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## The G-protein coupled receptor CMKLR1/ChemR23: Studies on gene regulation, receptor ligand activation, and HIV/SIV co-receptor function

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An academic dissertation

**The G-protein coupled receptor CMKLR1/ChemR23:**

*Studies on gene regulation, receptor ligand activation, and  
HIV/SIV co-receptor function*

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<p>In all higher organisms, there is a need for intercellular communication. G-protein coupled receptors (GPCRs), located on the cell surface, play an important role in this communication. Cells synthesize and release signalling molecules (ligands), which produce a specific response only in those cells that have a receptor for that ligand. The total number of GPCRs is estimated to be around 800 where approximately 160 still are "orphans", meaning that the endogenous ligands are unknown.</p> <p>CMKLR1/ChemR23 is a GPCR that was recently "de-orphanized" when the endogenous ligand, TIG2/chemerin, was isolated from inflammatory fluids and hemofiltrate. The receptor displays a high homology to chemoattractant-like receptors involved in inflammation processes. Sequence is implicated in osseous and cartilage development, and the receptor is also suggested to have a pathophysiological role as one of the co-receptors involved in human and simian immunodeficiency virus (HIV-1 and SIV)-infection of CD4<sup>+</sup> immune cells. We have described the genomic organization of <i>cmklr1</i> in mouse and analysed the regulatory mechanism behind the corresponding receptor expression. Two transcripts of mouse <i>cmklr1</i> have been identified, <i>cmklr1a</i> and <i>b</i>, which utilize alternative promoters for transcription. We show that four Sp1 transcription factors are involved in <i>cmklr1a</i> promoter activation in mouse NB4 1A3 cells, whereas the transcription factor NFY is important for transcription of <i>cmklr1b</i> in mouse BV2 cells. <i>cmklr1a</i> shows expression in organs such as heart and lung whereas <i>cmklr1b</i> is suggested to be an inducible transcript up-regulated by stimulation with all-trans retinoic acid (ATRA).</p> <p>The interaction between CMKLR1/ChemR23 and TIG2/chemerin in mouse has been studied. Mouse TIG2/chemerin was found to activate the mouse receptor, although to a lower degree than for the human receptor. Peptides corresponding to the C-terminus of mouse TIG2/chemerin could activate mouse CMKLR1/ChemR23 but to a lower extent than the human receptor. The results indicate that the peptide domains necessary for receptor activation differ for human and mouse TIG2/chemerin or that the maximal response of the mouse receptor is lower than in human.</p> <p>The importance of human CMKLR1/ChemR23 as a co-receptor for HIV and SIV has been investigated. We show that CMKLR1/ChemR23 can function as a minor co-receptor for select HIV-1 isolates as well as more generally for HIV-2 and SIV isolates. Among certain better-characterized co-receptors, the HIV-1 co-receptor function of CMKLR1/ChemR23 resembles that of the chemokine receptor, CCR3. Using a "humanized" hybrid rat CMKLR1/ChemR23 receptor as model, it was shown that the major determinants for HIV-1 and HIV-2 interaction with the receptor reside to a varying degree in the N-terminus and second extracellular loop, whereas the viral interaction in the case of SIV primarily involves the second extracellular loop.</p>		
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*To my parents, in memoria*

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## ABBREVIATIONS

### ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATRA	All-trans retinoic acid
BAC	Bacterial artificial chromosome
PBMCs	Peripheral blood mononuclear cells
BrdU-triphosphate	Bromo-deoxyuridine-triphosphate
CMKLR1	chemoattractant-like receptor 1
CHO-K1 cells	Chinese hamster ovary cells
DAG	Diacylglycerol
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Env	Envelope
GDP	guanosine diphosphate
gp120	Glycoprotein 120
gp41	Glycoprotein 41
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
GTF	General transcription factors
HEK293 cells	Human embryonic kidney cells
HeLa cells	Human cervix carcinoma cells derived from Henrietta Lack
HIV	Human immunodeficiency virus
IP <sub>3</sub>	Inositol 1,4,5 triphosphate
LiCl	Lithium chloride
mRNA	messenger RNA
NFY	Nuclear factor for Y-box
NTPs	nucleotide triphosphates
PTX	Pertussis toxin

## ABBREVIATIONS

PHA	Phytohemagglutinin
PLC	Phospholipase C
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
Pol II	RNA polymerase II
PtIns	Phosphatidylinositols
5' RACE	5' Rapid amplification of cDNA ends
RARs	Retinoic acid receptors
RT assay	Reverse transcriptase activity assay
RXRs	Retinoid X receptors
Sp	Specificity protein
Sp1	Specificity protein 1
SIV	Simian immunodeficiency virus
SIVmac	SIV isolated from macaque
SIVsm	SIV isolated from sooty mangabey
TAFs	TBP Associated Factors
TBP	TATA binding protein
TIG2	Tazarotene-induced gene 2

## LIST OF PUBLICATIONS

### LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

- I.    **Genomic organization and promoter analysis of the gene encoding the mouse chemoattractant-like receptor, CMKLR1.**  
Ulrika E. A. Mårtensson, Christer Owman, Björn Olde  
*Gene* 328:167-176 (2004)
  
- II.   **The mouse chemerin receptor gene, *mcmklr1*, utilizes alternative promoters for transcription and is regulated by all-trans retinoic acid.**  
Ulrika E. A. Mårtensson, Jesper Bristulf, Christer Owman and Björn Olde  
*Gene*; *in press*
  
- III.   **C-terminal domains of the TIG2/chemerin ligand, required for activation of its receptor CMKLR1/ChemR23, differ in mouse and human.**  
Ulrika E. A. Mårtensson, Knut Kotarsky, Niclas E. Nilsson, Christer Owman and Björn Olde  
*Manuscript*
  
- IV.   **Characterization of the human chemerin receptor – ChemR23/CMKLR1– as co-receptor for human and simian immunodeficiency virus infection, and identification of virus-binding receptor domains.**  
Ulrika E. A. Mårtensson, Eva-Maria Fenyő, Björn Olde and Christer Owman  
*Manuscript*

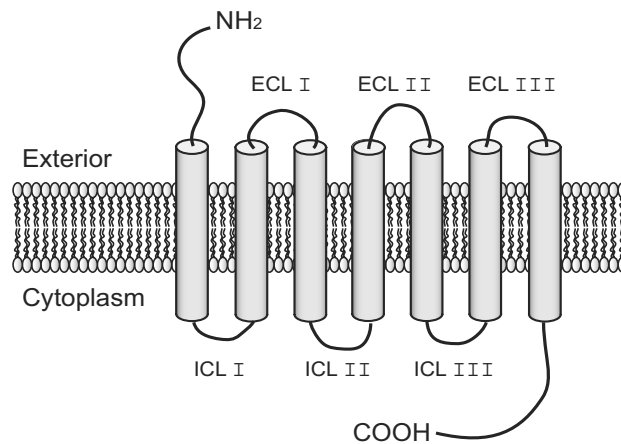


## BACKGROUND

## BACKGROUND

### The G-protein coupled receptor family

In all higher organisms, there is a need for intercellular communication. Cells synthesize and release signalling molecules (ligands), which produce a specific response only in the target cells that have a receptor for that ligand. There are three major families of cell surface receptors involved in signal transmission: the G-protein coupled receptors (GPCRs), the ion-channel receptors, and the enzyme-linked receptors. GPCRs constitute by far the largest and most diverse family of cell surface receptors. Based on structural and functional characteristics, this genetic superfamily is divided into five subfamilies – rhodopsin, glutamate, adhesion, frizzled/taste and secretin (1). GPCRs are activated by a variety of ligand stimuli, including neurotransmitters, chemoattractants, hormones, growth factors, odorants, pheromones and light. The superfamily of GPCRs constitutes an important portion of the genome. Indeed, it has been estimated that as much as 1% of all genes in fruit fly (*Drosophila melanogaster*), 5% in nematodes (*Caenorhabditis elegans*) and 2% in humans (*Homo sapiens*) are coding for GPCRs (2-5). Although the ligands for these receptors differ dramatically in size from large proteins to metal ions, the basic receptor structure is the same with seven highly conserved transmembrane helices, connected by three extracellular (ECL I-III) and three intracellular (ICL I-III) loops (Fig. 1).



**Figure 1. Representation of a GPCR.**

## BACKGROUND

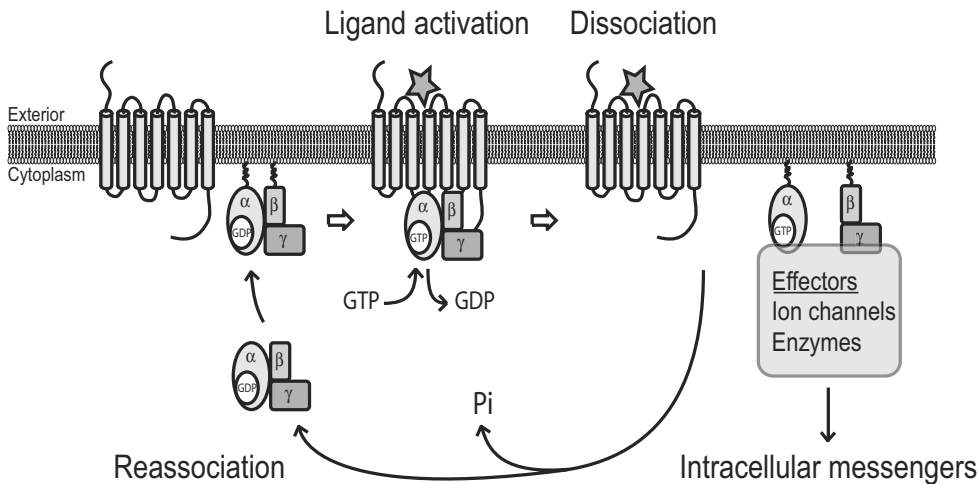
The important function of GPCRs in the body is emphasized by the fact that they are remarkably well conserved during evolution. For example, the muscarinic receptor of *C. elegans* displays 51% amino acid sequence homology with its orthologue in *D. melanogaster* and 42-44% with its human orthologue (in the alignment, a highly variable sequence in ICLIII is excluded) (6). A three-dimensional receptor structure would have been desirable in order to understand how ligand binding to a GPCR leads to conformational changes and transmission of a signal from the outside to the inside of the cell. Unfortunately, transmembrane proteins are difficult to crystallize, the main reason being that GPCRs contain highly hydrophobic regions. Another problem is the difficulty in obtaining the large quantities of pure receptor protein needed for crystallization. So far, the only three-dimensional structure of a GPCR described is the crystallized structure of the inactive state of bovine rhodopsin (7). The availability of the rhodopsin crystal information has greatly facilitates the construction of models of other GPCRs. However, the construction of realistic GPCR models is time-consuming and requires biological, pharmacological and biophysical data for verification.

### **Signal transduction through the cell membrane and the role of G-proteins**

The mechanism by which GPCRs transmit extracellular signals through the cell membrane to intracellular responses is mediated by heterotrimeric G-proteins. The activation of a GPCR by agonist binding causes the receptor to associate with an appropriate G-protein and to form a trimeric complex consisting of the agonist, the receptor and the G-protein (**Fig. 2**). The G-protein itself consists of three subunits: the  $\alpha$ -,  $\beta$ - and the  $\gamma$ - subunit. The binding of the activated receptor to the G-protein results in the replacement of GDP by GTP on the  $\alpha$ -subunit. The replacement induces a conformational change in the G-protein, leading to the dissociation of the G-protein into an  $\alpha$ -subunit and a  $\beta\gamma$ -complex. The free  $\alpha$ -subunit and  $\beta\gamma$ -complex then bind to and activate various effector proteins, thus propagating an intracellular signalling cascade. The G-protein is inactivated by hydrolysis of GTP to GDP through the intrinsic GTPase activity of the  $\alpha$ -subunit. The GDP- $\alpha$  subunit

## BACKGROUND

then reassociates with the  $\beta\gamma$ -complex to re-establish the trimeric G-protein, which is then ready for another round of receptor activation (**Fig. 2**).



**Figure 2. GPCR activation.**

In mammals, more than twenty different  $\alpha$ -subunits have been described. These are organized into four subfamilies,  $G\alpha_s$ ,  $G\alpha_{i/o}$ ,  $G\alpha_{q/11}$ , and  $G\alpha_{12/13}$ , based on structural and functional homologies (8-10). For a long time, it was believed that the  $\alpha$ -subunit of the trimeric G-protein alone was responsible for the regulation of all effectors. It has now become clear that also the  $\beta\gamma$ -complex is important in the regulation of effector proteins (11). In mammals, the  $\beta\gamma$ -complex can be combined from five subtypes of  $\beta$ -subunits and twelve subtypes of  $\gamma$ -subunits (12).

The four classes of G-protein  $\alpha$ -subunits interact with different specific effector proteins:  $G\alpha_{q/11}$  activates PLC (13), which catalyses the hydrolysis of  $PIP_2$  into  $IP_3$  and DAG, which in turn leads to the release of intracellular stored  $Ca^{2+}$ .  $G\alpha_s$  stimulates whereas  $G\alpha_i$  inhibits adenylyl cyclase leading to an increase and a decrease, respectively in cAMP (14-16).

## BACKGROUND

$G\alpha_{12/13}$  activates different pathways such as the effector protein phospholipase D (17). The  $\beta\gamma$ -complex stimulates a diversity of effector proteins including PLC (18) and certain isoforms of adenylyl cyclase (19, 20).

The bacterial toxin, pertussis toxin (PTX), produced by *Bordetella pertussis*, has been useful in the characterization of G-protein signalling pathways since it interferes with the  $G\alpha_i$  pathway whereas other G-protein pathways are unaffected. Pertussis toxin acts through ADP ribosylation of the  $G\alpha_i$  class, thereby uncoupling the receptor from the G-protein (21).

### **Regulation of gene transcription**

In order to investigate how GPCR expression is regulated, we need to study gene transcription, which takes place in the nucleus.

The genetic material is stored in genes, which are nucleotide sequences forming stretches of deoxyribonucleic acids, DNA, the universal genetic material. In the eukaryotic cell, DNA is located in the nucleus where it is wrapped around histone proteins, forming complexes called nucleosomes. Strings of nucleosomes are coiled up and folded into a highly condensed chromatin structure. The chromatin is further organized into larger units of up to thousands of kilobases in length called chromosomes. The total amount of genes in the human DNA has been estimated to approximately 25,000, where the total number of GPCR genes amounts to approximately 800 (22). Each gene provides the instructions for synthesizing a unique protein that has a specialized function in the cell. In order to transfer the information residing in a gene into a protein, the cell uses two processes: transcription and translation. Transcription occurs in the nucleus where the transcription machinery copies the gene into messenger RNA (mRNA), the template for protein synthesis. The mRNA is subsequently transported out of the nucleus and into the cytoplasm where it is translated into a protein. The whole process of transforming genetic information into a

## BACKGROUND

protein is known as expression. When and where a gene will be expressed is regulated primarily at the level of transcription but also partly by mRNA processing and translation. Transcription is dependent on the accessibility of the DNA. For a gene to be transcribed, the chromatin structure must be unpacked so that the transcription machinery can get access to the DNA. This is performed either by acetylation (23, 24), and/or phosphorylation of the NH<sub>2</sub>-terminal of the histones (23).

### *The transcriptional regulatory region of a gene*

A typical mammalian gene includes 1) the sequence coding for the protein, 2) a core promoter immediately upstream of the transcription start site containing “general” transcription binding sites for initiation of transcription, 3) a proximal or “regulatory” promoter upstream of the core promoter, containing gene-specific DNA elements, and 4) enhancer or silencer sequences, affecting transcription from large distances (**Fig. 3A**).

A core promoter that is localized up to about 60 bp upstream of the transcription start site is found in all genes coding for proteins. Core promoter elements are necessary for accurate initiating of transcription. The TATA-box (TATAA) is a common core element that is usually found at a conserved position. This element binds the transcription factor TBP, which is important in initiation of transcription. However, many eukaryotic core promoters have been shown to lack typical TATA-boxes. Instead, they contain a weakly conserved initiator element (5'P<sub>y</sub>P<sub>y</sub>A<sub>+1</sub>N(T/A)P<sub>y</sub>P<sub>y</sub> where P<sub>y</sub> stands for C or T and A<sub>+1</sub> is the transcription start site) which serves as the transcription start site (25, 26). It has been shown that TFIID, a complex consisting of TBP and TAFs (27), can bind to both TATA-boxes and initiator elements (28).

The proximal promoter region, situated approximately 60-500 bp upstream of the transcription start site, contains consensus binding sites for transcription factors that are tissue- or cell-specific as well as ubiquitously expressed proteins.

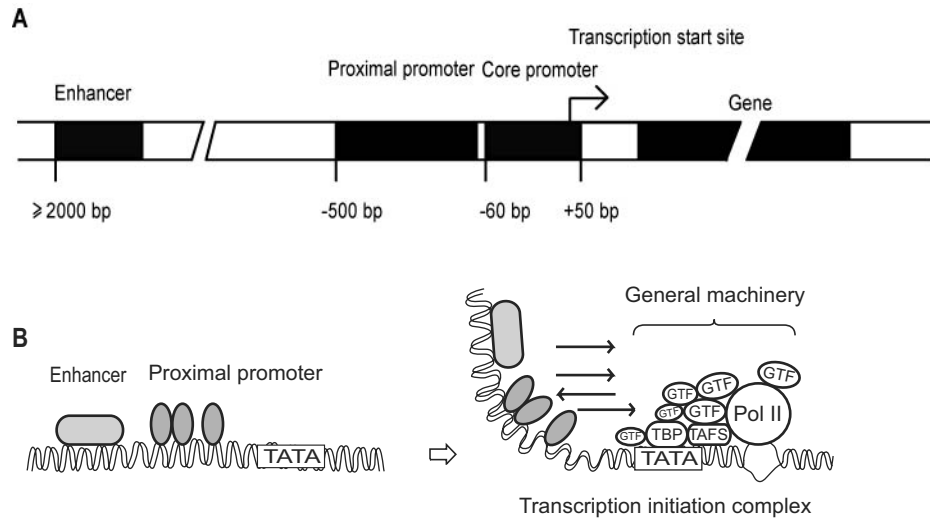
## BACKGROUND

Enhancers and silencers are sequences that are found thousands of base pairs upstream, downstream or even within the gene that they regulate (29). These regulatory regions affect transcription by binding the same kind of transcription factors as the promoters. Proteins that bind to enhancers/silencers also have themselves binding sites for the transcription factors that are assembled at the proximal promoter of the gene. This arrangement gives rise to large complexes bridging and forcing the DNA to make a loop and thus activating or repressing the target gene (29, 30).

### *A general model for regulation of a gene*

RNA polymerase II (Pol II) transcribes genes coding for proteins. For a gene to be transcribed, a cascade of events occurs that eventually leads to transcription. First, the opening up of the chromatin structure makes the regulatory region of the gene accessible for binding combinations of transcription factors. The bound transcription factors subsequently interact with the general transcription machinery recruiting it to the core promoter (31). The general machinery is then stepwise assembled on the core promoter forming the transcription-initiation complex. In short, TFIID initially binds the TATA-box or the initiator element, which is then followed by sequential recruitment of GTF, Pol II, co-activators and co-repressors (32-34) (**Fig. 3B**). The assembly of the transcription-initiation complex induces separation of the two DNA strands, and the presence of NTPs initiates transcription by starting RNA synthesis. The transcription factors then dissociate from the promoter, and a complex consisting of Pol II and certain transcription factors move along the DNA template strand, causing the synthesis of the mRNA. Termination of transcription occurs at a special stop sequence in the DNA (AATAAA).

## BACKGROUND



**Figure 3. (A) A model of a gene and its regulatory region. (B) Assembly of the transcription initiation complex.**

### *Retinoic acid receptors – nuclear transcription factors*

Retinoic acid (RA) is a biologically active metabolite of vitamin A (retinol), which plays an important role in differentiation and development processes of many organs including the skeleton (35-37) and the skin (38).

RA (all-trans or 9-cis RA) binds to a superfamily of nuclear receptors that function as transcription factors. This superfamily is composed of two subfamilies: RARs and RXRs, each having three members,  $\alpha$ ,  $\beta$ ,  $\gamma$ , of which several splicing variants exist. The existence of several forms of RA activating multiple receptors allows for a wide variety of possible responses. The RAR family is activated both by ATRA and 9-cis RA, whereas only 9-cis RA activates the RXR family. Upon RA-binding, the activated nuclear receptors act as transcription factors, either in the form of heterodimers (RAR/RXR) or homodimers,

## BACKGROUND

(RXR/RXR), by binding specific retinoic acid elements (RAREs) in the promoter and thereby regulating the transcription of the target gene (39). The final signalling cascade is more complex since the transcription factors can interact and be regulated by multiple co-activators and/or co-suppressors (40). In addition to binding directly to RAREs, the nuclear receptors also bind other transcription factors thereby indirectly regulate gene transcription from other motifs in the DNA (41, 42).

### *Sp1*

Sp1 belongs to a family of structurally related transcriptional proteins (43). The Sp family binds specific G-rich DNA elements such as the GC-box (GGGGCGGGG) and the GT/CACCC-box (GGTGTGGGG) located in promoters and enhancers of both house-keeping and tissue-specific genes. All Sp members contain a C-terminal DNA-binding domain consisting of three zinc fingers. Sp1 is ubiquitously expressed (44) whereas the expression of other members is either ubiquitous or more cell specific (45). Sp1 is an activator of transcription (46), whereas other Sp-family members repress Sp1-mediated transcription (47). Constitutive expression of many genes is dependent upon Sp proteins, which are able to interact with proteins associated with the basal transcription machinery such as TBP (48) and TAFs (49). Sp protein activation can also be cell- or tissue-specific (45, 50, 51) depending on differences in Sp protein expression and competition in binding between the Sp proteins to the GC-rich domains. The final effect on expression of different genes can be either synergistic or antagonistic depending on which of the Sp family proteins that bind to the promoter and which other DNA-dependent or DNA-independent transcription factors that are involved.

## BACKGROUND

### *NFY*

The transcription factor NFY binds to the CCAAT-box (5'YYRCCAATCAG3' or 5'CTGATTGGYYRR3' where Y=pyrimidines and R= purines), which is a transcription factor-binding element common in cell-cycle regulated (52), developmentally and tissue-specific (53, 54), house-keeping and inducible promoters (55, 56). The CCAAT box is usually located 60-100 bp upstream of the transcription start site. In addition to NFY, several other nuclear proteins are able to bind to CCAAT boxes including NF1, C/EBP and CP2. On the other hand, while the other CCAAT binding transcription factors are rather promiscuous in their binding to the consensus motif, NFY is the only factor that requires the intact CCAAT sequence as well as specific flanking sequences (57). NFY is a trimeric protein composed of three subunits, A, B and C, where all three subunits are needed for binding to the CCAAT box (58). All subunits contain sites also for protein-protein interactions. Oligomerization of the NFY protein with other transcription factors or co-activators results in positive cooperativity reflected in an increased affinity of NFY for the CCAAT box (59, 60). It has also been shown that NFY is able to increase the affinity of closely located transcription factors for their DNA elements (61, 62). In addition, NFY has been shown to interact with TBP in the transcriptional machinery (63).

### **Involvement of GPCRs in immunodeficiency virus infection of human cells**

Human immunodeficiency virus (HIV) is the cause of the severe disease called acquired immunodeficiency syndrome (AIDS). HIV infects and kills cells of the human immune system thereby destroying the body's ability to fight infections. Because of the impaired immune system, the infected individual becomes susceptible to non-pathogenic virus, fungi or bacteria (opportunistic infections), which eventually leads to death. HIV is passed between humans mainly through sexual contact (exposure to semen or vaginal fluids) or by blood-to-blood contact (through needles or syringes).

## BACKGROUND

The AIDS epidemic in the world is continuously growing. By the end of 2004 39 million people was estimated to live with HIV worldwide including 25.4 million people infected in Sub-Saharan Africa, which is the worst area affected (64).

### *Human and simian deficiency virus*

HIV and the closely related simian immunodeficiency virus (SIV) in monkeys are viruses that belong to the lentivirus subgroup of the retroviridae family. HIV and SIV are envelope viruses that store their genetic material as RNA. Upon infection of a host, the viral RNA is transcribed into DNA by the viral reverse transcriptase. Viral DNA becomes incorporated into the host DNA, where it is replicated together with the host genes. The majority of the HIV viruses integrate their DNA into the genome of activated immune cells, and new viruses are immediately produced. However, if the infected immune cells are in a latent state (which may last for a long time) they produce little or no virus. Upon proper stimuli, the cells are activated leading to an increase in viral production and dissemination.

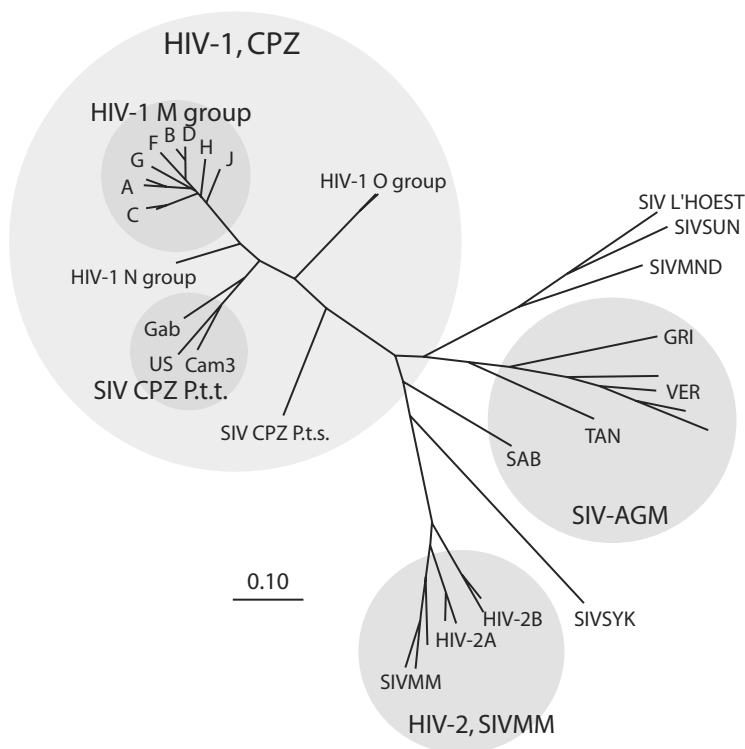
HIV is divided into two types, HIV-1 and HIV-2. The transmission route is the same for both types but they differ in their geographic distribution and pathogenicity.

HIV-1 originates in Central Africa. It is believed that the virus originated in a SIV-infected chimpanzee, and was spread to human by cross-species transmission (65). HIV-1 is divided into three phylogenetic groups, M (main), O (outliner) and N (non-M, non-O), where each group has been independently transmitted from chimpanzee to human (**Fig. 4**). The HIV-1 infections caused by group M are found worldwide whereas groups O and N are minor groups localized to Central Africa.

HIV-2 originates in West Africa where it is mainly located. HIV-2 is also thought to have originated by cross-species transmission from SIV-infected sooty mangabey to human (66, 67). Different genetic subtypes of HIV-2 (subtype A is the most common) exist, some

## BACKGROUND

probably appeared by independent transmissions from sooty mangabey to human (68) (**Fig. 4**). HIV-2 is less pathogenic than HIV-1, which may be due to a better human immune response to HIV-2 replication (69). This leads to a low viral load (70), a slower progression to disease (71), and a lower rate of transmission (72). The phylogenetic tree in Figure 4 shows that HIV-2 is more closely related to SIV than to HIV-1.



**Figure 4. A phylogenetic tree illustrating the relationship between HIV-1, HIV-2 and SIV (SIVMM; SIV of sooty mangabeys or macaques experimentally infected with SIVMM). The *pol* gene of the different viruses was used for the alignment.**

The SIV family of retroviruses is composed of several distinct branches originating from different simian species spread throughout Africa (**Fig. 4**). SIV infections result in

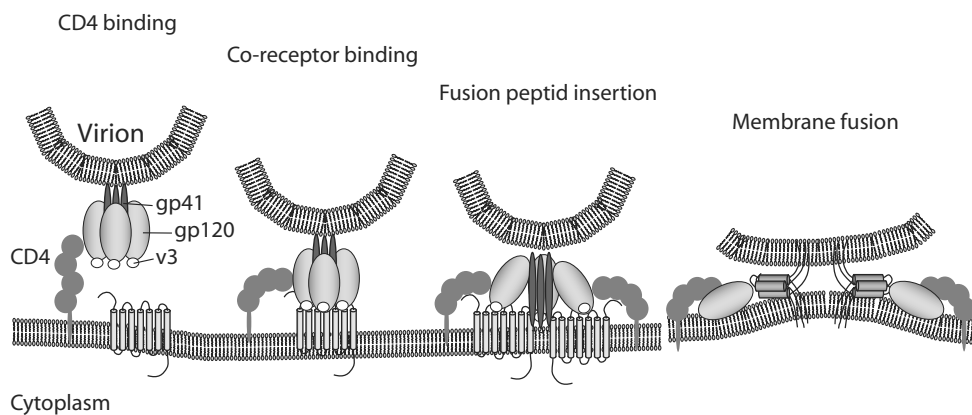
## BACKGROUND

different host responses depending on the type of monkey and virus. The African monkeys (*e.g.* sooty mangabeys) are natural hosts for SIV and upon natural infection they are infected but do not develop disease (73). Experimental infections with SIVmac239 (of macaque origin) cause an outcome similar to natural infection in the sooty mangabey whereas a disease similar to AIDS is developed in Asian monkeys (macaques) (74). The close relationship between HIV and SIV has resulted in the usage of SIV-infected monkeys, such as the Asian monkey (macaques), as a suitable model for studying the pathogenesis of AIDS (75).

### *HIV and SIV infection of host cells*

Usually, HIV and SIV isolates invade host cells that express the cell surface receptor CD4 and certain G-protein coupled receptors, so-called “co-receptors”. During viral entry, the envelope (Env) glycoprotein subunit 120 (gp120) initially binds to the host cell CD4 receptor. The CD4 binding leads to conformational changes in gp120 that enables the virus to bind to the co-receptor. The co-receptor binding elicits further changes in gp120 that exposes the viral Env glycoprotein subunit 41 (gp41), which penetrates the host cell membrane. This allows for fusion between viral and host membranes and subsequent viral entry (76-78) (**Fig. 5**).

## BACKGROUND



**Figure 5. HIV/SIV binding and entry into immune cells.**

### *GPCRs as targets for immunodeficiency viruses*

Both HIV and SIV isolates use GPCRs as co-receptors. The chemokine receptors CXCR4 and CCR5 are the major co-receptors *in vivo* (79, 80). HIV can use either or both CCR5 and CXCR4, whereas CCR5 is the main co-receptor for SIV isolates (81). HIV-1 uses CCR5 in the early stages of infection, whereas CXCR4 is used mainly in the later phases of infection (82). In addition, there are a large number of other GPCRs that can function as minor co-receptors for some HIV and SIV strains *in vitro*, but their *in vivo* significance is not yet clear. Many of these additional receptors are chemokine receptors like CCR1 (83, 84), CCR2b (85, 86), CCR3 (86, 87), CCR4 (88), CCR8 (89), CCR9, CXCR2 (90), CXCR5 (91), CXCR6/STRL33/Bonzo (86, 92, 93), and CX<sub>3</sub>CR1 (89, 94), chemoattractant/-like receptors such as BLTR (95) and ChemR23/CMKLR1 (96), the angiotensin-like receptor APJ (97, 98), orphan receptors such as GPR1 (99, 100), BOB/GPR15 (92, 100), or RDC1 (101) as well as viral chemokine receptor like CMV-US28, expressed in cytomegalovirus (CMV)-infected cells (102). HIV-2 and SIV are more promiscuous than HIV-1. A broad range of co-receptors is often used by HIV-2 (84, 86,

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88, 91) and SIV isolates (86, 103, 104) for cellular entry whereas few HIV-1 isolates are promiscuous in their co-receptors usage (96, 105, 106).

### Studies of the chemoattractant-like receptor CMKLR1/ChemR23

#### *CMKLR1/ChemR23*

The body's immune response against micro-organisms and other foreign agents depends upon the trafficking of the immune cells. This trafficking is regulated by chemical mediators, which activate certain GPCRs on the immune cells causing the cells to migrate towards the mediator concentration gradient. This process is called chemotaxis and the involved receptors belong to the GPCR subfamily of "classical leukocyte chemoattractants receptors", exemplified by the receptors for complement factor, N-formyl peptide, and leukotriene B<sub>4</sub> (107).

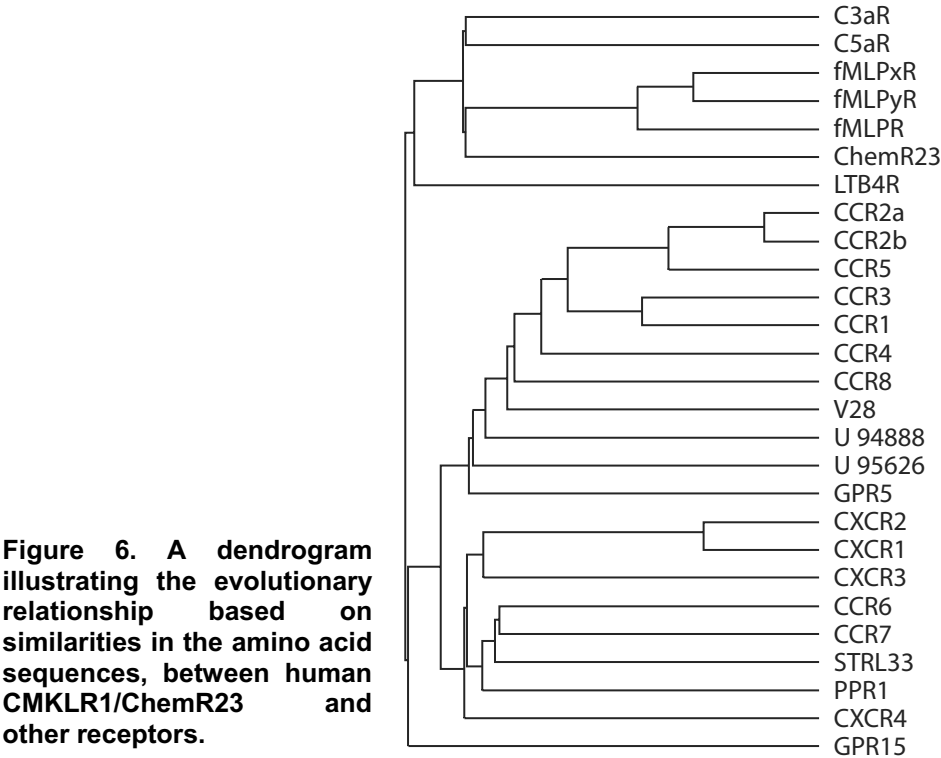
In this thesis project we have focused on a chemoattractant-like receptor that was cloned in our laboratory in 1996. The receptor has been referred to by alternative names in different species (**Table 1**) (96, 108-111).

**Table 1. Naming of CMKLR1/ChemR23 in different species.**

Receptor name	Species	Reference
CMKLR1	human	Gantz <i>et al.</i> , 1996.
CMKRL3/rAP	rat	Owman <i>et al.</i> , 1996
DEZ	mouse	Methner <i>et al.</i> 1997
ChemR23	human	Samson <i>et al.</i> , 1998
Chemerin receptor	human	Wittamer <i>et al.</i> , 2003

## BACKGROUND

We describe the receptor under the name CMKLR1/ChemR3. It displays high homology to other chemoattractant-like receptors as shown in **Figure 6**. CMKLR1/ChemR23 is expressed in immune cells such as macrophages and dendritic cells (96), and it has a pathophysiological role as one of the minor co-receptors involved in HIV-1/SIV infection of human CD4<sup>+</sup> cells (96). The receptor has also been suggested to be involved in osseous and cartilage development (110).



## BACKGROUND

### *The natural ligand, TIG2/chemerin*

CMKLR1/ChemR23 has been classified as an “orphan” receptor during the major part of the graduate research work described here in. In 2003, the human receptor was “de-orphanized” when two independent research groups isolated the natural ligand from human inflammatory fluids (111) and from hemofiltrate (112). The identified natural ligand is a 143 amino acid residue long protein, previously known as TIG2 (“tazarotene-induced gene 2”), and was tentatively named “chemerin” (111). The ligand is secreted as the precursor “pro-chemerin” which, upon proteolytic cleavage removing six to nine amino acids in the C-terminal end, becomes able to activate CMKLR1/ChemR23 (111, 112). In fact, the molecule itself was known before, under the name TIG2, a gene implicated in dermal physiology where it was suggested to be involved in keratinocyte differentiation and the skin disorder psoriasis (113). Tazarotene, a synthetic RA analogue (114), used for treatment of psoriasis, has been shown to up-regulate the TIG2 gene (113). Chemerin/TIG2 also seems to play a role in the mechanisms of bone modelling (115).

The identification of the natural ligand to CMKLR1/ChemR23 now makes it possible to explore the physiological relevance of this receptor. The role of CMKLR1/ChemR23 in chemotaxis could be confirmed when it was revealed that stimulation of immature dendritic cells and macrophages with TIG2/chemerin induces chemotaxis (111).

## AIMS OF THE STUDY

### AIMS OF THE STUDY

The aims of this project were to:

- 1) Characterize the genomic structure of CMKLR1/ChemR23 in mouse for comparison with the human sequence.
- 2) Study the regulation of receptor gene expression in mouse.
- 3) Investigate whether mouse TIG2/chemerin activates CMKLR1/ChemR23 in mouse.
- 4) Analyse if C-terminal peptides of mouse TIG2/chemerin can activate mouse CMKLR1/ChemR23.
- 5) Elucidate the importance of CMKLR1/ChemR23 as a co-receptor for human and simian immunodeficiency virus (HIV and SIV).
- 6) Identify the extracellular regions of the receptor needed for virus binding and entry into human immune cells.

## METHODOLOGY

### METHODOLOGY

This section gives a short summary and comments on the methods used in the papers included in this thesis. The paper where each method has been applied is indicated in roman letters. For more detailed instructions; see paper I-IV; Materials and methods.

#### Cell lines and receptor expression (I-IV)

NB4 1A3 cells (mouse neuroblastoma), endogenously expressing mouse *cmklr1a* and BV2 cells (mouse microglia), endogenously expressing *cmklr1b*, were used in the promoter studies. In these experiments, 3T3 clone A31 cells (mouse embryonic fibroblast), which does not express *cmklr1* endogenously, was used as negative control.

The HeLa-based reporter cell line, HFF11, stably expressing the reporter plasmid pcFUS3 was constructed by Kotarsky *et al.* (116). HFF11 cells, stably expressing mouse and human CMKLR1/ChemR23 were constructed for studies of receptor activation. HFF11 cells, expressing the reporter plasmid but no receptor (HFF11-sham) functioned as negative control.

The cell line HEK293 (human embryonic kidney) containing an effective apparatus for protein synthesis was used for expression of mouse wild-type chemerin. HEK293 cells and CHO-K1 cells (chinese hamster ovary) were used for expression of FLAG-tagged mouse TIG2/chemerin.

NP-2 cells (human glioma), stably expressing the human CD4 receptor, alone or in combination with CCR5, CXCR4, CCR3, FC-4b, hCMKLR1, rCMKLR1, hCMKLR1-EGFP, rCMKLR1-EGFP, rCMKLR1.hECL2-EGFP or rCMKLR1.hNterm-EGFP were constructed for studying human CMKLR1/ChemR23 as a co-receptor for HIV-1, HIV-2 and SIV or to map CMKLR1/ChemR23 domains important for virus binding. NP-2 cells

## METHODOLOGY

expressing the CD4 receptor alone, was used as negative control in the infection experiments.

### **Southern blot analysis (I)**

Southern blotting was used for isolation of the genomic region containing the *mcmklr1* gene. A mouse bacterial chromosome (BAC) containing the *mcmklr1* gene was digested with different enzymes and southern blot was performed according to standard procedures using a radioactively labelled probe containing the coding region of *mcmklr1*.

### **Northern blot analysis (I-IV)**

Expression of receptor transcript in different cell lines was confirmed by northern blot analysis. Total RNA was isolated by the guanidinium isothiocyanate method (117) and mRNA was selected using a commercial kit. The northern blot was performed according to standard procedures using a radioactively labelled probe containing the coding DNA region of the receptor to be analysed.

### **Rapid amplification of cDNA ends (5'-RACE) (I, II)**

The transcription start sites of *cmklr1a* and b were revealed by 5'-RACE. Total RNA and mRNA were isolated as for northern blot analysis. cDNA synthesis and the following PCR amplification of the 5' cDNA end including the transcription start site were performed using the Marathon<sup>TM</sup> cDNA amplification method.

### **Genome walking (I, II)**

Genome walking was applied for the genomic mapping of mouse *cmklr1a* and b and for obtaining the promoter regions of *cmklr1a* and b. The genome walking method is suitable

## METHODOLOGY

for amplifying DNA fragments that starts in a known sequence and extends into the unknown adjacent genomic DNA. Genomic walking was performed using the Universal Genome walker kit.

### **5' Deletions of promoter regions (I, II)**

5' Deletions of the *cmklr1a* and *cmklr1b* promoter regions cloned into the pGL3-Enhancer plasmid were performed according to the Erase-A-Base system developed by Henikoff (118). The promoter plasmids were linearized with *MluI* and *KpnI* resulting in one end with 5'overhang and one with 3'overhang. After purification, the linearized fragments were digested in their 5'ends with exonuclease III. The digestions were performed for different lengths of time whereafter the reactions were stopped by adding S1 Nuclease (the Nuclease digested the single stranded ends and the low pH in the buffer stopped the exonuclease III activity). The Nuclease activity was inactivated by heating whereafter Klenow DNA polymerase was added to blunt the ends. The plasmids were finally re-ligated with DNA ligase.

### **Site-directed mutagenesis (II)**

To establish if both CCAAT binding sites found in the *cmklr1b* promoter were functionally important for transcription, the elements were separately mutated. The mutations were performed using the site-directed mutagenesis method (QuickChange<sup>TM</sup>). Primers containing the desired mutation were used in a PCR reaction using a plasmid containing the *cmklr1b* promoter region as template. After PCR amplification, treatment with *DpnI*, an enzyme that specifically cleaves methylated DNA, digested the parental DNA (methylated in *E. coli*) and selected for the mutated non-methylated plasmid. Mutated plasmids were transformed into competent *E. coli*, and positive clones were identified by PCR screening using primers with the mutated element in the 3'end.

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### **Luciferase reporter assay (I, II)**

We applied a dual-luciferase reporter assay to confirm that the isolated putative promoter regions of *cmklr1a* and b possessed functional promoter activity. The dual luciferase reporter assay, based on transient transfection of cells, makes it possible to measure the activity of the two luciferases Firefly and *Renilla* in the same sample allowing for simultaneous promoter activity measurement (Firefly) and determination of transfection efficiency (*Renilla*). The enzyme activities are distinguished since they require different substrates and function at different pHs. In the presence of the substrates Beetle Luciferine and Coelenterazine, bioluminescent reactions occur catalysed by firefly and *Renilla* luciferases, respectively. Obtained luminescence signals are measured in a BMG Lumistar microplate luminometer.

In order to determine the transcriptional activities of *cmklr1a* and b, the putative promoter fragments were sub-cloned in front of the firefly luciferase gene using the reporter vector, pGL3-Enhancer. We used the pRL-TK vector, containing a herpes simplex virus thymidine kinase promoter in front of the *Renilla* luciferase, as control vector to compensate for differences in transfection efficiency.

The day before transfection, cells were seeded in white 96-well tissue culture plates. Luciferase constructs were co-transfected with pRL-TK as internal control. Forty-two hours after transfection, the cells were harvested in reporter lysis buffer. The dual-luciferase assay was applied to measure Firefly and *Renilla* luciferase activities using a luminometer.

### **Electrophoretic mobility shift assay (I, II)**

Electrophoretic mobility shift assay (EMSA) is a widely used method for studying gene regulation and determining protein-DNA interactions. The method is based on the fact that

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DNA that is bound to proteins (like transcription factors) migrates more slowly than free DNA when run on a non-denaturing polyacrylamide gel. The migration rate of the DNA is decreased or “shifted” upon protein binding. When an antibody also is present, for identification of the bound protein, the migration rate is further decreased or “super-shifted”. EMSA is used to identify the sequence-specific DNA-binding transcription factor in nuclear extracts and, in combination with mutagenesis, to identify the important binding motif within the gene’s regulatory region.

We used EMSA to investigate if transcription factors were able to bind the G-rich DNA elements in the *cmklr1a* promoter and the two identified CCAAT boxes in the *cmklr1b* promoter, and also to identify the nature of the binding proteins. The affinities of the transcription factors for the DNA elements were titrated by using increasing amounts of unlabelled probes containing the putative transcription binding elements. The specific proteins binding the DNA elements were identified using unlabelled DNA probes with or without mutations in the transcription-binding element, and by the use of antibodies.

Nuclear extracts containing transcription factors were prepared from cell lines endogenously expressing *cmklr1a* (NB4 1A3) and *cmklr1b* (BV2), essentially as described by Andrew and Faller (119). Synthetic radioactively labelled DNA oligonucleotides containing the putative transcription binding elements were used as probes. Binding was performed at 25°C and DNA-protein complexes were resolved on a non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film. For super-shift assays, either antibody (Sp1) and extract were incubated at 25°C before addition of labelled probe or labelled probe and extract were incubated before addition of antibody (NFY-A or NFY-B).

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### Real-time reverse transcription PCR (II)

Expression of *cmklr1a* and b was analysed by real-time reverse transcription PCR (real-time RT-PCR) in different tissues and in NB4 1A3 and BV2 cells with/without pre-treatment with ATRA. 3T3 clone A31 cells were used as negative control. The real-time PCR was performed in a LightCycler system using Sybr green, a dye that binds to double stranded DNA, for monitoring DNA synthesis. Primers specific to *mcmklr1a* and b were used in the PCR reaction. Copy-numbers of *mcmklr1a* and b were normalised to the copy-number of the house-keeping gene  $\beta(2)$ -microglobulin.

### Cloning of mouse wild-type- and FLAG-TIG2/chemerin (III)

To clone mouse wild-type chemerin, total RNA was isolated from mouse liver by the guanidinium isothiocyanate method (117). mRNA was prepared using a commercial kit and cDNA synthesized using the PCR-based first-strand synthesis system. Wild-type chemerin was amplified by PCR according to standard procedures using specific chemerin primers and the liver cDNA as template. Amplified wild-type chemerin was cloned into the expression vector, pEAK12.

In order to optimise TIG2/chemerin expression and simplify the purification process, the proximal part of the mouse TIG2/chemerin was replaced with a synthetic (mouse Igk V-J2-C) secretion signal, a TEV (tobacco etch virus)-recognition site and a FLAG epitope. The TEV site was included for possible removal of the FLAG-tag from the protein using TEV protease. Chemerin without the endogenous secretion signal was amplified by PCR using chemerin specific primers. The amplified product was ligated to a forward and a reversed oligo containing the synthetic secretion signal sequence, the sequence of the TEV-recognition site and the FLAG epitope sequence. The amplified FLAG-chemerin was ligated into the expression vector, pEAK12.

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### **HFF11 reporter assay (III)**

We used the HFF11 reporter cell line (116) for studying human and mouse CMKLR1/ChemR23 activation. Activation of different signalling pathways leads to activation of different transcription factors that can affect genes driven by promoters containing responsive elements for these transcription factors. HFF11 utilizes a reporter construct based on a luciferase gene driven by a multifunctional promoter including responsive elements for different transcription factors such as NF- $\kappa$ B, STAT and AP-1. Upon binding of transcription factors to the promoter, luciferase is expressed. The luciferase activity is measured in a luminometer. The HFF11 reporter cell line is suitable for studying activation of receptors that signal primarily through  $G\alpha_q$ ,  $G\alpha_{i/o}$ , and  $G\alpha_{12/13}$ . Consequently, it is a suitable system for studying activation of CMKLR1/ChemR23 since the human receptor has been described to signal through  $G\alpha_{i/o}$ .

On day one, HFF11 cells, either sham-transfected or transfected to express mouse or human CMKLR1/ChemR23, were seeded into a white 96-well plate. On day 3, the medium was changed to serum-free medium. On day 4, different activators were added to the wells and the cells were further incubated. After 7 hours, cells were harvested in lysis buffer and the plate assayed in a BMG Lumistar Galaxy microplate luminometer using a luciferase kit.

### **Phosphoinositide hydrolysis assay (III)**

Activation of human and mouse CMKLR1/ChemR23 was also studied using a phosphoinositide hydrolysis assay (PI assay) by monitoring the formation of inositol phosphates.

Phosphatidylinositols (PtIns) are components of the cell membrane and are important in signal transduction. By incorporating *myo*-[ $^3$ H]-inositol into PtIns in the membrane, it is

## METHODOLOGY

possible to monitor signal transduction and receptor activation. When a receptor is activated,  $G\alpha_q$  of  $G\alpha_q$ -coupled receptors or  $\beta\gamma$  of  $G\alpha_i$ -coupled receptors activates PLC in the membrane, which catalyses the hydrolysis of  $PIP_2$  into  $IP_3$  and DAG.  $IP_3$  is then stepwise dephosphorylated. By using LiCl to block further dephosphorylation into *myo*-inositol, the accumulation of radioactively labelled inositol phosphate may be monitored.

Cells were pre-labelled with *myo*-[ $^3H$ ]-inositol for 20 h. Stimulation with RA or PTX were performed for 16 h. At the day of analysis, medium containing *myo*-[ $^3H$ ]-inositol was removed, the plates washed and further incubated in medium containing LiCl with or without C-terminal peptides for 30 min. After incubation, medium was removed, and the cells were lysed in formic acid. [ $^3H$ ]-Inositol phosphates were isolated by extraction and anion exchange chromatography and counted in a Beckman LS6500 liquid scintillation counter.

### Flow cytometric analysis (IV)

Stable expression of recombinant receptors (CCR5, CXCR4, CMKLR1/ChemR23, CCR3, FC-4b) in NP-2.CD4 cells was verified by flow cytometric analysis using the FACS Calibur flow cytometer (Becton-Dickinson).

### Constructions of EGFP tagged receptors and receptor hybrids (IV)

To define extracellular receptor domains important for the virus interaction with CMKLR1/ChemR23 we applied a hybrid receptor model. This was based upon the fact that the rat receptor, although having high amino acid identity to human CMKLR1/ChemR23, is inefficient as viral co-receptor. Since no antibody is available to confirm rat receptor expression, we tagged the receptors and receptor hybrids with EGFP. EGFP was fused in-frame to the C-terminus of human and rat CMKLR1/ChemR23, respectively, using a stepwise PCR procedure according to a modified protocol (120) of

## METHODOLOGY

the single-overlap extension method (121). The same method was applied to replace the N-terminal and the second extracellular loop of the rat CMKLR1/ChemR23-EGFP with the corresponding human sequences.

### **Confocal microscopy (IV)**

Confocal microscopy (Zeiss LSM510) was used to verify that the EGFP-tagged recombinant receptors, stably expressed in NP-2.CD4 cells, were expressed at the cell surface. Cells stained with anti-CD4 mAb were used as positive control.

### **Amplification of virus isolates (IV)**

Virus stocks of HIV-1, HIV-2 and SIV were prepared by infection of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) mixed from two blood donors. Donor PBMCs were isolated by separation of buffy coats on a Ficoll gradient. Pooled PBMCs were infected with virus in RPMI medium containing 10% FCS, 5 U/ml of interleukin 2, 2 µg/ml polybrene, 50 U/ml penicillin and 50 µg/ml streptomycin. Cell-free supernatants were harvested at day 8, 10, and 12, assayed for virus antigen content and stored at -80 °C until used.

### **Virus detection in cell culture supernatants by enzyme-linked immunosorbent assay or by measuring reverse transcriptase activity (IV)**

Enzyme-linked immunosorbent assay (ELISA) or reverse transcriptase activity (RT assay) was performed to detect virus antigens in cell culture supernatants after virus infection.

The presence of HIV-1 was monitored by detection of p24 core antigen using a p24 ELISA (Vironostika HIV-1 Antigen, Biomérieux) based on the “sandwich” principle. ELISA plates were coated with antibodies (murine monoclonal) against the p24 core antigen.

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Disruption buffer was added to the wells to verify that all viruses were to be disrupted. Cell culture supernatants containing p24 antigens together with positive (p24 core antigen) and negative (human serum not containing p24 antigen) controls were added to the wells and the plate was incubated to allow formation of complexes between antibody and antigen. The wells were washed and p24 antibodies (human) coupled to horseradish peroxidase were added to the wells and the plate was further incubated allowing the secondary antibody to bind to the previously formed complex. After a final wash the tetramethylbenzidine substrate was added to the wells producing a blue colour that turned yellow upon stopping the reaction with sulfuric acid. The amount of p24 in the wells was proportional to the amount of produced colour, which could be measured photometrically at an absorbance of 450 nm.

The presence of HIV-2 and SIV were monitored either by p26 core antigen detection using an in-house p26 ELISA (122) or by measurement of virus reverse transcriptase activity using the Cavid HS-kit Lenti RT. To measure reverse transcriptase activity, plates coated with poly-rA were used as template. By adding oligo-dT primer and the BrdU-triphosphate substrate, reverse transcriptase was able to synthesize a new DNA strand, which could be detected by addition of a monoclonal anti-BrdU antibody coupled to alkaline phosphatase. After washing, the chromogenic substrate para-nitro-phenyl phosphate was added to the wells and the colour produced was measured photometrically at an absorbance of 405 nm.

### **Virus infection of NP-2 cells (IV)**

Virus infection studies were performed to elucidate the importance of CMKLR1/ChemR23 as a co-receptor for HIV-1, HIV-2 and SIV and to map CMKLR1/ChemR23 domains important for virus binding.

Two days before infection, NP-2.CD4 cells alone or in combination with human CMKLR1/ChemR23, rat CMKLR1/ChemR23, CCR5, CXCR4, CCR3, FC-4b, hCMKLR1-EGFP, rCMKLR1-EGFP, rCMKLR1.hECL2-EGFP or rCMKLR1.hNterm-

## METHODOLOGY

EGFP were seeded into 48-well plates. At the time of infection, medium was removed and virus was added to the wells in 200  $\mu$ l medium containing polybrene. Two hours after infection, medium with polybrene was added to a total volume of 500  $\mu$ l/well. After overnight incubation, cells were washed and medium without polybrene was added to each well. Twelve days after infection, medium was sampled from each well for detection of viral antigen. Cells were also evaluated microscopically for syncytia formation.

## RESULTS AND DISCUSSION

## RESULTS AND DISCUSSION

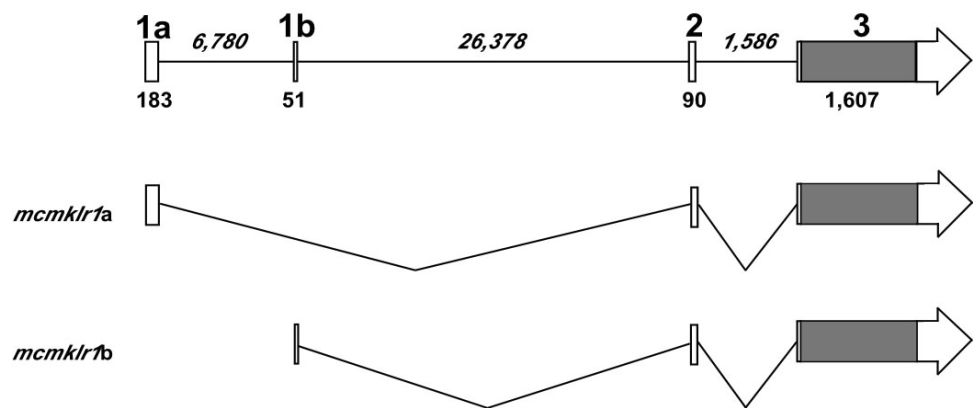
### **Genomic organization of CMKLR1/ChemR23 in mouse, and the regulatory mechanism behind receptor expression (Paper I and II)**

#### ***Genomic organization of mouse *cmklr1a* and *b****

Analysis of different mouse cell lines revealed mCMKLR1 expression in NB4 1A3 (neuroblastoma) and BV2 (microglia) cells. We show for the first time that the mouse *cmklr1* gene is spliced into two mRNA transcripts, *cmklr1a* (NB4 1A3) and *b* (BV2), containing alternative 5' ends. Mouse *cmklr1* is localized to chromosome 5 where *cmklr1a* spans approximately 36,000 bp and consists of three exons intercepted by one larger and one smaller intron. *cmklr1b* differ from *cmklr1a* in having an alternative exon 1, located downstream of the exon 1 of *cmklr1a* (**Fig. 7**). The fact that CMKLR1/ChemR23 lacks introns in its coding region is a common feature among GPCR genes. Despite the fact that less than 5% of the total genes are intronless in their coding regions, more than 90% of the mammalian GPCR genes lack introns in their open reading frames (ORF) (123). Since most GPCRs in nematodes contain introns in their ORF, lacking introns may be an evolutionary advantage for GPCR genes (123). The genomic organization of *cmklr1* is similar to some other GPCRs such as the chemoattractant receptor genes BLT1 and 2, which are also composed of 3 exons with the coding region located to the third exon (124, 125). The finding of two splice variants of the mouse *cmklr1* gene is not entirely unexpected in view of the existence of two splice variants also in the human gene (126). The human splice variants localized to chromosome 12 are also composed of 3 exons but with alternative second exons. Alternative splicing resulting in different receptor mRNAs is a common mechanism that has been found in many types of GPCRs (127, 128). Splicing within the coding region of GPCR genes results in protein isoforms that often differ in the C-terminus, the third intracellular loop or the extracellular N terminus (129) whereas

## RESULTS AND DISCUSSION

splicing in the uncoding region affects gene regulation thereby increasing the diversity and complexity of the receptor.



**Figure 7. Genomic organization of the mouse *cmklr1* gene and the alternative splicing variants *mcmklr1a* and *b*.**

### *Promoter analysis of mouse cmklr1a and b*

Since mouse *cmklr1a* and *b* utilize alternative exon 1, they utilize different start sites for transcription and are consequently transcribed from alternative promoters. The use of multiple promoters is an important mechanism among GPCR genes, which contribute to diversity and complexity in regulation of gene expression (130). The utilization of multiple promoters makes it possible for a gene to be expressed at different stages in development, specifically in certain cells or tissues, or to respond differently to a specific stimulus.

## RESULTS AND DISCUSSION

The mouse *cmklr1a* promoter lacks the canonical TATA and CCAAT boxes but contains several GC-rich regions. The same features are found in the human gene, suggesting that they are regulated in a similar manner. These results show that the *cmklr1a* gene has certain regulatory features in common with a house-keeping gene promoter, *i.e.*, the presence of several GC rich regions (131). The 5' flanking region of mouse *cmklr1b* lacks both TATA box and GC-rich regions but contains two CCAAT boxes in opposite directions. The lack of TATA and CCAAT boxes are usual features of many promoters of related human GPCR genes such as the chemokine- and major HIV co-receptor CCR5 (79, 128) and the chemoattractant- and minor HIV co-receptor BLT1 (95, 125). The widely expressed chemokine-receptor CXCR4 also has a promoter with features common with a house-keeping gene (132).

To functionally localize the promoter regions regulating *cmklr1a* and b, we applied a luciferase reporter gene assay. A reporter vector (pGL3E) containing a promoterless luciferase gene was used, where transcription of the luciferase gene was completely dependent upon the upstream cloned 5'-flanking region of *cmklr1a* or b, respectively. By testing the promoter constructs in NB4 1A3 and BV2 cells, endogenously expressing *cmklr1a* and b, respectively, we assured that necessary transcription factors were present for proper transcription. A luciferase signal confirmed that the cloned putative promoter fragments could actually work as promoters.

*Cmklr1a* requires a 280-bp region adjacent to the transcription start site for initiating transcription. Within this region, four putative GC-boxes all binding the transcription factor Sp1, may be active in the transcription of *cmklr1a*. The overall transcriptional activity of this region was low, suggesting that additional elements such as enhancers localized further upstream or downstream in the genome, are important for transcription of *cmklr1a*. This is verified by the fact that a transcriptional activity also was obtained when the *cmklr1a* promoter constructs were tested in cells not expressing *cmklr1a* endogenously.

## RESULTS AND DISCUSSION

This indicates that the promoter fragment is lacking elements for regulatory tissue selectivity.

A proximal and a distal region seem to be important for transcription of *cmklr1b*. The proximal promoter region includes two CCAAT boxes. However, site-directed mutagenesis separately within these elements revealed that only the forward CCAAT element binding the transcription factor NFY contribute to transcription of *cmklr1b*. Since the *cmklr1b* promoter constructs resulted in transcription activity also in cells not expressing *cmklr1b* endogenously, the proximal region seems to be controlled by a common mechanism, whereas the cell specificity may reside elsewhere in the genome.

When *cmklr1a* and *b* expression was investigated in different mouse organs (skeletal muscle, spleen, brain, kidney, liver, lung and heart), we found that *cmklr1a* is constitutively expressed in organs such as heart and lung, whereas *cmklr1b* expression is generally too low for reliable detection. The low detection levels of *cmklr1b* may be due to the fact that it is expressed only in a specific cell population, too few in number to give a proper signal, or that this transcript requires stimulation for transcription. We confirmed the later alternative by showing that *cmklr1b* is strongly up-regulated by ATRA, whereas *cmklr1a* is unaffected. A RARE element within the analysed *cmklr1b* promoter region could not be identified indicating that the element may be located further upstream or that ATRA works through an indirect mechanism affecting other transcription factors. Example of such other transcription factors are AP-1 and GATA-2, proteins that have been described to interact with ATRA (41, 42) and for which binding sites have been found in the promoter.

The emerging picture of the regulation of the *cmklr1* gene indicates a bi-functional mode of action including a basal and an inducible, possibly tissue- or cell- specific transcription. The results from the functional experiments with ATRA emphasises the potential involvement of the receptor in, *e.g.* bone modelling. An interesting notion is that ATRA

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up-regulates *cmklr1b* expression just as the synthetic RA derivate, tazarotene up-regulates the ligand TIG2/chemerin. In addition, *cmklr1* has shown to be expressed during bone development (110), where RA also has proved to play an important role (36).

### **Activation of mouse CMKLR1 using C-terminal peptides of the mouse TIG2/chemerin ligand (Paper III)**

We have searched for the natural ligand for human CMKLR1/ChemR23 by expressing the receptor in a HFF11 reporter cell system in order to monitor receptor activation (116). We tested a variety of different substances, and conditioned media from different cell lines, but without success. In 2003, two independent research groups identified the chemotactic protein TIG2/chemerin as the natural ligand for human CMKLR1/ChemR23. (111, 112). The ligand is secreted as a precursor, “pro-chemerin”, which upon proteolytic digestion in the C-terminus becomes capable of activating CMKLR1/ChemR23 (111, 112). Using peptides corresponding to various parts of the processed form of TIG2/chemerin, the active region important for human receptor activation was mapped to the C-terminus (133). Applying the HFF11 reporter cells expressing CMKLR1/ChemR23, we could confirm that a C-terminal peptide of the processed human ligand activates human CMKLR1/ChemR23. We also showed that receptor activation is inhibited by PTX, further verifying that the receptor signals through  $G\alpha_i$ .

Since we have been studying human as well as mouse CMKLR1/ChemR3, we also wanted to investigate if the mouse orthologue to human TIG2/chemerin can activate mouse CMKLR1/ChemR23, and if the C-terminus is the important region for receptor activation also, in mouse. We cloned and expressed mouse TIG2/chemerin in HEK293 cells. Conditioned medium containing TIG2/chemerin was found to activate mouse CMKLR1/ChemR23 expressed in HFF11 reporter cells, although to a lower degree than for the human receptor. In an attempt to obtain purified ligand, mouse TIG2/chemerin with a fused N-terminal FLAG-tag was expressed in HEK293 and CHO-K1 cells under a strong

## RESULTS AND DISCUSSION

promoter. The FLAG-tagged fusion protein was purified using ANTI-FLAG M2 affinity gel, but the amounts obtained were too small for use. The difficulty in expressing TIG2/chemerin has been experienced also by other investigators both when using CHO-K1 cells (134) and *E. coli* (135). We could show that C-terminal peptides of mouse TIG2/chemerin activate the mouse receptor, though to a lower extent than for the human receptor. These results indicate that the peptide domains necessary for receptor activation differ for human and mouse TIG2/chemerin. A possibility is that the mouse receptor requires a longer active peptide, including further upstream amino acid residues, than does the human receptor. Another possibility is that the mouse receptor responds less well than the human receptor to activation with TIG2/chemerin. This difference has also been reported when comparing orthologues of other receptors (136-138).

### **HIV/SIV co-receptor function of CMKLR1/ChemR23 (Paper IV)**

It is important to gain knowledge about the type of HIV and SIV that use a certain co-receptor for cellular entry and to investigate the receptor epitopes responsible for the interaction between receptor and virus. This will give an opportunity to design molecules that may intervene with the virus particle during its entry into and infection of the human cell, without disturbing the natural importance of the receptor in the immune system.

We have investigated the importance of CMKLR1/ChemR23 as co-receptor for HIV-1, HIV-2, and SIV primary isolates by expressing the receptor in human astroglia (NP-2) cells. HIV-1 isolates of genetic subtype B and D utilizes CMKLR1/ChemR23 for infection whereas the more promiscuous HIV-2 (84, 90) and SIV isolates use CMKLR1/ChemR23 to a wider extent for infection. Among certain better-characterized co-receptors, the HIV-1 co-receptor function of CMKLR1/ChemR23 resembles that of the chemokine receptor, CCR3. The FC-4b receptor chimera, which is a hybrid between CCR5 and CXCR4, has proved useful in elucidating certain evolutionary aspects of HIV-1 co-receptor use. This

## RESULTS AND DISCUSSION

chimera was therefore included in the present co-receptor characterization. However, it did not reveal any further distinct feature of CMKLR1/ChemR23 co-receptor function.

The general picture of the structural requirements for co-receptor function indicates that multiple extracellular epitopes are involved in virus binding (139-143). To define extracellular receptor domains important for the virus interaction with CMKLR1/ChemR23, we applied a hybrid receptor model. This was based upon the fact that the rat receptor, although having high amino acid identity to human CMKLR1/ChemR23, is inefficient as viral co-receptor. When we “humanized” the rat receptor to include either the human N-terminus or the second extracellular loop, exposure to HIV-1, HIV-2 and SIV resulted in efficient infection. HIV-1 and HIV-2 showed preference for the N-terminus and the second extracellular loop, whereas SIV was primarily dependent on the second extracellular loop for infection. The observation that the receptor domains important for virus binding differ for different virus isolates makes it harder to inhibit the HIV-infection at the receptor level.

## CONCLUSIONS

### CONCLUSIONS

In order to increase the knowledge of the previously identified orphan chemoattractant-like G-protein coupled receptor CMKLR1/ChemR23, we have focused our interest on various genetic and molecular biological aspects of this receptor as well as on its role as an immunodeficiency viral co-receptor. In this thesis work, the following conclusions were obtained:

Two transcripts of mouse *cmklr1* have been identified, *cmklr1a* and *b*, that contain alternative exon 1. The gene comprises three exons, intercepted by one larger and one smaller intron. The first and second exons contain untranslated sequence, while the coding region is localized to the third exon. Gene splicing into two variants also occurs in the human gene.

Mouse *cmklr1a* and *b* utilize alternative promoters for transcription. The transcription factor Sp1 is important for transcription of *cmklr1a*, whereas NFY is required for transcription of *cmklr1b*. ATRA strongly up-regulates *cmklr1b*, whereas *cmklr1a* is unaffected. The emerging picture of the regulation of the mouse *cmklr1* gene indicates a bi-functional mode of action, including a basal action through *cmklr1a* and an inducible, possibly tissue- or cell-specific, transcription through *cmklr1b*. The functional experiments with ATRA indicate a potential involvement of the receptor in, *e.g.* bone modelling.

The mouse chemotactic protein TIG2/chemerin activates mouse CMKLR1/ChemR23 but to a lower degree than the human receptor. A peptide corresponding to the C-terminus of the processed TIG2/chemerin in human activates the human receptor, whereas corresponding mouse peptides activate the mouse receptor to a lower extent. This indicates either that the peptide domain necessary for receptor activation differ for human and mouse TIG2/chemerin or that the maximal response of the mouse receptor is lower than the human.

## CONCLUSIONS

The human CMKLR1/ChemR23 receptor functions as a minor co-receptor for viral entry into human CD4<sup>+</sup> immune cells. Select HIV-1 isolates use the receptor for cellular entry whereas the receptor usage of HIV-2 and SIV is more general. The receptor domains important for virus interaction differ for HIV-1/HIV-2 and SIV, which should be taken into consideration when designing molecules that inhibit the HIV-infection at the receptor level.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

Popularized summary in Swedish

För att en organism ska fungera är det nödvändigt att alla de celler som bygger upp organismen kommunicerar med varandra. Denna kommunikation sker genom att speciella proteiner på cellens yta, s.k. receptorer, tar emot signaler från andra celler. G-proteinkopplade receptorer (GPCR) är den vanligaste typen av receptorer. Då en signalmolekyl binder till en GPCR ändrar den form vilket medför att den kan binda ett G-protein på insidan av cellmembranet. G-proteinet förmedlar signalen vidare till effektorproteiner inne i cellen och ett cellsvar uppkommer. GPCRs medverkar i de flesta fysiologiska funktioner som t.ex. immunförsvar eller uppfattning av ljus och lukt. GPCRs är involverade i många sjukdomar t.ex. HIV/AIDS där viruset använder sig av G-protein kopplade receptorer för att ta sig in i kroppens immunförsvarsceller. Av de närmare 800 GPCRs som finns i vår kropp är dock cirka 160 fortfarande okända, dvs man vet ej deras funktion i kroppen eller signalmolekylerna som aktiverar dem.

Vi har fokuserat på en GPCR som kallas CMKLR1/ChemR23. Receptorn är strukturellt lik andra receptorer involverade i immunförsvaret. CMKLR1/ChemR23 uttrycks bl.a. i ben, brosk och i immunförsvarsceller men dess fysiologiska roll i kroppen är nästan helt okänd. I sjukdomssammanhang har CMKLR1/ChemR23 visat sig ha betydelse vid HIV-infektion där viruset binder till receptorn för att sedan ta sig in i kroppens immunförsvarsceller. Signalmolekylen som aktiverar CMKLR1/ChemR23 har under större delen av mitt avhandlingsarbete varit okänd men nyligen identifierades molekylen, "TIG2" eller "chemerin", i inflammatoriska vätskor och i blodfiltrat. TIG2/chemerin är liksom dess receptor aktiv i inflammatoriska processer och verkar också ha en funktion i benbildning.

I syfte att öka kunskapen om denna länge okända receptor har vi studerat CMKLR1/ChemR23 i mus och människa. Med hjälp av molekylärbiologiska metoder har vi kartlagt hur receptorgen i mus ser ut i jämförelse med människa. Receptorgen i mus

## POPULÄRVETENSKAPLIG SAMMANFATTNING

kan se ut på två olika sätt och systemet som reglerar receptorns bildning kan också se ut på två olika sätt beroende på i vilken cell receptorn bildas.

I en studie av hur signalmolekylen TIG2/chemerin interagerar med CMKLR1/ChemR23 kan vi visa att mus TIG2/chemerin aktiverar musreceptorn i lägre grad än i människa.

Vi kan visa att HIV-1, HIV-2 och SIV (motsvarigheten till HIV i apa) binder CMKLR1/ChemR23 för att sedan infektera människans immunförsvarsceller. Av ett antal olika testade HIV-1 stammar är det endast två stammar som använder CMKLR1/ChemR23 för infektion. Flera HIV-1 isolat som använder den etablerade receptorn CCR3 kan utnyttja CMKLR1/ChemR23. HIV-2 och SIV använder CMKLR1/ChemR23 mer generellt. Vi har kunnat konstatera att vid infektion binder de olika virustyperna olika extracellulära receptorregioner.

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