment of all chemokines known to bind to US28 shows that an aromatic residue present in the N-loop region after the CX3C cluster is highly conserved (Fig. 2A), with CX3CL1/fraktalkine being the only exception. Within this study, we show that phenylalanine 13, the first residue after the CC cluster of CCL4, is an essential interaction partner for the high affinity binding of CCL4 to US28. Because of the high rate of conservation, it is likely that the results obtained with CCL4 are able to be extended to the other US28 ligands belonging to the CC chemokine family, and further studies are necessary in order to clarify this issue. Similarly, this aromatic residue has been shown to play a central role in the binding of other CC chemokines/receptors pairs, including CCL4 to CCR5 and CCL5 to CCR3 and CCR5 (40, 46). This aromatic residue is not shared by CXC and CX3C chemokines and may therefore represent a common binding site, specific for CC chemokines, that might contribute to the promiscuous binding of these ligands to CC chemokine receptors.

Chemokines possess several properties that might affect receptor binding and activation, such as interaction with GAGs and oligomerization in solution. How these properties influence chemokine biological responses is not clear at present. Several studies have shown that chemokine mutants with altered dimer interfaces, such as IL-8/CXCL8 N-methylated at Leu-25 (62) or CCL2-PSA (63), remain monomeric even at high protein concentrations and yet are fully functional in receptor binding and activation in vitro. Similarly, our data obtained with CCL4-PSA are indistinguishable from wild type CCL4, suggesting that the chemokine monomer is fully sufficient for binding to US28 and inducing a response.

Although there is general agreement that the ability of chemokines to bind GAGs has functional relevance for the generation of a gradient (8), the precise nature of the effect of GAGs on chemokine activity is not clear, particularly with respect to receptor binding. Results in the literature differ depending on the chemokine/receptor pair analyzed. For example, the presence of GAGs on the cell surface appears to be essential for CXCL1 binding and activation of its cognate receptor CXCR2 (10). In contrast, binding of CCL4 and CCL5 to CCR5 was shown to be largely unchanged in cells that do not express GAGs (64). The principal heparin-binding region of CC chemokines such as CCL3, CCL4, and CCL5 involves a classical BBXB cluster in the 40s loop that is also implicated in high affinity binding to cognate receptors, indicating a partial overlap in recognition site (48, 65). Most surprisingly, analysis of the 40s mutant CCL4-K45A/ R46A/K48A has shown that its affinity for US28 is comparable with WT-CCL4, highlighting differences in chemokine binding epitopes between US28 and CCR5. Furthermore, our results indicate that elimination of GAG binding does not influence the ability of CCL4 to bind and activate US28 in vitro.

According to the two-site model, extracellular domains of the receptor establish the high affinity interaction with chemokines. In particular, the N terminus of chemokine receptors, as shown for CCR3/CCR5, is an essential determinant in ligand binding (66–68). This is also the case for US28; we have shown previously that deletion of the first 22 amino acids (resulting in US28-Δ(2–22)) abrogates binding for all chemokines known to interact with this receptor (32). In order to understand which amino acids within the N terminus of US28 are involved, different truncation mutants were generated. Comparative analysis of their chemokine binding behavior indicated the importance of an hexapeptide sequence, which is conserved among US28, CCR1, and CCR2. Alanine-scanning mutagenesis of this region showed that none of the US28 receptors mutated at a single amino acid reproduced the binding behavior seen with the truncation mutant US28-Δ(2–16), i.e. complete loss of chemokine binding affinity, indicating that different residues within the hexad cooperatively participate in chemokine binding.

Because chemokines possess several positively charged residues and ionic interactions are thought to be important for high affinity binding (52, 53), we expected to see shifts in binding affinity when mutating the two acidic residues, Glu-13 and Asp-15, within the N terminus of US28. However, the mutants US28-E13A and -D15A showed an unaltered binding profile for all the tested chemokines (Table I). Although initially surprising, these results might be due to the fact that in the US28-N terminus there are six acidic residues all clustered together, and therefore mutation of a single one might not be sufficient to see changes in affinity. Alanine-scanning mutagenesis of US28 further indicated that residue Thr-12 is also partially involved in binding of CC chemokines. In silico analysis of the US28 sequence predicts O-glycosylation at this site (“NetOGlyc 2.0” program available at www.cbs.dtu.dk). Addition of O-linked glycans can occur at serine and threonine residues (69), and such modifications generally provide a larger and more flexible binding surface. O-Glycosylation has been shown to occur at the N terminus of CCR5 (70) and to affect high affinity binding of CCL3 and CCL4. Further experiments involving glycosidase treatment need to be performed in order to clarify the potential role of O-glycosylation for US28.

Most importantly, our data show that aromatic amino acids within this hexad play a fundamental role in the interaction of US28 with chemokines. Indeed, our results indicate that phenylalanine in position 14 of US28 is an important residue for CC chemokine binding, as can be seen by the shift in affinity for CCL3, CCL4, and CCL5. Similarly, mutagenesis performed on CCR2 showed that this conserved phenylalanine is essential for high affinity binding of CCL2 (50). Additionally, US28 shares several ligands, such as CCL3, CCL4, and CCL5, with CCR5. Scanning mutagenesis of CCR5 has identified several aromatic residues within its N terminus, which play an important role in CC chemokine binding (71). Although the overall sequence homology of US28, CCR2, and CCR5 is low, comparison of these results highlights the importance of aromatic residues within the N terminus of chemokine receptors for CC chemokine recognition.

Our results suggest that Phe-14 in the N terminus of US28 is the interaction partner for Phe-13 in the N-loop of CCL4, indicating that aromatic/aromatic interactions play a critical role in receptor/ligand interaction. Indeed, mutation of the receptor US28 or the chemokine CCL4 at these sites results in a similar shift in binding affinity, and combination of receptor mutant and chemokine mutant does not induce a further shift. This nonadditive behavior implies that the two aromatic residues possibly interact with each other.

Results obtained with alanine-scanning mutagenesis further
indicate clear differences in receptor epitopes involved in recognition of CC chemokines and the CX3C chemokine fractalkine, and possibly explain the lack of competitiveness among these different ligands, as reported previously (27). Indeed, mutation of both Thr-12 and Phe-14 within the US28 hexapeptide affects binding affinity for the US28 ligands belonging to the CC chemokine family, indicating overlapping epitopes for CC chemokines, without influencing fractalkine/CX3CL1 binding. Tyrosine 16 represents the only exception within the hexad; replacement of this residue with a phenylalanine has consequences for all chemokines tested.

Mutation of tyrosine 16 in phenylalanine retains the aromatic character of the amino acid but eliminates the possibility of secondary modifications occurring at the hydroxy group, such as sulfation. Tyrosine sulfation of proteins is a modification that widely occurs in multicellular eukaryotic organisms (72). This modification occurs post-translationally, in the trans part of the Golgi network (73). The most important feature of tyrosine sulfation consensus sequences is the presence of an acidic or neutral amino acid residue directly before a tyrosine to be sulfated (74). In silico analysis of US28 sequence predicts sulfation at Tyr-16 (55). Our results suggest that this secondary modification occurring in the N terminus of US28 contributes to high affinity chemokine binding. Blocking the sulfation process, either via mutation of Tyr-16 in Phe or treatment with sodium chlorate, results in a similar shift in binding affinity for chemokines. Sulfation is a common secondary modification occurring in chemokine receptors. For CCR2 and CCR5, similar experiments have shown that tyrosine sulfation is implicated in high affinity chemokine binding (50, 70). Additionally, the endogenous receptor of fractalkine, CX3CR1, has a tyrosine in its N terminus (Tyr-14) which is also sulfated (75). Mutation of Tyr-14 in phenylalanine for CX3CR1 has a limited effect in terms of affinity for fractalkine/CX3CL1, as measured in equilibrium binding assays, but resulted in a significant alteration of kinetics of interaction, determined by surface plasmon resonance (75). These results resemble data obtained with US28 and suggest that tyrosine sulfation of both these chemokine receptors is an important determinant in mediating a strong and long lasting interaction with fractalkine. It was shown previously that fractalkine, due to its unique properties such as the mucine stalk that anchors it to the cell surface and the chemokine domain itself, mediates firm adhesion of cells expressing CX3CR1 and US28 (76). In binding studies, fractalkine has a significantly slower off-rate from its receptors than any of the other tested chemokines, and the authors suggested that the unique ability of fractalkine to mediate adhesion under flow might be a function of its slow receptor off-rate (76). Our data confirm that fractalkine dissociation has a slower kinetic than the other tested chemokines and that its strong interaction with US28 depends on tyrosine sulfation of Tyr-16 at its N terminus. The strong interaction of US28 and fractalkine might have important consequences in vivo. US28 is expressed in infected monocytes (77), and the interaction of US28 with fractalkine expressed on the endothelium (78, 79) might increase the recruitment of monocytes at sites of inflammation (80). This monocyte recruitment could represent an additional way by which HCMV, through its receptor US28, facilitates the development of vasculopathies such as restenosis and atherosclerosis (20, 22, 23, 31).

Most interestingly, mutation of tyrosine 16 with phenylalanine, although impairing sulfation, still allows receptor expression at the cell surface, whereas replacement with an alanine results in intracellular receptor retention (Fig. 3B). These results suggest that an aromatic residue is essential in this position for proper receptor folding, and its absence probably causes significant perturbation in the conformation of the N terminus, resulting in intracellular retention.

In conclusion, this study represents the first attempt to understand how chemokines interact with the human cytomegalovirus-encoded US28 receptor. Our results show the importance of a hexad sequence in the US28 N terminus for chemokine recognition. Further alanine-scanning mutagenesis has indicated that several residues within this region, such as Thr-12, Phe-14, and Tyr-16, cooperate in determining high affinity chemokine binding. Moreover, in accordance with the two-site model, the N-terminal domain of chemokines is important for US28 receptor activation, whereas the N-loop plays a role for the high affinity interaction. In particular, we have shown that interaction of aromatic residues constitute points of tight binding in the CC chemokine/US28 receptor pair. Additionally, secondary modifications occurring at the receptor N terminus, such as sulfation and possibly glycosylation, play an important role in chemokine recognition and strongly affect kinetics of interaction. Moreover, by using CCL4 as model ligand, we have shown that chemokine properties such as dimerization and interaction with GAGs are not affecting chemokine binding and activation of US28 in vitro. By understanding how chemokines interact with the receptor at the molecular level will help in the design of specific ligands that can affect US28 binding and signaling profiles and represent potentially new tools in the study of cytomegalovirus-induced pathogenesis.

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