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TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function

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Background: Chemokines bind to specific receptors and mediate leukocyte migration to sites of inflammation. Recently, some chemokine receptors, notably CXCR4 and CCR5, have been shown to be essential fusion factors on target cells for infection by human immunodeficiency virus (HIV); the chemokines bound by these receptors have also been shown to act as potent inhibitors of HIV infection. Here, we describe the isolation of a novel, putative chemokine receptor.

Results: We have isolated the cDNA for a putative human chemokine receptor, which we have termed TYMSTR (T-lymphocyte-expressed seven-transmembrane domain receptor). The TYMSTR gene is localized to human chromosome 3 and encodes a protein that has a high level of identity with chemokine receptors. TYMSTR mRNA was selectively expressed in interleukin-2-stimulated T lymphocytes but not in freshly isolated lymphocytes and leukocytes or related cell lines. The natural ligand for TYMSTR was not identified among 32 human chemokines and other potential ligands. Cells co-expressing TYMSTR and human CD4 fused with cells expressing envelope glycoproteins of macrophage (M)-tropic HIV-1 as well as T-cell line (T)-tropic HIV-1 isolates. Addition of infectious, T-tropic HIV-1 particles to TYMSTR/CD4-expressing cells resulted in viral entry and proviral DNA formation.

Conclusions: Our findings demonstrate that TYMSTR, in combination with CD4, mediates HIV-1 fusion and entry. The high-level expression of TYMSTR in $CD4^+$ T lymphocytes and the selectivity of this receptor for T-tropic and M-tropic HIV-1 strains indicates that TYMSTR might function as HIV coreceptor at both early and late stages of infection.

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Background

Chemokines are essential mediators of the recruitment of leukocytes and lymphocytes to sites of inflammation [1–3] and are small inducible proteins of 68–103 amino acids containing four conserved cysteine residues. The arrangement of the first two cysteines defines the two subfamilies of CXC and CC chemokines. Chemokines act on all types of immune cells, including monocytes/macrophages, dendritic cells, granulocytes, natural killer cells and lymphocytes, and induce migration, increased adhesion and various immune defense functions. These target cells express chemokine receptors, which belong to the large family of G-protein-coupled, seven-transmembrane domain receptors [1,4,5]. Currently, 12 human chemokine receptors are known that are selective either for CXC chemokines (CXCR1–4) or CC chemokines (CCR1–8).

It has been known for a long time that CD4 molecules on host cells interact with the envelope (Env) glycoproteins on human immunodeficiency virus (HIV) [6–8]; however, CD4 alone is not able to mediate fusion and viral entry, which are critical steps in HIV infection, and additional host factors have been proposed to be involved. Recently, chemokines and their receptors have been found to play an important role as fusion cofactors [6–8]. Apparently, HIV strains, have evolved to recognize different human chemokine receptors on $CD4^+$ target cells. Notably, CCR5 is selective for macrophage (M)-tropic HIV-1 strains which predominate at early stages in infected individuals who lack symptoms of acquired immunodeficiency syndrome (AIDS) [9–13]. This coreceptor is expressed on interleukin-2 (IL-2)-treated $CD45RO^+$ T cells, which could be a primary host for HIV replication in infected individuals. Two additional CC chemokine receptors, CCR2 and CCR3, show a similar specificity [12,13]. By contrast, CXCR4 is the coreceptor for T-cell line (T)-tropic, syncytia-inducing HIV-1 strains, which are prominent in patients with AIDS [14]. The specificity for the

different chemokine receptors is determined by variable regions in the HIV-1 envelope glycoprotein (Env), whereas the constant regions in Env bind to CD4 [15,16]. The interaction of target cells with HIV Env glycoproteins leads to binding, fusion and viral entry. In addition, chemokines that bind to the coreceptors — RANTES and the macrophage inflammatory proteins MIP-1α and MIP-1β for CCR5, and the stromal-cell-derived factor SDF-1 for CXCR4 — block HIV-1 entry *in vitro* [17–19].

In a search for chemokine receptors that are selectively expressed on T lymphocytes in inflammation and disease, we have isolated the cDNA for a novel, putative chemokine receptor, termed TYMSTR for T-lymphocyte-expressed seven-transmembrane domain receptor. TYMSTR is selectively expressed in activated T lymphocytes and related cell lines but not in peripheral blood cells, and thus is a good candidate receptor for the regulation of the lymphocyte infiltrate typically seen in delayed-type immune responses. In combination with CD4, TYMSTR mediated fusion between cells expressing Env of T-tropic as well as M-tropic HIV-1 strains. Because of the selectivity of TYMSTR for certain HIV-1 strains and the restricted expression of this receptor in activated T cells, we conclude that TYMSTR represents a novel HIV coreceptor that may be important in disease progression.

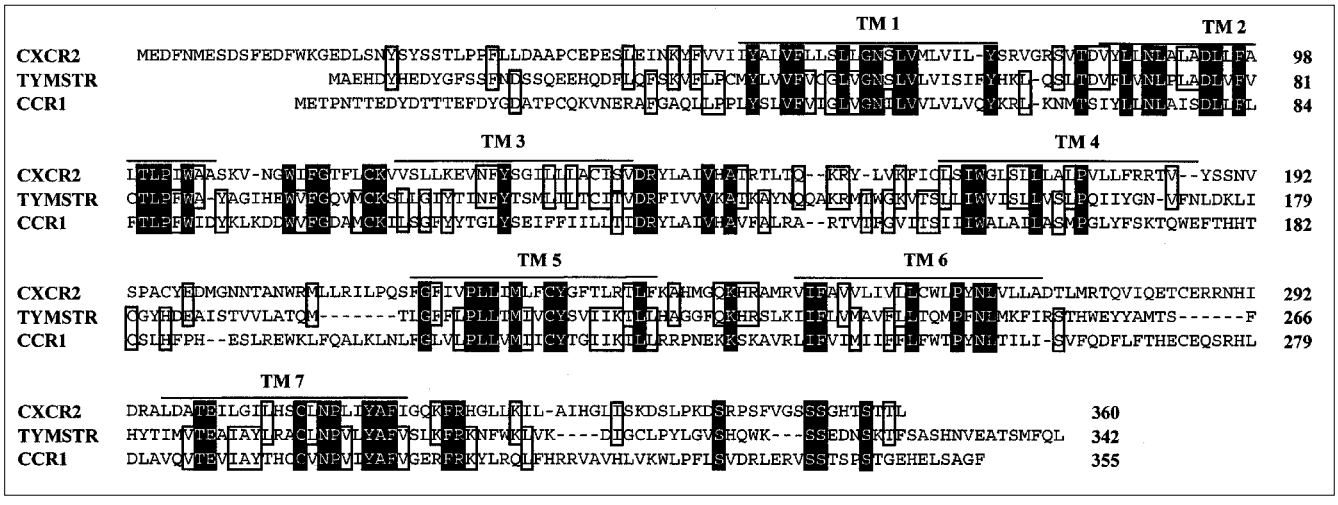
Results

Cloning of TYMSTR cDNA

In contrast to monocytes and granulocytes, the regulation of T lymphocyte recruitment to sites of inflammation and disease is not well understood. In order to search for chemokine receptors that are selectively expressed in T lymphocytes, we used a PCR-based strategy using degenerate oligonucleotide primers corresponding to chemokine receptor motifs [20]. One amplified DNA fragment, MLRB, with nucleotide similarity to known chemokine receptors hybridized selectively to RNA from activated but not freshly isolated peripheral blood lymphocytes. Using MLRB as hybridization probe, two cDNAs, TYMSTR1 of 2407 bp and TYMSTR2 of 1973 bp, were isolated from a $CD4^+$ T cell cDNA library; these cDNAs shared identical coding and 3′ sequences but differed in the 5′ untranslated regions. Divergency in the 5′ regions (494 bp and 60 bp in TYMSTR1 and TYMSTR2, respectively) was due to alternative mRNA splicing. TYMSTR2 (EMBL Database, accession number Y13248) corresponds to the completely spliced mRNA, whereas the 5′ region in TYMSTR1 contains an intron sequence, as assessed by PCR analysis using genomic DNA or cDNA as template and by northern blot analysis (data not shown). The flanking regions of the putative start codon AUG of the longest open reading frame conform well to the consensus sequence criteria for translation initiation in vertebrates [21]. The 3′ untranslated region contains a single polyadenylation signal sequence, and ends with a polyA tract.

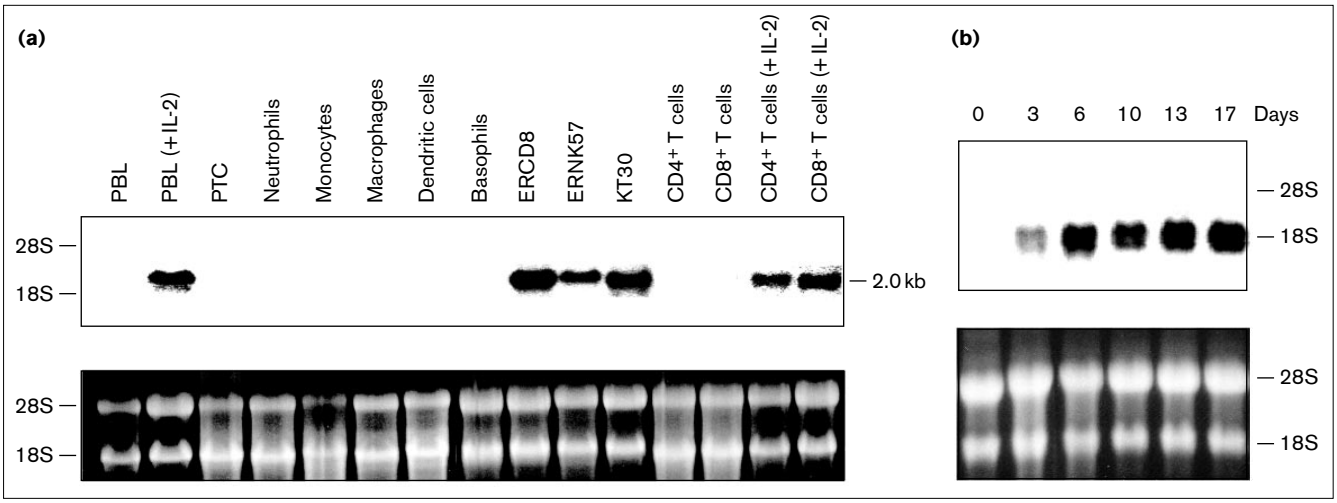
The open reading frame of 1026 bp encodes a polypeptide of 342 amino acids with a molecular weight of 39,280 Da and we have termed this protein TYMSTR (Figure 1). The amino-terminal, extracellular region is rich in acidic amino acids with a net charge of –7, which is typical for chemokine receptors, and contains a single potential N-linked glycosylation site (Asn^{16}–Asp–Ser). Protein kinase C substrate motifs (S/TXR/K in single-letter amino-acid code) and 11 serine/threonine residues are found in the third intracellular loop and the carboxyl terminus and phosphorylation at

Figure 1



Amino-acid sequence alignment of TYMSTR with the chemokine receptors CXCR2 and CCR1. Filled boxes with white letters highlight regions of sequence identity, and boxes with black letters designate residues in TYMSTR that are shared with either CXCR2 or CCR1; the putative transmembrane domains (TM 1–TM 7) are also indicated.

Figure 2



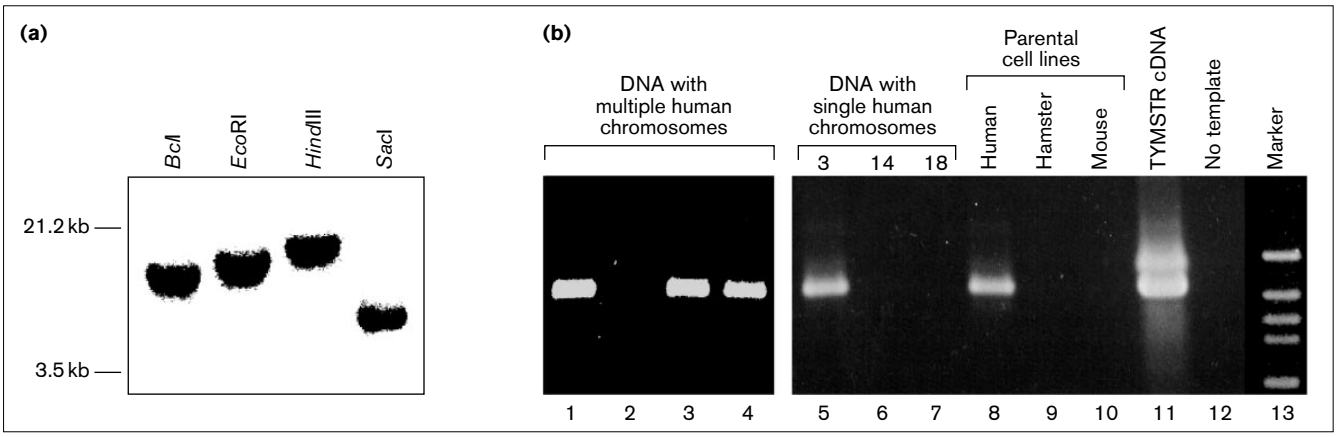
TYMSTR transcript expression in human leukocytes and lymphocytes. **(a)** Northern blot analysis was carried out with 10 μg total RNA from freshly isolated or cultured leukocytes and lymphocytes: PBL, peripheral blood lymphocytes; PBL (+ IL-2), IL-2-stimulated PBL; PTC, nylon-wool-purified T cells; ERCD8, CD8+ T-cell line; ERNK57, natural killer cell line; KT30, CD4+ T-cell line; CD4+ and CD8+ T cells were purified from fresh PBL. TYMSTR DNA was used as hybridizing probe (see Materials and methods). Lower panel, ethidium bromide staining of ribosomal RNA in the gel prior to transfer. **(b)** TYMSTR transcript expression in IL-2-stimulated T cell cultures. Freshly isolated PBLs were cultured in the presence of IL-2 for the number of days indicated and total RNA was analyzed by northern blot analysis. Lower panel, ethidium-bromide-stained ribosomal RNA prior to transfer.

these sites might be involved in receptor desensitization. Searches of known protein sequences revealed that TYMSTR is most closely related to chemokine receptors (33–36% sequence identity) and specifically has the highest level of identity with the type 2 IL-8 receptor, CXCR2, and the RANTES/MIP-1α receptor, CCR1 (Figure 1).

Expression of TYMSTR

The CD4+ T cell line KT30 that was used to prepare the cDNA library expressed a TYMSTR mRNA species of approximately 2 kb, indicating that the TYMSTR2 clone is complete (Figure 2a). Strong hybridization signals were also obtained with RNA from the CD8+ T cell line

Figure 3



Southern blot analysis and chromosomal localization of the TYMSTR gene. **(a)** Southern blot analysis. Genomic DNA from human PBL (10μg per lane) was digested with *Bcl*I, *Eco*RI, *Hind*III or *Sac*I, and the blot was hybridized with the same radiolabeled probe as used for the northern blot analysis in Figure 2. DNA fragments of 13 kb, 10.5 kb, 9 kb and 5.5 kb were detected and marker DNA fragments are indicated in kb. **(b)** Chromosomal localization of the TYMSTR gene by PCR. DNA from somatic cell hybrids containing single or multiple human chromosomes was amplified using specific primers for TYMSTR (see Materials and methods). Lanes 1–4, cell hybrids containing multiple human chromosomes (NA09930A, NA09932, NA09935A, NA09937); lanes 5–7, cell hybrids containing human chromosomes 3, 14 and 18, respectively (NA10253, NA10479, NA11010); lanes 8–10, parental human, hamster and mouse cell lines, respectively (NAIMR91, NA10658, NA05862); lane 11, TYMSTR cDNA; lane 12, no template DNA added; lane 13, DNA molecular weight markers.

ERCD8 and the natural killer cell line ERNK57. TYMSTR is highly expressed in peripheral blood lymphocytes (PBLs), $CD4^+$ and $CD8^+$ T cells after culturing these cells in the presence of IL-2, which leads to expansion of T cells with $CD45RO^+$ phenotype [22]. By contrast, unstimulated PBLs, $CD4^+$ and $CD8^+$ T cells did not contain TYMSTR transcripts. Importantly, TYMSTR transcripts were not expressed in other types of leukocytes, including neutrophils, monocytes, cultured macrophages, basophilic leukemia cells, monocyte-derived dentritic cells and many other related cell lines. Figure 2b illustrates the kinetics of TYMSTR expression in PBLs cultured in the presence of IL-2. The effect of IL-2 became evident after 3 days of culture and maximal level of TYMSTR expression was reached at day 6 and maintained up to day 17.

Chromosomal localization of the TYMSTR gene

TYMSTR is encoded by a single-copy gene, as demonstrated by Southern blot analysis with fractionated human genomic DNA (Figure 3a). Chromosomal localization of the TYMSTR gene was performed by PCR using TYMSTR-specific primers (see Materials and methods). As shown in Figure 3b, PCR products of the correct size were found in the three independently derived multiple chromosome-containing somatic cell hybrids that had human chromosomes 3, 14 and 18 in common (lanes 1, 3 and 4). Using cell hybrids that harbor single human chromosomes, the gene for TYMSTR could be assigned to human chromosome 3 (lane 5), coinciding with the chromosomal localization of the genes for all known CC chemokine receptors [23].

Chemokine selectivity of TYMSTR

In order to screen for ligands that interact with TYMSTR, murine pre-B cells (300-19) were stably transfected with TYMSTR cDNA, and clones that expressed high levels of TYMSTR transcripts were selected (data not shown). The agonists listed in Table 1, including 9 CXC chemokines and 17 CC chemokines, and unrelated ligands that bind to leukocyte G-protein-coupled receptors were tested for their activity in transfected 300-19 cells expressing TYMSTR. In addition, the C and CX_3C chemokines and four novel, unpublished chemokines have been tested. However, none of these substances induced $[Ca^{2+}]_i$ mobilization and *in vitro* chemotaxis, two typical chemokine-induced leukocyte responses. As a positive control, clones of 300-19 cells expressing CXCR3 responded strongly to the selective chemokines interferon-γ-inducible protein (IP10) and monokine induced by interferon-γ (Mig) [20], and parental 300-19 cells, which constitutively express murine CXCR4, responded to the CXCR4 ligand SDF-1 (data not shown).

TYMSTR is a coreceptor for HIV-1

The similarity of TYMSTR to chemokine receptors and its prominent expression in activated $CD4^+$ T cells prompted us to test this receptor for HIV coreceptor activity in Env-mediated cell fusion. For this purpose,

Table 1

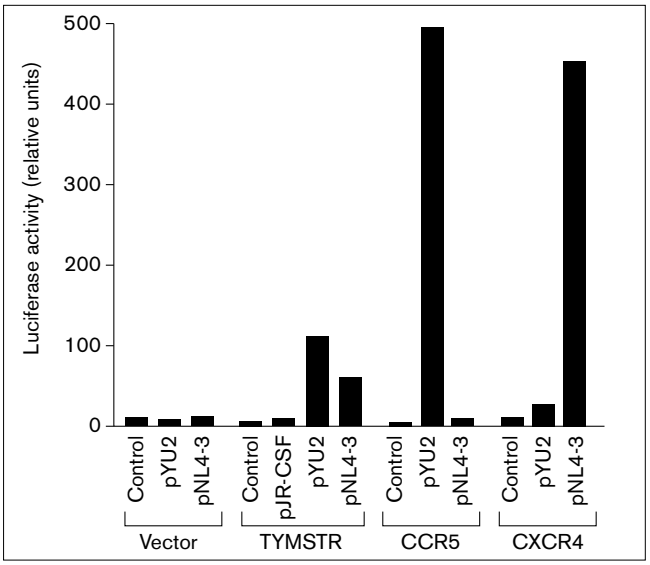
Ligands tested for $[Ca^{2+}]_i$ mobilization and chemotaxis

Agonist	$[Ca^{2+}]_i$*	Chemotaxis†	Control‡
RANTES	–	–	Monocytes
MCP-1	–	–	Monocytes
MCP-2	–	–	Monocytes
MCP-3	–	–	Monocytes
MCP-4	–	–	Monocytes
Eotaxin	–	–	Eosinophils
Eotaxin-2	–	–	Eosinophils
MIP-1α	–	–	Monocytes
MIP-1β	–	–	Monocytes
I-309	–	–	n.d.§
HCC-1	–	–	Monocytes
TARC	–	–	T cells
LARC	–	–	n.d.
ELC	–	–	n.d.
MIP-4	–	–	n.d.
MDC	–	–	CEM
CKβ8	–	–	Monocytes
IL-8	–	–	Neutrophils
GROα	–	–	Neutrophils
NAP-2	–	–	Neutrophils
GCP-2	–	–	Neutrophils
ENA-78	–	–	Neutrophils
Mig	–	–	T cells
IP-10	–	–	T cells
SDF-1	–	–	Monocytes
PF4	–	–	n.d.
C3a	–	–	HMC-1
C5a	–	–	Neutrophils
LTB_4	–	–	Neutrophils
Calcitonin	–	–	n.d.
NPY	–	–	SK-N-MC
Somatostatin	–	–	n.d.
Substance P	–	–	HMC-1

The ligands were tested in clones of 300-19 cells that stably expressed TYMSTR. *$[Ca^{2+}]_i$ responses to 100 nM agonist were measured in Fura2/acetoxymethylester-loaded cells. †Chemotaxis *in vitro* was analyzed with chemokine in the range 1 nM to 1 μM. ‡The cells listed were used as positive control for the activity of the corresponding agonists. §n.d., not determined.

NIH3T3 murine fibroblasts were cotransfected with recombinant plasmids encoding human CD4, a HIV-1 LTR-driven luciferase reporter gene and human chemokine receptors (CXCR4, CCR5) or TYMSTR. HEK293 human embryo kidney cells expressing proviral DNA from T-tropic or M-tropic HIV-1 particles were used as fusion partners. Cell fusion, occurring after binding of CD4 and coreceptors to HIV-1 Env glycoproteins, was quantified by measuring the luciferase activity present in the cell lysate. As shown in Figure 4, background luciferase activity was very low; background activity was assayed on parental HEK293 cells mixed with $CD4^+$, luciferase-positive NIH3T3 cells that were transfected with TYMSTR cDNA or plasmid alone. A 14-fold increase in luciferase activity was obtained, however, when NIH3T3 cells expressing TYMSTR were co-cultured with HEK293 cells expressing Env of the M-tropic HIV-1 YU2, but TYMSTR did not recognize the Env glycoprotein of the M-tropic HIV-1 JR-CSF.

Figure 4



TYMSTR mediates fusion with HEK293 cells expressing HIV-1 Env(YU2) and Env(Lai). NIH3T3 cells were transfected with a plasmid containing HIV-1 long terminal repeat (LTR)-driven luciferase reporter gene and plasmid DNA encoding human CD4 and either TYMSTR or other coreceptors (CXCR4, CCR5). These cells were mixed with HEK293 cells transfected with DNA for various molecular clones of HIV-1, and fusion was quantified by measuring luciferase activity (relative units) in the cell lysate. As a negative control, fusion partner cells [$CD4^+$ NIH3T3(*lucif*), HEK293] were transfected with empty plasmid vector DNA.

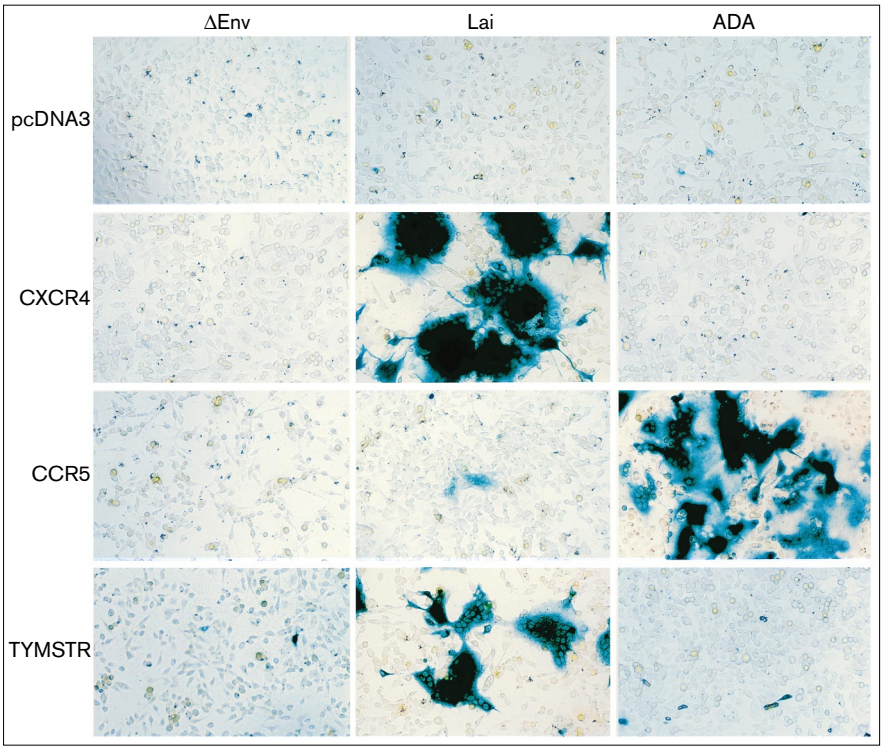
TYMSTR also functioned as a fusion receptor for the T-tropic viral clone pNL4-3, which carries the Env gene of the HIV-1 Lai.

Recognition of Env of HIV-1 Lai by TYMSTR was further documented in an alternative cell-fusion assay. NIH3T3 cells co-expressing human CD4 and TYMSTR or human CD4 and either CXCR4 or CCR5 as well as the HIV-1 LTR-driven *lacZ* gene were co-cultured for fusion with HeLa cells expressing Env(Lai) or Env of HIV-1 ADA. As revealed by intracellular staining for β-galactosidase activity, TYMSTR recognized Env(Lai) and fusion resulted in multinucleated cells (Figure 5). In contrast, TYMSTR failed to support fusion with HeLa cells expressing Env(ADA). As positive controls, CXCR4 mediated fusion with cells expressing the T-tropic Env(Lai), and CCR5 was active with cells expressing the M-tropic Env(ADA). Parental HeLa(ΔEnv) cells lacking viral Env glycoproteins were used as negative control and did not show any fusion activity.

Infection of TYMSTR-expressing cells by T-tropic HIV-1 particles

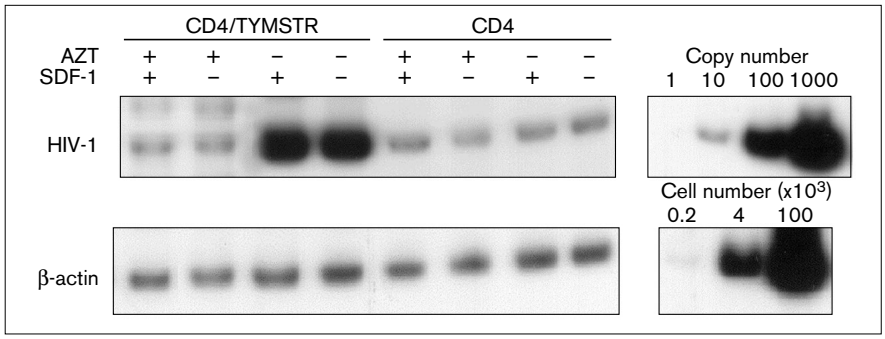
An early post-fusion event during viral infection is the formation of proviral DNA by the HIV-1 reverse transcriptase. This is required for expression of genes encoding viral proteins and, ultimately, for the generation of new infectious particles. Murine 300-19 cells expressing

Figure 5



TYMSTR mediates syncytia formation with HeLa cells expressing Env(Lai). NIH3T3 cells transfected with a HIV-1 LTR-driven β-galactosidase reporter gene and plasmids encoding human CD4 together with TYMSTR or other coreceptors (CXCR4, CCR5) were mixed with HeLa cells expressing the viral Env glycoproteins of HIV-1 Lai or HIV-1 ADA, and cell fusion was monitored by staining for intracellular β-galactosidase activity. The negative control monitored fusion between HeLa(ΔEnv) cells and NIH3T3 cells cotransfected with reporter gene DNA, CD4 DNA and empty vector DNA.

Figure 6



Entry of HIV-1 Lai in murine 300-19 cells transfected with TYMSTR and CD4. Clones of 300-19 cells that stably expressed human CD4 either alone or in combination with TYMSTR were infected with infectious HIV-1 Lai particles in the absence (–) or presence (+) of 10 μM AZT or 100 nM SDF-1, and newly formed proviral DNA was detected by PCR as described in Materials and methods. To evaluate the sensitivity of the assay, purified proviral DNA (1–10^3 copies) and DNA harvested from 200, 4×10^3 or 10^5 infected cells were included in the assay, and amplification of β-actin DNA was performed as an internal control. Amplification products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography.

TYMSTR together with human CD4 became infected with T-tropic HIV-1 Lai particles, as assayed by PCR detection of proviral DNA (Figure 6). Formation of proviral DNA was inhibited by the reverse transcriptase inhibitor AZT, indicating that the amplified DNA derived from newly reverse-transcribed HIV-1 Lai RNA. The chemokine SDF-1, which has been shown to block CXCR4-mediated infection by T-tropic HIV-1 strains [18,19], did not prevent proviral DNA formation, and 300–19 cells expressing human CD4 alone were not infected, demonstrating that viral entry was mediated by TYMSTR in combination with CD4. The weak AZT-insensitive amplification signals observed in all lanes were probably due to HIV-1 Lai-associated proviral DNA present from input virus. Our findings from cell fusion and viral entry assays demonstrate that TYMSTR has CD4-dependent HIV coreceptor function.

Discussion

Several features suggest that TYMSTR is a novel chemokine receptor. Firstly, TYMSTR has highest amino acid sequence identity (33–36%) with CXC and CC chemokine receptors whereas it has <30% similarity with other G-protein-coupled receptors, such as the receptors for the non-chemokine attractants fMet–Leu–Phe, anaphylatoxins C5a and C3a, the lipid mediator platelet-activating factor, and the thrombin receptor [24–28]. In addition, chemokine receptors are similar in size, ranging from 350–368 amino acids, which agrees well with the size of TYMSTR; most other G-protein-coupled receptors are over 400 amino acids in size [4,5,29].

Secondly, 25% of the amino acids in the extracellular amino-terminal region in TYMSTR are charged, having an eight-fold excess of acidic residues, which is typical for chemokine receptors [1,4,5,29]. The corresponding regions in the IL-8 receptors CXCR1 and CXCR2, for example, are composed of 22% and 26% charged residues with a nine-fold and six-fold excess of acidic residues, respectively [30,31]. These parts of the receptors have been shown to be essential for binding of chemokines, which themselves are highly charged and basic [32–34].

Thirdly, using a PCR-based method we could assign the TYMSTR gene to the human chromosome 3, the same chromosomal location of the genes for all five CC chemokine receptors [23], suggesting that the natural ligand for TYMSTR might be a CC chemokine. By contrast, the genes for the three CXC chemokine receptors CXCR1, CXCR2 and CXCR4 are localized to human chromosome 2 [35–37].

The selective expression of TYMSTR in activated T cells is notable with regard to its potential role in T-cell recruitment as well as HIV infection [38]. IL-2 treatment of PBLs or purified T cells induced prominent expression of TYMSTR, whereas non-activated T cells and any other type of leukocytes lacked detectable levels of TYMSTR transcripts. It is important to note that triggering of the T-cell receptor by means of immobilized anti-CD3, either alone or in combination with anti-CD28, or T-cell activation by phytohemagglutinin did not induce TYMSTR expression. These findings suggest that TYMSTR is involved in the selective recruitment of IL-2-expanded immunocompetent T cells to sites of inflammation.

For fusion and cell entry, HIV requires two key molecules on target cells, CD4 and an appropriate coreceptor [6,7]. CD4 is present on a subset of T cells thought to be the primary host cells for viral replication, and on certain antigen-presenting cells. By contrast, coreceptors belong to the growing family of chemokine receptors, which differ in their selectivity for chemokines and, equally important, in their range of cellular expression. Most chemokine receptors are present in large numbers in monocytes and granulocytes but not in circulating T lymphocytes [1–5]. In order

to express chemokine receptors, including CCR1, CCR2 and CCR5, and to become responsive to chemokines, T lymphocytes need to be activated, notably by stimulation with IL-2, which leads to expansion of $CD26^+$, $CD45RO^+$ T cells [20,22]. TYMSTR is selectively expressed in these activated cells and functions as an HIV-1 coreceptor. The lower potency of TYMSTR in cell fusion, compared with that of CXCR4 and CCR5, might reflect a lower affinity of TYMSTR for the Env glycoproteins of HIV-1 YU2 and HIV-1 Lai. It is likely that mutational changes in the HIV *env* gene, which occur rapidly during replication in infected individuals, could generate HIV-1 variants that have a high affinity for TYMSTR. Identification of such isolates *in vitro* might have been hampered by the procedures used for their amplification. In fact, TYMSTR is not expressed in phytohemagglutinin-activated peripheral blood mononuclear cells, which are routinely used as host cells for the culturing of primary viruses.

Conclusions

TYMSTR transcripts are abundant in activated T lymphocytes but are not detectable in resting lymphocytes or other leukocytes, suggesting that this putative chemokine receptor and its natural ligand are involved in the recruitment of immunocompetent T lymphocytes in T-cell-dependent immune responses. In addition, TYMSTR has HIV coreceptor function: its high-level expression in $CD4^+$ T lymphocytes, together with its ability to interact with viruses that predominate either at early stages in HIV^+ individuals or during disease progression, indicates that TYMSTR might be involved in HIV-1 infection. For a detailed understanding of disease progression, it is important to identify further HIV coreceptors with prominent expression in $CD4^+$ cells. This conclusion is consistent with our own observation (D. Rousset, D. Mathez, E. Oberlin, J. Leibowitch, F. Arenzana-Seisdedos and J-L. Virelizier, unpublished observations) that primary isolates from some patients with AIDS can not be blocked *in vitro* by the combined addition of known HIV suppressor chemokines, suggesting that novel HIV coreceptors are required. During the review stages of this manuscript, similar results were reported by Berger and colleagues [39].

Material and methods

Proteins

Chemokines and related proteins were prepared by chemical synthesis according to established protocols [40]. C3a and C5a were a gift of C. A. Dahinden, University Hospital, Bern. Somatostatin, calcitonin and substance P were from Sigma and LTB4 was donated by J. Rokach, Merck Frosst Canada Inc..

Cell preparation

Neutrophils, monocytes and human PBLs were isolated from donor blood buffy coats as described [41]. PBL and cloned human T and NK cells (KT30, ERCD8, ERNK57) were cultured in RPMI-1640 medium containing 2 mM glutamine, 1 × non-essential amino acids, 1 mM sodium pyruvate, 100 μg/ml kanamycin, 50 μM 2-mercaptoethanol and 5% human serum in the presence of 400 U/ml human recombinant IL-2. Murine 300-19 cells were maintained as described [22].

TYMSTR cDNA cloning

The cloning of TYMSTR cDNA was performed essentially as described for CXCR3 [20]. For PCR amplification on a Master Cycler 5330 (Eppendorf-Netheler-Hinz), the degenerate primers 5′-GGGCTGCAG CIIT(T/G)(T/G)C(C/A)GAC(A/C)TICTI(C/T)T-3′ and 5′-GGGTCTA-GAAA(A/G)GC(A/G)TA(G/C)AI(G/C)AIIGGGT-3′, I is inosine) were used with human genomic DNA as template. PCR products of the expected size (600–700 bp) were sequenced and examined for homology with known chemokine receptors and expression in leukocytes. The amplified DNA fragment, MLRB, was used as hybridization probe for isolation of TYMSTR clones out of a human $CD4^+$ T cell (KT30) library [20].

Northern and Southern blot analysis

Samples of 10 μg total RNA from human leukocytes and cultured cell lines (KT30, ERCD8, ERNK57) were analyzed as described [20]. PBL and fractionated $CD8^+$ and $CD4^+$ T cells were cultured for up to 10 days in the presence of IL-2 (400 U/ml) [22]. A 400 bp *Eco*RV/*Pst*I fragment of TYMSTR2 was radiolabeled and used as a probe for hybridization. The membrane was washed up to a stringency of 0.2 × SSC, 0.1% SDS at 65°C, and analyzed using a PhosphorImager (Molecular Dynamics). For Southern blot, 10 μg samples of genomic DNA from human PBL were digested with *Eco*RI, *Bcl*I, *Sac*I and *Hind*III, separated and vacuum blotted. The membrane was probed, washed and analyzed as in northern blot analysis.

Chromosomal localization

For chromosomal localization studies, PCR was performed with the TYMSTR-specific primers 5′-AAGACTATGGGTTCAGCAGT-3′ and 5′-CCACATATGAGCTTGTCGAG-3′ in a PTC-100 Thermocycler (MY Research). Genomic DNA from monochromosomal somatic cell hybrids and somatic cell hybrids harboring human chromosomes was used as template (Coriell Institute). Cell hybrids containing multiple human chromosomes (hc) were NA09930A (hc: 2–5,7,8,11–15,17, 18,20–22,X,Y), NA09932 (hc: 4–6,8,10–12,17,19,21), NA09935A (hc: 3–6,10,12–14,17,18,20–22), and NA09937 (hc: 3,4,6–8,10,12, 14,15,17,18); cell hybrids containing single human chromosomes were NA10253 (hc: 3), NA10479 (hc: 14), and NA11010 (hc: 18); parental cell lines were NAIMR91 (human), NA10658 (hamster), and NA05862 (mouse). After initial denaturation at 94°C for 3 min, 25 cycles of amplification were performed at 94°C for 45 s, at 58°C for 45 s, and at 72°C for 50 s with a final extension for 10 min at 72°C. Products were visualized by ethidium bromide staining of agarose gels.

Functional assays in TYMSTR transfectants

For the generation of stable transfectants, 4×10^6 mouse pre-B cells (300-19) were transfected by electroporation with 20 μg linearized pcDNA3-TYMSTR plasmid DNA. Stable TYMSTR transfectants were cloned by limiting dilution in the presence of 1.0 mg/ml G418 (Life Technologies). G-418-resistant clones were screened for TYMSTR transcript expression by RNA dot-blot analysis using the same hybridization probe as for the northern and Southern blots [20]. Changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell migration of murine 300-19 clones expressing TYMSTR were evaluated as described [20,22].

HIV-1 cell fusion assays

Subconfluent cultures of NIH3T3 murine fibroblasts were cotransfected with recombinant plasmids encoding human CD4, a HIV-1 LTR-driven luciferase reporter gene [42] and human chemokine receptors (CXCR4, CCR5) or TYMSTR. Coreceptor DNAs were cloned into the expression plasmid pcDNA3, and empty vector was used as a negative control. DNA transfections were performed with Lipofectamie (Gibco BRL) according to the manufacturer's instructions. After culturing for 24 h, NIH3T3 cells were mixed at a ratio of 1:1 with adherent human embryo kidney (HEK) 293 cells expressing infectious HIV proviral DNA of either NL4-3, YU2, or JR-CSF HIV-1 isolates. After co-culture for 24 h, cells were harvested and disrupted and luciferase activity was measured using a luminometer (Berthold). The β-galactosidase

reporter gene (*lacZ*) assay was used as a second cell-fusion assay [18]. Briefly, NIH3T3 cells were transfected with human CD4 cDNA, a HIV-1 LTR promoter-driven *lacZ* gene DNA construct and cDNAs for TYMSTR or chemokine receptors (CXCR4, CCR5). Vector DNA (pcDNA3) was used as negative control. Cells were cultured at 1×10^5 cells/well in 24-well plates for 18 h and were then mixed with cloned HeLa cells that stably expressed viral Tat protein and Env glycoproteins from HIV-1 Lai or HIV-1 ADA. A HeLa cell clone expressing Tat but lacking viral Env (ΔEnv) was used as control. Cells were fixed with 0.5% glutaraldehyde, and cell fusion was visualized by staining for β-galactosidase activity.

PCR amplification of proviral DNA

Murine pre-B 300-19 cells (5×10^6 cells/4 ml), stably expressing either human CD4 or CD4 and TYMSTR were infected for 3 h with 2.5 ml infectious HIV-1 Lai particles (350 ng/ml HIV-1 p24). After three washes, the cells were trypsinized, resuspended in fresh medium and cultured for a further 21 h. As control, the infection was performed in the presence of SDF-1 (100 nM) or AZT (10 μM) or a combination of both agents. After extraction of total DNA [43], 30 cycles of PCR were performed with ^{32}P-labeled primers for proviral DNA (3′-primer, 5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′; 5′-primer, 5′-GGCTAACTAGGGAACCCACTG-3′) in 25 μl 0.25 mM dNTPs, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mg/ml BSA and 1.5 U Taq DNA polymerase (Amersham), yielding amplified DNA of 200 bp. As control, β-actin DNA (244 bp) was amplified (5′-primer, 5′-GTGGGGCGCCCCAGGCACCA-3′; 3′-primer, 5′-CGGTTGGCCTTGGGGTTCAGGGGGG-3′). Amplification products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography.

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