Selective recognition of the membrane-bound CX₃C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28

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Abstract The 7TM receptor, US28, encoded by human cytomegalovirus binds a broad spectrum of endogenous CC chemokines with sub-nanomolar affinity as determined in homologous competition binding assays. We here find that US28 also recognizes the membrane-associated CX₃C chemokine, fractalkine, with sub-nanomolar affinity ($IC_{50} = 0.42 \pm 0.09$ nM). Importantly, although fractalkine could compete with high affinity against the binding of CC chemokines, the secreted CC chemokines were only able to compete for binding against radioactive fractalkine with very low affinity. It is concluded that US28, which is known to enhance cell-cell fusion processes through interaction with an as yet unidentified, human cell-specific factor, has been optimized by cytomegalovirus to selectively recognize the membrane-associated fractalkine. It is suggested that US28 expressed on the surface of infected cells and possibly on the envelope of the virion is involved in transfer of the virus from cell to cell.

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Key words: Chemokine; Cytomegalovirus; Fractalkine; Viral receptor; Receptor state; Cell entry

1. Introduction

Human cytomegalovirus encodes four 7TM, G-protein-coupled receptors – US27, US28, UL33, and UL78 [1,2]. When the chemokine system was characterized at the molecular level, it became clear that at least US27 and US28 were homologues of human chemokine receptors. Accordingly, US28 but not US27 was shown to bind several human CC chemokines such as RANTES, MIP-1α, and MCP-1 [3,4]. Nevertheless, it was still unclear what benefit the virus could have from expressing such receptors. Knock-out of the UL33 gene in the mouse and rat cytomegalovirus (CMV) indicated that this receptor is important for the virulence of the virus. UL33 was apparently involved in the targeting and/or replication of the virus in salivary glands, which is a crucial point in the life cycle of the virus since spreading of virus from individual to individual occurs through the saliva [5]. Since US27 and US28 are only found in human CMV it has been impossible to perform similar gene knock-out experiments with these receptors in vivo. However, based on experiments performed in vitro, it has been suggested that the ability of the US28 receptor to bind and sequester a broad spectrum of CC chemokines could be a mechanism by which the virus eliminates these chemoattractants from the surroundings of infected cells [6,7]. However, this does not appear to be a very efficient process and it will furthermore only work at a short range.

Recently a new class of chemokines having three residues between the first two cysteines and therefore named CX₃C was discovered. Fractalkine or neurotactin is as yet the only known member of this CX₃C chemokine 'family' [8,9]. Fractalkine is not a secreted chemokine but a membrane protein consisting of a chemokine-like domain covalently associated through a mucin-like stalk to a transmembrane segment. The endogenous receptor for fractalkine is a previous orphan receptor called V28, now CX₃CR1 [10], only distantly related to the US28 receptor, as shown in Fig. 1. Nevertheless we find in the present study that US28 is able to recognize the CX₃C chemokine, fractalkine, very selectively. US28 at the same time functions as a broad-spectrum CC chemokine receptor. However, none of the secreted CC chemokines can compete with the binding of the radioactive CX₃C chemokine domain of fractalkine to the virally encoded US28 receptor. Since enveloped viruses such as CMV get access to their target cells through interaction of proteins in their membrane envelope with membrane proteins on their target cells [11] it is suggested that the highly specialized US28-fractalkine interaction is involved in the cell to cell transfer of human CMV.

2. Materials and methods

2.1. Materials

The human CC chemokines MIP-1α, MIP-1β, MCP-1 and RANTES were purchased from Peprotech, Rocky Hill, NJ. The CX₃C chemokine domain of fractalkine, corresponding to amino acids 1–69 of the mature peptide as well as the DNA encoding the US28 receptor were kindly provided by Timothy N.C. Wells, Serono, Geneva. The 'CX₃C+stalk', i.e. amino acids 1–317, corresponding to the CX₃C chemokine domain extended at the C-terminal end with the mucin-like stalk but lacking the transmembrane segment was kindly provided by Thomas J. Schall, ChemoCentryx, San Carlos, CA. Monoiodinated ^{125}I-MIP-1α, ^{125}I-MIP-1β, ^{125}I-RANTES and ^{125}I-MCP-1 were purchased from Amersham (Little Chalfont, UK). The ^{125}I-labeled CX₃C domain of fractalkine was prepared in-house using Na^{125}I (IMS30) (Amersham, Little Chalfont, UK) and the oxidizing agent Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) (Pierce, Rockford, IL). The ^{125}I-labeled CX₃C chemokine was HPLC purified before use.

2.2. Transfection and tissue culture

COS-7 cells were grown at 10% CO_2 and 37°C in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method [17].

2.3. DNA constructs

The US28 DNA was inserted into the pTEJ-8 expression vector [12] and its sequence was confirmed (ALFexpress, Pharmacia Amersham). The US28 clone used in the present study differed by 25 nucleotides

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from the one deposited in GenBank, accession number L20501, which was originally cloned from the *VHL/E* strain of human CMV. These substitutions correspond to five relatively conservative amino acid substitutions (D18E, E19D, L25F, K267R, and A346V), indicated with asterisks in Fig. 1.

2.4. Binding experiments

COS-7 cells were transferred to 24 well culture plates 1 day after transfection. The number of cells seeded per well was such as to obtain 5–10% specific binding of the added radioactive ligand. Two days after transfection competition binding was performed on whole cells for 3 h at 4°C using 12 pM of either ^{125}I-MIP-1α, ^{125}I-MIP-1β, ^{125}I-RANTES, ^{125}I-MCP-1 or ^{125}I-CX$_3$C plus variable amounts of unlabeled ligand in 0.5 ml of a 50 mM HEPES buffer, pH 7.4 supplemented with 1 mM CaCl$_2$, 5 mM MgCl$_2$ and 0.5% (w/v) bovine serum albumin. After incubation the cells were quickly washed four times in 4°C binding buffer supplemented with 0.5 M NaCl to reduce unspecific binding and interference caused by ligand dimerization. Non-specific binding was determined as the binding in the presence of 0.1 μM unlabeled chemokine. Determinations were made in duplicate.

2.5. Calculations

IC$_{50}$ values were determined by non-linear regression and K_D and B_{max} values were calculated from competition binding experiments using the equations $K_D = IC_{50} - L$ and $B_{max} = B_0(1+(K_D/L))$ using the Inplot 4.0 software (GraphPad Software, San Diego, CA).

3. Results

The US28 receptor was expressed transiently in COS-7 cells and competition binding experiments were performed on whole cells at 4°C using four different radioactively labeled human CC chemokines as tracer: ^{125}I-RANTES, ^{125}I-MCP-1,

^{125}I-MIP-1α, or ^{125}I-MIP-1β. US28 bound all of these soluble CC chemokines with almost similar, sub-nanomolar affinity as determined in homologous displacement experiments: RANTES (IC$_{50}$ = 0.50 nM), MCP-1 (IC$_{50}$ = 0.76 nM), MIP-1α (IC$_{50}$ = 0.71 nM), and MIP-1β (IC$_{50}$ = 0.72 nM) (Fig. 2 and Table 1). This confirms previously published results using ^{125}I-MIP-1α, ^{125}I-RANTES and ^{125}I-MCP-1 [3,4,13]. Thus, in homologous binding experiments the human CMV-encoded US28 receptor appears to be a broad-spectrum receptor for the CC chemokines of its host.

When the CX$_3$C chemokine domain of fractalkine was applied in competition binding assays against the radioactively labeled RANTES, MCP-1, MIP-1α, or MIP-1β, the CX$_3$C domain, just like the CC chemokines, was able to bind to the US28 receptor with sub-nanomolar affinity, IC$_{50}$ being between 0.29 and 0.51 nM (Fig. 2 and Table 1). In all cases the Hill coefficient of the competition curve for the CX$_3$C domain was higher than the Hill coefficient for the corresponding homologous displacement curve (Fig. 2 and Table 1).

When the ^{125}I-CX$_3$C chemokine domain of fractalkine was used as tracer, the CX$_3$C domain itself displaced this with an IC$_{50}$ of 0.42 nM in homologous competition experiments, corresponding closely to the IC$_{50}$ values observed for the CX$_3$C domain in heterologous competition against the soluble CC chemokines on the US28 receptor (Fig. 3A and Table 1). Surprisingly, however, all the CC chemokines displayed a very poor affinity in heterologous competition binding experiments against ^{125}I-CX$_3$C despite the high affinity of these peptides determined in homologous binding assays with the US28 re-
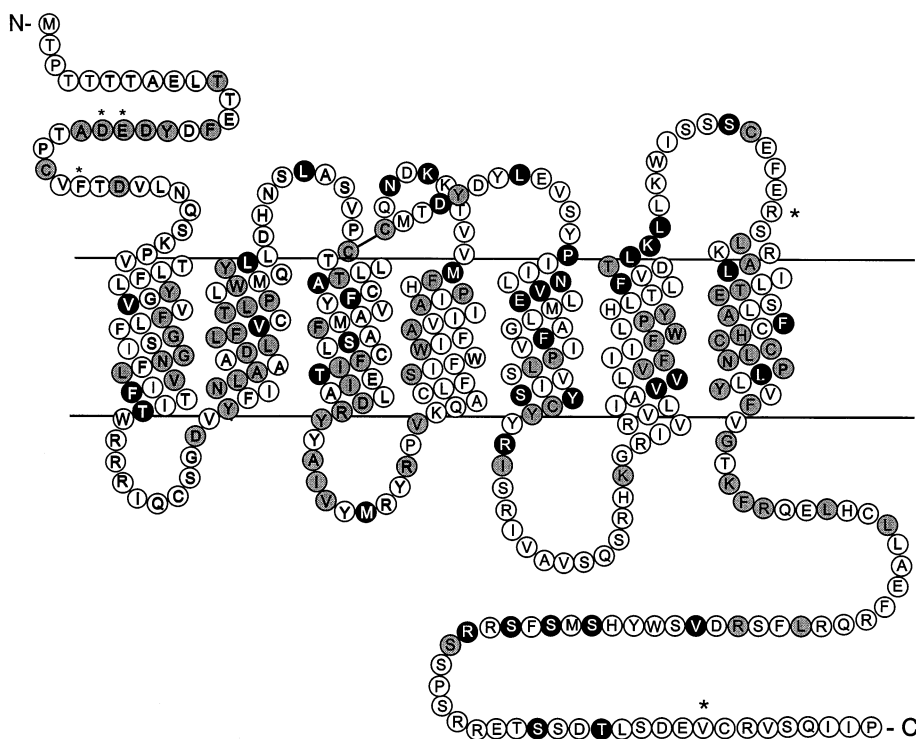

Fig. 1. Serpentine diagram of the CMV-encoded US28 receptor. Residues that are identical in US28 and in the endogenous receptor for fractalkine, CX$_3$CR1 (previously V28), but are not shared with the CC -chemokine receptors are indicated in white on black. Residues conserved among CC chemokine receptors (CCR1, CCR2, CCR3, and CCR5) and US28 are indicated in black on gray. The US28 receptor used in this study differed from GenBank accession number L20501 in five amino acids, indicated with asterisks (see Section 2).
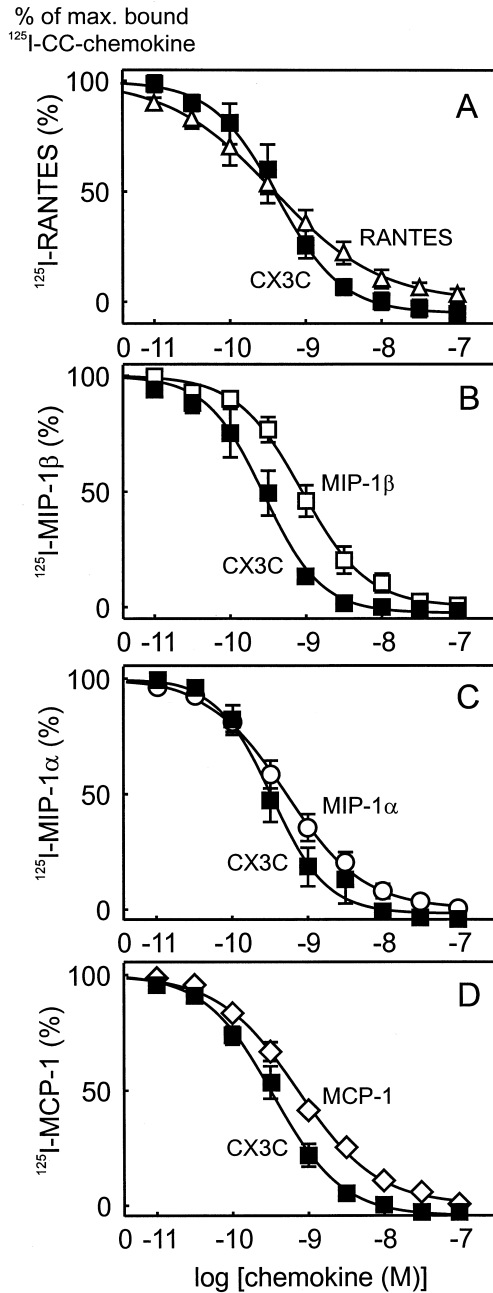
Fig. 2. Competition binding experiments with US28 using radioactive CC chemokines as tracer. Binding was performed on whole COS-7 cells transiently expressing US28 with either ^{125}I-RANTES (A), ^{125}I-MIP-1β (B), ^{125}I-MIP-1α (C) or ^{125}I-MCP-1 (D). Competitor ligands were the CC chemokines RANTES (△), MIP-1β (□), MIP-1α (○), and MCP-1 (◇) as well as the CX$_3$C chemokine domain of fractalkine (■).
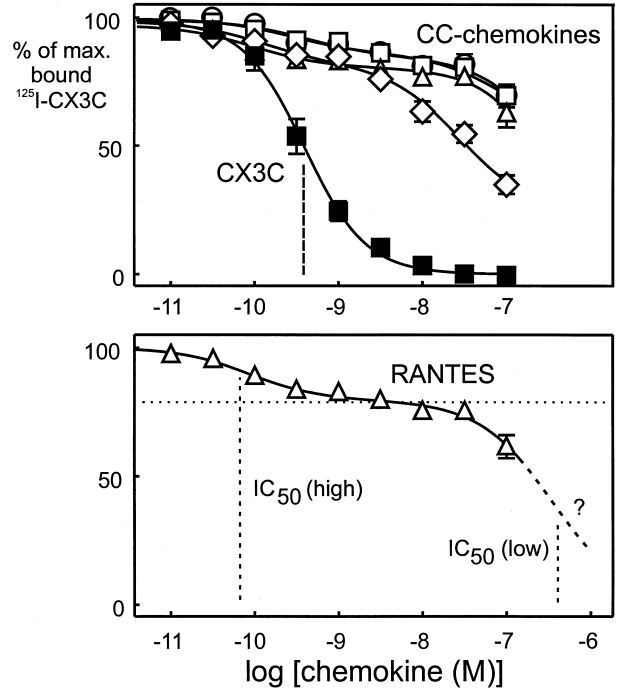

Fig. 3. Competition binding experiments with US28 using the radioactive CX$_3$C chemokine domain from fractalkine as tracer. Binding was performed on whole COS-7 cells transiently expressing US28. A: Competition binding curves for RANTES (△), MIP-1β (□), MIP-1α (○), MCP-1 (◇), and the CX$_3$C chemokine domain of fractalkine (■). B: The presumed high affinity displacement mode (IC$_{50}$-high) and low affinity displacement mode (IC$_{50}$-low) for the CC chemokines against the radioactive CX$_3$C chemokine domain of fractalkine are shown here for RANTES.

ceptor (Fig. 3A,B). The heterologous competition curves for the CC chemokines against ^{125}I-CX$_3$C were in fact each composed of two components (Fig. 3A,B). Thus, 10–20% of the ^{125}I-CX$_3$C was displaced by the CC chemokines with the sub-nanomolar IC$_{50}$ values known from the homologous competition assays, whereas the remaining 85% was displaced by the CC chemokines with IC$_{50}$ values 100–1000-fold higher (Fig. 3A,B and Table 1).

It was not possible with the available tools to determine the

affinity of the US28 receptor for the native fractalkine molecule as such since both fractalkine and US28 are integral membrane proteins. However, US28 did bind the CX$_3$C chemokine domain of fractalkine with high affinity when it was attached to its mucin stalk (IC$_{50}$ = 2.8 nM) as determined in competition against ^{125}I-CX$_3$C (Fig. 4). This affinity is only approximately seven times lower than the affinity determined for the free CX$_3$C chemokine domain alone. Conceivably, a
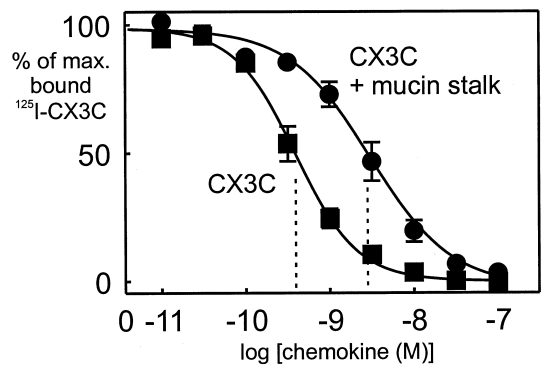

Fig. 4. Competition binding experiments with US28 using the radioactive CX$_3$C chemokine domain of fractalkine as tracer. Binding was performed on whole COS-7 cells transiently expressing US28. Competitor ligands were the CX$_3$C domain (■) and the CX$_3$C domain covalently associated with the mucin-like stalk of fractalkine, CX$_3$C+stalk (●).

Table 1
Binding constants for selected CC chemokines and the CX_3C chemokine domain of fractalkine on the human CMV-encoded US28 receptor using different radioactive ligands

	B_{max} (fmol/10^5 cells ± S.E.M.)	IC_{50} in nM ± S.E.M. (n); (Hill coefficient ± S.E.M.)				
		CX_3C	MIP-1α	MIP-1β	RANTES	MCP-1
^{125}I-CX_3C	19 ± 3.6	0.42 ± 0.09 (6) (1.24 ± 0.08)	0.4/488 (7)	0.1/477 (4)	0.1/356 (5)	0.2/74 (4)
^{125}I-MIP-1α	14 ± 4.5	0.50 ± 0.21 (4) (1.23 ± 0.15)	0.71 ± 0.20 (5) (0.88 ± 0.09)	–	–	–
^{125}I-MIP-1β	32 ± 12	0.29 ± 0.09 (3) (1.34 ± 0.23)	–	0.72 ± 0.26 (3) (0.89 ± 0.14)	–	–
^{125}I-RANTES	41 ± 17	0.51 ± 0.17 (4) (1.17 ± 0.10)	–	–	0.50 ± 0.14 (4) (0.69 ± 0.08)	–
^{125}I-MCP-1	46 ± 7.4	0.37 ± 0.07 (5) (1.05 ± 0.09)	–	–	–	0.76 ± 0.12 (5) (0.84 ± 0.06)
Fold difference		1	687	663	712	97

The US28 receptor was transiently expressed in COS-7 cells and competition binding experiments were performed on whole cells as described in the text. Both homologous competition experiments – indicated in the diagonal of the table – and heterologous competition experiments against ^{125}I-CX_3C – indicated in the upper row – and heterologous competition for CX_3C against the CC chemokines – indicated in the first column – were performed. For the heterologous displacement of ^{125}I-CX_3C by CC chemokines IC_{50} values for both the minor, high affinity displacement mode and the major, low affinity displacement mode are indicated (see Fig. 3) and consequently no Hill coefficients are indicated in those cases. 'Fold difference' indicates the ratio between the IC_{50} value determined for the CC chemokines in homologous versus the major, low affinity mode determined in heterologous binding experiments.

multivalent interaction mode involving several molecules on each side will in fact occur in an envisioned interaction between US28 located either on the envelope of a CMV virion or on the surface of a CMV infected cell and fractalkine on the surface of the presumed target cell.

4. Discussion

4.1. Selective US28-fractalkine recognition

The phenomenon that a receptor can function at the same time as a broad-spectrum receptor and as a highly selective
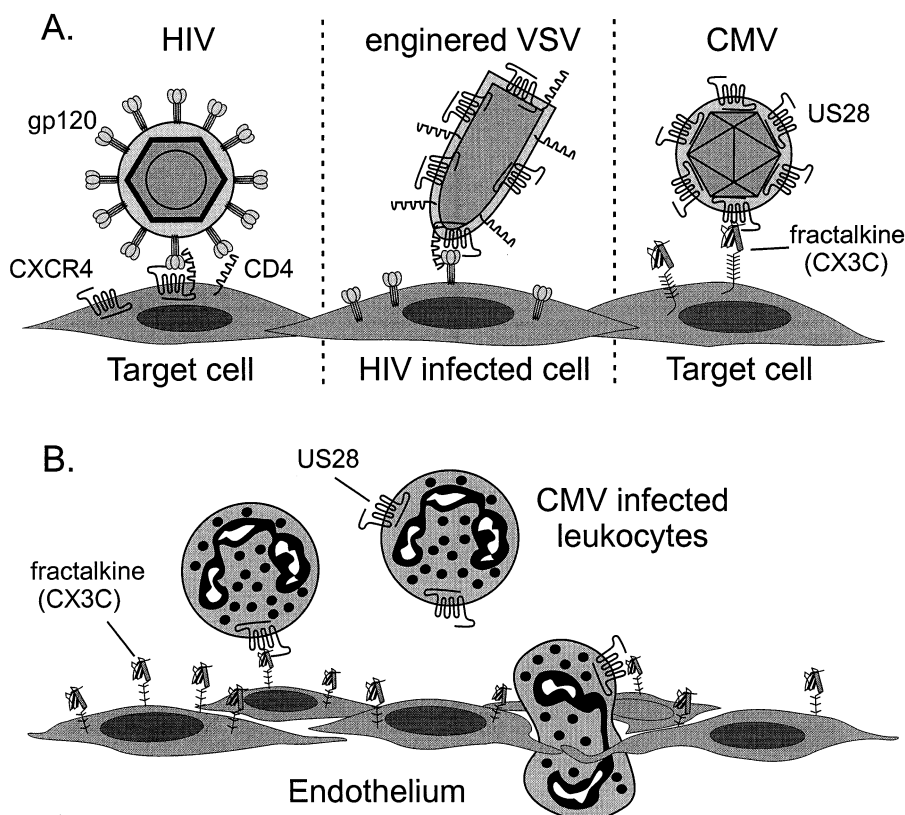

Fig. 5. Diagram of proposed membrane protein interactions in the binding and fusion process between various virus and target cells. A: Left panel: HIV enveloped protein gp120 binding to CD4 plus one of several chemokine receptors, here CXCR4. Middle panel: targeting of vesicular stomatitis virus (VSV) to HIV-infected target cells expressing gp120 after substitution of the VSV normal spike protein G with CD4 and CXCR4 [24,25]. Right panel: proposed target cell binding of CMV virion through interaction of its US28 chemokine receptor to the membrane associated fractalkine with its CX_3C chemokine domain. B: Proposed involvement of the US28-CX_3C binding in the dissemination of the virus via tight cell-cell interactions/fusion. CMV-infected leukocytes express US28 in their cell membrane and could facilitate the interaction of the infected cells with a target endothelial cell expressing fractalkine with its CX_3C chemokine domain.

receptor has previously been studied especially in the tachykinin system. The NK_1 receptor, which originally was considered to be highly selective for the neuropeptide, substance P, was recently found to bind basically all tachykinin peptides with sub-nanomolar affinity [14,15]. Moreover, all these peptides could function as agonists on the receptor. This observation could explain, for example, the stimulatory function of NKA at sites in the CNS where no 'normal' NK_2 receptor was present. The problem had previously been that NKA and other non-substance P tachykinins competed only very poorly against radioactive substance P [14] – just as the CC chemokines all compete poorly against the radioactive CX_3C on US28 in the present study. Also in the case of the NK_1 receptor, the 'selectively' recognized ligand, in this case substance P, competes readily against the non-substance P tachykinin peptides [14] – just as fractalkine competes readily against the secreted CC chemokines on US28.

The structural or cell biological reason for this non-classical competitive behavior of different ligands – usually agonists – for the same receptor is still unclear [15]. However, it has been suggested that it may be related to the interaction of the receptor with different G-proteins, which may be compartmentalized in the cell. Another possibility is that monomeric versus dimeric forms of the receptor could selectively recognize certain G-proteins and/or ligands. Whatever the structural reason, the phenomenon appears to be observed in many different 7TM receptor systems.

4.2. Is fractalkine a target cell receptor for human CMV?

The life cycle of CMV within its host is complicated and has still not been totally clarified. Most certainly it involves viral transfer between several cell types. A number of not very well defined proteins of various molecular sizes have been suggested as target cell receptors for human CMV [11,16,17]. But none of these have yet been generally accepted. Both fractalkine and US28 are located appropriately for being involved in cell targeting and/or cell entry of CMV. Thus, fractalkine is expressed on, for example, endothelial cells as well as several other putative target cells for CMV [8,9,18]. US28 is expressed on the surface of CMV infected cells [6,7,19,20]. And – although it has not yet directly been demonstrated for US28 – two of the other CMV-encoded 7TM receptors, UL33 and US27, have been shown by immunochemistry to be located in the envelope membrane of human CMV ([2] and H. Browne, personal communication). Interestingly, although US28 can recognize a number of CC chemokines with high affinity, none of these secreted, endogenous chemokines can in fact hinder the binding of US28 to the membrane-associated fractalkine, i.e. they cannot block the hypothetical virus entry/transfer process (Fig. 5).

The US28 receptor co-expressed with CD4 has recently been shown to be able to mediate cell fusion with HIV-1 gp120 expressing cells [21,22]. Importantly, US28 can function as a co-receptor mediating cell fusion together with not only CD4 but also, for example, envelope proteins from other viruses such as vesicular stomatitis virus (VSV) and human T-cell lymphoma virus-1 (HTLV-1) [22]. It was concluded by Pleskoff and coworkers that US28 enhanced the cell fusion process mediated by different viral proteins through a specific interaction with an as yet unidentified membrane protein found on human cells [22]. We would suggest that this membrane protein is fractalkine and that the US28-fractalkine interaction has been optimized as part of the cell-cell transfer mechanism for CMV in man (Fig. 5B). Chemokine 7TM receptors expressed on the target cells are obviously already known to be efficient coreceptors in the cell entry process for HIV-1 [23]. It is known from genetically engineered rhabdovirus, e.g. VSV, that the glycoprotein-7TM receptor complex functions equally well in cell entry when the 7TM is expressed on the virus and the viral envelope protein 'ligand' is expressed on target cells [24,25]. Thus, we here suggest that such a system has already been developed naturally in human CMV (Fig. 5).

In the CMV-target cell interaction several proteins on each side are probably involved just as is the case for many other viral cell entry processes [11]. Thus, although US28 can assist in the cell fusion process with human cells – supposedly expressing fractalkine – cell fusion does not take place in these in vitro systems without the co-expression of additional known viral envelope proteins, for example proteins from HIV-1, VSV, or HTLV-1 [22]. Moreover, human CMV in which US28 has been genetically eliminated can still infect tissue culture cells equally efficient as wild-type virus, e.g. under the special circumstances normally used in vitro [6,7]. Although we here propose that fractalkine may function as a target cell receptor for human CMV, it should be noted that several other proteins having characteristics significantly different from those of fractalkine have previously been suggested to be receptors for CMV [11,16, 17,26].

4.3. Why is US28 also a broad-spectrum CC chemokine receptor?

Not only membrane proteins as such but also heparan sulfate has been suggested to be involved as target cell receptors for human CMV [11]. In the present context, this could be interesting since chemokines are known to bind to glycosaminoglycans [27,28]. An interesting, albeit speculative possibility could be that US28 recognition of glcosaminoglycan-bound CC chemokines is involved in this process.

A second possibility could be that the binding of the endogenous soluble secreted CC chemokines could serve to protect the US28 receptor from being recognized as a foreign molecule by the immune system. Chemokines are relatively large ligands, which could rather effectively shield the receptor.

Finally, US28-CC chemokine interaction may be involved in tissue targeting of the virus. In this connection it may be important that CMV at a certain stage in its life cycle infects neutrophil granulocytes [29,30], which normally respond only to CXC chemokines and not to CC chemokines. Thus, since US28 functions as a signaling G-protein-coupled receptor in CMV-infected cells in vitro [20], it is possible that the endogenous granulocytes after infection with CMV become responsive to CC chemokines and thereby obtain the signalling machinery for transporting the virus to a supposedly appropriate CC-secreting tissue.

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