The influence of CXCR4 on Maedi visna virus-induced syncytium formation

by

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## Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CA</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBL</td>
<td>Foetal bovine lung</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>GSM</td>
<td>Goat synovial membrane</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HO6T1</td>
<td>Crandell feline kidney</td>
</tr>
<tr>
<td>HOS</td>
<td>Human osteosarcoma cells</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>MA</td>
<td>Matrixprotein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MVV</td>
<td>Maedi visna virus</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SCP</td>
<td>Sheep choriod plexus</td>
</tr>
<tr>
<td>SDF</td>
<td>Stomal derived factor</td>
</tr>
<tr>
<td>SU</td>
<td>Surface unit</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane unit</td>
</tr>
<tr>
<td>U87</td>
<td>Human glioblastoma cells</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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</tbody>
</table>
Abstract

Maedi visna virus (MVV) is a retrovirus that is member of the Lentivirus genus. MVV infects sheep and goats and causes progressive pneumonia or paralysis, leading to death. Since the discovery of the receptor for Human immunodeficiency virus (HIV), Simian immunodeficiency virus (SIV) and Feline immunodeficiency virus (FIV) all include the chemokine receptor CXCR4, it has been postulated that all members of the Lentivirus share a common mechanism of entry that involves the use of CXCR4.

With the use of syncytia assays, infection-, inhibition- and enhancement studies, it was shown that CXCR4 is not a common lentivirus receptor. U87 and HOS cells, both cell-lines lacking CXCR4, were susceptible to infection. However, cells transfected with CD4 and CXCR4 showed an increased syncytia formation and the presence of CD8 and CXCR4 augments virus-induced cell fusion. The nature of MVV receptor is still not known, but our data suggest the use of CD4 and/or CXCR4 as accessory molecules or as part of a receptor complex.
1. Introduction

1.1. Retrovirus

Retroviruses are diploid single stranded RNA viruses with an icosahedral capsid structure, surrounded by a spherical envelope and belong to the family Retroviridae. The name of this family is due to an enzyme, reverse transcriptase (RT). Reverse transcriptase uses the RNA viral genome as a substrate that is converted to DNA, the proviral form which then integrates into the host genome. All the viral proteins are translated from the DNA provirus form. "Retro" comes from Latin and means "backwards". Retroviruses differ from all other known RNA viruses in their ability to integrate into the host genome. Retroviruses are also the only viruses that are diploid (two identical RNA molecules) and the only viruses where the (+) sense RNA genome does not serve directly as an mRNA.

The retroviral genome consists of at least three or four genes, including both structural and enzymatical proteins, namely gag (capsid), pro (protease), pol (reverse transcriptase and integrase) and env (envelope glycoproteins). In some retroviruses pro is part of either gag or pol. In more complex retroviruses like HIV one finds additional genes like rev, tat, nef, vpr, vpu and vif, all of which are translated from post-transcriptional multispliced RNA.

The first retrovirus to be identified, was Rous sarcoma virus (RSV) in 1911 (Rous 1911) although the enzyme that gives the family its name, was not discovered before 1970 (Baltimore 1970; Temin & Mizutani 1970). Every RNA virus containing RT was in 1982 included in the family Retroviridae (Weiss 1982). Retroviruses were originally called RNA tumour viruses and before the 1980s were mainly studied because of their ability to cause cancer. Human T-cell leukaemia virus (HTLV) was associated with adult T-cell leukaemia in humans (Poiesz et al. 1980) and RSV caused cancer in chickens. An immunosuppressive virus Mason Pfizer monkey virus (M-PMV) (Chopra & Mason 1970; Jensen et al. 1970) was the first primate retrovirus to be isolated in 1970, but was of little interest before the discovery of HIV in 1983. When HIV was identified as the cause of Acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Gallo et al. 1983), work on retroviruses was intensified in order to
understand the mechanism of immunosuppression and to gain an understanding of retroviruses as a whole. SIV (Daniel et al. 1985) was discovered in 1985, and represents a primate model for AIDS. Immunosuppressive viruses have also been isolated from other species, like Bovine immunodeficiency virus (BIV) (Van der Maaten et al. 1972) from cows and FIV (Pedersen et al. 1987) from domestic cats.

Taxonomy
Virions of the Retroviridae family are spherical with a diameter of 80-100 nm. All viruses in this family have an envelope consisting of a lipid bilayer membrane with two glycoproteins, SU (surface) and TM (transmembrane) as seen in figure 1.1.1. Under the membrane there is a matrix protein (MA), which anchors the transmembrane protein. The internal core consists of the capsid protein (CA). Within the capsid are two identical RNA molecules, and the enzymes RT, Integrase (IN) and a protease (PR). There are also nucleoproteins (NC) that help stabilize RNA.

The virion of retroviruses consists of about 60 per cent proteins, 35 per cent lipids and about 3 per cent carbohydrates. The SU and usually TM proteins are glycosylated, and the virion also has cellular components on its membrane. The SU protein is also responsible for the interaction with the cellular receptor that leads to entry. The virion must be modified by a protease to become mature. This occurs during or just subsequent to budding (See life-cycle, figure 1.2.1.)
1. Introduction


The family *Retroviridae* is divided into seven genera (Van Regenmortel 2000) and the nucleocapsid is concentric for *Alpharetrovirus*, *Gammaretrovirus*, *Deltaretrovirus* and *Spumavirus*, and rod or truncated coneshaped, for *Lentivirus* and *Betaretrovirus* (Table 1.1.1.). The *Epsilonretrovirus* is a recently discovered group that is not well understood, although it appears to group with the mammalian type C retroviruses. The form of assembly was used as means of classification, where *Betaretrovirus* assembled A-type particles (immature capsids) in the cytoplasm and budded as a B-type (MMTV, Mouse mammary tumour virus) or as a D-type (M-PMV). *Alpharetrovirus* and *Gammaretrovirus* that assemble their capsids on the plasmamembrane, were classified morphologically as C-type viruses.

The early classification had three subfamilies, *Oncovirinae*, *Lentivirinae* and *Spumavirinae*, having three different outcomes of infection; leukaemia and sarcomas from the oncoviruses, immunodeficiencies and neurological disorders from
Table 1.1.1. The family Retroviridae

<table>
<thead>
<tr>
<th>Genus</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpharetrovirus</td>
<td>ALV (Avian leukosis virus)</td>
</tr>
<tr>
<td>Betaretrovirus</td>
<td>MMTV (Mouse mammary tumor virus)</td>
</tr>
<tr>
<td>Gammaparetrovirus</td>
<td>MLV (Murine leukemia virus)</td>
</tr>
<tr>
<td>Deltaretrovirus</td>
<td>BLV (Bovine leukemia virus)</td>
</tr>
<tr>
<td></td>
<td>HTLV (Human T-cell leukemia virus)</td>
</tr>
<tr>
<td>Epsilonretrovirus</td>
<td>WDSV (Walleye dermal sarcoma virus)</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>HIV (Human immunodeficiency virus)</td>
</tr>
<tr>
<td></td>
<td>MVV (Maedi visna virus)</td>
</tr>
<tr>
<td>Spumavirus</td>
<td>HFV (Human foamy virus)</td>
</tr>
</tbody>
</table>

lentiviruses and spumaviruses that are not presently associated with any known disease in vivo. The genomes have a similar genomic organization and vary in size from 8.3 kb to 11 kb.

1.2. The lentiviruses

History and nomenclature

This genus can be divided into five different groups based on the host they infect: Bovine lentivirus group (BIV and Jembrana disease virus (JDV) (Kertayadnya et al. 1993)), Equine lentivirus group (EIAV, Equine infectious anemia virus), Feline lentivirus group (FIV and Puma lentivirus (PLV) (Olmsted et al. 1992)), Ovine/caprine lentivirus group (MVV and Caprine arthritis-encephalitis virus (CAEV)) and Primate lentivirus group (HIV-1, HIV-2 and SIV). The pathogenesis of these viruses involves immunodeficiencies (HIV, FIV and SIV) or pneumonia and/ or paralysis (MVV). The genus can also be divided in two groups, those that cause immunosuppression (e.g. HIV, FIV, SIV and BIV) and those that do not (e.g. MVV, CAEV and EIAV).

The term lentivirus is used because of the long incubation period from infection to the disease caused by the virus (Sigurdsson 1954). Lent is from Latin, meaning "slow." Lentiviruses differ from other retroviruses in their ability to infect and replicate in non-dividing cells, e.g. neuron cells.
Figure 1.2.1. Viral life-cycle of lentiviruses. Binding of virion to a receptor leads to entry and uncoating of the particle. RT reaction takes place inside the capsid followed by transcription of dsDNA. The dsDNA then migrates to the nucleus and integrate in the genome. The viral genome is then transcripted by cellular enzymes and is translated on free ribosomes or on ribosomes bound to endoplasmatic reticulum (env, TM
1. Introduction

and SU). The virion is assembled on the plasma membrane from where it buds, and then becomes mature by the PR. Arrows show the sequence of events from entry to integration and from budding to maturation.

One of the oldest viruses lentiviruses known is the Maedi visna virus. The virus was first described in Iceland in 1957 in sheep (Sigurdsson et al. 1957). It is also thought it may be one of the oldest lentiviruses in evolution. That makes it interesting to study closer, because it may hold the answer to understanding the more "recently" isolated viruses, like HIV and SIV. The genomic organization is also not so complex and it may therefore be easier to reveal its secrets.

Table 1.2.1. Members of the genus Lentivirus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 and -2</td>
<td><em>Human immunodeficiency virus</em></td>
</tr>
<tr>
<td>SIV</td>
<td><em>Simian immunodeficiency virus</em></td>
</tr>
<tr>
<td>BIV</td>
<td><em>Bovine immunodeficiency virus</em></td>
</tr>
<tr>
<td>JDV</td>
<td><em>Jembrana disease virus</em></td>
</tr>
<tr>
<td>PLV</td>
<td><em>Puma immunodeficiency virus</em></td>
</tr>
<tr>
<td>FIV</td>
<td><em>Feline immunodeficiency virus</em></td>
</tr>
<tr>
<td>CAEV</td>
<td><em>Caprine arthritis-encephalitis virus</em></td>
</tr>
<tr>
<td>MVV</td>
<td><em>Maedi visna virus</em></td>
</tr>
<tr>
<td>EIAV</td>
<td><em>Equine infectious anemia virus</em></td>
</tr>
</tbody>
</table>

Genomic organization and expression

The gene organization in lentiviruses is similar, all viruses carry four structural genes; 5’ *gag*, *pro*, *pol* and *env* 3’. When the virus is inside the cell, the capsid proteins are degraded by cellular proteases. The reverse transcriptase reaction (the RT enzyme is packed within the virion) takes place using a host-derived tRNA as primer, while the virion has a partially uncoated core (Figure 1.2.1.). The RT has RNAse H activity that degrades RNA in a RNA:DNA helix. The RT polymerase then makes the dsDNA that migrates to the nucleus, still with virus protein attached. The virus genome is integrated in what looks like a random manner and the genome is now called a provirus. The provirus is transcribed using host enzymes to generate RNA that serves as a genome and that can be translated to generate viral proteins.
In HIV *gag* genes are translated as a polyprotein precursor protein which is later cleaved by the viral protease during maturation to p24 (CA) and p17 (MA) and two small nucleoproteins (NC) p7 and p9. The *env* gene is also translated as a precursor that is cleaved by a cellular enzyme into the surface protein gp120 (SU) and the transmembrane protein gp41 (TM). A -1 framshifting event results in the Gag-Pol polyprotein precursor. The *pol* gene consists of integrase (IN), RT and protease (PR). In addition there are six non-structural proteins derived from multiply spliced RNA species (Figure 1.2.2.).

The genomic organization of MVV is quite similar (Figure 1.2.2.) and is under strict regulation. This is shown by the fact that monocytes become infected but show low levels of mRNA that increase significantly when the monocytes become mature.
macrophages (Gendelman et al. 1986). The MVV Gag-Pol precursor is also derived from a -1 ribosomal framshifting event. Unlike HIV there are only three non-structural genes in the MVV genome, called Q, S and L (Davis et al. 1987; Tiley et al. 1990). The Q protein is a 29-kDa late protein that is found in the cytosol. There is no evidence of accumulation at the cell membrane or that it is packed in the virion (Audoly et al. 1992). This gene is similar to HIV vif and is therefore termed vif (Skraban et al. 1999). Vif proteins inhibit the cleavage of Gag and Gag-Pol and inhibit premature activation of the protease.

The L protein shows great similarity with HIV Rev and is therefore referred to as Rev-V (Tiley et al. 1990) or Rev (Skraban et al. 1999). The Rev-V protein shows the same effect as HIV Rev and can actually rescue HIV-1 rev− mutants (Tiley et al. 1990). Before Rev is synthesised only fully spliced mRNAs are transported to the cytoplasm. Rev then migrates to the nucleus and binds a specific sequence, which leads to transport of unspliced and singly spliced viral mRNAs to the cytoplasm.

The S protein is a 11-kDa protein that has later been named Tat (Gourdou et al. 1989) and shows the same effect as HIV Tat. Tat is a regulatory protein that regulates the transcription of viral mRNA and increases the amount of full length mRNA. Tat binds to the transcriptional complex in a positive feedback loop and stimulates mRNA production as much as 100 fold.

**Ovine/caprine lentivirus group**

The closest relative of MVV is CAEV and the two make this group. These viruses are similar in structure and genomic organization. Both viruses have been cloned and sequenced. Gag and pol show great sequence homology, 75 and 78 per cent identity respectively (Braun et al. 1987). Env is more divergent with 60 per cent identity, but shows the same hydrophilicity. This is an indication that MVV and CAEV are evolutionary related. CAEV is tropic for synovial tissues, has a low virulence for tissue cultures of sheep origin and interacts with the immune system resulting in failure to induce neutralizing antibody. MVV causes lytic infection in goat and sheep cell culture and induces neutralizing antibody production in infected sheep (Narayan & Clements 1989).
1.3. Lentiviruses receptors

In order to produce virus particles, the retrovirus has to infect cells. Susceptible cells express a cell surface-molecule that is capable of specifically binding and internalising the virion. This molecule can be a sugar, a lipid or a protein and is called a receptor. The receptor carries out specific functions for the host cell and the virus exploits this molecule(s) to gain entry (Figure 1.3.1.). When the virus binds the receptor, it can get in close proximity to the plasma membrane that surrounds the cell and fusion between the cell membrane and the virion membrane can occur. This fusion is specific and involves interactions between the virus and the receptor. Once the virus is inside the cell, its capsid is broken down and the genome is free for the replication process to start.

![Figure 1.3.1. Binding of a lentivirus to a receptor complex.](image)

**History**

Research on virus receptors accelerated with the discovery AIDS (Gottlieb *et al.* 1981; Masur *et al.* 1981) and HIV that caused it (Barre-Sinoussi *et al.* 1983; Gallo *et al.* 1983). In 1984 CD4 was found "as an essential component of the HIV receptor" (Dalgleish *et al.* 1984; Klatzmann *et al.* 1984). Later work revealed that CD4 did not function alone and an extensive search for the molecules that comprise the HIV receptor complex began. Two different HIV types, HIV-1 and -2, are recognized based on genomic sequence, neutralisation, virulence and geographical distribution. After a
period of 10 years two reports came independently, identifying two molecules where each one together with CD4, gave rise to HIV-1-induced membrane fusion. That was the α-chemokine receptor CXCR4 (Feng et al. 1996) and the β-chemokine receptor CCR5 (Deng et al. 1996). Expression of CD4 and CCR5 allowed binding and entry of predominantly macrophage tropic HIV-1 strains (also called slow/low (Asjo et al. 1986)). Co-expression of CXCR4 and CD4 allowed binding and entry of T-cell tropic HIV-1 strains (also called rapid/high).

Soon after, other chemokine receptors were found to work for HIV-2. All the chemokine receptors are G-coupled receptors that span the membrane seven times. For certain strains of HIV-2 the presence of CD4 is not required, even though entry is enhanced after binding soluble CD4 (sCD4). These HIV-2 strains use CXCR4 alone (Endres et al. 1996).

This extensive work also led to the investigation of other lentivirus. Both primary and laboratory adapted strains of FIV (Richardson et al. 1999) were found to use feline CXCR4 alone (Willet et al. 1997a). FIV was also able to use the human homologue of CXCR4 with as great efficiency as feline CXCR4. And the feline CXCR4 could support cell fusion between feline CXCR4 transfected cells, and either HIV-1 or HIV–2 env expressing human cells. This discovery led to speculation that CXCR4 is a common lentivirus receptor, because primate and non-primate lentiviruses could utilise the same chemokine receptor, suggesting a common mechanism of entry (Willett et al. 1997b). Lentivirus and retrovirus receptors are reviewed by Sommerfelt (1999).

SIV receptor usage was thought not include CXCR4 with the exception of one strain (SIVmnd(GB-1)) that uses CXCR4 as a co-receptor (Schols & De Clercq 1998). Later work has revealed that several strains of SIV can use CXCR4, including a stumptail macaque (SVSTM), a sooty mangabey (SIVSm) and a African green monkey SIV(AGM) (Owen et al. 2000). Other SIV strains were found to utilise CD4 in conjunction with a number of other chemokine receptors, including the HIV-1 receptor CCR5 (Schols & De Clercq 1998; Owen et al. 2000).
The role of CXCR4 in vivo

CXCR4 is a seven transmembrane protein that was discovered when the search for a coreceptor for HIV was ongoing. It is a receptor that is widely distributed on different cell types. The natural function of the receptor is not exactly known, but it is highly likely that it is involved in the regulation of leukocyte migration and activation. It is a G coupled protein and the mechanism inside the cell is fairly well understood. Upon ligand-receptor binding, the receptor activates a signal transducing protein (the G protein), which activates an effector protein and induces a second messenger (e.g. cyclic AMP, cAMP).

Knockout mice which have CXCR4 deleted have been made and the embryos died perinatally or developed abnormally (Ma et al. 1998; Zou et al. 1998). This suggests that CXCR4 is crucial for development. That is also shown by the conservation of
CXCR4 between feline and human, to the extent that FIV and HIV can use each other’s receptors. (Willet et al. 1997a). Even murine and rat CXCR4 together with human CD4 have been shown to support HIV-1 entry (Simon et al. 1994; Tachibana et al. 1997), but they can not be infected in vivo. Murine NIH3T3 cells transfected with huCD4 were originally thought to be resistant (Maddon et al. 1986), but later work revealed that this cell-line does not express CXCR4 (Tachibana et al. 1997). (See results for an alignment of CXCR4 from different species).

Much work has been done on CXCR4 since its discovery as an HIV coreceptor. It has been found that the N-terminus and the third extracellular domain of CXCR4 are important for binding HIV (Figure 1.3.2.), but with some strain variation (Brelot et al. 1997; Chabot et al. 1999). These domains are also shown to be involved in binding the ligand, stromal cell-derived factor (SDF) (Doranz et al. 1999; Brelot et al. 2000) and have been found to determine FIVs use of the feline CXCR4 homologue (Willett et al. 1998).

The natural ligand for CXCR4 is stromal cell-derived factor, SDF-1, (Bleul et al. 1996; Oberlin et al. 1996). SDF-1 is a chemokine of the CXC type, with motifs of two cysteines and an amino acid that can vary in the middle, hence the name for its receptor. The chemokine is a highly efficacious lymphocyte chemoattractant (Bleul et al. 1996). The ligand is well conserved with an amino acid conservation of 97% between mouse and human in SDF-1α (Shirozu et al. 1995). This chemokine is essential for correct development of the immune system in mouse, where mutants lacking SDF-1 died in utero (Nagasawa et al. 1996). It is therefore possible that both CXCR4 and SDF-1 have no redundancy, meaning that there are no receptors and ligands that can compensate the loss of function of CXCR4 or SDF-1. This is in contrast to most chemokines and chemokine receptors where each chemokine can use more than one receptor and each chemokine receptor can bind more than one chemokine (Ma et al. 1998). Two forms of SDF-1 exist, named SDF-1α and SDF-1β, arising from differential splicing from a single gene. The β-form has four additional amino acids in the carboxy-terminal end. Both forms are found in humans.

When SDF-1α binds to CXCR4, CXCR4-SDF-1α is internalized by endocytosis and a significant part of the receptor is targeted for lysosomes and the degradation pathway.
Without the ligand, CXCR4 is not found in lysosomes. This suggests that only ligand-induced endocytosis leads to receptor depletion at the cell surface. The recycling of CXCR4 is not efficient and varies from 30% in HeLa cells to 5% in U937 (Tarasova et al. 1998) and 10-25% in CEM cells (Amara et al. 1997). The HIV protein gp120 also interacts with the CXCR4 and has been shown to be as potent as SDF-1α in inducing rapid endocytosis, both in the presence and absence of CD4 (Tarasova et al. 1998).

1.4. The Maedi visna virus

History
The first report of Maedi and Visna was in Iceland in the late 1930's (Sigurdsson 1954). In the Maedi form, this disease causes progressive pneumonia and in the Visna form attacks the central nervous system and causes paralysis. Maedi Visna was apparently introduced to Iceland in 1933 when sheep were imported from Germany. In the 1940's the disease took on epidemic proportions, probably due to centuries of sheep isolation and inbreeding, and the practice of gathering of large flocks for winter housing. Late in the 1950's the infections were brought under control by the extensive slaughter of infected sheep (Haase 1975). The nature of the infection was claimed in 1957 by Sigurdsson et al. (Sigurdsson et al. 1957) to be a virus although no evidence of a virus was presented. This claim was repeated in 1958 (Sigurdsson & Palsson 1958) before the agent was finally grown in cell culture in 1960 (Reviewed by Thormar et al. in 1974) and the final proof of a viral etiology was made (Sigurdsson et al. 1960).

Maedi and Visna were originally thought to be caused by two viruses because of the two distinct pathways of disease progression. It was not until 1974 (Gudnadottir 1974) that it became apparent that the same virus caused both Maedi and Visna. The nature of the two forms of disease progression is still unclear. The complete MVV genome was sequenced in 1985 (Sonigo et al. 1985). MVV is cytopathic in cell culture and unlike HIV, chronically infected cell lines cannot be generated.

Today Maedi and Visna are not large problems, although the MVV has been found on all continents, except Australia and New Zealand. In Norway it has been reported as late
as 1995 and there are a few outbreaks every decade. The practice of slaughter of infected flocks has however limited the spread. There is no vaccine currently available and due to the relatively small economic losses that are involved, it is unlikely that a vaccine will be generated.

**Tropism**

MVV is tropic for cells of the macrophage lineage. Monocytes have a low susceptibility to infection, with low level of viral RNA transcription (Gendelman et al. 1986). The susceptibility of the cells to infection increases and virus replication is greatly amplified in mature macrophages. *In vivo* the replication is strictly regulated shown by the fact that macrophage infection is confined to specific tissues, such as lung and spleen but not liver. This is in contrast to the wide range of cells that become infected *in vitro*. MVV is able to infect every cell-line tested (Human, simian, syrian hamster, murine, chicken, goat, sheep), with the exception of a particular hamster line (Chinese hamster ovary, CHO) and lung cells (V79 Tor). This means that the receptor for MVV must exist on virtually every cell and it has to have strictly conserved domains that the virus recognizes. The strict regulation *in vivo* is therefore likely to lie at a post-entry stage. Another interesting feature is that a reduction of CD4+ cells takes place in sheep with advanced disease (Kennedy-Stoskopf et al. 1987), which is also seen with advanced HIV infection (AIDS). This reduction in sheep takes place without a collapse of the immune system as seen for the immunodeficiency viruses (e.g. HIV).

The nature of the MVV receptor is not known. It was thought that the Major histocompability complex (MHC) II could be a part of the receptor because soluble ovine MHC II could inhibit MVV infection (Dalziel et al. 1991). Antibodies against MHC II, however, could only partly block infection. The problem with MHC II as a receptor candidate, is that it is only present on antigen presenting cells (APC) whereas a wide range of MHC II negative cells can become infected *in vitro*. Another study excluded mouse CXCR4 and MHC II as MVV receptors and maps the MVV receptor gene to mouse chromosome 2, 4, 6 or X (Lyall et al. 2000).

Using immunoprecipitation three membrane-associated proteins of 30-kDa, ~45-kDa and ~56-kDa have been identified. The ~45-kDa protein exhibits a serine/threonine kinase activity (Barber et al. 2000) which has been shown to be important for MVV
binding. Anti-serum against the 30-kDa protein and the ~45-kDa protein significantly reduces virus production (Bruett et al. 2000). Furthermore, a β-D-xyloside (Xylβ4MU), which inhibits chondroitin sulphate glycosaminoglycan (GAG) addition to proteoglycans, reduced infection (Bruett et al. 2000). This could suggest that GAG is a non-specific molecule that promotes virus attachment, which in turn leads to stable virus-receptor interactions resulting in virus entry. This has been shown to be the case for HIV-1 and other viruses, where the virion binds heparan sulphate proteoglycans prior to its receptor (Ohshiro et al. 1996).

Transmission

MVV is primarily transmitted in milk. The virus is also present in saliva, and nose-to-nose contact and the use of breeding rams in different farms, has been suggested as a route of infection. Other infections can increase the number of macrophages in mammary glands and these cells are present in both colostrum and milk (Narayan & Clements 1989). Lambs are therefore especially exposed to infection.

1.5. Methods of virion-cell studies

There are different methods to study virus-cell interactions. The first is usually infection studies. These can be done in vivo by infecting an animal and monitoring the progression of the infection or in vitro infection in cell culture. In vivo infection is an important method because it provides knowledge about how an infection attacks the organism, which organs are affected and shows if any disease is associated with infection. But this approach has ethical aspects that have to be addressed. For example the availability of susceptible animals or as in case of HIV, the impossibility of the use of such. With the use of in vitro studies the ethical aspects are no longer a problem. In vitro studies depend on the existence of a cell-culture system. With an established method for infection in cell-culture, studies of virus-cell interactions are significantly easier and more controllable, due to the fact that more parameters can be monitored. In vitro methods are also a very good tool if one wants to manipulate virus entry, which is important for the determining of receptor dependence.
When the infection in cell culture is established, it is important that one is able to specifically block the potential receptor to demonstrate entry through that receptor. If it is not possible to block the receptor and see a reduction or no infection at all, a clear evidence of entry is not made. This inhibition could be done with the natural ligand, monoclonal antibodies or polyclonal antiserum, or synthetic peptides binding the receptor. Infection studies are easy to do in vitro when the cells show signs of infection, e.g. the formation of multinuclear cells (syncytia), rapid cell death or plaque formation. Syncytia formation is a consequence of virus and cell-receptor interactions where cell membranes fuse, either from without (receptors on adjacent cells binding the same virion) or from within (viral envelope glycoproteins on the surface of one cell interacting with receptors on a neighbouring cell.). Plaque formation is a method where a serial dilution of lytic virus is plated on susceptible cells and forms a “plaque” when the infected cells die. Or one can manipulate the cells with a reporter gene that is activated upon entry (e.g. luciferase or β-gal). This makes an infection easy to follow and control.

A different approach to verify the entry of the virion, is PCR. This can be done whether the virus genome is integrated in the cell genome or exists episomally. With the proper controls this makes a strong case, but the problem could be the extreme sensitivity, which makes it easy to contaminate samples or solutions. Primer design can be a problem, especially if the genome is not sequenced. If that is the case, one can make DNA libraries and sequence the genome. Another problem can be some viruses with high mutation rates, which makes potential mismatches between primers and gene of interest. In this case degenerate primers can be made or conserved domains identified for PCR primer design.
1. Introduction

1.6. Aims of study

The expanding knowledge on the mechanics of HIV entry is raising questions about other members of the lentivirus genus. Receptors have been identified for HIV, SIV and FIV, but for the rest of the lentiviruses very little is known about receptor identity and use. The identity of known lentivirus receptors spurred the notion that CXCR4 was a common lentivirus receptor (Willett et al. 1997b). On that background, we sought to answer the following questions:

• Is huCXCR4 a component of the MVV receptor?
• Can any effect of huCXCR4 on MVV syncytia formation be strain dependent?
• Is the effect of huCXCR4 transfected cells on MVV conserved for the related CAEV and the unrelated BIV?
2. Materials

2.1. Viruses

Table 2.1.1. Viruses used in this thesis.

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Provided by</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVV-88</td>
<td>Espen Rimstad (Veterinary College, Norway)</td>
</tr>
<tr>
<td>MVV-EV1</td>
<td>Barbara Blacklaws (University of Cambridge, UK)</td>
</tr>
<tr>
<td>MVV-SA</td>
<td>Gilles Querat (University of Marseilles, France)</td>
</tr>
<tr>
<td>HIV-1 Lai</td>
<td>In House</td>
</tr>
<tr>
<td>FIV-Gi8</td>
<td>Brian Willett (University of Glasgow, UK)</td>
</tr>
<tr>
<td>BIV-R29</td>
<td>Mary Collins (The Royal Veterinary College, UK)</td>
</tr>
<tr>
<td>CAEV-NVH1</td>
<td>Espen Rimstad, Veterinary college, Norway</td>
</tr>
<tr>
<td>CAEV-ATCC</td>
<td>American type culture collection (USA)</td>
</tr>
<tr>
<td>VSV</td>
<td>Miklos Degré (Rikshospitalet, Oslo, Norway)</td>
</tr>
</tbody>
</table>

* MVV, Maedi visna virus; HIV-1, Human immunodeficiency virus-1; FIV, Feline immunodeficiency virus; BIV, Bovine immunodeficiency virus; CAEV, Caprine arthritis-encephaliti virus; VSV, Vesicular stomatitis virus

2.2. Cell lines

Table 2.2.1. Cell lines used in this thesis.

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Full Name</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCP</td>
<td>Sheep choroid plexus</td>
<td>10% FBS EMEM</td>
</tr>
<tr>
<td>HOS</td>
<td>Human osteosarcoma</td>
<td>10% FBS DMEM</td>
</tr>
<tr>
<td>HO6T1</td>
<td>Crandell feline kidney</td>
<td>10% FBS DMEM</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovaries</td>
<td>10% FBS in a 50:50 F12/ DMEM</td>
</tr>
<tr>
<td>CHO CXCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mink</td>
<td></td>
<td>10% FBS DMEM</td>
</tr>
<tr>
<td>PBL</td>
<td>Foetal bovine lung</td>
<td>10% FBS MEM</td>
</tr>
<tr>
<td>GSM</td>
<td>Goat synovial membrane</td>
<td>10% FBS DMEM</td>
</tr>
<tr>
<td>U87</td>
<td>Human glioblastoma</td>
<td>10% FBS DMEM</td>
</tr>
<tr>
<td>U87 CXCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87 CD4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All cells are adherent
2. Materials

2.3. Primers

Table 2.3.1. Primers used in PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Sequence 5' -3' *</th>
<th>Product size</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVV PolF1</td>
<td>1009-1030</td>
<td>AAG-AGC-ACA-GGG-GTA-TCG-TGA-A</td>
<td></td>
<td>MedProbe</td>
</tr>
<tr>
<td>MVV PolR1</td>
<td>1600-1580</td>
<td>TCT-TTT-TCC-ACC-TCA-CCG-AGC</td>
<td>680</td>
<td>MedProbe</td>
</tr>
<tr>
<td>MVV PolF2</td>
<td>1113-1133</td>
<td>GGA-TTT-GAA-TTG-CAT-CCG-GAG</td>
<td></td>
<td>MedProbe</td>
</tr>
<tr>
<td>MVV PolR2</td>
<td>1669-1552</td>
<td>TCT-TCC-CTT-CCC-GGC-AAC</td>
<td>487</td>
<td>MedProbe</td>
</tr>
</tbody>
</table>

* Based upon the MVV 1514 strain sequence, Genebank nr. M60609

2.4. Solutions for cell-culture

*PBS 10x solution*

85 g NaCl  
8.6 g Na₂HPO₄ x 2H₂O  
2.5 g KH₂PO₄

This is mixed with 1 litre of deionised water and pH is adjusted to 7.2. Before use, it is diluted in deionised water to a concentration of 1x and autoclaved.

*X-gal solution*

80 ml PBS (1x)  
400 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside) in 50 mg/ml DMSO  
0.1 g K₃Fe(CN)₆ (3mM)  
0.13 g K₄Fe(CN)₆ (3mM)  
0.03 g MgCl₂ x 6H₂O (1.3 mM)

Adjusted to 100 ml with PBS. Stored at room temperature and protected from light.
2. Materials

_Syncytia stain (200 ml)_

1 g Methylene blue
0.33 g Basic Fuschin
200 ml Methanol
Stored at room temperature.

_Neutral red_

10 g Neutral red
100 ml PBS
Solution was made directly before use.

_Tryptsin- Versene EDTA_

Bio Whittaker, cat. no 17. 161E
Aliquoted in 5 ml and stored at –20 °C.

_Agarose SeaPlaque_

3 g SeaPlaque (FMC Bioproducts, cat. no 14-701F)
100 ml of distilled water
Directly before use, 1 vol. of agarose was mixed with 2 vol. of prewarmed (37 °C) medium.

_Solution for fixing of cells before immunostaining(100 ml)_

50 ml Acetic acid
50 ml Methanol
Stored at 4 °C.

_Antibiotics in cell-culture medium_

Penicillin (5000 U/ml) + Streptomycin (5000 µg/ml). Gibco, cat. no. BE 17-603E.
2.5 ml per 500 ml of medium (DMEM or EMEM).
2. Materials

**Antibiotics for selection in transfected cell lineages**

Puromycin, Sigma, cat. no. P8833. Aliquoted in 500 µg/ml and stored at –20 °C. Final concentration, 1 µg/ml. Used to select CXCR4 and CCR5 in U87 cells.

Geneticin, Sigma G 1279. Aliquoted in 100 mg/ml and stored at –20 °C. Final concentration, 300 µg/ml to select CD4 in U87 cells and 800 µg/ml to select CXCR4 in CHO cells.

**Medium**

Minimal Essential Medium Eagle, BioWhittaker, cat. no. 12-611F (EMEM).

Complete Dulbeco's modified Eagle's medium, BioWhittaker, cat. no. 12-601 F/U1 (DMEM).

MEM (Eagle) with Glutamax-1, Gibco, cat. no 42360-024.

F12, Gibco, cat. no. 21765-029.

**Fetal Bovine Serum (FBS)**

Bio Whittaker, cat. no 14-701F.

**Freezing mixture for cells**

10% DMSO

90% FBS

Mixed and stored not more than one week at 4 °C.

**2.5. Solutions for PCR**

**DNA extraction solution (phenol:chloroform)**

5.5 ml Phenol (saturated with water)

4.5 ml Chloroform

50 µl 5M NaOH

Stored at –20 °C.
2. Materials

**PCR Lysis buffer**

1 mM EDTA (Stock autoclaved)  
10 mM Tris.HCl pH 8.3 (Stock autoclaved)  
1% (v/v) NP-40  
1% (v/v) Tween-20  
in ddH2O  
pH adjusted to 7.3.  
Stored in 5 ml aliquots in –20°C. Prior to use 15 µl of proteinase K (20 mg/ml) was added per ml of lysis buffer.

**50x TAE**

242 g/l Tris base  
57.1 ml/l Glacial acetic acid  
100 ml/l 0.5 M EDTA, pH 8.0  
in ddH2O and pH adjusted to 8.0. Mixed with dH₂O to 1x.

**10x PCR buffer II**

Perkin Elmer, cat. no. N808-0167. Stored in –20°C.

**MgCl₂ solution for PCR**

Perkin Elmer, cat. no. N808-0167, 25 mM. Stored in –20°C.

**Agarose gel 1% (TAE)**

1.0 g Agarose, FMC Bioproducts, cat. no. 50101  
100 ml 1x TAE buffer pH 8.0.

**dNTP mix**

2.5 mM of each dNTP, Pharmacia, Ultrapure dNTP.  
In 20 µl aliquots, stored at –20°C.
2. Materials

**PCR amplification mix**

per reaction:
5 µl PCR Buffer (10x)
1 µl dNTP mix (2.5 mM of each dNTP)
1 µl Forward primer (10 mM in dH2O)
1 µl Reverse primer (10 mM in dH2O)
0.5 µl Taq (5U/µl)
7 µl MgCl2 (25 mM)
5 µl template
29.5 µl ddH2O
Total volume: 50 µl
Prepared in PCR room with no DNA present. Template added just before start of the PCR.

**DNA standard**

BioRad, cat. no. 170-8200. Stored at 4 °C.

2.6. Solutions for antibody binding and reconstitution

**1 % Fetal Bovine Serum (FBS) in PBS**

1 ml FBS
100 ml PBS
Solution was made directly before use.

**2 % FBS in PBS**

2 ml FBS
100 ml PBS
Solution was made directly before use.
2. Materials

2.7. RT kits

Boehringer- Mannheim, non-radioactive kit, cat. no. 1 468 120.
Cavidi Tech. High sensitive lentivirus kit.

2.8. Proteins

Taq polymerase

Perkin Elmer AmpliTaq, 5U/µl, cat. no. N808-0167. Stored at –20 °C.

SDF-1α

R&D systems, cat. no. 350-NS.
Reconstituted in PBS with 2 % FBS and stored at –20 °C.

Anti CXCR4 monoclonal antibody

R&D systems, Mab 171, 172, 173.
Reconstituted in PBS at 5 µg/ml and stored at –20 °C.

Anti mouse monoclonal antibody conjugated with β-gal

Southern Biotechnology associates, cat. no. 1010-06. Supplied in 100 mM borate buffered saline, pH 8.2. Stored at –20 °C.

Anti CD4 monoclonal antibody

Q4120, Centralised Facility for AIDS Reagents (ARP318).
Reconstituted in PBS at 200 µg/ml aliquoted and stored at –80 °C.
2. Materials

*Soluble CD4*

sCD4, Centralised Facility for AIDS Reagents (ARP608/609). Reconstituted in PBS at 1mg/ml, aliquoted and stored at –80 °C.

*HIV recombinant gp120 (HIVIIIb)*

rgp120, Centralised Facility for AIDS Reagents (EVA 657). Reconstituted in PBS at 200 µg/ml, aliquoted and stored at –80 °C.
3. Methods

3.1. Cell culture

Culturing

All cells were grown in a humidified incubator with 5 % CO₂ at 37 °C. Cells were maintained in medium with various quantities (5-15 %) (Table 2.2.1.) of FBS depending on the cell type. Infected cells were normally grown in 5 % FBS.

Cell passage

Cells were passaged twice a week. Medium was removed and the cells were washed with sterile PBS. Trypsine/versene was added to just cover the cells (1 ml for T-25 flask and 1.5 ml for T-75 flask). The cells were incubated briefly at 37 °C before the adherent cells loosened from the flask's surface. Medium containing FBS was added to inactivate the trypsine. The cells were split in accordance with the rate they were growing. Generally SCP were split 1:4, GSM 1:4, HO6T1 1:12, HOS 1:12, and U87 cells (U87, U87 CXCR4, U87 CD4 CXCR4, U87 CD4 CCR5 and U87 CD4) about 1:8.

Freezing and thawing

Typically, 2-6x10⁶ cells would be centrifuged for 5 minutes at 1000 rpm in a Kubota KS-8000 and then resuspended in 2 ml of 10 % dimethyl sulphoxide (DMSO) in FBS and aliquoted (ca 1 ml) in Nunc cryotubes (1.8 ml). The tubes would be put at −80 °C, in polystyrene to ensure slow freezing. For long-term storage the cells were transferred to liquid nitrogen (-196 °C).

Cells were thawed in warm water (ca 37 °C), until a small lump of ice was left. Medium (ca 9 ml) was added drop wise before the cells were centrifuged for 5 minutes at 1000 rpm in a Kubota KS-8000, in order to remove the DMSO. The cells were then resuspended in fresh medium and transferred to a culture flask and incubated at 37 °C.
3. Methods

Selection in transfected cell lineages

At regular intervals all transfected cells were selected. The cells were maintained in medium with antibiotics (Materials 2.4.) for a period up to two weeks. To ensure that all cells that had the transfected genes, a positive control was included. The control was cells not carrying resistance gene and these cells went through the same selection. Selection was at a minimum maintained until all cells in the control died. This was carried out for U87 CD4, U87 CXCR4, U87 CD4 CXCR4, U87 CD4 CCR5 and CHO CXCR4.

Immunostaining

To verify the presence of surface proteins, monoclonal antibodies were used. This was done to detect either CXCR4 or CD4. The cells were seeded in 24 well plates and grown until confluent. Then they were fixed with a methanol: acetone mixture (1:1) for 10 minutes at –20 °C. The wells were then washed with PBS and PBS with 1 % FBS (Materials 2.4.) to improve binding of the second antibody. The first antibody (anti CD4 or anti CXCR4) from mouse was added, 125 µl of a 5 µg/ml solution. After one hour incubation in room temperature, the wells were washed three times with 1 % FBS in PBS. The second antibody was then added, 150 µl of goat anti-mouse conjugated with β-galactosidase at a dilution of 1:400. After 1 hour incubation in room temperature, cells were washed three times with 1 % FBS in PBS and two times with PBS. Then the substrate analog for β-galactosidase, X-gal, was added. After two hours at 37 °C, followed by overnight incubation at 4 °C, positive cells could be viewed as blue cells.

3.2. Virus culture

Virus stocks

To make virus stocks, 2 ml inoculum was added (of which normally 1 ml was virus supernatant), for MVV, to SCP cells (Thormar et al. 1974). The inoculum was allowed to adsorb for two hours, when an additional 3 ml of serum free medium was added. After 24 hours all medium was removed and cells washed with 5 ml of sterile PBS. Then fresh medium was added. In T-25 bottles 5 ml medium were used and in T-75 14
ml were used. Sometimes smaller volumes were used in T-75 flask to concentrate virus and thereby increase virus titre per ml. Culture supernatant was harvested once or twice weekly by removing the supernatant and centrifuged at 2000 rpm for 10 min in a Kubota KS-8000 centrifuge to remove cell debris. The amount of virus produced could be estimated from the rate of cell-death/syncytia formation. This could be verified later by RT-testing. Virus supernatant was stored at −80 °C until used. This was done for MVV-88, MVV-EV1, MVV-SA, HIV-1 Lai, CAEV NVH1, CAEV ATCC, BIV R29 and VSV. Table 3.2.1. show which cell lines that was used. With FIV which was cell associated, stocks were not made. Instead persistently infected HO6T1 cells were used directly in the assays.

Table 3.2.1. Stock production: Viruses and cell-lines used.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVV-88</td>
<td>SCP</td>
</tr>
<tr>
<td>MVV-EV1</td>
<td>SCP</td>
</tr>
<tr>
<td>MVV-SA</td>
<td>SCP</td>
</tr>
<tr>
<td>HIV-1 Lai</td>
<td>U87 CD4 CXCR4</td>
</tr>
<tr>
<td>FIV-Gi8</td>
<td>HO6T1</td>
</tr>
<tr>
<td>BIV-R29</td>
<td>PBL</td>
</tr>
<tr>
<td>CAEV-NVH1</td>
<td>GSM</td>
</tr>
<tr>
<td>CAEV-ATCC</td>
<td>GSM</td>
</tr>
<tr>
<td>VSV</td>
<td>Mink</td>
</tr>
</tbody>
</table>

Tissue culture infectious dose (TCID50)

This method uses the fact that infective agents can be diluted to a point where no sign of infection can be seen. A TCID50 value, is the dilution of virus where half of the wells infected, show signs of infection. This was particularly important in the experiment were different stocks or different viruses were used. The enhancement and blocking experiments (Section 3.6.) where the syncytium inducing abilities to MVV and HIV were compared, it was crucial that the inoculum used, had a common TCID50 value which was monitored as syncytium induction and cytopathic effect.

To calculate the amount of virus needed in an experiment, a dilution series of virus was made. Then the virus was added to semiconfluent wells of the cells that should be used in the experiment (e.g. if U87 cells were used in an experiment, the TCID50 test were
done on U87 cells) and incubated at 37 °C (usually 24-48 h). After incubation, the syncytium inducing abilities to the different dilutions could be seen under a microscope and a suitable dilution could be identified for other experiments.

**Reverse Transcriptase (RT) assay**

A productive infection of lentiviruses will show some level of RT activity and a significant RT value demonstrates entry and replication. During infection studies medium was harvested, aliquoted and stored at –80 °C.

Two RT assays were used in this study. The first assay used a non-radioactive kit from Boehringer- Mannheim, where the activity of the viral RT of interest was used to make DNA from a template/primer hybrid Poly A/dT(15). First 1.5 ml of virus supernatant was pelleted for 13000 rpm for 99 min in a Heraeus Biofuge 15 centrifuge. The virus supernatant was then carefully removed. The virus pellet could be stored at –80 °C until needed. The invisible virus pellet was then dissolved in 40 µl lysis buffer supplied with the kit. After 30 minutes at room temperature the lysed viruses were transferred to a new eppendorf tube. A serial dilution of quantified HIV-1 RT as a positive control was made in parallel. This could serve as a standard curve. Twenty µl reaction buffer (included in kit) was added to the HIV-1 RT dilution series and to each sample. The RT reaction incubated for up to 15 hours at 37 °C. The reaction incorporated an optimised amount of digoxigenin and biotin.

The ELISA wells were provided coated with streptavidin allowing the newly synthesized helix to bind to the wells for one hour at 37 °C. After washing the ELISA plates five times using a washing buffer (supplied with the kit) an antibody conjugated with peroxidase (POD) was allowed to bind to the digoxigenin for one hour at 37 °C. The washing procedure was then repeated, before the substrate, o-Phenyldiamine (OPD), was added.
3. Methods

Figure 3.2.1. The principle of the RT-kit. Adopted from the Boehringer-Mannheim protocol.

Absorbance values increase proportionally with the amount of RT present. The absorbance was measured at 492 nm. Because it is not known whether MVV RT shows the same activity as HIV-1 RT, the amount of MVV RT could not be calculated from the HIV-1 RT standard curve. Instead the absorbance, displayed as optical density (OD), was used to compare samples that were run simultaneously. This kit could detect HIV-1 RT values of ca 1 pg per well. Significant values were three times the background.

A highly sensitive RT kit, supplied from Cavidi Tech was also used. This is also an ELISA-based non-radioactive kit that incorporates Bromo-deoxyuridine triphosphate (BruUTP). This used 10 µl virus supernatant, to which was added 100 µl reaction mixture that had preincubated for 20-60 minutes at 33 °C. The viral RT synthesizes a DNA strand from an immobilized template/primer construct during a 3 to 15 hour incubation at 33 °C. After a washing procedure with 5 containers, each filled with a litre of washing buffer, the conjugated antibody was added and incubated for 90 minutes at 33 °C. BruUTP is then recognized by an antibody that is conjugated to alkaline phosphatase (AP). The absorbance is measured at 405 nm. This kit was used to detect HIV-1 RT down to ca 0.0018 pg well. HIV-1 RT was used as control and to produce a standard curve. This kit used supernatant directly, with no need for virus pelleting. A
significant measurement was achieved when the RT activity was two times the background.

### 3.3. Computer analysis

To perform computer analysis of gene sequences, ClustalX 1.7 and 1.8 were used on default settings. All sequences were prepared in FASTA format. The sequences were retrieved from SWISSPROT (http://www.ebi.ac.uk/swissprot/). The alignment of CXCR4 was done on a predicted amino acid sequence because only mutations resulting in changes in amino acid sequence were of interest.

**Table 3.3.1.** The CXCR4 sequences used with accession numbers in swissprot

<table>
<thead>
<tr>
<th>Species</th>
<th>Full Name</th>
<th>Acc. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Homo sapiens</td>
<td>P30991</td>
</tr>
<tr>
<td>Sheep</td>
<td>Ovis aries</td>
<td>Q28553</td>
</tr>
<tr>
<td>Cow</td>
<td>Bos taurus</td>
<td>P25930</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mus musculus</td>
<td>P70658</td>
</tr>
<tr>
<td>Crab eating macaca</td>
<td>Macaca fascicularis</td>
<td>Q28474</td>
</tr>
<tr>
<td>Rat</td>
<td>Rattus norvegicus</td>
<td>O08565</td>
</tr>
<tr>
<td>Rhesus macaque</td>
<td>Macaca mulatta</td>
<td>P79394</td>
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<tr>
<td>Sooty mangabey</td>
<td>Cercocebus torquatus atys</td>
<td>O62747</td>
</tr>
<tr>
<td>Cat</td>
<td>Felis silvestris catus</td>
<td>P56498</td>
</tr>
</tbody>
</table>

### 3.4. Syncytia assays

**Syncytia induction**

Syncytia assays were performed to detect syncytia formation. This was done in two different ways either cell-cell, fusion by mixing infected and uninfected cells, or cell-virus when viral supernatant was added to indicator cells. Cell-virus fusion was used for all virus strains with the exception of FIV where cell-cell fusion was used because FIV remained mainly cell associated. Typically 1/2 ml virus was incubated with a culture of $10^5$ cells in 24 well plates. The culture was then stained with syncytia stain no more
3. Methods

than 3 days post infection. Each experiment was treated individually and any comparison between amounts of syncytia on different cells was from the same experiment only. In cell-cell assays for FIV, HO6T1 cells persistently producing FIV were mixed with indicator cells at a 50/50 ratio.

**Syncytium inhibition and enhancement**

**SDF-1α:**
To block CXCR4 as a potential receptor, its natural ligand SDF-1α was used (Bleul et al. 1996). Lyophilised SDF was reconstituted in PBS with 2 % FBS (Materials 2.4.), to a stock of 10 µg SDF/ml. The stock was then kept at –20 ºC. Before the SDF was used, a twofold dilution series was made, ranging from 10 µg/ml to 20 ng/ml. The dilution was prepared using serum free medium. From these dilutions, 50 µl was used in each well. Cells that were to be investigated were trypsined and counted. Both CXCR4 positive and negative cell lines were included in the test (U87, U87 CXCR4, U87 CD4 CXCR4, U87 CD4 CCR5 and U87 CD4). Between 1-1.5 x10⁴ cells were used per well in a 96 well cell-culture plate. The cells were pipetted into the wells. Every dilution of SDF-1α was done in duplicate.

All assays were done with three uninfected controls and three controls without SDF-1α addition for each cell-line used. HIV-1 was used as a positive control to determine the dose response in this system. After the trypsined cells and SDF-1α were added, the plate was incubated for 10 minutes at 37 ºC. Then 50 µl virus was added in each well except for the uninfected control, to which medium was added. The 96 well plate was wrapped in plastic an incubated for 24 hours before it was stained. Then it was examined under the microscope for syncytia formation.

**Antibody Q4120:**
To investigate the potential use of CD4 in entry, a monoclonal antibody called Q4120 was used. Inhibition of entry by use of monoclonal antibodies was how the HIV-1 receptor, CD4, originally was found (Dalgleish et al. 1984). Q4120 has been found to inhibit entry of HIV (Healey et al. 1990). The experiment was performed in a 96 well plate. Cells were trypsined, counted and 1x10⁴ cells were added each well. This was
3. Methods

done using U87, U87 CXCR4 and U87 CD4 CXCR4 cells. The controls were uninfected wells of the mentioned cells (negative control) and U87 CD4 CXCR4 with HIV-1 and 50/50 of HO6T1 FIV Gl8/U87 CD4 CXCR4 (positive control). The FIV control was included to verify that FIV does not use CD4 alone.

The amount of Q4120 was 15 µl and 5 µl of a 200 µg/ml stock, in duplicate on all cells. Virus was added at 50 µl per well, with the exception of FIV, which is cell-associated. The cells, virus, medium and antibody were added at the same time and incubated in a humidified incubator at 37 °C and 5 % CO₂. The total volume in all wells was adjusted to 150 µl. The following day the cells were washed, stained and examined under the microscope.

Gp120:
Soluble HIV envelope glycoprotein gp120 has been used to block HIV entry as the glycoprotein can bind CD4 and compete with the virus for the CD4 receptor. The cells were counted and 1x10⁴ cells were added each well. After 24 hours the medium was removed and the subconfluent cells washed with sterile PBS. From a 200 µg/ml stock stored at –80 °C, different amounts of gp120 were diluted in medium and added to the wells in duplicate (at final concentrations of 2.5 µg, 1 µg, 0.5 µg, 0.1 µg and 0 µg per well). The cell lineage used for MVV-88 was U87 CD4 and for the HIV control, U87 CD4 CXCR4. The cells were preincubated with gp120 for 30 minutes at 37 °C after which time 100 µl of virus was added to each well. The total volume in each well was 150 µl. The plates were then incubated overnight, washed using PBS, stained and examined for syncytia formation the following day. If MVV-88 interacted directly with the CD4 receptor as HIV does, the MVV-88 wells would show as increase in syncytia formation as the level of gp120 decreased.

Soluble CD4 (sCD4):
The sCD4 protein interacts with HIV gp120 causing a conformational change that allows HIV-2 to enter CD4 negative cells (Clapham et al. 1992; Bandres et al. 1998). If MVV-88 interacted with CD4 in the same way, preincubating MVV with sCD4 should increase the amount of syncytia on U87 cells to the equivalent of that seen on U87 CD4
3. Methods

...cells. The cell-lines used were U87 for MVV-88 (using U87 CD4 as a control) and U87 CXCR4 for HIV (using U87 CD4 CXCR4 as control).

The viruses were thawed and 100 µl was incubated with various amounts of sCD4 (1 µg, 0.5 µg 0.25 µg and 0 µg per well) in 50 µl medium, at 37 °C for 30 minutes. Medium was used as a negative control. sCD4 is kept at –80 °C as stock of 1 mg/ml and the dilutions were made in medium. 1x10^4 cells were added to the wells with the preincubated virus/sCD4 solution. The total volume was 200 µl. When the cells had visible syncytia after 24 or 48 h, the wells were washed, stained and examined under a microscope.

3.5. Virus pseudotype

In order to isolate and investigate the MVV env-receptor interaction specifically, pseudotype virus was made. The goal was to make virus with MVV env and the core of Vesicular stomatitis virus (VSV). This pseudotype virus could then be titrated on cell-lines expressing the receptors that were to be examined and clear plaques would be seen where a specific virus-pseudotype-receptor interaction had taken place due to the great lytic effect of VSV.

After infecting SCP cells with MVV (Methods 3.2.), one ml of VSV was added at different time points before the peak in the MVV infection. Non-adherent virus was removed after one hour. The VSV pseudotype was harvested after 15 hours post-infection. The pseudotype virus was aliquoted and stored at –80 °C until used.

To see the effect the pseudotype virus had on the different receptors, subconfluent cells were set up in 6 well plates and incubated overnight. A serial dilution of virus was then made and 0.4 ml of virus was placed in the wells. After one hour incubation with gently rocking the cells every twenty minutes, the virus supernatant was pipetted away.

Then 2 ml of 1 % seaplaque agarose using a 2:1 mixture of medium and distilled water, was added each well. The agarose was prewarmed to 37 °C. When the agarose had
solidified, two ml of medium was placed on top. The plates were then wrapped in plasticwrap and incubated at 37 °C 24-48 hours. Visualising the plaque could be done with neutral red, where the living cells took up the neutral red and thereby showing the plaque appearing as clear patches in a red monolayer. One ml of dye from a 10 % neutral red in PBS solution, was added each well for one hour at room temperature. This method has been used previously to study retrovirus receptors (Sommerfelt & Weiss 1990).

These experiments were carried out together with my supervisor Prof. M Sommerfelt Grønvold for safety reasons.

3.6. Infection studies

When infecting different cell lines, same procedure was used as when making MVV stocks. Controls were included, both positive (usually by infecting SCP) and negative (uninfected cells of the line that was to be investigated). Infected cells were maintained until all were dead or for a maximum of 22-25 days. Medium was harvested throughout the infection, for RT testing. The supernatant was centrifuged at 2000 rpm for 10 min in a Kubota KS-8000 centrifuge, to remove cell debris. The supernatant was then aliquoted and stored at −80 °C until an RT assay was performed.

Duplicate bottles were also stained with a syncytia stain (Materials 2.4.) to look for syncytia formation. The cells were washed once with sterile PBS before the syncytia stain was added to cover cells and incubated at room temperature for 10 minutes. The stain was then removed and cells washed in PBS or dH2O. Syncytia were then clearly visible under the microscope. Cells were also harvested for PCR analysis at the time of maximum infection (syncytia/ cell death) or at day 25 (if there was no sign of infection).

Polymerase chain reaction (PCR)

To detect stably integrated proviral MVV in infected cells, PCR was used as described (Mullis & Faloona 1987). A cell-lysate was made at the peak of infection or at day 25
(if there was no sign of infection). Cells were washed with sterile PBS and trypsinised before counting. The cells were then pelleted at 2000 rpm for 10 min in a Kubota KS-8000, resuspended in sterile PBS and pelleted again, before being resuspended in 100 µl lysis buffer containing proteinase K. Using a drop of mineral oil to prevent evaporation, the lysate was incubated at 37 °C overnight. The proteinase K was then inactivated at 95 °C for 15 minutes. The lysate was stored at –20 °C until used.

Two sets of primers, specific for the MVV polymerase were used (Materials 2.3.) in a nested reaction. The primers were designed according to a published sequence of the MVV-88 genome, genebank accession number M60609 (Staskus et al. 1991). To optimise the reaction, primers were tested on a plasmid (pACkv 72-67r) containing the MVV genome (Andresson et al. 1993). This plasmid also served as a positive control (0.5 µl per reaction of a 10 pg/µl concentration). Negative control was dH2O. The outside reaction was found to work at an annealing temperature of 54 °C with an Mg2+ concentration of 3.5 mM. The outside primers were designated MVV PolF1 and MVV PolR1. Nested primers, called MVV PolF2 and MVV PolR2, were found to work at an annealing temperature of 47 °C and 3.5 mM Mg2+.

The PCR machine used was a Perkin Elmer Gen amp PCR system 2400. The PCR mixture (Materials 2.5.) without template, was prepared in a premix room to avoid contamination of DNA. All the solutions were added in a mix and then aliquoted to 45 µl per reaction in an Oxygene 0.2 ml, thin walled PCR tubes. The DNA template was added just before the tubes were put in the PCR machine. Negative controls used ddH2O as template.

The outside reaction was carried out with a 7-minute hot start at 95°C, followed by 1-minute denaturation at 95 °C, 1 minute annealing at 54 °C, elongation at 72 ºC for 1 minute and a final elongation at 72 ºC for 7 minutes. The outside reaction was normally run with 30 cycles. The nested started with a hot start for 2 minutes at 95 °C and then the same as the outside, with the exception of annealing temperature at 47 °C. The template used in the nested reactions was 5 µl of the outside reaction product. PCR products were electrophoresed on a 1 % agarose gel containing TAE buffer and 1.5 µl EtBr (10mg/ml). The gel was ten centimetres long and the products were
3. Methods

electrophoresed at 100 V for ca 30 minutes, before being visualized using UV light and photographed. A size marker from Biorad was used (2 kb-50 bp).

![Figure 3.6.1. Primer localisation on Pol gene](image)

**DNA purification from PCR lysis buffer**

The cell-lysate that had been incubated overnight at 37 °C and 15 minutes at 95 °C, was transferred to a new eppendorf tube. An equal volume of phenol:chloroform (Material 2.5.) was added and mixed by vortexing, followed by centrifugation at 12 000 g at 4 °C for two minutes. The supernatant was transferred to a new tube and the process was repeated. The supernatant from the second extraction was added an equal volume of chloroform, mixed by vortexing and centrifuged at 12 000 g at 4 °C for two minutes. The supernatant was transferred and twice the volume of 100 % ethanol was added. The tube was then incubated at –80 °C for one hour.

The DNA was pelleted at 12 000 g at 4 °C for 20 minutes, taking care to orient the tubes for easy localization of the pellet. The pellet was washed with ice-cold 70 % ethanol and dried in a vacuum dessicator. The DNA was resuspended in ddH2O and stored at –20 °C until used.
4 Results

4.1. Alignments

In order to find out whether CXCR4 could represent a common lentivirus receptor, it is of interest to find out how conserved the gene is between species. If the gene is very different between species, it reduces the possibility that CXCR4 is a common receptor. It should at least have conserved regions that the virus could utilize as a virus-binding site that could lead to virus entry. CXCR4 amino acids sequences from different species were retrieved from the database Swissprot (Methods 3.3.). The amino acids sequences were used to mask silent substitutions. The only differences that were interesting were those that gave new amino acids, since silent mutations would not have any effect on the processed receptor.

Sequences from human (352 aas), rhesus macaque (352 aas), cat (353 aas), sooty mangabey (352 aas), crab eating macaque (352 aas), cow (353 aas), rat (349 aas) and mouse (359 aas) were aligned using ClustalX software, version 1.7 and 1.8 for windows on default settings. The result is shown in figure 4.1.1. It is striking how conserved CXCR4 is between species (Figure 4.1.1 and 4.1.2). A star on top of the alignment show that CXCR4 from all species have identical amino acid in the same position suggesting the gene is very conserved throughout evolution. The major differences that exist are a duplication of 5 amino acids in the mouse between amino acid 180 and 190 (the numbers refers to figure 1.3.1.), and “some” diversity at N terminus.
Figure 4.1.1. Black line at the bottom show how similar the amino acids are. A straight line shows the same amino acid in exactly the same position. The amino acids are coloured according to their properties. A star at the top of the alignment shows that all sequences have the same amino acids in that position.
4. Results

Unfortunately sheep CXCR4 is only partly sequenced. For this reason only, an alignment of amino acids 14-210 was made (Figure 4.1.2.). The sheep sequence is however, very similar to the other sequences almost without mutations. In the third extra cellular domain, starting at position 168 to position 192 (Referring to figure 4.1.2.) there are in total six amino acid substitutions in sheep contra human, although the sheep sequence has the same net charge as the human sequence in this region.

Comparing sequences for sheep and cat there are five amino acid substitutions giving the cat sequence a higher net charge (+2) compared to sheep. The cat sequence has five substitutions and a higher net charge (+2) compared to the human sequence (Figure 4.1.2.). The cat sequence is more positively charged in this domain than either human or sheep. The sheep sequence has a substitution in position 168 resulting in loss of a potential glycosylation site (Chabot et al. 1999) while the cat and human sequences both have the same amino acid in that position.

The alignment that included sheep was used to make a phylogenetic tree, to show the relationship between the amino acid sequences from different species. This could be used to correlate the known use of CXCR4 for lentiviruses (e.g. the viruses that have known receptor use), with the difference in sequence and thereby showing how probable the shared use of CXCR4 is for lentiviridae.
Figure 4.1.2. Sheep contra human has six substitutions, D_{168}N, I_{169}V, K_{170}S, E_{179}D, S_{189}N and L_{193}V. Sheep contra cat has five substitutions, D_{168}N, I_{169}V, K_{170}R, E_{179}G and L_{191}S. Cat contra human has five substitutions, R_{170}S, G_{179}D, S_{189}N, S_{191}N, and L_{193}V. If positive and negative amino acids are summarized in the third extra cellular domain, sheep has a net charge of $-3$, human $-3$ and cat $-1$. 
4. Results

Figure 4.1.3. A unrooted, bootstrapped phylogenetic tree of CXCR4 from a selection of species made with ClustalX 1.8.

4.2. Virus stocks and cells used.

Viruses
To make MVV stocks SCP cells were used. MVV show little syncytia formation on SCP cells, the effect was mainly cell death. (Figure 4.2.1.) The harvest that gave the best titre was late in infection, at day 22. This was done allowing the virus time to concentrate with only one harvest a week. The three different MVV strains, 88, SA and EV1, that were used in the syncytium assays and infection studies showed the same characteristics in infection on SCP cells.
4. Results

Figure 4.2.1. MVV-88 infected SCP cells to the left and uninfected control to the right.

HIV-1 Lai stocks was prepared on U87 CD4 CXCR4 cells. These stocks were prepared the same way as MVV. For the enhancement and inhibition studies a TCID50 assay was performed to ensure that the supernatant used had the same syncytium inducing capabilities. For MVV a 1:4 dilution was as good as undiluted HIV-1 Lai in inducing syncytium in indicator lines. MVV used U87 CD4 for titration and HIV-1 used U87 CD4 CXCR4.

FIV Gi8 used HO6T1 cells for stock production. Due to a high degree of cell association, persistently infected cells were used directly, mixed 50:50 with the indicator line. Both strains of CAEV (NVH1 and ATCC), stocks were made using GSM cells, with harvest once a week. It was difficult to achieve a good titre with the ATCC strain compared to NVH1 strain. At maximum sensitivity ATCC had an OD$_{405}$ at 0,376 and NVH1 2,928. Only NVH1 were used in the infection studies (See section 4.7.)

BIV-R29 stocks were made using a primary cell line PBL. Virus supernatant was harvested when 50 percent of the cells were involved in syncytia. At maximum sensitivity the supernatant had an OD$_{405}$ at 3,443. VSV stocks were prepared infecting mink cells. The titre was calculated with titration on mink cells, which was positive control.

Transfected cells

The expression of CD4, CXCR4 and CCR5 on U87 cells was confirmed on all cells tested by immunostaining and by the use of viruses as positive control. Also CHO CXCR4 cells were stained for the surface expression of CXCR4. All transfected cells were regularly selected for the transfected genes using antibiotics (See methods 3.1.).
4. Results

4.3. Syncytium induction on U87 cells

The experiments using U87 cells were performed to determine whether expression of either CD4 and/or CXCR4 could influence syncytium formation on these cells. MVV-88 was applied to U87, U87 CXCR4, U87 CD4 CXCR4, U87 CD4 CCR5 and U87 CD4 cells, using HIV Lai (Figure 4.3.3) and FIV Gl8 as controls (Figure 4.3.4). After 24 hours extensive syncytia formation was detected (Figure 4.3.1). A qualitative estimate ranged the number of nuclei involved in syncytia formation from 40 % in U87 to 97 % (virtually all) in U87 CD4 for MVV-88. This was verifiable during repeated testing. There were some variations as indicated in figure 4.3.2. (Vertical bars showing maximum and minimum). As seen on figure 4.3.2. U87 CXCR4 had 70 % of cells involved in syncytia formation. U87 CD4 CXCR4 had less syncytia than either U87 CD4 and U87 CXCR4 with just fewer than 50 % of cells in syncytia. Least syncytia were on untransfected U87 cells with 40 %. For HIV-1 there was only syncytia on U87 CD4 CXCR4 (Figure 4.3.3) and for FIV there was roughly the same amount of syncytia on U87 CXCR4 and U87 CD4 CXCR4 (Figure 4.3.4).

Figure 4.3.1. MVV-88 infection on U87 cells. Panel A show U87, panel B show U87 CD4, panel C show U87 CXCR4 and panel D show U87 CD4 CXCR4. Stained after 24 hours.
4. Results

Figure 4.3.2. Graphical display of the amount of syncytia on different U87 cells for MVV-88. Bars showing maximum and minimum. The result displayed, is mean of three experiments done in duplicate.

Figure 4.3.3. HIV-1 infection on U87 cells. Panel A show U87, panel B show U87 CD4, panel C show U87 CXCR4 and panel D show U87 CD4 CXCR4. Stained after 24 hours.
4. Results

The same pattern appeared for the two other MVV strains that were tested, MVV-EV1 and MVV-SA. All three strains displayed this extensive syncytia formation on U87 CD4 cells. The cells were dead at day 3 - 7 after infection. To make sure that it was really the virus that caused these syncytia, negative controls were included, both the use of medium as inoculum and conditioned medium from uninfected SCP cells.

These results show that both CD4 and CXCR4 expressing cells had more extensive syncytia than untransfected U87.

Virus pseudotype

In order to investigate the MVV env-receptor interaction in isolation and to see if MVV had higher titre on U87 CD4 compared to U87, pseudotype virus was made by phenotype mixing (Sommerfelt & Weiss 1990). These pseudotypes were tested on near confluent Mink, HOS, BHK and SCP cells with wildtype (WT) VSV as control, at different dilutions, from undiluted to $10^{-8}$. The infections were monitored at 24 hours and 48 hours. SCP should be the positive control and show a good titre.
4. Results

VSV (MVV) show no titre on any cell type whereas VSV (WT) show a titre on $10^8$ on all cell lines (Table 4.3.1.). This shows that VSV (MVV) was not made. VSV did not incorporate MVV glycoproteins. Future pseudotypes will have to be made by molecular means.

**Table 4.3.1. Result virus pseudotype**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pseudotype*</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mink</td>
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</tr>
<tr>
<td></td>
<td>WT</td>
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</tr>
<tr>
<td>HOS</td>
<td>$\Psi$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>$1.5 \times 10^{9}$</td>
</tr>
<tr>
<td>BHK</td>
<td>$\Psi$</td>
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</tr>
<tr>
<td></td>
<td>WT</td>
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<td>SCP</td>
<td>$\Psi$</td>
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</tr>
<tr>
<td></td>
<td>WT</td>
<td>$4.8 \times 10^{8}$</td>
</tr>
</tbody>
</table>

* $\Psi$ is pseudotype virus VSV (MVV). WT is wildtype VSV

4.4. Syncytium inhibition and enhancement

Because of the surprising results in the syncytia formation assay, antibodies and/or ligands to CXCR4 and CD4 were used to try and reduce syncytium formation.

**SDF-1α**

To block CXCR4 as a potential receptor, its natural ligand SDF-1α was used. (Bleul et al. 1996). The assay was set up in 96 well plates (See methods 3.6.) and incubated 24 hours. The cells were stained and a qualitative estimate was made as to the extent of the syncytia. This is displayed in figure 4.4.1. It is evident that the blocking of CXCR4 does not affect the amount of syncytia for MVV-88 compared to the HIV control (Figure 4.4.2.). This experiment was also done for FIV and showed a decrease in FIV-induced syncytia. This is a bit harder to see, because the FIV is cell-associated and therefore a mix of HO6T1 and U87 CXCR4 cells was necessary to induce syncytia. The syncytia
4. Results

Figure 4.4.1. Amount of syncytia after 24 hours on U87 cells infected with MVV, with decreasing SDF-1 concentration. One representative experiment in duplicate is shown.

Figure 4.4.2. Amount of syncytia after 24 hours on U87 cells infected with HIV-1, with decreasing SDF-1 concentration. One representative experiment in duplicate is shown. U87 CD4 CXCR4 is the only cell line that shows any syncytia formation when challenged with HIV-1.
from FIV are not as extensive as MVV. All three viruses with and without SDF-1α are shown in figure 4.4.3., and only HIV and FIV show a marked reduction in syncytia.

Figure 4.4.3. Cells with and without addition of SDF-1, syncytia formation after 24 hours. Panels to the left are without SDF and panels to the right show the effect of SDF-1 on syncytia formation. Panel A is HIV-1 on U87 CD4 CXCR4 and B with SDF-1. Panel C is FIV on HO6T1 and U87 CXCR4 cells and D with SDF-1. Panel E is MVV-88 on U87 CXCR4 and panel F is with SDF-1.

Antibody Q4120

To investigate the large syncytia formation in U87 CD4 and the potential use of CD4 in entry, a monoclonal antibody against CD4, called Q4120, was used. This antibody has been found to inhibit entry of HIV. The experiment was performed in a 96 wells plate. The cells used are U87 CD4 CXCR4 for HIV, U87 CD4 CXCR4 for FIV mixed with HO6T1 FIV cells (See methods 3.4.), U87 CD4 for MVV. As the figure 4.4.4. show, there was an effect on HIV as predicted because HIV depend on CD4 upon entry, FIV
show no effect as predicted and on MVV no reduction in syncytium formation was apparent. This was carried out using MVV-88 only.

Figure 4.4.4. Cells with and without addition of Q4120. Panels to the left are without Q4120 and panels to the right show the effect of Q4120 on syncytia formation. Panel A is HIV-1 on U87 CD4 CXCR4 and B with Q4120. Panel C is FIV on HO6T1 and U87 CD4 CXCR4 cells and D with Q4120. Panel E is MVV-88 on U87 CD4 and panel F is with Q4120.

Gp120
The HIV protein gp120 has been used to block HIV entry as the protein binds CD4 if it is provided soluble in the medium. The protein then competes with the virus for the CD4 receptor. The cells were trypsinised and $10^4$ cells per well were allowed to settle over-night. The cell–lineage used for MVV-88 was U87 CD4 and for the HIV control U87 CD4 CXCR4. The cells with gp120 were then incubated for 30 minutes in the incubator giving the gp120 time to bind the CD4.
After over-night incubation with gp120 and virus, HIV had an almost complete inhibition with 2.5 µg gp120 compared to 55 percent syncytia in the control without gp120 as seen on figure 4.4.5. There was not possible to see any inhibitory effect on MVV at the gp120 amounts used. MVV had syncytia involving almost 100 per cent of the nuclei.

**Figure 4.4.5.** Graphical display on the amount of syncytia after 24 hours with decreasing gp120 concentration. One representative experiment in duplicate.

**Soluble CD4 (sCD4)**

The CD4 protein interacts with HIV causing a conformational change on gp120, allowing HIV to interact with its co-receptor CXCR4 which will then mediate the necessary fusion events resulting in virus entry. If MVV-88 used CD4 the same way as HIV, the use of sCD4 should increase the amount of syncytia on U87 cells. The cell-lineage used was U87 for MVV-88 and U87 CXCR4 HIV.

After incubation it was not possible to see any enhancing effect on MVV-88. Because we used HIV-1 as control, it was not possible to determine the effect on HIV either. Only certain strains of HIV-2 can use sCD4 this way. Such strains were not available.
4. Results

4.5 Syncytium induction on HOS and CHO cells

HOS infection

To see whether MVV entry was dependent of α or β chemokine receptors and CD4, HOS cells, which are devoid of chemokine receptors, were challenged with virus. The cells were infected as described in methods. After 3 days large syncytia were observed (See figure 4.5.1). The syncytia were not as extensive as on the U87 cells(Figure 4.4.1.), but they were clearly visible after staining. All three strains, MVV-88, MVV-EV1 and MVV-SA, all showed this syncytia formation. This demonstrates that MVV entry can take place without an absolute requirement for α or β chemokine receptors or CD4.

![Figure 4.5.1](image)

**Figure 4.5.1** Panel A show uninfected HOS (negative control); Panel B show infected HOS with MVV-88; Panel C show HOS infected with MVV-SA; Panel D show HOS infected with MVV-EV1. Stained after 72 hours.

CHO cells

To investigate the role of human CXCR4 further, CHO cells was used because of the cell-line’s natural inability to support entry and subsequent production of particles (Lyall *et al.* 2000). This made the cells ideal to see whether CXCR4 could represent a
receptor for entry, with the use of stably transfected cells, expressing CXCR4. Cells were obtained from B. Moser (University of Bern) and tested. However, due to a large degree of spontaneous syncytia formation (Figure 4.5.2.) and RT activity in uninfected cells (Table 4.6.1.) it became apparent that these cells arrived contaminated with a retrovirus. These cells could not be used for further analysis. We did not have the CXCR4 gene and could not recreate the cell line.

![Figure 4.5.2.](image)

**Figure 4.5.2.** Panel A show uninfected CHO cells; Panel B show uninfected CHO CXCR4 cells.

### 4.6. Infection studies

**SCP**

A positive control, SCP cells were used. At peak of infection the cells started to die, normally at 8 - 12 days post infection, with all cells dead at day 16 - 22. SCP cells did not make extensive syncytia. After staining a few syncytia were visible with only 4-6 nuclei in each. With high titre inoculum the cells died sooner, at 6 – 8 days, but the high titre also meant low production, due to the increased cell-death. The three different MVV strains that were used in the infection studies (MVV-88, MVV-SA and MVV-EV1) showed the same characteristics in infection on SCP cells. Infection studies summary shown in table 4.6.1.
<table>
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<th>Virus</th>
<th>Syncytia*</th>
<th>RT activity**</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>+</td>
<td>xxx</td>
</tr>
<tr>
<td>U87</td>
<td></td>
<td>++</td>
<td>x</td>
</tr>
<tr>
<td>U87 CXCR4</td>
<td></td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
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</tbody>
</table>

* Syncytia are given as -, < 1% nuclei in syncytia; +, > 5% nuclei in syncytia; ++, > 25% nuclei in syncytia; ++++, > 50% nuclei in syncytia; ++++, > 90%. ** RT activity is given as -, less than two times background; x, > 3 times background; xx, > five times background; xxx > ten times background. § uninfected cells

**U87 family**

MVV had a quick effect on U87 cells. Due to a high degree of cell death/syncytia formation it was difficult to harvest supernatant more than once during the infection. This extensive syncytia formation led to low RT production compared to SCP cells as seen in figure 4.6.1. All cells were dead in 3-7 days. Only U87 supernatants RT activity are significant with 3.6 times the background (uninfected U87) whereas SCP supernatant activity is 27 times background (uninfected SCP). In the U87 CXCR4 culture, the RT activity not exceeded the background (Figure 4.6.1)
Figure 4.6.1. Mean RT activity in U87 and U87 CXCR4 from duplicate supernatant harvests from MVV-88 infected cells compared to infected SCP cells. Controls are supernatant from uninfected cells in the same experiment. Bars show maximum. This was carried out using the Boehringer-Mannheim kit.

**HOS infection**

To see whether MVV entry was dependent of α or β chemokine receptors and CD4, HOS cells, which are devoid of chemokine receptors, were challenged with virus as described in 3.6. A significant RT value (Figure 4.6.2.) demonstrate a productive infection and is proof of entry. The lowest RT activity measured was at day 20 with 6.8 times the background (uninfected HOS). It is possible that the high RT activity at day 4 is in part due to residual virus inoculum. In HOS cells a significant RT activity mean, that MVV entry can take place in cells lacking known chemokine receptors or CD4.
4. Results

**Figure 4.6.2.** Mean RT activity in MVV-88 infected HOS cells from one experiment with duplicate supernatant harvests. Bars show maximum. This was carried out using the Cavidi kit.

**CHO cells**

The use of CHO in infection studies was not possible due to some sort of retrovirus contamination (See table 4.6.1. and section 4.5.).

**PCR**

To detect stable integrated proviral MVV in infected SCP cells, PCR was used. A cell-lysate was made at the peak of infection (syncytia/ cell death) or at day 25 (if no sign of infection). The primers, MgCl2 and temperature were optimised on a plasmid, containing an infectious clone of the MVV genome (Methods 3.6.).

The outside reaction were found to work at an annealing temperature of 54 °C with an Mg²⁺ concentration of 3,5 mM. The outside primers were designated MVV PolF1 and MVV PolR1. Nested primers, called MVV PolF2 and MVV PolR2, were found to work with annealing at 47 °C and 3,5 mM Mg²⁺. It was not possible to get a positive result on anything but the plasmid.
When PCR analysis this did not give a positive result on infected SCP cells, a number of approaches were tried to solve this problem. A longer “hot start” of up to 10 minutes was tried to see if that could yield a positive outcome. DNA was purified from cell lysate using phenol: chloroform extraction to see if that could remove any inhibitors. This also gave a negative result.

Another method that was tested was to “spike” the cell-lysate with plasmid to determine if inhibitors were present in the cell lysate. The lysate was diluted at 1:5, 1:10, 1:15, 1:20, 1:30, 1:50 and 1:100. In each dilution, 0.5 µl of plasmid was used together with 4.5 µl of diluted cell-lysate. If there had been inhibitors, a dilution of the cell-lysate should dilute away the inhibitors and at high dilutions a band would show on the stained gel.

A step temperature gradient combined with “spiked” lysate, for the outside reaction was also tried, where the annealing temperature started as low as 40 – 45 °C raising the temperature 0.5 °C each cycle for ten cycles before a normal outside reaction was run. This was done to increase the number of targets for the primers followed by a hot start at 97 °C for 5 minutes. After this outside a normal nested reaction was run, still without the expected result. It was not possible to get a band on infected SCP cells in any of the approaches tried, either alone or using a combination of approaches in the same experiment.

4.7. Other lentiviruses

The importance of CXCR4 in entry is not known for lentiviruses other than HIV, SIV and FIV. In order to determine this, CAEV and BIV were also tested on the U87 family to see whether these viruses showed the same characteristics as MVV.

CAEV, which is the closest phylogenetic relative to MVV, showed no sign of infection or syncytium induction on either U87 or U87 CXCR4. The infection was done as a normal infection over 25 days. At maximal sensivity some RT activity was detected at day 6 of infection, but the activity dropped below the significance limit during the
course of infection. Due to the high sensivity of the kit used, this suggests that the activity obtained resulted from the initial virus inoculum.

The BIV was tested on four U87 cell-lineages (U87, U87 CXCR4, U87 CD4 CXCR4 and U87 CD4). This virus also showed no syncytia formation or cell death during a 14 days infection period.

This however does not mean that these viruses do not infect this family of cells. With other methods it could have been possible to detect an infection (e.g. PCR, anti serum and others). But it shows that these viruses do not share a similar infection progression.
5. Discussion

The discovery of HIV receptor usage (Dalgleish et al. 1984; Deng et al. 1996; Feng et al. 1996), FIV receptor usage (Willet et al. 1997a) and SIV receptor usage (Schols & De Clercq 1998) leads to an investigation of other members of the Lentivirus genus. MVV is a prototype lentivirus, which makes its receptor use interesting to establish. Particularly since it has been suggested that primates and non-primate lentiviruses share the use of chemokine receptors (Willett et al. 1997b).

The aim of the study was to see the importance of human CXCR4 in MVV entry and to determine any strain differences of MVV in the effect of huCXCR4 on syncytium induction. It was also interesting to determine the effect on syncytium induction by other lentiviruses -namely CEAV and BIV- to see if they showed the same characteristics as MVV.

5.1. Alignments of CXCR4 from different species

Since MVV has an in vitro host range that includes almost every cell line tested and CXCR4 is widespread on different cell lines, this makes CXCR4 a good candidate receptor for MVV. If CXCR4 really represents a common lentivirus receptor, the gene should be conserved or at least have conserved domains.

The alignment shows that CXCR4 differs only slightly in length in closely related species (Results 4.1.); all primates investigated had a length of 352 amino acids. More distant species like cat and cow had 353 amino acids, whereas rat and mouse had 349 and 359 amino acids respectively. When it comes to conserved amino acids, figure 4.1.1. shows a very conserved gene where the largest diversity is at the N-terminus. Also when the partly cloned sheep CXCR4 is aligned (Figure 4.1.2.), it shows how conserved the gene is throughout evolution. It is unfortunate however, that the sheep CXCR4 sequence does not include the N-terminus or the C-terminus. If there is any
diversity, figure 4.1.1. gives us reason to believe that N-terminus would be the least conserved region.

With HIV-1, it is the N-terminus together with the third extra cellular domain that interacts with the virion (Brelot et al. 1997) and this variation between species N-terminally could therefore be important. HIV has been shown to utilize a wide range of CXCR4 molecules from different species. In addition to human, HIV can use feline CXCR4 (Willet et al. 1997a) and more distant murine CXCR4 (Tachibana et al. 1997) and rat CXCR4 (Simon et al. 1994). Murine CXCR4 shows only a 67 per cent identity in the third extra cellular domain compared to human CXCR4 (Nagasawa et al. 1996). This suggests that it is CD4, which is the limiting factor, not CXCR4, in HIV entry. This is expected if one takes into account how conserved the CXCR4 gene is and the fact that CXCR4 and SDF have no redundancy, it is likely that that is the case. The restricted tropism of HIV is therefore controlled by CD4. How MVV enters the cell is unclear, but if MVV shows any similarity with HIV, it implies that perhaps CD4 is also a candidate MVV receptor to be investigated. This is also in line with the results shown on U87 CD4 (results 4.3., discussed in 5.2. and 5.3.)

The six amino acid substitutions in the third extra cellular domain, sheep compared human CXCR4, could be important due to the fact that HIV has been shown to depend on this domain for entry (Chabot et al. 1999). The substitutions do not change the overall amino acids charge and it is interesting to see that this area has the most variation in the part of sheep CXCR4 that is sequenced. In cat contra human there are five substitutions, cat with a net charge of minus one and human minus three (Figure 4.1.2.). Therefore there must be a tolerance for a decreased negative charge since HIV can utilize cat CXCR4. But there is a difference that could be important; sheep has a substitution in position 168 (Position 176 on figure 1.3.1.), which is a glycosylation site and where cat and human have the same amino acid (Substitution shown in figure 4.1.2.) A potential loss of a glycosylation could very well alter the topography in this region and perhaps deny MVVs use of human CXCR4. This could theoretically mean that MVV uses CXCR4, but cannot use human CXCR4. On the other hand this substitution may not necessarily be important, because HIV can use rat and mouse CXCR4, which has the same amino acids in position 168 as sheep.
5. Discussion

The question of N-terminally sheep CXCR4 and the glycosylation site could be resolved with cloning and sequencing of the entire gene. Even so, the overall picture is that CXCR4 is greatly conserved and it could therefore be a basis to claim CXCR4 as a common lentivirus receptor. This is underlined in figure 4.1.3, which is a phylogenetic tree based on the alignment. It shows that primates group together with bovine and cat as a separate group closer to primates than mouse and rat.

The restricted in vivo host range can not be explained with a well conserved CXCR4 as a receptor. But that could be due to aspects of the immunosystem or post entry inhibition that make only animals of the ovine/caprine group susceptible to MVV infection. The specific cellular tropism can not be controlled by the use of CXCR4 alone since the receptor is widespread on different cells, but also macrophages express CXCR4 and MVV could utilise this receptor to gain entry.

5.2. Syncytium assays

Human U87 cells were used as a model system to test MVV for its receptor use. This cell-lineage has been extensively used as a model system for HIV and different cell clones transfected with human chemokine receptors were readily available. The use of human chemokine receptors for lentivirus from other species is established (Willet et al. 1997a) and it is showed that FIV can use huCXCR4 with as great efficiency as feline CXCR4 homologue. And when the gene is very conserved as seen in the alignment, the species barrier should not stop MVVs potential use of huCXCR4 when one see how HIV use of CXCR4 from different species (Tachibana et al. 1997; Willet et al. 1997a).

There is a difference in the amount of syncytia on U87 cells with regard to which of the receptors that are expressed, as seen in figure 4.3.1 and figure 4.3.2. U87 CD4 show an extensive syncytia formation where almost every cell are involved in syncytia. Co expression of CD4 and CCR5 or CXCR4 shows a reduction in syncytia formation compared to U87 CD4. This reduction could be the result of a steric hindrance, following co-localisation of CD4 and either CXCR4 or CCR5 as reported
(Xiao et al. 1999). It is also possible that the dual-transfected clones show a lower expression of the receptor due to the fact the transfected cells are experiencing significant stress. This could be verified with a FACS (Fluorescence-activated cell sorter) analysis where the true expression of the transfected genes would be revealed.

MVV, as seen on figure 4.3.1., produces large syncytia, involving from 40% to virtually all cells compared to uninfected controls, in five different U87 clones transfected with CD4, CXCR4 and CCR5 (See figure 4.3.2.) and untransfected U87. This shows that MVV can produce syncytia even in cells that do not express any α- or β-chemokine receptors or CD4, ruling out that MVV is dependent on CXCR4 and CD4 for entry. This means that these molecules do not act as primary receptors for MVV strains MVV-88, -EV1 and –SA.

The large syncytia formation in U87 CD4 led to a closer investigation of the role CD4 has, as discussed in 5.3. Here both FIV and HIV were used as control (Figure 4.3.4. and 4.3.3.) with HIV induced syncytia only on U87 CD4 CXCR4 and FIV induced syncytia on U87 CXCR4 and on U87 CD4 CXCR4 as expected (Willet et al. 1997a), both cell-lines with equal amount of syncytia. This also shows that CD4 does not block the use of CXCR4 in FIV entry. The FIV syncytia are not as extensive as HIV or MVV, the reason for which is that FIV is cell-bound and show a lower titre. In Willet, (1997a), they used an env construct to achieve large amount of syncytia.

Since syncytium induction is a consequence of virus-receptor interactions and we have shown that neither CD4 nor CXCR4 are absolutely required, the enhancement of syncytium induction by CD4 and CXCR4 respectively suggest that the molecules serve as co-receptors or accessory molecules for MVV-induced cell fusion.

5.3. Inhibition and enhancement studies

Due to the increased syncytia formation on cells expressing CXCR4 and CD4, it was interesting to see it this effect could be blocked. To find the role CXCR4 plays in entry, the receptor was inhibited with the use of its natural ligand, SDF-1α. This is an
established method (Bleul et al. 1996; Oberlin et al. 1996) and HIV was set up as a control and to adjust the system. This is displayed in figure 4.4.2. U87 CD4 CXCR4 HIV reduces syncytia from near 100 per cent of nuclei involved in syncytia to 25 per cent with 0.5 µg SDF-1α. The HIV control shows a dose dependency as expected (Figure 4.4.2.), although complete inhibition not was achieved. This is difficult to achieve due to the competitive nature of the experiment. Bleu et al. (1996) reached an inhibition of 80 per cent in Peripheral blood mononuclear cells (PBMC) infection at maximum SDF-1 concentration used. The FIV control also shows an effect as could be seen in figure 4.4.3. MVV show however no drop in syncytia with the addition of SDF (Figure 4.4.1.) and no significant increase in syncytia with decreasing amount of SDF compared to the HIV control. The curve for U87 CD4 CCR5, which is not affected by SDF-1α, show the same variation as U87 CD4 CXCR4 and U87 CXCR4.

It is possible that MVV uses a different binding site on CXCR4 than HIV, FIV and SDF, and therefore elude the blocking. There are however published data (Tarasova et al. 1998) showing out that CXCR4 is internalized when bound to SDF-1α, with only a fraction of the population reaches the cell surface again and therefore are unavailable for binding MVV at the surface. The rate of recycling varies between cell-lines and the recycling of CXCR4 in U87 cells is not known. It is also possible that the U87 cells act differently because they are transfected and the plasmid expression is not under cellular regulation. To check this, it would have been worth trying the use of mAb against huCXCR4, much the same as with the Q4120 against CD4.

The massive syncytia formation on U87 CD4 cells raised the question if CD4 played any role in syncytium formation. A blocking experiment using a monoclonal antibody Q4120 was carried out. This has found to have an effect on HIV entry as seen on figure 4.4.4. FIV was included as a negative control and showed no apparent reduction in syncytia as expected. MVV showed no reduction in syncytia with concentration of antibody that was found to affect syncytia formation on HIV.

It is possible that MVV uses a different binding site than HIV on CD4. But due to the fact that the antibody blocks HIV entry and the large antibody, there could be some sort of blocking or steric interference that resulted in a reduced syncytia formation, although there was not a apparent reduction on MVV induced syncytia. To investigate
5. Discussion

the potential use of CD4 further, soluble gp120 was used. This HIV protein has been demonstrated to block HIV's use of CD4 when provided soluble in the medium. With 2.5 µg per well there was a strong effect on U87 CD4 CXCR4 HIV, reducing syncytia from 55 per cent in the control to almost none, whereas there was no effect on U87 CD4 MVV at maximum gp120 concentration (Figure 4.4.5.). This suggest that a direct interaction CD4 is unlikely. It is however possible that MVV binds CD4 in a different manner than HIV and therefore elude the blocking.

The CD4 protein interacts with HIV causing a conformational change on gp120 allowing interactions with CXCR4 resulting in virus-cell membrane fusion (Clapham et al. 1992). If MVV used CD4 on a similar manner, the use of sCD4 should increase the amount of syncytia on U87 cells. This is however not true for all strains of HIV, only certain strains of HIV-2 can utilize sCD4 this way. When this method did not produce any increased syncytia formation for MVV on U87 cells, it is not possible to, using this result alone, to conclude whether MVV uses CD4 as a receptor. But it was still interesting experiment to see if MVV followed the HIV-2 strains in this use of sCD4. If the result had been positive, the use of CD4 at some level would be established.

These experiments was done using MVV-88 alone and there would be natural to try other strains to see if there are any strain variation in receptor use as seen in HIV and SIV (Deng et al. 1996; Feng et al. 1996; Schols & De Clercq 1998), but not in FIV. It is possible that there is a strain variation, even if it was not seen any strain variation during the infection studies or syncytium assays.

5.4. Infection studies

Positive signs of infection are also seen in HOS, which do not express any known chemokine receptors. This fact is important because many of the important receptors found for lentiviruses, are chemokine receptors (Sommerfelt 1999). The syncytia inducing capabilities of MVV in HOS compared to uninfected controls, is seen in figure 4.5.1. RT activity measured with the Cavidi kit in the HOS supernatant with the
lowest RT activity was 6.8 times the background as seen in figure 4.6.2. This shows a significant production of RT even in HOS. As figure 4.6.1. shows, there is a significant RT (3.6 times the background) on U87 cells. The low activity compared to SCP is probable due to the extensive syncytia formation and early cell-death. U87 cells are dead in three days as to SCP 14-21 days. This is also the reason for the fact that no significant RT activity was achieved on U87 CXCR4. The slower syncytia formation in HOS (three days compared to 24 hours on U87) and why the syncytia is not as extensive, is not clear, but it is evident that HOS and U87 cells do express receptor(s) for MVV.

The CHO cell-lineage’s inability to support entry and production of MVV makes it a good system to prove the role of CXCR4. It would be a convincing experiment if CHO CXCR4 was found to be susceptible to MVV infection compared to nontransfected controls. Unfortunately these cells had a large amount of spontaneous syncytia formation and as it later turn out, uninfected cells had a large RT activity. It then became apparent that these cells were infected with some kind of retrovirus, prior to their arrival. However this would be a natural approach to work along with new cells, due to the strong evidence it would create if unsusceptible cells became infected with the MVV when transfected with the potential receptor(s). However, our results show that CXCR4 is not a primary receptor and so entry into CHO CXCR4 may not have occurred because the other relevant components of the receptor were missing. This is probable, due to the fact that the results show that a complex receptor is likely.

The infection studies results are true for all the three strains tested: MVV-88, MVV-SA and MVV-EV1. The experiments suggest that MVV can use a non-chemokine for entry, even though both CXCR4 and CD4 augment virus-induced syncytia. The controls included, uninfected with medium as inoculum and uninfected with supernatant from uninfected SCP cells, and make it unlikely that these findings are some kind of artifact. These findings are strengthen by the fact that the results are confirmed with three different strains of MVV from different geographical origin. Each virus strain was propagationated at different times and the possibility for cross contamination should be minimal.
5. Discussion

5.5. PCR

A positive result on PCR would have confirmed the RT and syncytia formation results and demonstrated entry and integration. The primers were optimised on a plasmid carrying an infectious clone of MVV (pACKv72-67r) and were found to work. Unfortunately it was not possible to detect a MVV genome in the infected SCP cells that were used to produce stocks of virus. There could be several reasons for the failure to detect MVV using PCR. It is possible that there are differences from the published sequence to the virus that was used, even though it was the same strain (1514). If there were base substitutions and/or deletions, the primers would not be able to recognize the binding site. Both primer pairs, outside and nested, recognized the plasmid, which was not used as a basis for primer construction. Still it is quite possible that is the reason a positive result not was achieved, was the fact that the published sequence the primers were based upon was sequenced in 1991.

Inhibitors in the cell-lysate are also a reason it would not work. There could be inhibitors that somehow interfered with the PCR reaction. Attempts were made to resolve this by diluting the cell-lysate. However, at the dilutions tried (down to 1:100) there was still no detection of MVV. The cell-lysate was diluted and spiked with plasmid to test the lysate for inhibitors. Even a 1:1000 dilution of the MVV infected SCP lysate with the plasmid, did not give a positive result. This leads to the conclusion that the primers that were used did not recognize the MVV genome. Therefore new primers should be designed based on a region of the genome that has a low mutation rate, using the the latest MVV sequence published.

5.6. Other lentiviruses and pseudovirus

The other lentivirus tested, CAEV and BIV, showed no sign of infection on U87, U87 CXCR4 (CAEV, BIV) and U87 CD4, U87 CD4 CXCR4 (BIV). The virus strains that were used (CAEV-NVH1 and BIV-R29) showed significant RT, but a too low titre can not be ruled out entirely. These negative results do not mean that these viruses do not infect this family of cells. With other methods it could have been possible to
detect an infection (e.g. PCR, anti serum and others). But it shows that these viruses do not share a similar infection progression in vitro and it is apparent that these viruses do not show the same characteristics as MVV. This does not support the hypothesis of the common use of CXCR4 for all lentiviruses.

To isolate the virus-receptor interaction, an attempt was made to create virus particles with a VSV genome and MVV envelope. The strong lytic effect of VSV would make the reading of the result easy. Unfortunately these pseudotypes were not possible to make. It is however an excellent method to demonstrate entry and should be investigated further. An alternative to this method could be to clone MVV env and stably transfect cell-lines and mix that cell-line with cells that express potential receptors. If there were any env-receptor interaction, large syncytia would have formed.

5.7 Future prospects

This work is important to understand the receptor use in lentiviruses and this thesis represents a basis for further work in this area. A better knowledge on the members of the genus could be important in several fields, such as design of anti-viral drugs to fight HIV infection and the use of retroviral vectors in gene-therapy.

This work with MVV should be carried on, especially with the techniques that where not successful or not done, e.g. the use of PCR with new primers and new blocking experiments using mAbs against huCXCR4. It would also be interesting to clone and express sheep CXCR4 to see if there is any species variation and to see if the mutations in the third extra-cellular domain are important. It would also be a clear advantage if MVV env constructs could be made and expressed on cells. This could of course be done with the other lentiviruses as well. With the use of env constructs as compared to viruses, it is easier to verify the env-receptor interaction due to the fact that one excludes the possibility of post-entry interference of viral replication. It is also easier to control the level of expression with the use of selection markers compared to control the virus titre. Such constructs were however beyond the scope of
this project. Receptor interference assays with other lentiviruses where the receptor is known would also be a method worth trying (Sommerfelt & Weiss 1990).

5.8. Summary

The inhibition and enhancement studies show no effect on blocking CXCR4 with SDF-1α. If CXCR4 were used as a receptor for MVV one would expect a reduction in syncytia formation as seen in HIV and FIV. It is still a possibility that MVV uses a different binding site distinct from that of SDF, HIV and FIV. If CD4 was involved as a receptor, blocking with Q4120 and gp120 should have been seen with a marked reduction of syncytia formation as the HIV control. This points to a not critical role for CD4. This is consistent with the infection studies, which also show an entry of MVV independent of CD4 and CXCR4.

The increase of syncytia formation on transfected cell-lines indicate a role for CD4 and CXCR4, but the HOS results demonstrate that they are not absolutely required. This is in agreement with published material where mouse CXCR4, using mouse somatic cell hybrids, is excluded as a primary receptor for MVV (Lyall et al. 2000). The fact that HOS MVV and U87 MVV demonstrate a significant RT activity also shows that there is not a need for CD4 or CXCR4 as a primary receptor.

It seems like if MVV can use an unknown receptor X that is widespread on almost every cell-line, either alone or with use of CXCR4/CD4 as co-receptors and a complex receptor seems highly likely. This is in agreement with Barber et. al. (2000) and Bruett et. al. (2000). In HOS cells transfected with CD4, syncytium induction was enhanced by all three MVV strains compared to untransfected HOS, and HOS CD4 CXCR4 with less syncytia than HOS (article submitted, Hovden & Sommerfelt, 2001). This show that CD4 plays a part in syncytia formation, but is not absolutely required. This work should be followed by an investigation with the same methods that were used on U87 cells.
Syncytium induction results from interaction between receptor and virus. It is possible that the increased syncytia on U87 cells expressing CD4 and CXCR4 is due to the use of these proteins as accessory molecules. In such a case it would be a new way of explaining the syncytia formation. It is also possible that the expression of CD4 or CXCR4 on this cell-line lead to a different expression of other surface molecules or molecules that interact with such. The expression of a new receptor would explain why ligands to CD4 and CXCR4 had no effect. This would be difficult to establish, but perhaps a 2D electrophoresis could reveal the expression of different surface molecules.

This result shows a different receptor use than postulated for Lentivirus (Willett et al. 1997b). It has not been possible to verify the use of CXCR4 as an essential component of the receptor and it must be concluded that CXCR4 is not absolutely required. This genus, Lentivirus, can be divided in the viruses that causes immunodeficiencies (e.g. HIV, FIV, BIV and SIV) and those that do not (e.g. MVV, CAEV and EIAV). Since the viruses that have their receptor use discovered (HIV, FIV and SIV) all are immunosuppressive, it is possible that this groups dependency on chemokine receptors is not shared with all members of the genus and that the group that does not give immunodeficiencies use a different mechanism of entry.

These findings for MVV are all in vitro phenomenons and cannot explain the restricted cell tropism found in vivo. But these results show, together with the large number of cell-lines that become infected in vitro, that this restriction is likely to depend on a post entry inhibition. In vivo the MVV infects the macrophage linage, confined to specific tissues. It is not known what cause the to distinct forms of MVV, Maedi and Visna, but it is possible that this could be due to different receptor use. This is however difficult to decide before a final identification of the receptor.

**Conclusions**

The aims of these studies were to determine the importance of huCXCR4, which in the experiments that were carried out, showed not to be a principal receptor for MVV. MVV showed a marked increase in syncytia formation in cells expressing CD4. It was not possible to see a reduction in the amount of MVV-induced syncytia using
5. Discussion

measures to block CD4 and CXCR4 that were effective at inhibiting FIV and HIV-induced syncytia formation. Both CXCR4 and CD4 show an effect on syncytia formation and this suggest that CD4 and CXCR4 can serve as accessory molecules or coreceptor. The sequence of events leading to MVV entry may involve multiple components in a receptor complex where CD4 and/or CXCR4 are dispensable molecules that interact indirectly with MVV to augment virus-induced cell fusion. It was not possible to see any divergence between the three strains of MVV tested, and CAEV and BIV did not show the same characteristics on U87 cells as MVV suggesting alternate mechanisms of entry for these viruses.

The results of this study show that CXCR4 is not a common lentivirus receptor although both CD4 and CXCR4 will augment virus-induced cell fusion and are dispensable for entry. The principal receptor for MVV is not a chemokine receptor since cells lacking expression of both CC and CXC chemokine receptors are susceptible to infection. Further work is needed to elucidate the nature of the MVV receptor.
References:


References


References


