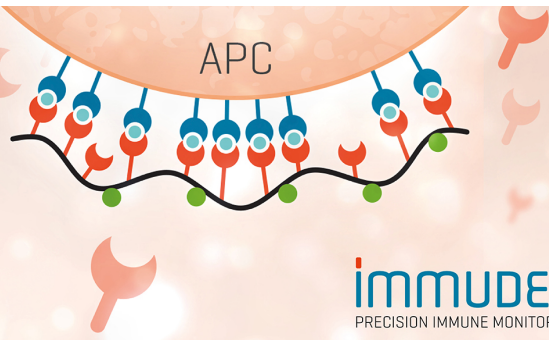


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Cutting Edge: Identification of a Novel Chemokine Receptor That Binds Dendritic Cell- and T Cell-Active Chemokines Including ELC, SLC, and TECK

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Searching for new receptors of dendritic cell- and T cell-active chemokines, we used a combination of techniques to interrogate orphan chemokine receptors. We report here on human CCX CKR, previously represented only by noncontiguous expressed sequence tags homologous to bovine PPR1, a putative gustatory receptor. We employed a two-tiered process of ligand assignment, where immobilized chemokines constructed on stalks (stalkokines) were used as bait for adhesion of cells expressing CCX CKR. These cells adhered to stalkokines representing ELC, a chemokine previously thought to bind only CCR7. Adhesion was abolished in the presence of soluble ELC, SLC (CCR7 ligands), and TECK (a CCR9 ligand). Complete ligand profiles were further determined by radiolabeled ligand binding and competition with >80 chemokines. ELC, SLC, and TECK comprised high affinity ligands ($IC_{50} < 15$ nM); lower affinity ligands include BLC and vMIP-II ($IC_{50} < 150$ nM). With its high affinity for CC chemokines and homology to CC receptors, we provisionally designate this new receptor CCR10. *The Journal of Immunology*, 2000, 164: 2851–2856.

The chemokines ELC² (also called MIP-3 β) and SLC (also called 6CKine), and their cognate receptor, CCR7, have profound effects on the regulation of dendritic cells (DC) and T cells. ELC and SLC have been shown to be major attractants

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² Abbreviations used in this paper: ELC, EB11 ligand chemokine; EST, expressed sequence tag; MIP, macrophage-inflammatory protein; ORF, open reading frame; DC, dendritic cell; SLC, secondary lymphoid tissue chemokine; BLC, B lymphocyte chemoattractant; CKR, chemokine receptor; CCX, chemocentryx; BLAST, basic local alignment search tool; TECK, thymus-expressed chemokine; HEK293, human embryonic kidney 293 cells; v, viral; -SK, -stalkokines; HHV8, human Kaposi's sarcoma herpesvirus.

of mature (although not immature) DC, and they have been suggested to control the migration of the newly postulated T central memory lymphocytes. Natural or targeted genetic deletions of ELC, SLC, or CCR7 result in marked deficiencies in DC, T and B cell trafficking, as well as morphological disruption of secondary lymphoid organ architecture (1–7). CCR7 is related to another chemokine receptor, CCR9 (formerly the orphan clone GPR9.6), shown to be a receptor for the CC chemokine TECK (8–9). The CCR9/TECK pairing has been reported to be important for the regulation of thymocytes, as well as lymphocytes with intestine-targeted homing patterns (10). To date, CCR9 has been the only reported TECK receptor and CCR7 the only credible receptor for ELC and SLC, despite contradictory reports (11, 12) surrounding SLC binding.

A comprehensive map of the constellation of chemokine and chemokine receptor interactions requires the assignment of ligands to orphan receptors, i.e., receptors known only by their predicted amino acid sequences. However, such ligand assignments have been a major challenge in the study of G protein-coupled receptors in general and chemokine receptors in particular. We have developed new technology to address this issue by constructing an array of immobilized chemokines on stalks (which we have designated stalkokines) and interrogating this array with cells stably transfected with orphan chemokine receptor candidates. We have used this methodology to more fully understand the biology of DC- and T cell-active chemokines such as ELC and SLC.

One such orphan receptor candidate comprises a new human sequence which we have identified, CCX CKR, not previously known in totality in any of the publicly available databases, but related to what had been thought to be a bovine gustatory receptor. We detect CCX CKR mRNA expression in human DC, T cells, spleen, and lymph node, as well as in several nonhemopoietic organs. Through a combination of approaches, we have assigned the spectrum of ligands that bind to CCX CKR. These include ELC, SLC, and TECK with high affinity, and BLC and vMIP-II with lower affinity. The identification of this new receptor, provisionally designated CCR10, may reveal characteristics for potential cross-over binding of CXC and CC ligands and adds to the understanding of the molecular mechanisms of action for DC and T cell-active chemokines.

Materials and Methods

Reagents and cells

Human, viral, and murine recombinant chemokines were obtained from R&D Systems (Minneapolis, MN). 125 I-labeled ELC and TECK were obtained from Amersham Pharmacia Biotech U.K. (Buckinghamshire, U.K.). Full length CCX CKR expression constructs were made in pIRESpuro expression vector (Clontech, Palo Alto, CA) with a FLAG epitope tag (DYKDDDDK) and prolactin signal sequence and were used to generate stable transfectants in human embryonic kidney 293 (HEK293) cells. Transient and stable transfections for CCX CKR and stalkokines were done using Superfect reagent (Qiagen, Valencia, CA) following the manufacturer's protocol. Stables were generated by selecting in 2 μ g/ml puromycin for 7 days, and expression was confirmed by FACS analysis of the FLAG epitope using anti-FLAG M1 (Sigma, St. Louis, MO) and 2' anti-mouse PE conjugate (Coulter Immunotech, Miami, FL).

PCR; screening of cDNA libraries

BLAST analysis of known chemokine receptors identified a related bovine receptor, PPR1, designated as a gustatory receptor. A search of the human EST database using PPR1 resulted in two noncontiguous ESTs: H67224 (5') and A1131555 (3'). Primers were designed against the 5'-end of H67224 (5'-AAT TTG GCT GTA GCA GAT TTA CTC C-3') and in the reverse orientation for the 3'-end of A1131555 (5'-GCT AAA AGT ACT GGT TGG C-3'), and used in PCR (5% DMSO, annealing 58°C) of genomic DNA isolated from human buffy coats. The reaction resulted in a 855-bp product containing the ESTs and connecting sequences. A Rapid Screen arrayed spleen cDNA library (Origene, Rockville, MD) was then screened using PCR, yielding a 5'-extended clone; this clone was finally used to screen a human genomic library by filter hybridization. The full length cDNA sequence was determined by sequence analysis of genomic clones using reverse primer from the 5'-sequence of the Origene clone, PCR with proofreading *Pfu* (Stratagene, La Jolla, CA) enzyme and then reconstituted full length predicted open reading frame (ORF) cloned into pIRESpuro expression vector (Clontech) with a FLAG epitope tag and prolactin signal sequence. Sequence was confirmed on several clones.

Orphan receptor interrogation by adhesion to stalkokines

Technology was developed to identify ligands for orphan chemokine receptors via adhesion. Briefly, immobilized native chemokines alone are incapable of capturing cells bearing cognate receptors (13). We have developed non-native chemokine structures, stalkokines, comprising chemokine moieties engineered as N-terminal attachments to extended modified mucins (14), details of which will be published elsewhere (Z. Maio et al., manuscript in preparation). Stalkokines, harvested in the supernatants of HEK293 cells after transient transfection, are anchored to solid substrates via Abs against carrier domains (e.g., poly(His) epitopes) engineered to the carboxyl terminus, leaving the chemokine domain free to interact with candidate orphan receptors. Stalkokines were interrogated using 8-well chamber slides coated first with anti-His anchoring Ab (10 μ g/ml in PBS overnight at room temperature), which were washed and "blocked" (2% FBS/0.5% BSA in PBS); treated with 250 μ l HEK293 cell stalkokine supernatants (1 h at 37°C), and incubated with 500,000 HEK293-CCX CKR transfectants (1.5 h at room temperature). Inhibition of adhesion by competition with soluble chemokines was done by incubating cells with 5–10 μ g/ml recombinant chemokines. In all cases, nonadherent cells were removed by washing in PBS; remaining adherent cells were fixed with 1% glutaraldehyde, photoimaged, and counted.

Binding analysis

We have recently developed a technique for global profiling of chemokine receptor/chemokine ligand interactions, designated DisplaceMax. This technology uses expanded, efficiency-maximized radioligand binding using filtration protocols (15). In these assays, DisplaceMax used the simultaneous interrogation of CCX CKR transfectants by >80 distinct purified chemokines in the ability to displace radiolabeled ELC or TECK, by the protocol described (15). The competition dose-response curves were analyzed using GraphPad Prism software (San Diego, CA) to determine IC₅₀ values. Additionally, a Scatchard transformation using WaveMetrics Igor software (Lake Oswego, OR) was used to estimate the receptor sites per cell.

Results and Discussion

Identification and cloning of CCX CKR

In an attempt to map comprehensively the receptors relating to the biology of DC and T cell-active chemokines, we have searched for

fragments of new chemokine receptor-like sequences from various sources, including searches of the EST database of the National Center for Biotechnology Information. BLAST analysis of known chemokine receptors identified a related bovine receptor, PPR1, previously identified as a gustatory receptor. The bovine sequence was used to search a human EST database; this yielded two non-contiguous ESTs: H67224 (5') and A1131555 (3'). To obtain a full length clone for the human receptor, we first tested whether the two ESTs were part of a single ORF. Primers were designed against the 5'-end of EST H67224 and in the reverse orientation for the 3'-end of EST A1131555. Using these complementary primers in PCR, we obtained a single 855-bp amplification product from human genomic DNA (not shown), indicating that the two EST likely represented segments of a single gene. The 855-bp fragment product was used to design additional primers for use in an anchored PCR screen of a human spleen library. This yielded a longer, still incomplete fragment, which was radiolabeled and used as a probe to screen a human genomic DNA library to isolate and construct the complete ORF. The sequence of the full length cDNA and its predicted protein, which we initially designated CCX CKR, suggested that it was the human homologue of bovine PPR1, because they are nearly 80% identical (not shown). The predicted CCX CKR protein sequence is shown in Fig. 1A aligned with the human chemokine receptors likely to be most closely related, CCR7 and CCR9. Multiple sequence alignment of the protein encoded by CCX CKR with these and other human chemokine receptor sequences showed amino acid identities ranging from 29 to 35%.

Expression of CCX CKR in leukocytes and various tissues

The expression of CCX CKR mRNA was determined by PCR analysis of human cDNAs as well as by RT-PCR of RNAs isolated from various tissues. First, CCX CKR expression in hemopoietic cells and tissues was investigated. Receptor expression was apparent in immature DC (derived from monocytes after treatment with GM-CSF and IL-4), in primary T cells from two of three donors, and in spleen and lymph node tissue (Fig. 1B). Additionally, expression was detected in nonlymphoid tissues such as heart, kidney, placenta, trachea, and brain; unfractionated leukocytes on the same panel were also positive (Fig. 1B). Control PCR products for GAPDH confirmed the integrity of all starting RNA. The observed pattern of CCX CKR overlaps with and complements the distribution of human EST in the National Center for Biotechnology Information databases, which have been isolated from kidney, fetal heart, olfactory epithelium, and tonsillar B cells. Thus, CCX CKR seems expressed in motile cells in the periphery, as well as in lymphoid and nonlymphoid tissues.

Stable expression of CCX CKR protein

To assess the functional properties of the protein encoded by the CCX CKR cDNA, including its potential chemokine-binding profile, we constructed expression plasmids encoding CCX CKR with an added N-terminal FLAG epitope. This allowed for detection and selection, using an anti-FLAG mAb, of the most highly expressing stable transfectants. HEK293 cells stably expressing the M1 FLAG epitope-tagged CCX CKR were confirmed by FACS (Fig. 1C) and were selected for further analysis.

Adhesion of CCX CKR transfectants to ELC stalkokines

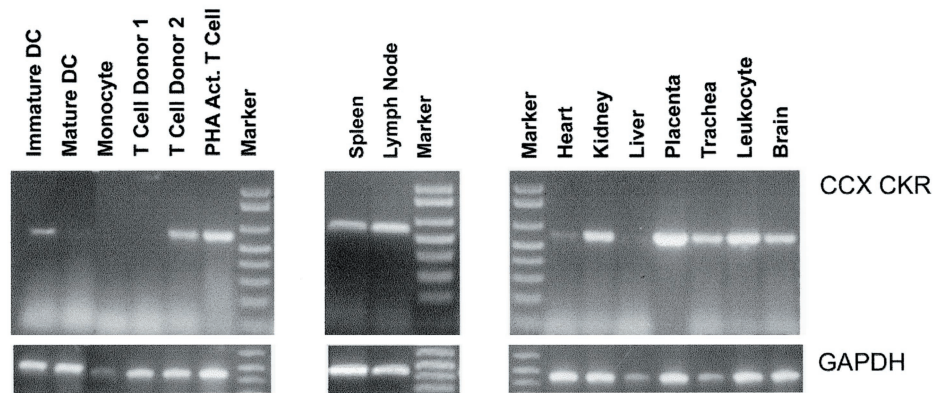
HEK293-CCX CKR cells were used to interrogate chemokine stalkokines, i.e., molecules in which discrete chemokine domains were engineered to be tethered to the end of an extended stalk structure. Stalkokines were immobilized to glass slides via an Ab specific to an anchoring domain, leaving the chemokine domain

A

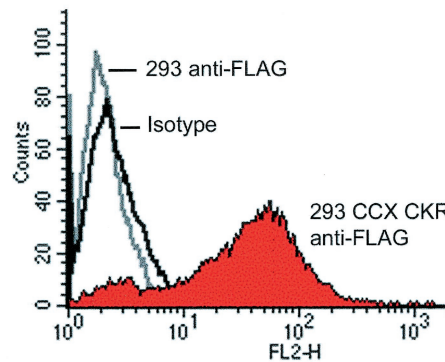


FIGURE 1. CCX CKR sequence, tissue expression, and stable cell line generation. *A*, Sequence homology of the CCX CKR coding region with other chemokine receptors. The deduced amino acid sequence encoded by the CCX CKR cDNA was compared with other human chemokine receptors using the sequence alignment program CLUSTAL (GeneWorks). Shown is the CCX CKR amino acid sequence aligned with the human CCR7 and CCR9. The positions of the predicted hydrophobic membrane spanning regions TM1 to TM7 are indicated by bars above the sequence. Amino acids identical between CCX CKR and other chemokine receptors are boxed. *B*, Cells and tissues expressing CCX CKR RNA, as analyzed RT-PCR of cytoplasmic RNA from cultured primary cells and whole tissues from various organs as indicated. *C*, Stable cell surface expression of CCX CKR protein. Shown is a selected population of transfected HEK293 cells stably expressing CCX CKR protein containing an N-terminal FLAG epitope, comparing intensity of anti-FLAG mAb staining relative to wild-type HEK293 cells. GenBank accession no. AF 233281.

B



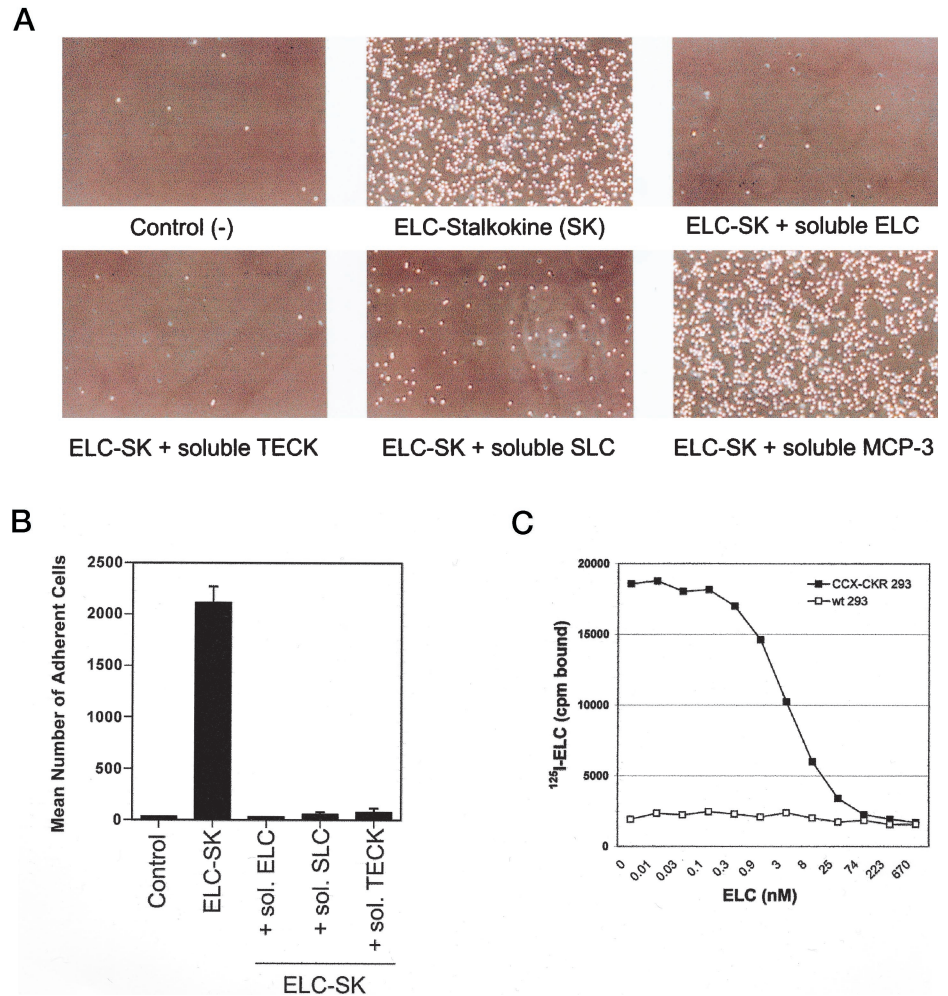
C



free to interact with candidate receptors on the surface of transfected cells. The presumption was that interactions of sufficient affinity would cause cells bearing a given orphan receptor to ad-

here to the stalkokine representing that orphan receptor's cognate ligand. As a primary screen this adhesion would reveal putative receptor-ligand interactions. CCX CKR cells adhered well to ELC

FIGURE 2. Screening for ligands for CCX CKR by adhesion to stalkokines. *A*, Interrogation of immobilized stalkokine (SK) by HEK293-CCX CKR cells, where control = background adhesion of HEK293-CCX CKR cells to wells containing no stalkokine (anchoring Abs alone are present); ELC-SK = strong adhesion of HEK293-CCX CKR cells to locations containing ELC-SKs immobilized via anchoring Abs; ELC-SK + soluble ELC, soluble TECK, or soluble SLC = ablation of adhesion in the presence of excess concentrations of soluble recombinant “native form” chemokines as shown; ELC-SK + soluble MCP-3 = no diminution in adhesion in the presence of MCP-3 as representative of many noncompeting chemokines. Wild-type (wt) HEK293 cells showed no adhesion to any of the sites (not shown). *B*, Quantitation of adhesion of HEK293-CCX CKR cells to ELC-SK in the absence and presence of soluble chemokines from a representative experiment. *C*, Homologous competition binding assay using radiolabeled ELC in the presence of increasing concentrations of cold ELC on either HEK293-CCX CKR cells (■) or wild-type HEK293 cells (□).



stalkokines (ELC-SK; Fig. 2A), but not other stalkokines tested (not shown). Furthermore, ELC-SK-mediated adhesion was abolished in the presence of soluble native ELC as a competitor (Fig. 2A, top). We also observed a significant reduction in ELC-SK-mediated adhesion of HEK293-CCX CKR cells in the presence of soluble SLC, as well as soluble TECK, but not soluble MCP-3 (Fig. 2A, bottom). These experiments were performed and quantitated over several independent trials, an example of which is given in Fig. 2B, and were found to be highly reproducible. Moreover, radiolabeled ELC was used in a traditional homologous competition assay in the presence of increasing concentrations of unlabeled ELC. The results revealed significant binding of ELC to HEK293-CCX CKR cells, but not to wild-type HEK293 cells (Fig. 2C). Nearly identical results were obtained in homologous competition of radiolabeled TECK with cold TECK (not shown). Taken together, the stalkokine-based adhesion and radiolabeled ligand binding/homologous competition assays suggested that CCX CKR represented a new chemokine receptor that bound a novel complement of chemokines.

Complete ligand binding fingerprint of CCX CKR

To rapidly and thoroughly define the ligand binding fingerprint of a given chemokine receptor, we have established an approach to comprehensively profile chemokine receptors using a large array of purified chemokines and chemokine variants (15). We used this approach independently to confirm the interaction of ELC and other chemokines with CCX CKR. We used radioligand binding of

^{125}I -labeled-ELC or ^{125}I -TECK to CCX CKR-stable transfectants, chemokine specificity for the new receptor was exhaustively determined. Approximately 80 distinct purified chemokines and chemokine variants were used as cold competitors (initially at a saturating final concentration of 200 nM), against ^{125}I -labeled ELC (Fig. 3) or ^{125}I -TECK (not shown) in binding experiments; the results were comparable for each. The radiolabeled ligand binding displacement data confirmed that CCX CKR bound well to human and murine ELC, SLC, TECK, and moderately to mMIP-1 γ (although its human homologue did not bind). Moreover, other potential lower affinity chemokine ligands were revealed including the CXC chemokine BLC, and the virally encoded vMIP-II from the human Kaposi's sarcoma herpesvirus HHV8 (Fig. 3). All other chemokines tested failed to compete consistently with radiolabeled ELC.

Determination of binding constants

The binding interactions identified in the primary screen were examined quantitatively by extensive radioligand binding competition to CCX CKR stable transfectants (Fig. 3 and Table I). The results confirmed the high affinity binding of human ELC, SLC, and TECK with affinities (IC_{50}) between ~ 5 and 15 nM. In each case, the murine versions of these chemokines also bound, and with even greater affinity; the IC_{50} values are listed in Table I. Intriguingly, the CC chemokine BLC, although of lesser affinity, also bound well, showing a steeply inflected competition curve. The viral chemokine vMIP-II showed moderate to low affinity and

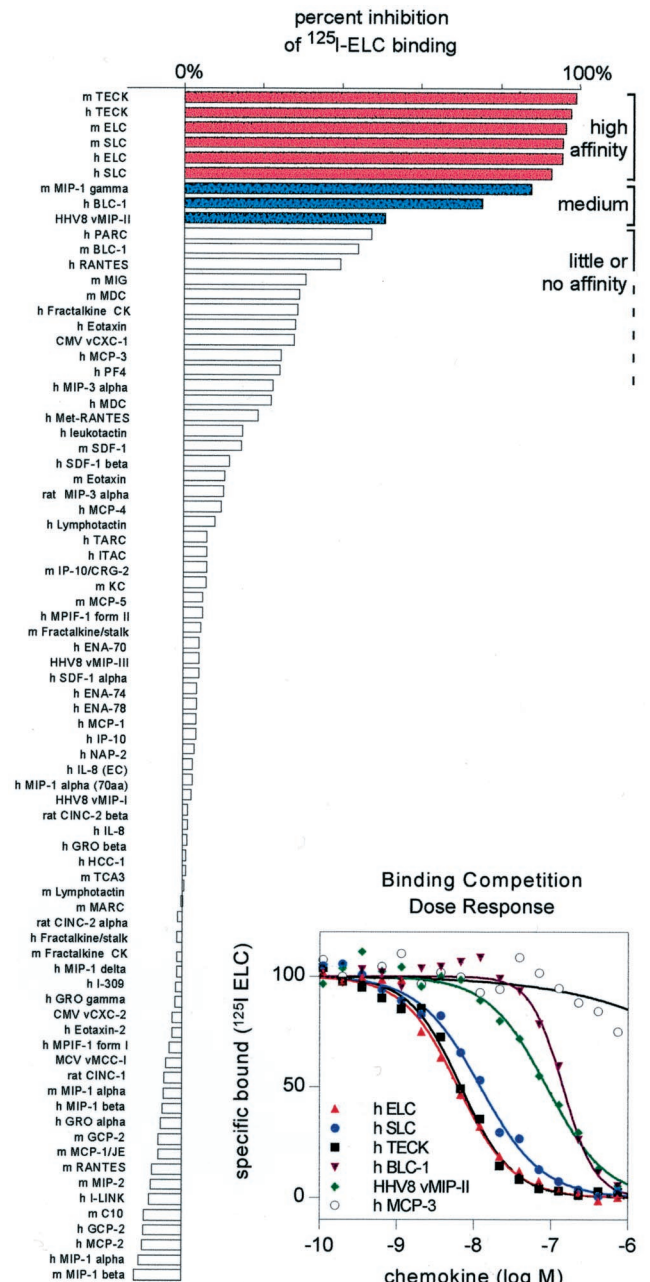


FIGURE 3. Ligand binding fingerprint of CCR10. Definition of CCX CKR protein as CCR10, as indicated by using ¹²⁵I-ELC against a comprehensive array of viral, human (h), and murine (m) chemokines in binding competition. The percent inhibition of specific binding (~3600 cpm total bound; ~350 cpm nonspecific bound) is shown as a bar graph to emphasize that chemokines can be classed in categories as potential high affinity (red bars), potential medium affinity (blue bars), or little or no affinity (open bars). The results are means of three determinations; the SEM in all cases is ≤20%; error bars are omitted for clarity. Because intraassay experimental error was ± ~20%, determinations within this range to the left or right of the 0% meridian are not likely to be statistically significant. Rank order of high affinity CCR10 ligand binding. Multipoint determination reflecting the competition of unlabeled chemokines against ¹²⁵I-ELC binding to CCR10.

was the only viral chemokine to show any interaction with CCX CKR. The HEK293-CCX CKR cells did not exhibit robust cytoplasmic calcium signals in several tests, but this may be due to G protein dilution, because the transfectants stably express CCX

Table I. IC₅₀ values of chemokines that bind to CCR10

	IC ₅₀ (nM)
Human chemokines	
hELC ^a	6
hSLC	12
hTECK	7
hBLC-1	140
HHV8 vMIP-II	90
Murine chemokines	
mELC	1
mSLC	4
mTECK	2
mMIP-1γ	70

^a h, human; m, murine.

CKR protein at >250,000 sites per cell (not shown). Also, in preliminary chemotaxis analyses, the CCX CKR transfectants showed moderate migration in response to ELC and SLC, but not to chemokines having no binding activity (not shown). Taken together, these data suggest that the physiologically relevant spectrum of ligands for CCX CKR includes ELC, SLC, and TECK, with possible lower affinity interactions with the CXC chemokine BLC and the viral chemokine vMIP-II.

In summary, this report describes a novel combination of approaches used to match chemokine ligands and orphan receptors. The study focuses on one such chemokine receptor clone, CCX CKR, previously unknown in humans and related to an apparently erroneously assigned bovine gustatory receptor candidate. Using a novel adhesion-interrogation screen coupled with radiolabeled ligand binding, we have assigned a unique complement of ligands that bind with high affinity to CCX CKR. These ligands include the DC- and T cell-active chemokines ELC, SLC, and TECK, previously reported to bind monogamously to CCR7 and CCR9, respectively. We have also identified a class of potential lower affinity CXC and viral chemokine ligands for CCX CKR. The expression of CCX CKR is found in T cells, immature DC, spleen, and lymph nodes, as well as nonlymphoid tissues, and it may regulate the migration of transfected cells expressing it. Thus, we provisionally designate this receptor CCR10. The identification of CCR10 extends and refines the understanding of ELC, SLC, and TECK chemokines, potentially providing new perspectives on the biology of DC and T cell regulation.

Note added in proof. An orphan receptor termed D6 was at one time erroneously given the chemokine receptor designations CCR9 and CCR10 (16, 17). Although D6 interacts with a number of chemokines of the CC family, these designations have since been rescinded. The CCR9 designation now refers to the chemokine receptor previously termed GPR9.6 that has TECK as its primary ligand (8–10). CCR10 now refers to CCX CKR and should not be confused with D6.

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