Yaba-like disease virus protein 7L is a cell-surface receptor for chemokine CCL1

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Received 21 August 2003
Accepted 15 September 2003

Yaba-like disease virus (YLDV) genes *7L* and *145R* are located on opposite ends of the genome and are predicted to encode 7-transmembrane proteins (7-TM) that share 53 and 44 % amino acid identity, respectively, to human CC chemokine receptor 8 (hCCR8). In this report, we demonstrate that early after infection with YLDV, cells acquire the ability to bind human CCL1. By expression of genes *7L* and *145R* in vaccinia virus, we demonstrated that each protein is glycosylated and is exposed on the cell surface with the N terminus outside the cell. Protein 7L, but not 145R, is able to bind hCCL1 ($K_d = 0{\cdot}6 \pm 0{\cdot}13$ nM) and couple to heterotrimeric G-proteins and to activate the extracellular signal-regulated kinases (ERK1/2). 7L binds several chemokines including the viral chemokines vMIPI and vMIPII and hCCL7/MCP3. This binding seems species-specific as 7L does not bind the murine orthologues of CCL1 and CCL7 in the assays used. This represents the first example of a poxviral 7-TM chemokine receptor that has functional interactions with a human chemokine.

INTRODUCTION

During evolution large DNA viruses, such as poxviruses and herpesviruses, have acquired genes to counter the host responses to virus infection. In particular, these viruses have acquired many genes that target the cytokine and chemokine network (for reviews see Murphy, 2001; Alcamí, 2003). Virus-encoded chemokines (vCKs) may function as agonists or antagonists and aid virus dissemination and growth (Murphy, 2001). Virus-encoded, secreted chemokine-binding proteins (vCKBP), which are unrelated to host chemokine receptors, block binding of chemokines to glycosaminoglycans (GAG), chemokine receptors or both (Alcamí, 2003). Virally encoded 7-transmembrane (7-TM) proteins with similarity to host chemokine receptors (vCKR) may interfere with the normal receptor function or signal constitutively (Rosenkilde *et al.*, 2001).

Herpesvirus vCKRs include those encoded by human cytomegalovirus (HCMV) gene *US28* (Neote *et al.*, 1993; Gao & Murphy, 1994) and by the gammaherpesvirus ORF74 family (Arvanitakis *et al.*, 1997). The mechanisms of action of these vCKRs include constitutively activated receptors that induce cell proliferation (Bais *et al.*, 1998) and receptors with high affinity for chemokines that cause depletion of endogenous ligands (Bodaghi *et al.*, 1998). vCKRs localize to endosomes and/or the plasma membrane and this may enable their incorporation into the viral particles during virus assembly (Fraile-Ramos *et al.*, 2001, 2002). Several vCKRs from poxviruses have been predicted from DNA sequence data (Massung *et al.*, 1993; Cao *et al.*, 1995; Afonso *et al.*, 2000, 2002; Lee *et al.*, 2001; Tulman *et al.*, 2002) but hitherto these were uncharacterized. This report concerns two 7-TM proteins encoded by Yaba-like disease virus (YLDV) (Lee *et al.*, 2001).

YLDV belongs to the *Yatapoxvirus* genus of the *Chordopoxvirinae* and causes infections in primates (Knight *et al.*, 1989) that are characterized by an acute febrile illness accompanied by localized skin lesions. These viruses and the genes they encode are poorly characterized. YLDV genes *7L* and *145R* are of particular interest because the predicted proteins show 53 and 44 % amino acid identity, respectively, to the human CC chemokine receptor 8 (CCR8).

CCR8 was identified as a receptor for CCL1 (I-309) (Roos *et al.*, 1997; Tiffany *et al.*, 1997) although CCL4 (MIP-1β) and CCL17 (TARC) have subsequently been described as agonists (Bernardini *et al.*, 1998; Garlisi *et al.*, 1999). Chemokines of viral origin such as the viral macrophage inflammatory proteins 1 and 2 (vMIP-I and vMIP-II) encoded by human herpesvirus 8 (HHV8) (Sozzani *et al.*, 1998; Dairaghi *et al.*, 1999; Endres *et al.*, 1999) and the

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chemokine 1 (vMCC-1) (Luttichau *et al.*, 2001) of *Molluscum contagiosum virus* (MCV) also bind CCR8.

CCR8 is expressed by thymocytes ($CD4^+$ cells) (Kremer *et al.*, 2001), monocytes (Tiffany *et al.*, 1997), polarized Th2 cells (D'Ambrosio *et al.*, 1998), NK cells, skin-homing CLA-positive T cells and regulatory T cells (Colantonio *et al.*, 2002). CCR8 influences the positive selection of murine thymocytes during T cell maturation (Kremer *et al.*, 2001) and its expression on T cells in the allergen-challenged lung implies a role in the pathogenesis of allergen-induced late asthmatic responses. CCR8 knockout mice ($CCR8^{-/-}$) showed defective Th2 immune responses and impaired eosinophil recruitment (Chensue *et al.*, 2001). However, these results have not been reproduced fully by other groups (Bishop & Lloyd, 2003; Chung *et al.*, 2003; Goya *et al.*, 2003).

Here we show that YLDV-infected cells, but not uninfected cells, bind hCCL1. This binding activity is mediated by YLDV protein 7L, which binds hCCL1 with high affinity and leads to functional responses. This is the first demonstration of a poxvirus 7-TM protein that binds chemokines.

METHODS

Cells and viruses. Owl monkey kidney (OMK), CV-1, BSC-1, TK^- 143B and Jurkat cells were grown as described previously (Alcamí & Smith, 1992; Lee *et al.*, 2001; Auger *et al.*, 2002). YLDV (Davis strain, ATCC VR-937) was obtained from the ATCC and grown and purified as described (Lee *et al.*, 2001). *Vaccinia virus* (VV), strain Western Reserve (WR) mutant vΔB8R, lacking gene *B8R* encoding the interferon-γ binding protein, was described previously (Symons *et al.*, 2002).

Reagents. Radioiodinated (^{125}I-labelled-) hCCL1 (2200 μCi $mmol^{-1}$) was purchased from DuPont-NEN. [^{35}S]GTP-γ-S (>1000 μCi $mmol^{-1}$) was from Amersham. Recombinant human, murine and viral chemokines were from Peprotech Inc. except for TCA-3/mCCL1, vMIP-I and vMCC-II, which were purchased from R&D Systems.

Plasmid and recombinant virus construction. Oligonucleotide primers to amplify genes *7L* and *145R* from YLDV genomic DNA also encoded an (HA) epitope to enable detection of recombinant protein with an anti-HA mAb, and *Sma*I, *Eco*RI and *Xba*I sites to facilitate cloning into different expression vectors. Restriction sites are underlined, the HA sequence is in bold and start and stop codon are in italics. Oligonucleotides (1) 5′-GCCCCCGGGGAATTCGCCACC*ATG*GAA**TACCCATACGATGTTCCAGATTACGCT**AAATACGTTATTACTATAAAC-3′ and (2) 5′-CACCCCGGGTCTAGA*TTA*AAGAGCATTTTGAGCACATGTGCTTTTTG-3′ generated a fragment encoding 7L with the HA-tag in the N terminus ($7LHA_N$). Oligonucleotides (3) 5′-GCCCCCGGGGAATTCGCCACC*ATG*GAAAAATACGTTATTACTATAAAC-3′ and (4) 5′-CACCCCGGGTCTAGA*TTA***AGCGTAATCTGGAACATCGTATGGGTA**AAGAGCATTTTGAGCAC-3′ generated a fragment encoding 7L with the HA-tag in the C terminus ($7LHA_C$). Oligonucleotides (2) and (3) generated a fragment encoding 7L (7L). Similarly for 145R oligonucleotides (5) 5′-GCCCCCGGGGAATTCGCCACCATGGAATACCCA**TACGATGTTCCAGATTACGCT**GAAACAACGGTTTTCGTAG-3′ and (6) 5′-GCGCCCGGGTCTAGATCATATAACATTATTAGAAGATTGTCTAATAAAAATG-3′ generated a fragment encoding 145R with the HA-tag in the N terminus ($145RHA_N$); oligonucleotides (7) 5′-GCCCCCGGGGAATTCGCCACCATGGAAACAACGGTTTTCGTAG-3′ and (8) 5′-GCGCCCGGGTCTAGATCA**AGCGTAATCTGGAACATCGTA**TGGGTATATAACATTATTAGAAGATTG-3′ generated a fragment encoding 145R with the HA-tag in the C terminus ($145RHA_C$) and oligonucleotides (6) and (7) generated a fragment encoding 145R (145R). Platinum Taq (Gibco-BRL)-amplified products were cloned into the *Sma*I site of VV expression vector pSC11(Chakrabarti *et al.*, 1985). The pSC11-derived plasmids (pSC11-7L, pSC11-$7LHA_N$, pSC11-$7LHA_C$, pSC11-145R, pSC11-$145RHA_N$, pSC11-$145RHA_C$) were transfected individually into cells infected with vΔB8R (Symons *et al.*, 2002) to obtain the recombinant viruses vΔB8R-7L, vΔB8R-$7LHA_N$, vΔB8R-$7LHA_C$, vΔB8R-145R, vΔB8R-$145RHA_N$ and vΔB8R-$145RHA_C$. TK-negative, β-galactosidase-positive recombinant viruses were isolated as described (Chakrabarti *et al.*, 1985). For transient expression assays HA-tagged versions of hCCR8 and 7L were ligated into expression vector pcDNA 3.1 from Invitrogen to generate pcDNA-CCR8HA and pcDNA-$7LHA_N$.

Binding assays. OMK or TK^- 143B cells were infected at the indicated p.f.u. per cell for 6 or 16 h. Cells were detached from the plastic support with 0·5 mM EDTA pH 8·0 in PBS, resuspended in binding buffer (MEM, 1 % FBS, 20 mM HEPES pH 7·4) at 10^6 cells ml^{-1} and incubated with ^{125}I-labelled hCCL1 as described below. To separate cells from unbound ligand, cells were centrifuged through an oil cushion (dioctyl phthalate/dibutyl phthalate, 2 : 3, v/v) for 1 min at 13 000 r.p.m in a Beckman tabletop centrifuge at 20 °C. The bottom of each tube containing the cell pellet was excised and the radioactivity was measured in a gamma counter (LKB). Experiments were performed in mock-infected and infected cells in parallel and each datum point was obtained in duplicate.

For kinetic binding experiments, ^{125}I-labelled hCCL1 at 200 pM was added to the cell suspension and samples were taken at various times thereafter to measure specific binding and the association rate. Once equilibrium was reached, further binding was blocked by addition of 100-fold excess of unlabelled ligand and 20-fold dilution of the mixture. After dissociation was initiated the remaining cell-associated ligand was measured over time to obtain the 'off rate'. In saturation binding experiments ^{125}I-labelled hCCL1 was added to the cells at increasing concentrations and binding was for 2 h at 4 °C. In competitive binding experiments, cells were incubated with ^{125}I-labelled hCCL1 at 100 pM with or without increasing concentration hCCL1 (homologous competition curve) or a 10^4-fold excess of various unlabelled chemokines. Data were analysed using Prism 3.0, GraphPad Software Inc.

Preparation of membranes. TK^- 143 cells were collected and washed in ice-cold PBS. After resuspension in hypotonic buffer (10 mM Tris/HCl, pH 7·4, 10 mM NaCl, 1·5 mM $CaCl_2$) cells were swollen for 10 min at 4 °C and ruptured by shearing in a Dounce homogenizer. An equal volume of 0·68 M sucrose, 20 mM Tris/HCl pH 7·4 was added and nuclei were removed by centrifugation at 1000 ***g*** for 10 min at 4 °C. Membranes in the supernatants were sedimented at 49 000 ***g*** for 20 min at 4 °C and resuspended in 0·34 M sucrose, 10 mM Tris/HCl pH 7·4, aliquoted and stored at −80 °C. Protein concentration was determined by the Bradford method (Bradford, 1976).

^{35}S[GTP-γ-S] binding to membranes. Membranes from cells infected at 1 p.f.u. per cell for 16 h were isolated as above and 4 to 10 μg of protein per sample was incubated with CCL1 for 1 h at 28 °C in binding buffer (20 mM HEPES pH 7·4, 10 μM GDP, 100 mM NaCl, 5 mM $MgCl_2$, 0·2 % BSA). Then [^{35}S]GTP-γ-S was added to 100 pM and reactions were incubated for 1 h at 28 °C. Samples were collected on a GF/B Unifilter plate (Perkin Elmer) using a cell harvester and radioactivity was measured by liquid scintillation with a TopCount microplate scintillation and luminescence counter (Packard). Nonlinear regression analysis of the data was done with Prism 3.0.

Immunofluorescence. BSC-1 cells were infected with vΔB8R, vΔB8R-7LHA$_N$, vΔB8R-7LHA$_C$, vΔB8R-145RHA$_N$ or vΔB8R-145RHA$_C$, at 5 p.f.u. per cell for 18 h. Surface staining on live cells and on fixed and permeabilized cells was done as described previously (Bartlett *et al.*, 2002).

Immunoblotting. OMK cells were mock-infected or infected with YLDV or VV WR strain at 10 p.f.u. per cell for the times indicated. Additional treatments during adsorption and infection were cytosine β-D-arabinofuranoside (AraC) at 40 μg ml^{-1} and tunicamycin at 1 μg ml^{-1} added during adsorption and infection where stated. Cells were resuspended in lysis buffer [10 mM Tris/HCl pH 8·0, 1 mM $MgCl_2$, 150 mM NaCl, Miniprotean proteinase cocktail inhibitor (Roche) and 3 % NP-40] and incubated on ice for 1 h at 4 °C. Cell lysates were clarified by centrifuging at 10 000 ***g*** for 10 min at 4 °C. Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were probed with mouse anti-HA mAb (1 : 1000) (Covance), mouse anti-D8L (1 : 1000) (Parkinson & Smith, 1994), rabbit anti-activated ERK1/2 (1 : 1000) or rabbit anti-ERK1/2 (1 : 1000) (both from Promega) followed by the corresponding peroxidase-labelled anti-mouse or anti-rabbit Ig Ab (Sigma) (1 : 1000). Blots were developed with the enhanced chemiluminescence detection system (ECL).

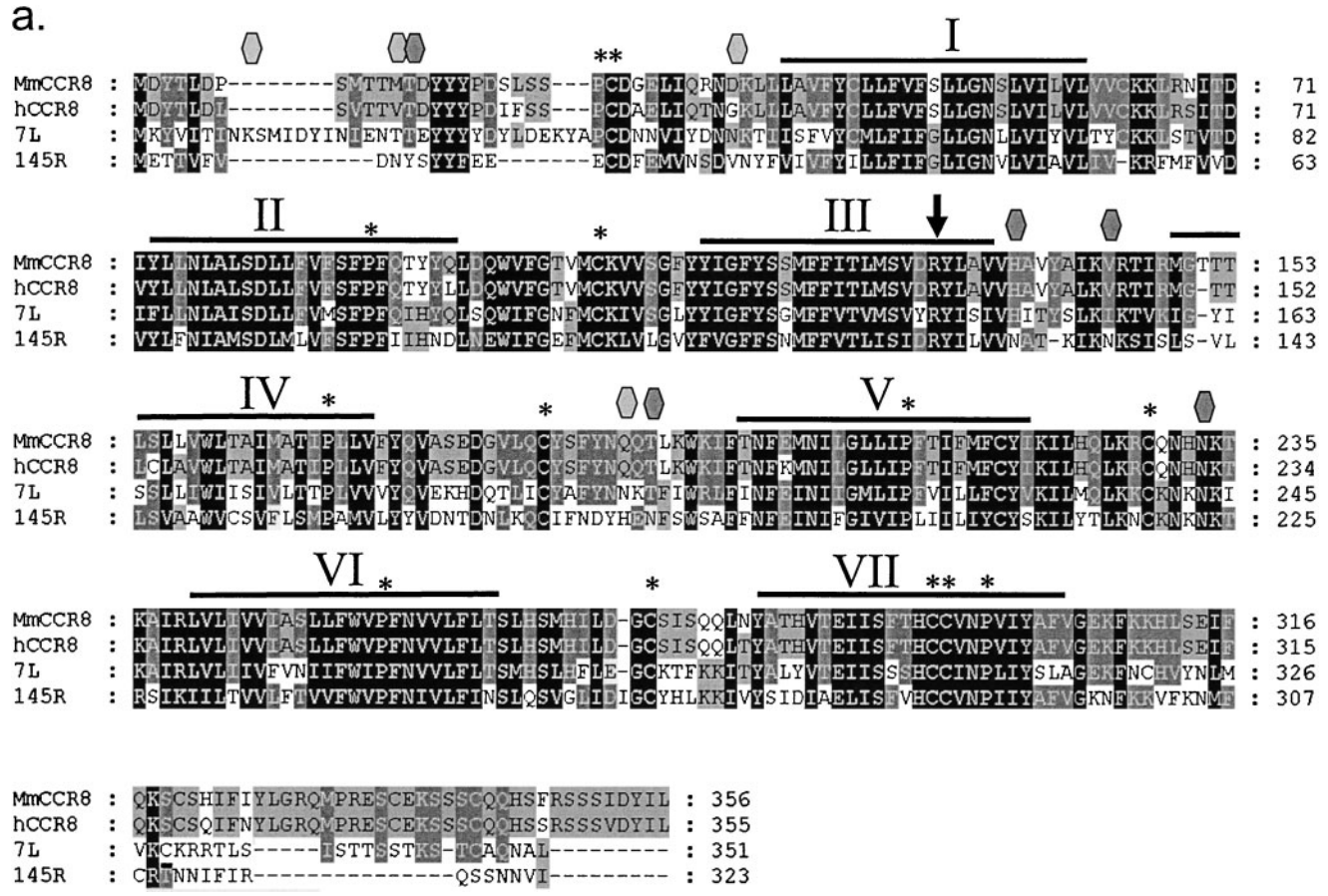
RESULTS

Bioinformatics analysis of the YLDV 7L and 145R proteins

YLDV proteins 7L and 145R are related to human and *Macaca mulatta* CCR8 (Lee *et al.*, 2001) and an alignment is shown in Fig. 1(a). The virus proteins share 59 % similarity and 45 % identity with each other. Compared to human CCR8, 7L has 73 % similarity and 53 % identity, whereas 145R is slightly more divergent and has 68 % similarity and 44 % identity. When compared to the other primate CKRs, 7L is most similar to CCR8 from *Macaca mulatta*.

Chemokine receptors share a similar membrane topology, overall sequence identity and several conserved motifs (Baggiolini *et al.*, 1997). Both 7L and 145R share many of these features. First, there are seven transmembrane domains as well as N- and C-terminal regions that are predicted to be extracellular and cytoplasmic, respectively (Fig. 1a, b). The transmembrane domains share the greatest similarity with the primate CCR8 proteins. Second, there are two conserved cysteine residues, one in the N-terminal domain and one in the third extracellular loop (Fig. 1a, c), which are predicted to form a disulphide bond crucial for the overall conformation of the receptor. Third, there is a DRY motif in the second intracellular loop that is important for receptor signalling and functioning (Gosling *et al.*, 1997; Auger *et al.*, 2002). This is conserved in 145R and present as YRY in 7L.

The main differences between 7L and 145R compared to the primate counterparts are the presence in the viral proteins



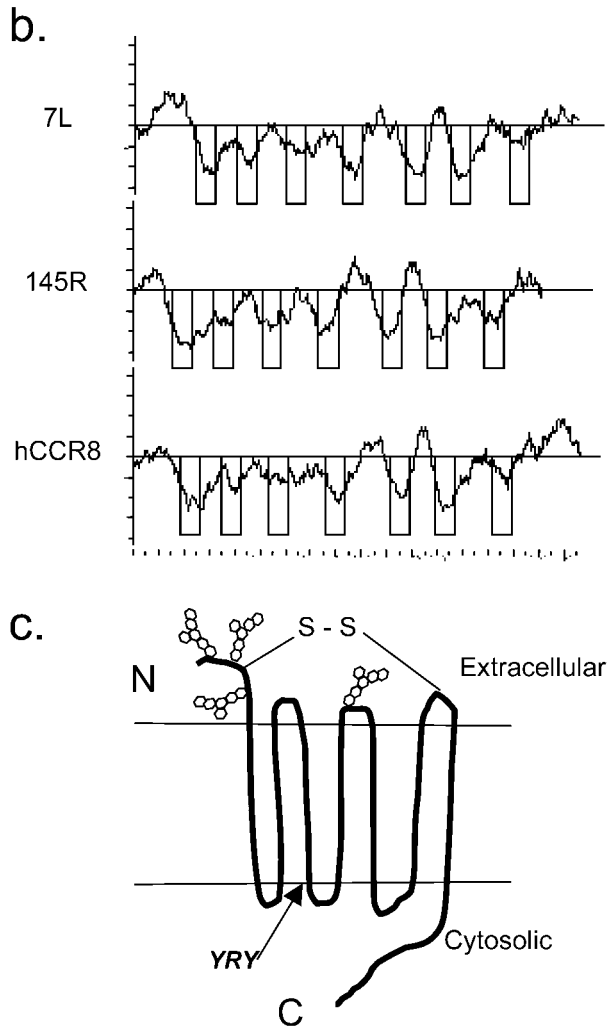


Fig. 1. Sequence alignment of YLDV 7L and 145R with *Macaca mulatta* (Mm) and *Homo sapiens* (h) CCR8. The protein accession numbers used in the alignment are: 7L (CAC21245.1), 145R (CAC21383.1), MmCCR8 (AAC72403) and hCCR8 (NP005192). (a) The alignment was made using CLUSTALW (Thompson *et al*., 2000). Conserved cysteine and proline residues are indicated with asterisks. Outlined above the sequences are the putative transmembrane domains (I–VII) with intervening extracellular and intracellular loops. The DRY motif is indicated with an arrow. Hexagons correspond to potential *N*-glycosylation sites; light shaded for the 7L sequence and dark shaded for the 145R sequence. (b) Hydrophobicity profiles of 7L, 145R and hCCR8 according to Kyte and Doolittle. Segments corresponding to putative transmembrane domains are in boxes. (c) Scheme depicting the experimentally determined topology of YLDV 7L at the plasma membrane.

of *N*-glycosylation motifs (NXS/T) in the N-terminal domain and the second extracellular loop (Fig. 1a, c) and the length of the N- and C-terminal regions. In 7L there are three predicted NXS/T motifs in the N-terminal extracellular domain and one in the second extracellular loop. In 145R there are six NXS/T motifs although only two are predicted to be accessible to the glycosylation enzymes in the lumen of the endoplasmic reticulum. 7L has a longer N-terminal domain compared to both 145R and primates CCR8 proteins, whereas both 7L and 145R have shorter C-terminal cytoplasmic domains. Recently, the crystal structure of murine gammaherpesvirus 68 (MHV-68) chemokine binding protein M3 was resolved alone and in complex with CCL2/MCP-1 (Alexander *et al*., 2002). A key residue in the structural mimicry of this viral protein is P-272, which interacts directly with the chemokine invariant disulphide bond. This proline is represented in 7-TM G-protein-coupled receptors by P-29 and is highly conserved in CCR and CXCR receptors. In the YLDV proteins, P-29 is conserved in 7L but absent in 145R. The overall similarity of 7L and 145R with each other and primate CCR8 indicates that each gene was most likely derived from the host. However, the degree of divergence between these proteins indicates that the genes were likely to have either been independent acquisitions, or the acquisition occurred long ago followed by divergence within the virus genome.

Expression of CCL1 binding activity on YLDV-infected cells

Based on the similarity of 7L and 145R to CCR8 we investigated whether cells infected with YLDV were able to bind to the CCR8 ligand CCL1. OMK cells were infected at 5 p.f.u. per cell with YLDV or WR for 16 h, incubated with ^{125}I-labelled hCCL1 and bound isotope was determined as described in Methods. Fig. 2 shows that YLDV-infected cells bind CCL1 and that this binding was specific because it was competed by a 50- and 200-fold molar excess of cold ligand. Binding of CCL1 to either mock-infected OMK cells or cells infected with VV WR gave similar background counts and the latter was subtracted from the c.p.m. bound to YLDV-infected cells.

Forty nucleotides upstream of the start codon of gene *7L* there is a TAAAT motif, suggesting transcription late during infection, but for *145R* the time of expression is difficult to predict (Lee *et al*., 2001). To investigate the phase during infection at which the CCL-1-binding activity is expressed, ^{125}I-labelled hCCL1 binding was investigated at different times post-infection (p.i.) in the presence or absence of AraC, an inhibitor of viral DNA replication that blocks the expression of late genes. CCL-1-binding to WR-infected cells was analysed in parallel. In the absence of AraC, binding activity was detected as early as 2 h p.i. and increased steadily with time (Fig. 3a). In the presence of AraC, binding activity was similar at all time-points, except 24 h p.i. when it was reduced 26 %. Immunoblot analysis to detect VV protein D8L showed that treatment with AraC was effective in preventing the expression of late VV genes (Fig. 3b). Overall, these data demonstrate that the YLDV-associated CCL1 binding activity is expressed early and probably also late during YLDV infection.

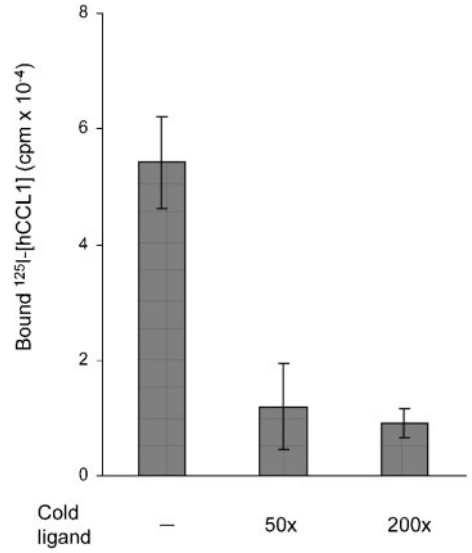


Fig. 2. YLDV-infected cells bind to hCCL1. OMK cells were infected with YLDV or VV strain WR at 10 p.f.u. per cell for 16 h. Detached cells were incubated with 100 pM ^{125}I-labelled hCCL1 alone or in the presence of a 50- or 200-fold molar excess of unlabelled ligand for 2 h at 4 °C. Samples were processed as described in Methods. Non-specific binding from vΔB8R-infected cells was subtracted to calculate specific binding and represented 20–30 % of total binding in different experiments.

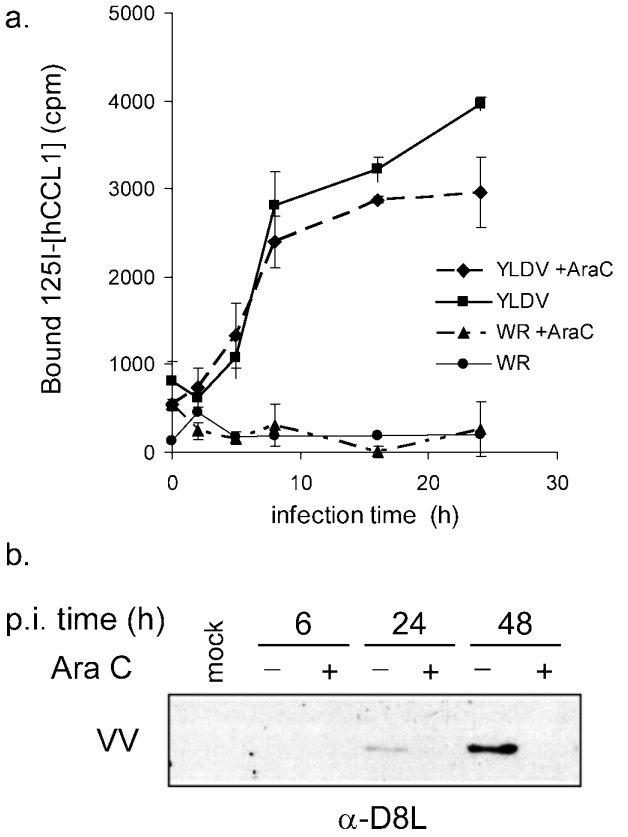


Fig. 3. Expression of CCL1 binding activity by YLDV-infected cells. (a) OMK cells were infected with YLDV or VV strain WR at 10 p.f.u. per cell for the times indicated in the presence or absence of AraC. Cells were resuspended in binding buffer and incubated with 100 pM ^{125}I-labelled hCCL1 for 2 h at 4 °C. (b) Immunoblotting showing the expression of VV WR D8L in the presence or absence of AraC.

Expression of 7L and 145R by VV

To determine whether the expression of 7L, 145R or both was responsible for YLDV-associated CCL1 binding activity, each gene was cloned and expressed individually from VV recombinants. Three different constructs were produced for each gene: the *wt* gene (vΔB8R-7L and vΔB8R-145R) and versions containing an N- (vΔB8R-7LHA$_N$ vΔB8R-145RHA$_N$) or C-terminal (vΔB8R-7LHA$_C$ vΔB8R-145RHA$_C$) HA epitope that can be detected by an anti-HA mAb. To determine the membrane topology of 7L and 145R, VV-infected cells were stained with anti-HA mAb before or after membrane permeabilization and were analysed by indirect immunofluorescence (Fig. 4a, b). The HA epitope was detected only in live non-permeabilized cells expressing 7LHA$_N$ or 145RHA$_N$. Staining with anti-α-tubulin confirmed the membrane integrity of live cells because only those cells permeabilized prior to addition of Ab were stained. The distribution of the HA signal obtained coincides with the plasma membrane and confirms the localization of the N-terminal domain on the cell surface. In contrast, 7LHA$_C$ and 145RHA$_C$ could only be visualized when cells were permeabilized prior to addition of antibody indicating a cytoplasmic location of the epitope.

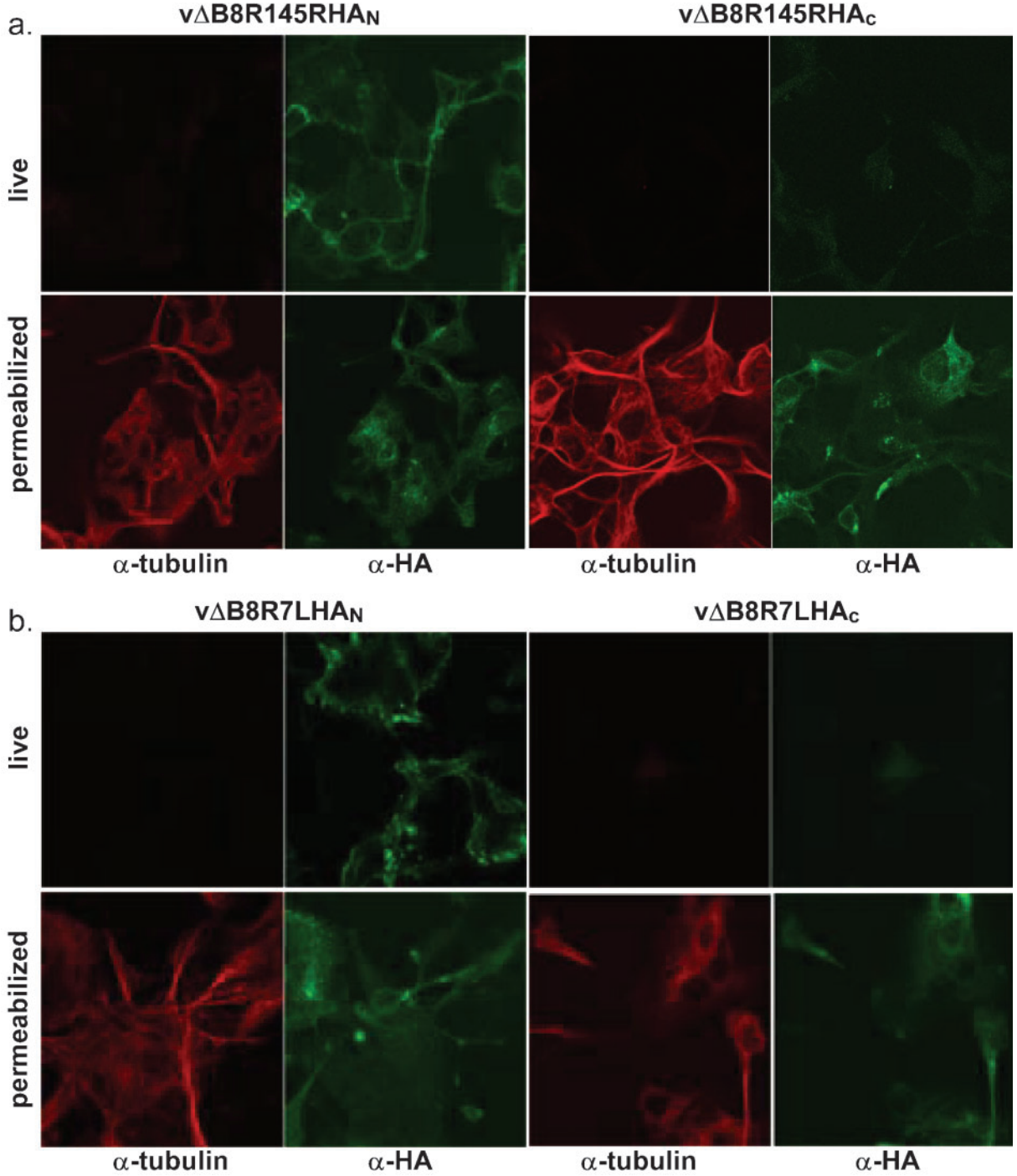
Expression of 7L and 145R in the presence or absence of tunicamycin (an inhibitor of addition of *N*-linked carbohydrate) was also analysed by immunoblotting (Fig. 4c). Without tunicamycin, 7L and 145R were detected as proteins of approximately 56 and 43 kDa, respectively. In the presence of tunicamycin, the proteins were reduced in size to 41 kDa for 7L and 37 kDa for 145R. Inhibition of *N*-glycosylation by tunicamycin treatment appeared incomplete as some full-size protein was also detected. Tunicamycin did not affect the level of VV D8L protein (Fig. 4c, lower panel). In summary, these data show that 7L and 145R are glycosylated, 7-TM receptor proteins expressed with the predicted topology on the plasma membrane.

7L binds to hCCL1

To test if 7L or 145R was responsible for hCCL1 binding on YLDV-infected cells (Fig. 2), ligand-binding assays were repeated using VV-infected cells. Fig. 5 shows that 7L but not 145R bound hCCL1. HA-tagged versions of both proteins gave similar results: C- and N-terminal HA-tagged 7L bound to hCCL1, while 145R did not. Cold hCCL1 displaced the binding of the ^{125}I-labelled hCCL1 to 7L and

slightly decreased the background level of binding observed for 145R. As a positive control, a parallel infection with YLVD was used in the same assay. These data indicated that under the conditions used in this assay (750 pM ^{125}I-labelled hCCL1) only 7L binds to hCCL1.

To characterize further the binding of 7L and hCCL1, time-course analysis, saturation and displacement assays were performed. In Fig. 6(a), cells infected with vΔB8R-7L were incubated with 200 pM ^{125}I-labelled hCCL1 and binding was analysed at different times thereafter. After 30 min at 4 °C, hCCL1 binding to 7L reached equilibrium (Fig. 6a) and this binding was largely reversible, because more than 75 % of the specific binding is dissociable. This dissociation occurs rapidly and the half-life of the binding was calculated to be 12 min. From the association and the dissociation curves the experimental K_d was determined as 0·7 nM. Fig. 6(b) shows the saturation curve obtained for the same receptor/ligand interaction 6 h p.i. with vΔB8R-7L and from this a Scatchard analysis gave a K_d of 0·5 nM ± 0·135 nM.



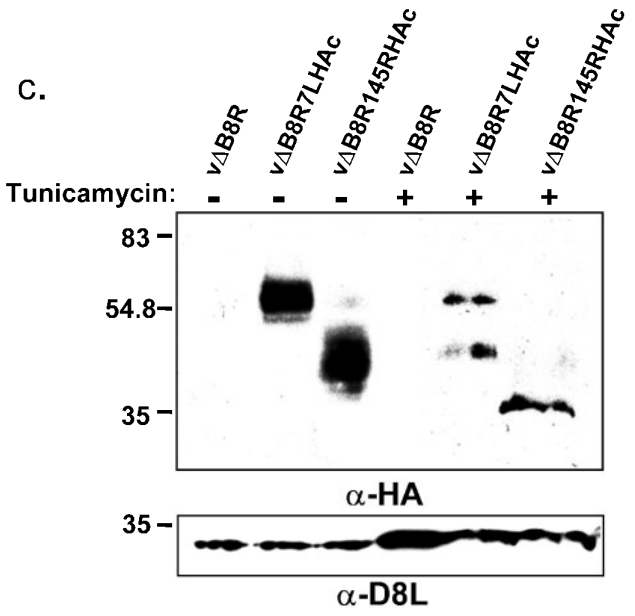


Fig. 4. Immunofluorescence showing surface expression of HA-tagged 7L and 145R. (a and b) BSC-1 cells were infected with the indicated viruses at 10 p.f.u. per cell for 16 h. Live or permeabilized cells were incubated with an anti-HA mAb or anti-tubulin mAb and bound mAbs were detected with secondary Abs conjugated to FITC or TRIC. (c) OMK cells were infected as in (a, b) in the presence or absence of 1 μg tunicamycin ml^{-1}. After separation of the soluble proteins by SDS-PAGE (10 % gel) and transfer to nitrocellulose membranes, the HA tag and D8L were detected with the corresponding mAb and developed using ECL reagents.

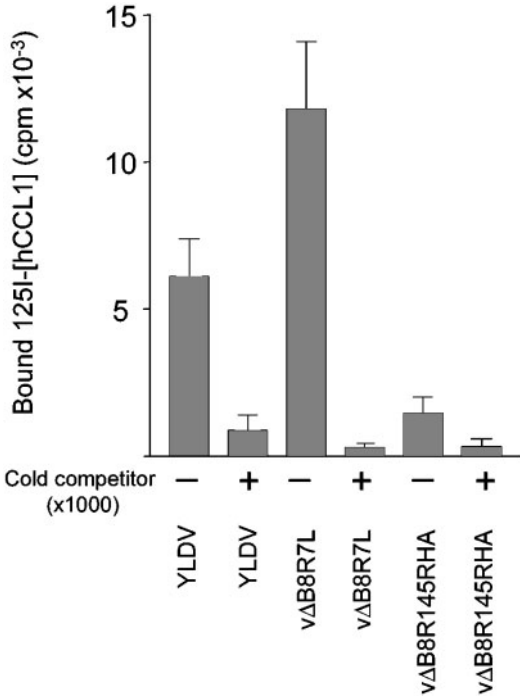


Fig. 5. YLDV 7L binds to hCCL1. OMK cells were infected with the indicated viruses at 5 p.f.u. per cell for 16 h. Cells were incubated with ^{125}I-labelled hCCL1 as in Fig. 3 in the presence or absence of a 1000-fold molar excess of hCCL1. Specific binding was calculated by subtracting the background obtained for vΔB8R-infected cells (approximately 1000 c.p.m.).

Lastly, the displacement of hCCL1 in equilibrium with 7L was measured with increasing concentration of cold hCCL1 and a sigmoid curve was obtained (Fig. 6d). From this curve the K_d was calculated to be 0·3 nM ± 0·15 nM. From all the above assays the K_d of hCCL1 binding to 7L range from 0·3 to 0·6 nM.

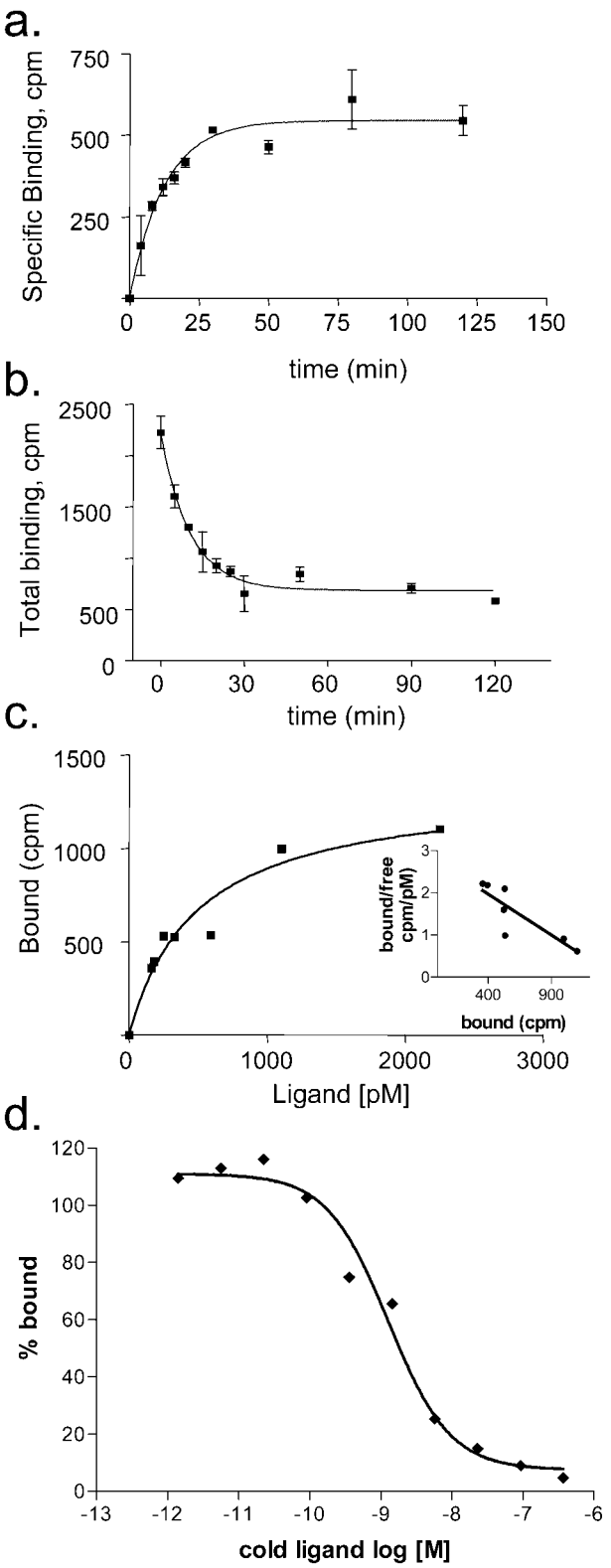
To investigate which other chemokines might bind to 7L, a collection of cold ligands was used to compete with ^{125}I-labelled hCCL1 binding. Fig. 7 shows that vMIPI, vMIPII and hCCL7 (hMCP3) bind well to 7L, hCCL4 (MIP-1β) and CCL17 (hTARC) bind poorly, but vMCCII, murine counterparts of hCCL1 and hCCL7, and human and murine CCL3 (MIP1-α) did not bind to 7L. These results indicate that 7L is a fairly promiscuous receptor, because it can be engaged by different viral and human chemokines. Interestingly, it shows species specificity because none of the murine chemokines tested bind to 7L.

Functional analysis of the 7L receptor

The fact that hCCL1 can bind to 7L with high affinity is not directly demonstrative of productive receptor–ligand interactions. We were mindful of the fact that the DRY motif, shown to be crucial for chemokine receptor function (Gosling *et al.*, 1997; Auger *et al.*, 2002) is represented by YRY in 7L. To determine if binding of 7L to hCCL1 could induce the coupling of heterotrimeric G-proteins to the receptor (the first step in the functional activation of G-protein-coupled receptors), membranes from vΔB8R-7LHA$_N$-infected cells were isolated and examined for basal and hCCL1-promoted [^{35}S]GTP-γ-S binding. Fig. 8(a) shows that the level of G-protein activation rose with increasing CCL1 concentration in the low nanomolar range, reaching a plateau at CCL1 concentrations of 10 nM and above. No dose response was seen for cells infected with vΔB8R, indicating that the activity was specifically mediated via 7L. Moreover, it was evident that in the absence of CCL1, the levels of G-protein activation were around 2-fold higher than for cells infected with vΔB8R, suggesting that 7L may also signal constitutively.

To determine if CCL1 binding to the 7L protein induced downstream signalling, Jurkat cells were infected in serum-free medium for 16 h at 1 p.f.u. per cell and then incubated with CCL1 for zero or 5 min. Total cell extracts were then examined by immunoblotting using antibodies that recognize the activated (phosphorylated) forms of ERK1/2 (Fig. 8b). In the absence of CCL1, the level of activated

ERK1/2 from vΔB8R-infected cells was similar to that when using membranes from vΔB8R-7LHA$_N$-infected cells. But after 5 min in the presence of CCL1 the level of activated ERK1/2 increased when 7L was expressed. The total level of ERK1/2 (anti-ERK1/2, bottom panel) remained constant.



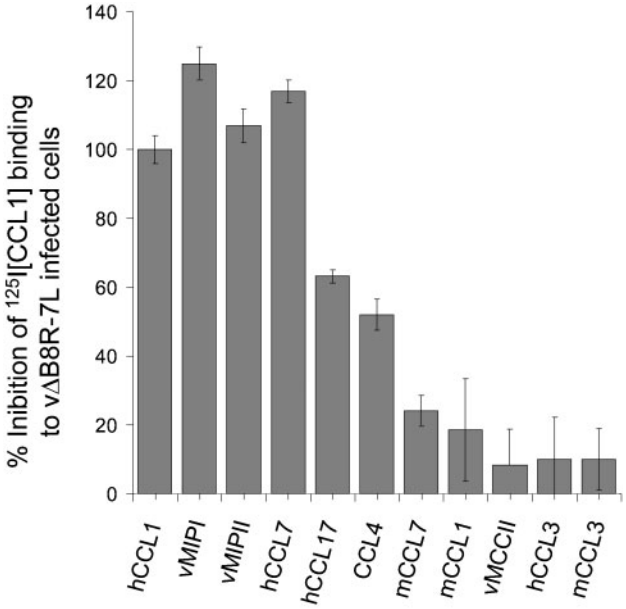


Fig. 7. Binding of different chemokines to 7L. Cells expressing 7L after infection with vΔB8R-7L were incubated with ^{125}I-labelled hCCL1 in the presence of a 100-fold molar excess of the various chemokines indicated. The reduction of ^{125}I-labelled hCCL1 bound to 7L after addition of cold ligand CCL1 was taken as 100 % inhibition of binding. The relative displacements caused by other chemokines are shown in decreasing order.

Finally, the ability of 7L to induce chemotaxis of L1·2 cells in response to CCL1 was measured (data not shown). Cells were transfected with plasmids designed to express either 7L or hCCR8 (each tagged with the HA-epitope) and the chemotactic index in response to CCL1 was measured for cells expressing either protein compared to cells treated with empty vector. CCR8-expressing cells showed a maximum chemotactic index of about 100, whereas those expressing 7L showed a lower response with a maximum chemotactic index of about 10. The lower level of chemotaxis induced

Fig. 6. Binding kinetics of CCL1 to 7L. (a) Association curve. TK$^-$ 143B cells were infected at 10 p.f.u. per cell for 16 h. Then ^{125}I-labelled hCCL1 was added and cells were incubated to determine the kinetics of association. (b) Dissociation curve. For dissociation experiments, further binding was prevented by the addition of a 100-fold molar excess of unlabelled hCCL1; the reaction mixture was then diluted 20-fold and the dissociation of ^{125}I-labelled hCCL1 was monitored over time. (c) Saturation curve of CCL1 binding to 7L. Cells were infected with vΔB8R-7L at 1 p.f.u. per cell for 6 h and incubated with increasing concentrations of ^{125}I-labelled hCCL1. Linear (inset) and non-linear regression curves for the specific binding are shown. Data were analysed with GraphPad prism software. (d) Homologous displacement curve. Cells infected with vΔB8R-7L were incubated with 100 pM ^{125}I-labelled hCCL1 in the presence of increasing concentrations of cold hCCL1.

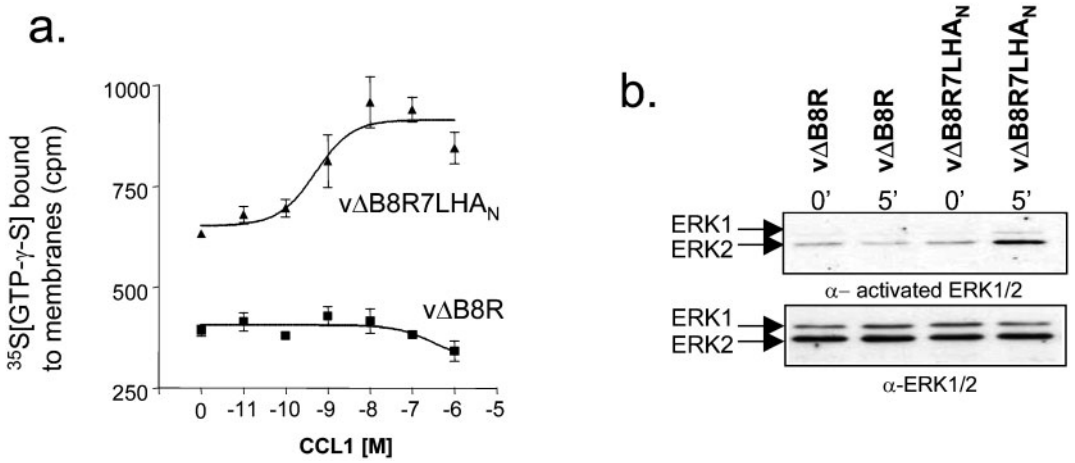


Fig. 8. Functional signalling of 7L receptor. (a) 7L binds to heterotrimeric G-proteins. [^{35}S]GTP-γ-S binding was assayed on membrane fractions derived from TK$^-$ 143 cells infected with vΔB8R-7LHA$_N$ (▲) or vΔB8R (■) in the presence of hCCL1 at the indicated concentrations. (b) Phosphorylation of ERK1/2 proteins. Jurkat cells were infected at 1 p.f.u. per cell for 16 h in serum-free medium and stimulated with hCCL1 for zero or 5 min. Total cell lysates were separated by SDS-PAGE (10 % gel) and transferred to nitrocellulose membranes. Activated ERK1/2 were detected with Abs that recognize the phosphorylated forms of the proteins and loading was monitored with probing parallel blots with anti-ERK1/2 Ab. Bound Abs were developed using ECL reagents.

by 7L may reflect the lower levels of expression of 7L compared to CCR8 as indicated by flow cytometry (data not shown), or be due to the presence of the YRY motif rather than DRY that is important for CCR3-mediated chemotaxis.

DISCUSSION

Although several potential 7-TM chemokine receptors have been recognized in poxviruses, until now these have been uncharacterized and it was not known if they bound chemokines or whether they signal with or without ligand binding. Here we report the characterization of YLDV proteins 7L and 145R and show that 7L binds CCL1 and several other chemokines whereas 145R does not. Moreover, binding was associated with an increase in receptor signalling via heterotrimeric G-proteins and mitogen signalling pathways.

YLDV proteins 7L and 145R show a remarkable degree of amino acid sequence similarity to CCR8 (Lee *et al.*, 2001) and this suggested either protein might bind the natural ligand for CCR8, CCL1. Consistent with this proposal, YLDV-infected cells bound CCL1 from early after infection. To determine which of the YLDV proteins conferred the CCL1-binding activity, each protein was expressed in isolation using recombinant VV. The addition of an N- or C-terminal epitope tag enabled the proteins to be characterized and their locations studied. Indirect immunofluorescence on live cells infected with the recombinant VVs expressing 7L or 145R showed that only proteins with the HA epitope tag at the N terminus exposed this epitope on the cell surface. This confirmed that the topology of each protein in the plasma membrane was the same as that for other 7-TM chemokine receptors, namely an extracellular N terminus and an intracellular C terminus. Treatment of the cells with tunicamycin revealed that both proteins are glycosylated, whereas human and monkey CCR8 are not. The significance of the glycosylation of the viral proteins is unknown, but carbohydrate chains around the ligand-binding pocket might influence binding specificity or reduce recognition of the protein by the immune system.

To assess the phase during YLDV infection at which the CCL1-binding activity is expressed, cells were treated with AraC to block DNA replication and late gene expression. CCL1 binding activity was not diminished early during infection, but was reduced at 24 h p.i. by 26 %. This demonstrates that the 7L protein is expressed early during infection and suggests it is also expressed late, consistent with the presence of a TAAAT motif upstream of the ORF. YLDV has a much slower replication cycle that VV (Knight *et al.*, 1989), but the 7L protein resembles the soluble CC chemokine-binding protein encoded by several strains of VV in being expressed throughout infection (Alcamí *et al.*, 1998), suggesting that early and sustained expression of these proteins is beneficial to these viruses.

The 7L protein but not 145R was found to bind CCL1 in addition to several other chemokines reported to bind to CCR8 (Roos *et al.*, 1997; Tiffany *et al.*, 1997; Dairaghi *et al.*, 1999; Luttichau *et al.*, 2001).

The affinity of CCL1 for CCR8 has been determined in several cell types and expression systems with K_d values

ranging form 0·17 to 1·2 nM (Roos *et al.*, 1997; Dairaghi *et al.*, 1999). In comparison, vCKR 7L binds to hCCL1 with a K_d of 0·6 nM suggesting that it would be an effective competitor for CCL1 binding *in vivo*. Moreover, binding was associated with an increase in receptor signalling via heterotrimeric G-proteins and mitogen signalling pathways. Notably, some signalling was evident without CCL1 ligand binding, indicative of constitutive activity. In the related G-protein-coupled receptor, the β-adrenergic receptor, the DRY motif has been postulated to form an ionic lock that holds the receptor in an inactive conformation (Ballesteros *et al.*, 2001). It is plausible that such a lock holds the hCCR8 conformation in an inactive state and that disruption of this lock in the YLDV orthologue lacking the aspartate residue, leads to the observed constitutive activity.

Expression of the 7L protein in a murine pre-B cell line was feeble in comparison to levels observed for hCCR8 using the same system. Analysis of the nucleotide sequence of 7L showed some splicing acceptor and donor sites that are not present in the CCR8 sequence, which may explain the comparatively low expression levels observed following transient transfection. Such sites would not affect expression from YLDV (or VV) because of the cytoplasmic transcription of poxviruses. This might explain why only modest but detectable chemotactic responses were observed. Alternatively, the DRY to YRY mutation in 7L might diminish chemotaxis as reported for CCR3 (Auger *et al.*, 2002).

Since the binding of CCL1 to CCR8 can block apoptosis via the RAS/MAPK pathway (Louahed *et al.*, 2003), it is possible that 7L expression enables infected cells to respond to the anti-apoptotic and chemotactic signals triggered by CCL1 and thus enhance cell survival and virus dissemination. The signalling might also enhance virus replication within the cell and vary depending on the type of cells infected by YLDV. Conversely, it may be argued that a local decrease in CCL1 concentration *in vivo*, due to sequestration by 7L, might impair recruitment of CCR8-bearing immune cells (e.g. Th2 cells). This could be detrimental to the virus, which might benefit from a Th2 rather than a Th1 type host response.

CCR8 is among the few CC chemokine receptors with a unique high-affinity ligand. The significance of the virus acquiring two similar receptors out of the many encoded by the host is intriguing. Although the battery of ligands that we tested for 7L binding is not as extensive as that reported for CCR8 (Dairaghi *et al.*, 1999) our findings draw a parallel with the ligand-binding fingerprint reported for hCCR8. Notably, like hCCR8, 7L shares a high affinity binding site for CCL1, vMIPI and vMIPII. However, 7L did not bind to the MCV chemokine-like protein vMCCII. vMCCI (from MCV type 1) and vMCCII (from MCV type 2) share 89 % amino acid identity but only vMCCI competes with hCCL1 for binding to hCCR8 (Luttichau *et al.*, 2001). So it remains possible that vMCCI can bind to 7L. CCL17 and CCL4 have also been proposed as ligands for CCR8, but this remains controversial (Bernardini *et al.*, 1998; Garlisi *et al.*, 1999). These ligands could displace some of the binding of ^{125}I-labelled hCCL1 to 7L, suggesting a moderate to low affinity of these chemokines for the viral receptor. In the assays used, 7L showed a preference for human rather than murine chemokines, but whether this is evident *in vivo* remains to be determined. Firstly, hCCL7 but not mCCL7 displaced the binding of CCL1 to 7L. More importantly, mCCL1 did not compete with ^{125}I-labelled hCCL1 for binding to 7L. YLDV has a restricted host range and can only infect primates and is closely related to *Tanapox virus*. Recently, it was shown that a tumour necrosis factor-binding factor encoded by *Tanapox virus* bound human but not murine TNF (Brunetti *et al.*, 2003). These observations may have implications when using murine infection models to study the *in vivo* effect of 7L during VV infection.

In summary, we have demonstrated that transcription of the YLDV *7L* gene results in a functional CCR8-like receptor, with many of the characteristics of hCCR8. Although several poxviruses and herpesviruses express soluble proteins that bind a wide range of chemokines (Alcamí, 2003), it is notable that several of these viruses have targetted the interaction of CCL1 and its receptor CCR8 by the expression of chemokines and chemokine receptors specific for CCR8. Our findings underscore CCR8 and its ligands as potential key players in viral defence although the role of this receptor and its ligand CCL1 in this setting still require elucidation.

ACKNOWLEDGEMENTS

This work was supported by The Wellcome Trust and the UK Medical Research Council. J. F. is an MRC research student and G. L. S. is a Wellcome Trust Principal Research Fellow.

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