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Identification and characterization of two novel proteins interacting with the chemokine- and HIV-1 co-receptor CCR5

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Zusammenfassung

Der Chemokin-Rezeptor und HIV-1 Korezeptor CCR5 gehört zur überaus bedeutsamen Familie der G Protein-gekoppelten Rezeptoren. Die in letzter Zeit erfolgte Identifizierung neuer Rezeptor-interagierender Proteine hat zur Aufklärung der Funktionsmechanismen dieser Rezeptoren und deren Regulation beigetragen.

In einem Hefe 2-Hybrid System konnten wir α-catenin und JM4 als neue CCR5-interagierende Proteine identifizieren.

Alpha-catenin ist ein essentieller Bestandteil und Organisator des zellulären Zytoskeletts und verbindet als Adaptormolkül verschiedene Proteinkomponenten miteinander. Die Assoziation mit CCR5 wurde mit exogenen und endogenen Proteinen gezeigt. Zudem interagierte nicht nur CCR5, sondern auch CXCR4, ein weiterer wichtiger HIV-1 Korezeptor, mit α-catenin. Die Aktivierung von Chemokin-Rezeptoren bewirkt eine strukturelle Reorganisation des Zytoskelett. Die Chemokin-Rezeptoren sind über α-catenin möglicherweise mit dem Zytoskelett verbunden. Somit kann die Bindung von α-catenin an CCR5 bedeutsam sein für Rezeptorfunktion, für Multimerisierung und intrazellulären Transport des Rezeptors, Modulation des Zytoskeletts sowie für die HIV-1 Infektion. Im Hefe 2-Hybrid System wurde hier gezeigt, dass die Assoziation von α-catenin und CCR5 durch die C-terminalen Enden der beiden Proteine vermittelt wird. In Säugertierzellen interagierten sowohl α-catenin, als auch Deletionsmutanten. Dieser Befund deutet darauf hin, dass weitere Bindungststellen auf dem α-catenin Molekül vorhanden sein könnten. Da α-catenin oligomerisien kann, ist es auch möglich, dass die Interaktion der C-terminalen Deletiontsmutante mit dem Rezeptor indirekt, nämlich über endogenes CCR5-gebundenes α-catenin, erfolgen könnte.

Das zweite hier neu identifizierte und charakterisierte CCR5-interagierende Protein ist JM4. Dieses Protein ist in der Datenbank deponiert, bisher konnte jedoch keine biologische Funktion für JM4 gezeigt werden. JM4 ist mit zellulären Membranen assoziiert, hat vier putative Transmembran-Domänen und oligomerisiert. In der Protein-Datenbank wurden Proteine beschrieben, die auf Aminosäureebene Sequenzhomologien von 35 % bis 92 % mit JM4 teilen. Diese JM4-ählichen Proteine sind konserviert in verschiedenen Spezien und putative Vier-Transmembran-Proteine. Wir konnten die

übereinstimmende zelluläre Lokalisation und Verteilung von JM4 und nah verwandten Proteinen, wie dem humanen JWA und seinem homologen Protein, dem GTRAP3-18 aus der Ratte, zeigen. JM4, JWA und GTRAP3-18 bilden Homo- und Hetero-oligomere. Es könnte also sein, dass diese Proteine sich auch gegenseitig funktionell ersetzen können. Dies wird zudem dadurch unterstrichen, dass JWA ähnlich effizient wie JM4 mit dem CCR5-Rezeptor assoziierte. GTRAP3-18 bindet an den Glutamat-Transporter EAAC1 und reguliert über diesen die zelluläre Aufnahme von Glutamat, wie in der Gruppe von Prof. Rothstein, Johns Hopkins Medical Institutions, USA, gezeigt wurde. In einer Zusammenarbeit mit dieser Gruppe haben wir kürzlich zeigen können, dass auch JM4, vergleichbar mit GTRAP3-18, die Transporter-vermittelte zelluläre Aufnahme von Glutamat regulieren kann. Basierend auf diesen Ergebnissen zeichnet sich ab, dass JM4, JWA und GTRAP3-18 eine neue Proteinfamilie bilden, deren Mitglieder an der Regulation von Transmembran-Rezeptoren und –Transportern beteiligt sind. Die Zusammenarbeit mit Prof. Rothstein wird fortgesetzt, um die biologische Funktion von JM4 mit der von GTRAP3-18 zu vergleichen.

Die Interaktion von JM4 und CCR5 wurde im Hefe Zwei-Hybrid System durch den C-Terminus der beiden Proteine vermittelt. Weitere CCR5-intramolekulare Bindungsstellen des Rezeptors könnten eine Rolle als zusätzliche "docking sites" spielen, wie Versuche mit verschiedenen Deletionsmutanaten gezeigt haben.

In weiteren Studien wurde der Effekt von α-catenin und JM4 nach Überexpression auf verschiedene Rezeptorfunktionen, wie Chemokin-induzierte Aktin-Polymerisation, Internalisierung des Rezeptors und CCR5-vermittelte HIV-1 Infektion, getestet.

Retroviren anderer Spezien benutzen zur zellulären Infektion transmembrane Aminosäure-Transporter, die Sequenz- und Struktur-homologien mit dem Glutamat-Transporter EAAC1 haben. In einer begonnenen Zusammenarbeit mit Prof. Weiss, University College London, England, wird getestet, ob JM4, JWA und GTRAP3-18 eine generelle Rolle in der Rezeptor-vermittelten Virisinfektion haben.

Summary

The chemokine- and HIV-1 co-receptor CCR5 belongs to a very important family of membrane receptors, the class of G protein-coupled receptor (GPCRs). Recent approaches have identified new receptor-interacting proteins that have shed new light on mechanisms of function and regulation of GPCRs.

Using the cytoplamic C-terminus of CCR5 as a bait for a yeast two-hybrid screen we identified α-catenin and JM4 as novel interaction partners of CCR5.

The α-catenin protein is an essential component and organizer of the cytoskeletal network and links different protein components. We demonstrated endogenous protein interactions in mammalian cells and additionally showed an endogenous interaction of α-catenin with the second major HIV-1 coreceptor CXCR4. Chemokine receptor activation induces the structural reorganization of the cytoskeleton. Our data suggest that α-catenin connects the chemokine receptors with the cellular cytoskeleton, thereby affecting chemokine receptor function, clustering, trafficking, modulation of the cytoskeleton, and possibly HIV-1 infection. The association of α-catenin and CCR5 is mediated by the C-termini of the proteins, as shown here in yeast two-hybrid analyses. In mammalian cells, full-length α-catenin and mutants were able to interact with the receptor. This indicates that α-catenin has further interaction sites. Alpha-catenin is known to form oligomers, it is hence also conceivable that the C-terminally deleted α-catenin mutant is bound indirectly via endogenous CCR5-associated α-catenin to the receptor.

In this study we identified JM4 as a second novel interaction partner of CCR5 and further characterized this protein. JM4 is deposited in the database, but no biological function was assigned to it. JM4 is a membrane-associated protein with four putative membrane-spanning domains and has a tendency to form oligomers. In the database are proteins described that share sequence homologies of 35 % to 92 % with JM4. These proteins are conserved throughout different species and are putative four-transmembrane spanning proteins. We showed similar localization and distribution of JM4 and closely related proteins, such as human JWA and its rat homologue GTRAP3-18, in mammalian cells. JM4, JWA and GTRAP3-18 did form homo- and hetero-oligomers. Therefore, it is possible that these proteins can substitute for each others biological function. This is

furthermore indicated by the fact that JWA associated with similar efficiency as JM4 to CCR5. GTRAP3-18 binds to and regulates the cellular glutamate transporter EAAC1 as shown by group of Prof. Rothstein, Johns Hopkins Medical Institutions, USA. In a recent collaboration with this group we could show that, like GTRAP3-18, JM4 can regulate the transporter-mediated cellular uptake of glutamate. Based on these observations, we hypothesize that JM4, JWA and GTRAP3-18 constitute a new protein family, of which the members are involved in the regulation of transmembrane receptors and transporter proteins. To compare the biologal functions of JM4 and GTRAP3-18 the collaboration with Prof. Rothstein will be continued.

The interaction of JM4 and CCR5 was dependent on intact C-termini of both proteins in yeast. Additional intramolecular docking sites of CCR5 could play a role in mediating the interaction with JM4, as studies using deletion mutants have shown.

In other studies we analyzed the effect of overexpression of α-catenin and JM4 on CCR5 activities, such as chemokine-induced actin polymerisation, receptor internalization, and receptor-mediated HIV-1 infection.

Other species of retroviruses utilze multi-transmembrane amino acid transporters, that share sequence and structure-based homologies with the JM4- and GTRAP3-18-regulated glutamate transporter EAAC1, for cellular entry. We have initiated a collaboration with Prof. Weiss, University College London, UK, to test for a general role of JM4, JWA, and GTRAP3-18 in virus infection.

1. Introduction

1.1 G protein-coupled receptors

The G protein-coupled receptors (GPCRs) constitute a family with more than 1000 members and are the most diverse group of signaling proteins encoded by mammalian genome (1). The biological properties of GPCRs are diverse and involve a wide range of physiological processes including the sensory perceptions of pain, light, odors and tastes, cognition, muscle contraction, endocrine and exocrine secretion, metabolism, inflammation, and immunity.

The regulation of these seven transmembrane (7 TM) receptors involves activation, signaling, desensitization, and trafficking. These events are highly complex and involve many different molecules. Despite of some redundancy among different receptors and interacting proteins the processes are tightly and specifically regulated (2-4).

Ligand binding induces a conformational change in the receptor and allows coupling with the heterotrimeric G protein complex, consisting of three subunits, α, β, and γ (Fig. 1).

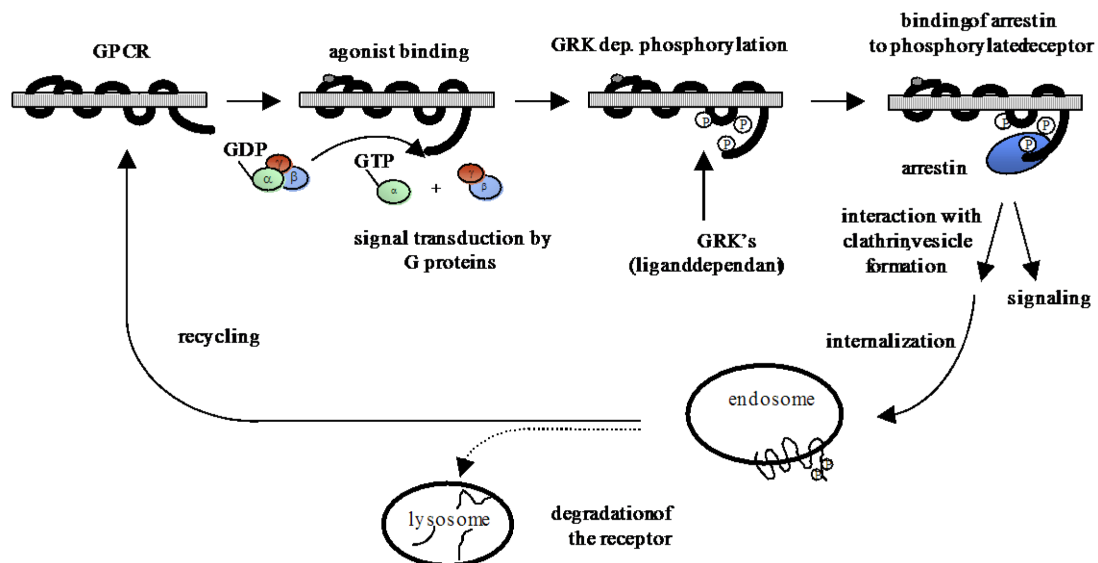

Fig. 1. Ligand binding induces conformational changes of the receptor and association of heterotrimeric G proteins. Subsequent signaling cascades lead to phosphorylation of the receptor by GRKs and recruitment of arrestin, resulting in internalization of the receptor through clathrin-coated pits. The receptor is either degraded or recycles to the plasma membrane. Abbreviations used are: GPCR, G protein-coupled receptor, GRK, G protein-coupled receptor kinase.

The receptor-coupling of the heterotrimeric G proteins activates the exchange of the α-subunit-bound GDP with GTP and leads to dissociation of the trimeric complex into an α-subunit and a βγ-dimer, both of which activate several separate downstream effectors (5). Subsequent hydrolysis of GTP to GDP by an intrinsic GTPase activity results in reassociation of the heterotrimer and termination of the activation cycle. This process is regulated by GTPase-activating proteins, which are named regulators of G protein signaling (RGS) (6). So far 16 α, five β, and five 12γ proteins have been cloned. The α-subunit family consists of four distinct subfamilies and receptor-coupling to G proteins is referred to these α-subunits. G_s and G_i proteins are linked with stimulation and inhibition of adenylyl cyclase, respectively. G_q proteins couple to the activation of phospholipase C, and G_{12} proteins couple to the activation of Rho guanine-nucleotide exchange factor (GEF) (2). Still, relatively little is known about specific subunit composition and subsequent function in specific pathways.

Stimulation of GPCRs activates several intracellular signaling pathways, leads to phosphorylation of GPCRs at their third intracellular loop and the C-terminus by second messenger-dependent protein kinases and specific G protein-coupled receptor kinases (GRKs) (7-10). Phosphorylation has been shown to be crucial for the desensitization of many GPCRs and promotes the binding of regulatory proteins, the most prominent being β-arrestin, to the cytoplamsmic C-terminus of the receptors (11-14). This in turn sterically inhibits a further association of trimeric G proteins, leading to uncoupling of the receptor. Beta-arrestin plays a pivotal role in the biology of GPCRs receptors. It acts as a multifunctional adaptor and scaffold molecule that links the C-terminal domain of GPCRs with diverse proteins (14, 15). Beta-arrestin directs internalization of receptors as it recruits components of clathrin-coated pits and phosphoinositides to the receptor (16).

The direct interaction of β-arrestin with ADP-ribosylation factor 6 (ARF6), a small G protein, its nucleotide exchange factor, ARNO, and Mdm-2, an E3 ubiquitin ligase, has been shown to enhance endocytosis of the GPCR β_2-adrenergic receptor (β_2-AR) (17-19). Furthermore, β-arrestin binds the signal transducer and tyrosine-kinase c-Src (20) that phosphorylates dynamin, which in turn is involved in the release of clathrin-coated pits from the plasma membrane into the cytoplasm (21, 22). In addition β-arrestin acts as a

scaffold protein in the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and Jun N-terminal kinase 3 (JNK3) pathways (23-25), bringing kinases into close proximity, therefore enhancing and ensuring specificity of signal transduction pathways.

Multiple signal transduction cascades are triggered by agonist-occupation of GPCRs (26). These events are thought to regulate the cytoskeleton reorganization and subsequent polarization/migration, cell survival, differentiation and activation of the cell (Fig. 2).

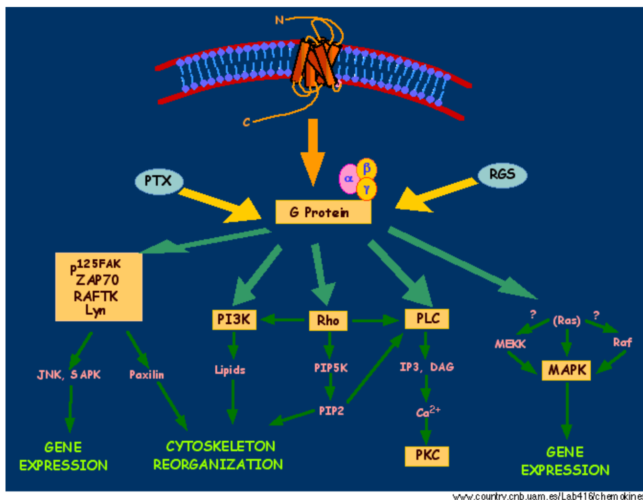

Fig. 2. Schematic representation of GPCR signaling. Pertussis toxin (PTX) can cross-deactivate the receptor, regulators of G protein signaling (RGS) affect signaling through hetertrimeric G-proteins. Downstream pathways can lead to various responses, some of them crosstalk, (arrows). Abbreviations used are: FAK, focal adhesion kinase; RAFTK, related adhesion focal tyrosine kinase; JNK, c-Jun-N-terminal kinase; SAPK, stress-activated protein kinase; PI3K phosphoinositide 3-kinase; PIP, phosphatidylinositol; PLC, Phospholipase C; PKC, Protein kinase C; DAG, Diacylglycerol; MEKK, Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) kinase.

Differences in the activation and subsequent cellular outcome of G protein-coupled receptor activation might be attributed to redundancy and the diversity of ligands. Recently it became apparent that the composition and set of cellular proteins involved in mediating outcome of the ligand-receptor interaction can be crucial (7).

GPCR have been shown to associate with cytoplasmic scaffolding proteins. For example, the C-termini of many receptors are known to interact with proteins containing PDZ domains (27, 28). These PDZ domain-containig proteins mediate protein-protein interactions and play a central role in the organization of signaling complexes or “signalosomes”. They are also involved in regulating receptor activity and trafficking (3, 4). The name PDZ derives from the first three identified proteins harbouring such domains: PSD-95 (post-synaptic density protein of 95-kDa) the *Drosophila* tumor suppressor Dlg (Discs large protein) and the tight junction protein ZO-1 (Zonula

Occludens 1) (29). They consist of six α-helices and eight β-strands and interact with a short C-terminal peptide motif, most often found in cytoplasmic tails of transmembrane receptors and channels (30, 31). PDZ domain-containing proteins can be divided into three classes on the basis of their binding preference for C-terminal peptide sequences: class I S/T-X-hy-COOH, class II hy-X-hy-COOH, and class III XXC-COOH, where X is any, hy is an hydrophobic amino acid, usually L/I/V (32). However, some PDZ domains recognize also an internal sequence motif, an extended β-hairpin fold. This so-called β-finger consists of a sharp β-turn resembling the normally required C-terminus (33). PDZ proteins interacting with GPCR might act as a scaffold, affecting the efficiency of receptor signaling. They might also have a direct allosteric action on receptor conformation. The regulation of interactions by agonist occupation and differential protein distribution or activity in distinct cellular backgrounds might play a significant role.

Homo- and hetero-dimeritzation of GPCRs has been described (26). The dimerization of receptors seems not to be essential for their function, but still might modulate it and contribute to the diversity of signaling (34) (35-37). In the case of chemokine receptors heterodimerization increased the sensitivity to agonist-triggered calcium responses and invoked distinct G protein coupling and signaling, thereby triggering adhesion rather than chemotaxis of the cells (38). The significance of oligomerization has furthermore been demonstrated for internalization, kinase activation and signal transduction of different chemokine receptors (39, 40). Heterodimers of GPCRs did not only show increased agonist-sensitivity but also exhibited distinct functional properties and further studies might even unravel novel binding and functional properties distinct from those of monomeric receptors.

1.2 The CCR5 receptor

Chemokines (*chemo*tactic cyto*kines*) are small soluble chemoattractant peptides and are subdivided into four subfamilies, on the basis of the first conserved cysteine residues: α (C-X-C, β (C-C), γ (C) and δ (C-X_3-C) (41, 42). They promote recruitment of various types of leukocytes to sites of inflammation and to secondary lymphoid organs (43, 44).

Accumulating evidence suggests that their function is not restricted to an effective inflammatory immune response through induction of polarization and recruitment of immune cells but also cell maturation, macrophage activation, neutrophil degranulation, B cell antibody switching, and T cell activation (26, 45-48).

The chemokine receptor family is the largest subfamily of peptide-binding GPCRs described thus far (49) and consists of to date 18 family members. The individual expression of the chemokine receptors, their regulation and chemokine binding show a broad functional diversity (Fig. 3). They play a role in organogenesis, hematopoiesis, neuronal communication, and leukocyte trafficking. Some chemokine - receptor systems are involved in fighting specific infections, while others are being exploited by viruses such as HIV-1 or herpesviruses, e.g. EBV.

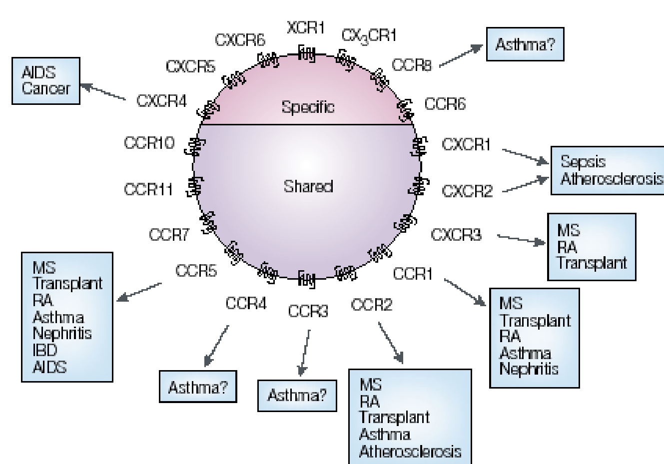

Fig. 3. Role of chemokine receptors in inflammatory disorders. Receptors are either activated by a specific chemokine or can bind different ligands. Abbreviations used are: MS, multiple sclerosis; Transplant, transplant rejection; RA, rheumatoid arthritis; IBD, inflammatory bowel disease; AIDS, acquired immunodeficiency syndrome (48).

from: Proudfoot, NatureReviews Immunology 2, 106 (2002)

In 1995 it was shown that the chemokines CCL3 (MIP-1α; marcophage inflammatory protein-1α), CCL4 (MIP-1β) and CCL5 (RANTES; regulated upon activation, normally T-cell expressed and secreted) were able to interfere with macrophage / monocyte-tropic (M-tropic) HIV-1 infection (50). The human CC chemokine receptor 5 (CCR5) cDNA was isolated in 1996 from a genomic library by homology hybridization cloning strategy based on its similarity to a murine chemokine receptor and shown to functionally respond to the β-chemokines CCL3, CCL4, and CCL5 (51). It belongs to the superfamily of the heptahelical G protein-coupled receptors (GPCR's). CCR5 has been implicated to play a

role in inflammatory disorders such as multiple sclerosis, rheumatoid arthritis, asthma, nephritis, inflammatory bowel disease, organ transplant rejection (48, 49, 52). In addition to its role as a chemokine receptor, CCR5 was shown to be a key cofactor for cell entry of human immunodeficiency virus 1 (HIV-1).

In 1984 the CD4 receptor molecule was identified to be essential for HIV-1 infection (53, 54), but it soon became obvious that further components were required for efficient virus entry. This led to the identification of the two major HIV-1 coreceptors CXCR4 (CXC chemokine receptor 4) and CCR5 (55-61). Primary HIV-1 infection mainly occurs through M-tropic HIV-1 via CCR5 (R5 virus) (59), which is preferentially expressed on memory T lymphocytes, macrophages, and dendritic cells (62). Only at later stages of the disease the virus switches to utilize T-cell tropic (T-tropic) CXCR4 (X4 virus) (63, 64).

A non-functional mutant of CCR5, termed CCR5Δ32, has been described (65, 66). The *CCR5Δ32* gene harbors an internal deletion of 32-base pairs that leads to a premature stop codon, thereby encoding a severely truncated version of the CCR5. The key role of CCR5 in HIV-1 pathogenesis is demonstrated by the fact that individuals that are homozygous for *CCR5Δ32* have been shown to be highly resistant against HIV-1 infection. They have been termed long-term non-progressors (LTNP) and are not known to display any pathological phenotype whatsoever. The CCR5Δ32 protein was shown to be defective in some aspects of post-translational processing, which resulted in retention / accumulation in intracellular compartments, most likely the endoplasmic reticulum (67). This leads to a lack of functional CCR5 at the plasma membrane for binding and entry of HIV-1 (68). Furthermore, CCR5Δ32 expression resulted in reduced cell surface expression of coexpressed wild-type CCR5, indicating the capacity of the receptor to form homo-oligomers (68). These findings explain the delayed onset of the acquired immune deficiency syndrome (AIDS) in *CCR5/CCR5Δ32* individuals. Heterozygosity for *CCR5Δ32* was reported to delay the disease progression (69, 70).

Another naturally occurring mutant of CCR5 has been observed exclusively in Asians (71, 72). A single nucleotide deletion in the *CCR5* gene leads to the expression of a receptor with a truncated cytoplasmic C-terminus, termed CCR5-893(-). Again, this receptor revealed reduced cell surface expression and reduced coreceptor function for HIV-1 infection.

Evidence for the role of the cytoplasmic C-terminus in receptor trafficking has recently been shown (73). The CCR2 receptor shares more than 75 % sequence homology with CCR5. Two isoforms of CCR2, 2A and 2B, have been described (74). They differ only in their cytoplasmic tail. CCR2B is about 76 % homologous to the C-terminus of CCR5 and readily expressed at the plasma membrane. In contrast wheras CCR2A shares little homology with CCR5 within its C-termini and it was predominantly cytoplasmic in transfected cells (73). Furthermore, the C-terminus of CCR5 has been shown to facilitate proper function of the receptor. Palmitoylation of the C-terminus of CCR5 has a significant impact on targeting the receptor to the plasma membrane, on signaling, internalization and intracellular trafficking (75-78).

The importance of CCR5-induced signaling for HIV-1 entry is controversial.

Chemokines activate chemokine receptors and induce signaling via a number of different pathways, e.g. the Ras-Raf-MEK-ERK pathway. Other pathways involve focal adhesion kinase or related kinases, Src-related kinases, the PI3Kinase, Rho, PLC, PKA, and PKC (39). These pathways, either in concert or individually regulate gene expression and cytoskeletal reorganization. The changes within the cytoskeletal network may affect chemotaxis or migration, adhesion, cellular polarization, internalization, recycling or degradation of the receptor. HIV-1 envelope protein gp120 also activates the chemokine receptors and stimulation of receptor-linked pathways may contribute to dysregulation of cellular functions, new target cell recruitment or modulation of HIV-1 infection and/or replication (79).

Two artificial mutations in CCR5, a C-terminal deletion and a mutation in the highly conserved DRY-sequence located at the second cytoplasmic domain, resulted in a block of chemokine-dependent activation of signal transducers, while none of these mutations altered the ability of CCR5 to act as coreceptor for HIV-1 (7, 80).

Whereas other reports, using primary T cells, showed that entry of X4 virus correlated with actin-dependent cocapping of CD4 and CXCR4, suggesting that signaling plays a role in the process of viral entry (81). Moreover, treatment of peripheral blood mononuclear cells with the B-Oligomer of pertussis toxin led to cross-desensitization of CCR5. At the same time this resulted in an inhibitory effect on HIV-1 infection and replication, blocking CCR5 signaling and cocapping of CCR5 and CD4 (82). During the

viral entry gp 120 interacts with CD4 and CCR5. Binding of gp120 to CD4 activates e.g. the tyrosine kinase Lck, the CCR5- and CD4 dependent signals cooperate in the regulation of biological functions as depicted in Fig. 4. A broad spectrum of other signaling effects can modulate the activation status of the cell and/or affect post-entry stages of HIV-1 replication (83).

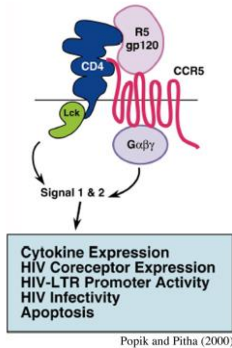

Fig. 4. During the course of viral entry the HIV-1 envelope glycoprtotein 120 (gp120) interacts with CD4 and CCR5. This results in stimulation of CD4- and/or CCR5 dependent signaling pathways, therby affecting cellular activation status and possibly viral entry or replication (83).

Signaling events caused by chemokines or gp120 are not identical and may further differ from HIV-1-induced cascades. Cellular events may allow HIV-1 infection or trigger apoptosis, which may contribute to HIV-1 pathogenesis. CCR5 but not CXCR4 efficiently signals through MEK-ERK or JNK-p38 MAPK, which may support CCR5-tropic virus entry or replication during early stages of the infection (84).

Identification of chemokine receptor-interacting proteins can contribute to further unravel the precise mechanisms responsible for efficient receptor regulation. An alignment of different CC chemokine receptors is shown in Fig. 5.

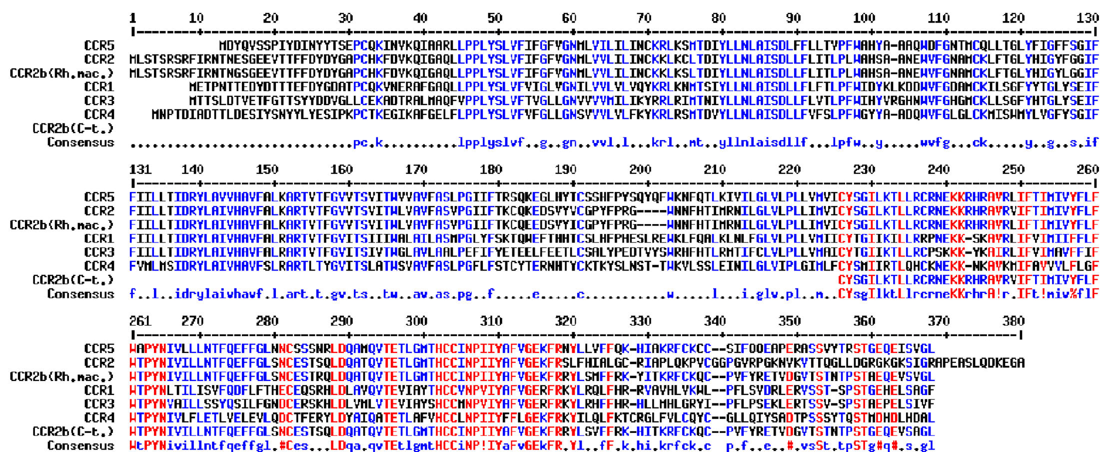

Fig. 5. Alignment of CC chemokine receptors. Red color indicates homology, blue color similarity of aligned amino acids.

The very C-terminal leucine (L) of the CCR5 receptor corresponds to a PDZ-domain binding motif type II, indicating that CCR5 might bind to PDZ-domain containing proteins. PDZ proteins are known to regulate the activity and trafficking of diverse receptor molecules. As described above the cytoplasmatic C-terminus of GPCRs is involved in the regulation of the receptor function.

CCR5 plays a major role in inflammatory diseases and acts as a HIV-1 coreceptor, furthermore the receptor-mediated signaling influences viral replication. Therefore, we chose to use the C-terminal domain of CCR5 to identify novel interacting proteins. We identified JM4 and α-catenin to interact with the C-terminus of CCR5. Our and further studies might contribute to unravel the precise mechanisms responsible for efficient regulation of the receptor, involving signaling, desensitization, internalization, trafficking, dimerization, or HIV-1 infection.

1.3 Alpha-catenin

Alpha-catenin is one of the two novel binding partners for CCR5 that are described in this work.

Alpha-catenin is often found in either cell-matrix contacts or cell-cell contacts. These contacts are assembled through multiprotein complexes. Actin filaments are linked through cytoplasmic adaptor proteins to transmembrane proteins that interact with neighboring cells or the extracellular matrix. The assembly of specific types of cellular junctions involves different compositions of adaptor, regulatory proteins, and transmembrane proteins, reflecting the different biological functions of the junctions. In the case of cell-matrix contacts the adaptor protein vinculin associates with the transmembrane protein integrin and forms so-called focal adhesions. In cell-cell junctions the transmembrane protein cadherin is linked via catenins with the cytoskeleton (85, 86) (Fig. 6). Cadherins comprise a superfamily of Ca^{2+}-binding transmembrane proteins located at the plasma membrane. They are essential in the stabilization of cell-cell-contacts (87). Cadherin-mediated intercellular adhesion is critical for normal embryonic development and maintenance of mature epithelial tissues (88, 89).

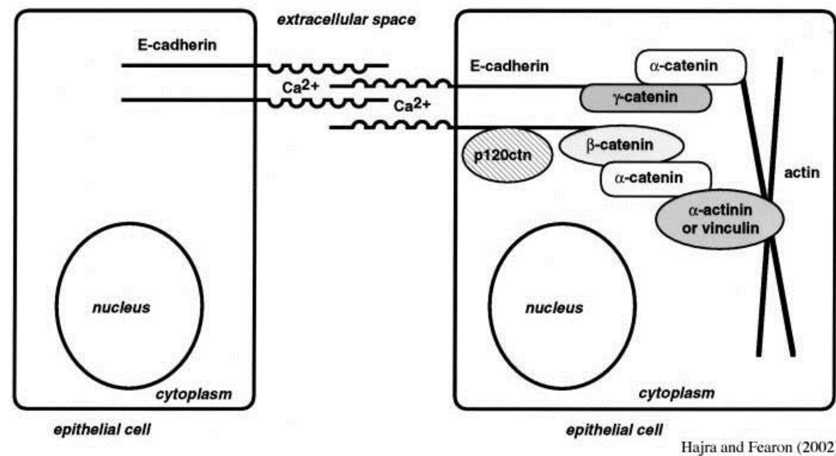

Fig. 6. The role of α-catenin in the formation of cell adhesion complex. E-cadherin forms intercellular, calcium-dependent, homotypic interactions. The cytoplasmic tail of E-cadherin interacts with β-catenin and/or γ-catenin. These link E-cadherin to α-catenin, which is associatd with the actin cytoskeleton either directly or indirectly through a number of actin-associated proteins, including α-actinin and vinculin. Additional proteins, such as δ-catenin (p120ctn] bind to a more membrane-proximal cytoplasmic domain of the E-cadherin (109).

The physiological importance of cellular adhesion is reflected in cases of malfunction during development, carcinogenesis and metastasis. Reduced expression of E-cadherin is regarded as one of the molecular events involved in dysfunction of the cell-cell adhesion system and is thought to be involved in cancer invasion and metastasis (90, 91). The extracellular domain of cadherins is responsible for specific homotypic binding (92). The cytoplasmic domain facilitates adhesion through interaction with three proteins known as catenins - α, β, and γ (93, 94).

Catenins are cytoplasmic proteins initially identified to be complexed with cadherins. The cadherin molecules bind either β- or γ-catenin (plakoglobin) which in turn bind to the N-terminus of α-catenin, which is linked to the cytoskeleton actin bundle (94-100) (Fig. 6). This complex is considered as a functional unit. At cell-cell junctions this complex is colocalized with and connected to actin filaments, which constitute the cytoskeletal backbone and are crosslinked by dimers of α-actinin. Alpha-catenin is an actin-binding protein, appears to be a homodimer in solution and forms heterodimeric complexes with β-catenin (101). Due to sequence similarities within three extended domains, α-catenin and vinculin are related, they are both are multiligand binding proteins and can interact with each other.

Distinct domains of α-catenin independently regulate the association with cadherin and with the cytoskeleton (Fig. 7).

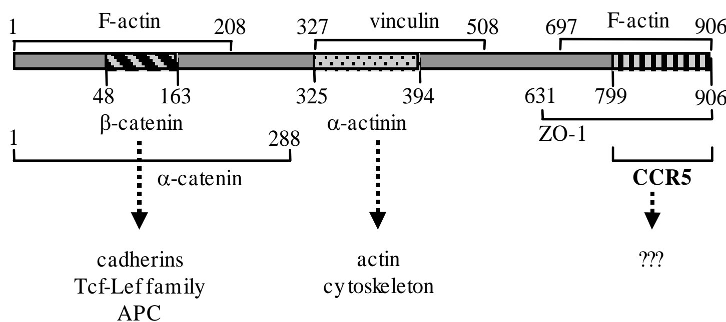

Fig. 7. Schematical representation of the multiligand-binding protein α-catenin. The interactions with multiple proteins of the cytoskeleton are involved in regulation of cell-cell adhesion. The interacting protein β-catenin has been shown to bind transcription factors of the Tcf-Lef family and the tumor suppressor adenomatous polyposis coli (APC). In addition, α-catenin has been shown to mulitimerize through its N-terminal domain.

Especially the C-terminus of α-catenin plays a pivotal role in connecting the cadherins with cytoskeleton-associated proteins. Alpha-catenin interacts directly with α-actinin, ZO-1 (zonula occludens 1), vinculin and actin (102-106). Like vinculin, the cytoskeletal PDZ protein ZO-1 colocalizes with E-cadherins at cell-cell contacts (107). The recruitment of these proteins is dependent on α-catenin (91, 93, 94). Several functional domains of α-catenin are identified (Fig. 7). Vinculin is recuited to α-catenin by amino acids 327-508 (108). The same domain is involved in binding of α-actinin (103). The C-terminal region, amino acids 631-906, mediates the interaction with the N-terminus of ZO-1 (108) and amino acids 609-643 are regulating the cell-cell adhesion-activity of the molecule complex (108).

Furthermore, α-catenin has been characterized as a tumor suppressor, suggesting that it has a regulatory function in addition to its structural role. This tumor suppressor function correlates with a function in cell-cell contact, namely the linking of actin to the membrane. Alterations in cadherin-catenin complexes, loss-of-function and defects in expression of α-catenin contribute to cell-adhesion defects in carcinoma of many different tissues (109). Furthermore, tyrosine phosphorylation of catenins is likely to regulate the cadherin-catenin complexes (110-112). Also protein tyrosine phosphatases have been shown to be associated with the complex (113-118). Therefore, protein mutations may alter kinase/phosphatase localization and/or function, resulting in aberrant phosphorylation events.

Recently α-catenin was identified to interact with the HIV-1 viral envelope glycoprotein 41 (gp41) (119, 120). After expression of the envelope precursor protein gp160 in the infected cell, this protein is cleaved into its subunits gp120 and gp41. These are incorporated from the cellular plasma membrane into budding virions. Both, gp120 and gp41 are displayed at the surface of mature HIV-1 virus and facilitate HIV-1 entry into the host cell (53, 54). The interaction of gp41 and α-catenin was suggested to regulate the alignment of viral envelope proteins in the plasma membrane in the process of HIV-1 virion budding.

1.4 JM4

JM4 is the second binding partner for CCR5 identified in this study

So far no biological function has been attributed to the JM4 protein. Databases from NCBI (LocusLink) mapped the *JM4* gene to chromosome Xp11.23. It contains 3 exons. Several JM4-related proteins were found in different species by sequence analysis using standard protein-protein BLAST (blastp) (121). An alignment of JM4 and similar proteins is shown in Fig. 8.

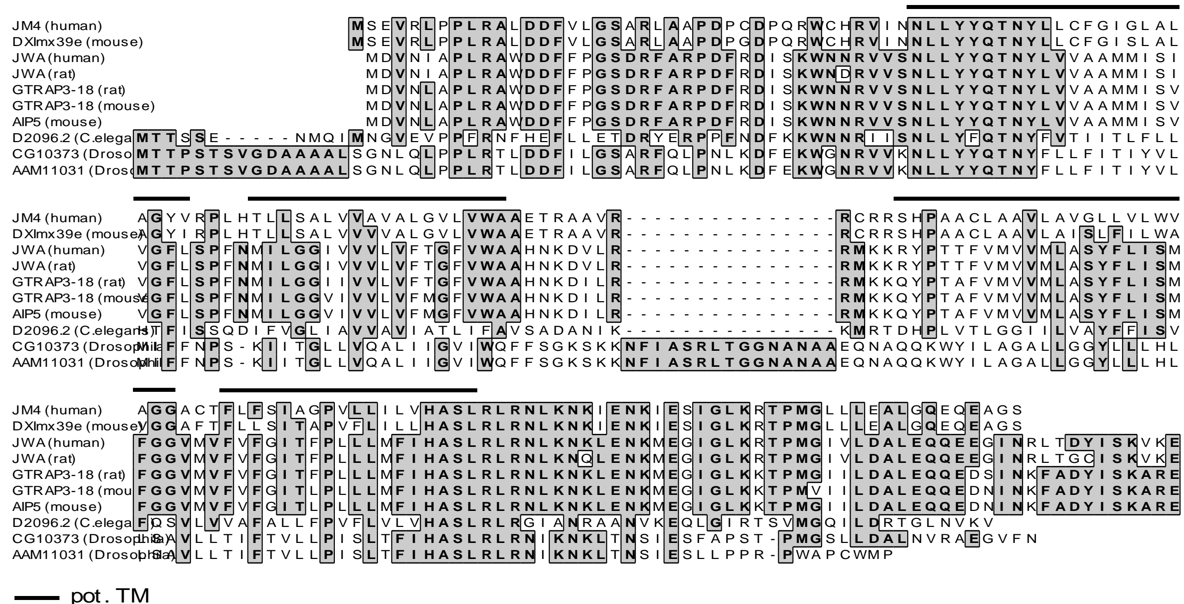

Fig. 8. Alignment of JM4 and proteins found to share sequence similarities. The four potential transmembrane (pot. TM) domains of JM4 are indicated by black bars.

Of this group of proteins to date only GTRAP3-18, a protein identified in rat, has been attributed a biological function to. GTRAP3-18 binds to a ten-transmembrane glutamate transporter and regulates its acticity (122). The human homologue of GTRAP3-18, JWA, has been described only in the database. It was predicted to be cytoskeleton-associated and its gene expression to be vitamin A responsive.
Prediction analyses of structure and function at amino acid level suggested that all three proteins are hydrophobic encoding four potential transmembrane regions (Fig. 9).

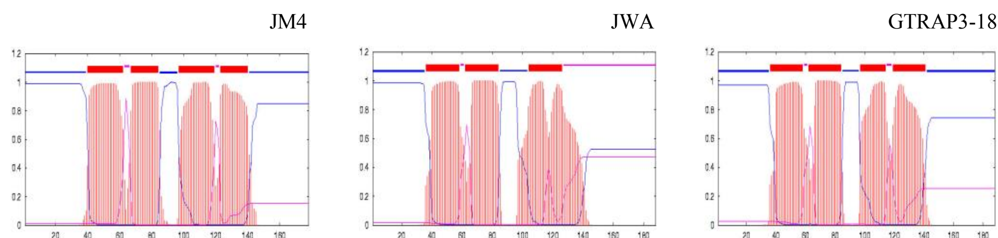

Fig. 9. Transmembrane prediction plots of JM4, JWA, and GTRAP3-18 reflect the structural similarity of the proteins. Potential transmembrane helices of JM4, JWA, and GTRAP3-18 are shown in red (TMHMM (v. 2.0) (123), (y-axis: TM probability, x-axis: amino acid position).

The human JWA protein has not functionally been characterized, but shares 90 % sequence identity with the murine Aip-5 (ADP-ribosylation-like factor 6 (ARL6)-interacting protein-5). Aip-5 was found to interact with ARL6 in a yeast two-hybrid screen (124). The ADP-ribosylation factor (ARF) group of proteins, subdivided into ARF and ARF-like (ARL) molecules, belong to the Ras-superfamily of small GTPases. ARFs seem to be involved in vesicle formation, in intracellular traffic, and associate with membranes on the secretory and endocytic compartment (125). ARF6 has recently been shown to enhance endocytosis of the β_2-adrenergic receptor (β_2-AR) through its interaction with β-arrestin (17).
Rothstein and colleagues at Johns Hopkins Medical Institutions published a study on GTRAP3-18 (122). These authors showed that GTRAP3-18 binds to an amino acid transporter for glutamate, EAAC1, a protein with ten transmembrane regions. GTRAP3-18 is the rat homologue of human JWA with 95 % identiy at amino acid level. The function of GTRAP3-18 is to regulate EAAC1-mediated amino acid transport. Binding of GTRAP3-18 to the C-terminus of EAAC1 interferes with glutamate transport by

lowering the transporters affinty to its substrate, possibly by changing its confirmation due to binding. Therefore, overexpression of GTRAP3-18 interferes with EAAC1-mediated glutamate uptake.

The glutamate transporter EAAC1 is a ten-transmembrane spanning cell surface molecule that is regulated by GTRAP3-18, resembling JM4 that interacts with the seven-transmembrane chemokine- and HIV-1 coreceptor CCR5. It is known, that other retroviruses bind to transmembrane spanning amino acid transporters, e.g. ecotropic mouse leukemia virus binds to a specific 14 transmembrane spanning cationic amino acid transporter (CAT). By sequence analysis of various retroviral receptors we found that the human sodium-dependent neutral amino acid transporters type 1 and 2 (hASCT1/2) share sequence and structure-based homologies with the EAAC1 glutamate transporter. The human cell surface molecules ASCT1/2 have been identified as cellular receptors for the human endogenous retrovirus type D (HERV-W) (126). Additionally, ASCT2 is a common cell surface receptor for viruses including the feline virus RD114, the baboon endogenous retrovirus BaEV, type D simian retroviruses, and avian reticuloendotheliosis viruses (127, 128).

The chemokine- and HIV-1 co-receptors CCR5 and CXCR4 interact with α-catenin, a component of the cellular cytoskeleton

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Abbreviations: CCR5, CC chemokine receptor 5; CXCR4, CXC chemokine receptor 4, GPCR, G protein-coupled receptor; HIV, human immunodeficiency virus

Abstract

The chemokine receptors CCR5 and CXCR4 belong to the versatile family of heptahelical G protein-coupled receptors. Chemokines and their receptors play an important role in surveillance of host cell defense and have been implicated in numerous diseases. CCR5 and CXCR4 are renowned as necessary coreceptors for cellular entry of HIV-1. In this study the intracellular C-terminus of CCR5 was identified to interact with the C-terminus of α-catenin in a yeast two-hybrid screen. This initial result was confirmed and further analyzed in mammalian cells by coimmunoprecipitation studies. Endogenous interaction of α-catenin with CCR5 and CXCR4 was detected in PM1 T-lymphocytes, a HIV-1 host cell line. Our results suggest that α-catenin is involved in the regulation of chemokine receptor function, thereby possibly affecting HIV-1 infection.

1. Introduction

Chemokines are a family of small chemotactic cytokines and elicit diverse biological effects through different chemokine receptors that belong to the superfamily of G protein-coupled receptors (GPCRs) (129, 130). Important roles for chemokines and their receptors have been demonstrated in acute and chronic inflammatory diseases, hematopoiesis, angiogenesis, tumor rejection, and human immunodeficiency virus 1 (HIV-1) infection (47, 48). The chemokine receptors CCR5 and CXCR4 were identified as essential coreceptors for HIV-1 entry and represent attractive targets to interfere with HIV-1 infection (55-60). Binding of chemokines to their receptors triggers the activation of a complex network of signal transducers that regulate redistribution of membrane receptors, integrins, and adhesion proteins. The concomitant rearrangement of the cellular actin cytoskeleton affects morphology and polarization required for cellular migration (131). The HIV-1 engagement with cellular receptors activates cellular signaling cascades as well (79, 83, 132). Whatsoever, the outcome of virus-induced cellular signaling on viral infection remains controversial (133, 134).

The biological properties of chemokine receptors are regulated by cytoplasmic interactions with various cellular adaptor / scaffold proteins, but also at the level of cell surface expression, desensitization, internalization, recycling, or degradation (2, 3).

Natural mutants of the HIV-1 coreceptor CCR5 interfere with viral infection. The most frequent mutation in the *CCR5* gene, termed *CCR5Δ32,* is associated with a loss-of-function of the protein. This gene encodes a severely truncated receptor with only the four N-terminal trans-membrane domains expressed. This results in retention and accumulation of the mutant CCR5 in intracellular compartments, most likely the endoplasmic reticulum (67). Individuals that are homozygous for *CCR5Δ32* are highly protected against HIV-1 infection and have been named long-term non-progressors (LTNP) since they hardly ever develop acquired immune deficiency syndrome (AIDS) (65, 66). Heterozygosity was shown to delay disease progression (69, 70). Another natural receptor, CCR5-893(-) was found to be deleted for its entire C-terminal cytoplasmic tail (72). Again, CCR5 cell surface expression was greatly impaired implying an inhibitory effect on HIV-1 infection.

Since the C-terminus of CCR5 is involved in the multifaceted receptor function and regulation, cell surface expression, and internalization, this domain of CCR5 was screened for interacting proteins by yeast two-hybrid analysis. In this study we describe a physical interaction of the two HIV-1 coreceptors CCR5 and CXCR4 with α-catenin. The cytoplasmic protein α-catenin acts as connector that attaches the plasma membrane-associated cadherin adhesion-complex to the matrix of the cellular cytoskeleton (98, 99).

Our data show that α-catenin links the chemokine receptors to the cytoskeletal network, thereby possibly affecting the chemokine receptor properties such as trafficking and receptor-mediated HIV-1 infection.

2. Material and methods

Plasmid construction

The C-terminus of CCR5 was excised from pBABE.CCR5 (AIDS Research and Reference Reagent program) via restriction sites *SfaN*I / *Sal*I and subcloned into *EcoR*I / *Sal*I restriction sites of yeast bait vector pGBT9pheS using an *EcoR*I-*Sfa*I linker. The pGBT9pheS is a modified version of yeast bait vector pGBT9 (Clontech) and contains a Gal4-DNA binding domain and positive selection properties (M. Buchert, unpublished data) (135). The CCR5 C-terminus was mutagenized at four specific sites to introduce

stop codons after amino acid (aa) positions 336, 320, and 308 and to substitute the very C-terminal leucine at position 352 with a proline.

The *CCR5* gene was excised from pcDNAI.CCR5 (AIDS Research and Reference Reagent program) and inserted into *Hind*III / *Xba*I restriction sites of pcDNA3 (Invitrogen) using a *Hind*III-*BamH*I linker including an HA-epitope to create pcDNA3-HA-CCR5, which encodes a N-terminally HA-tagged full-length CCR5 for expression in mammalian cells.

The plasmid DNA encoding the codon-optimized, C-terminal tagged CCR5 (synCCR5-C9) was received from J. Sodroski (Dana-Farber Cancer Institute, USA) (136). The C-terminal C9-epitope tag was removed by mutagenesis to generate a codon-optimized plasmid encoding wild-type CCR5 (synCCR5).

The coding sequence of α-catenin missing nine 5' nucleotides was received from Y. Nakamura (Institute of Medical Science, University of Tokio, Japan) and subcloned into *Kpn*I / *EcoR*I restriction sites of pcDNA3 using a *Kpn*I-*Xba*I linker encoding a N-terminal Flag-epitope and the missing three amino acids. The resulting pcDNA3-Flag-α-catenin vector was used to generate two deletion mutants of α-catenin. A *PshA*I fragment was excised, the backbone religated, encoding the Flag-α-catenin C-terminus (aa 624-906). The second mutant was generated by excision of a *Cla*I / *EcoR*I fragment from pcDNA3-Flag-α-catenin, fill-in reaction and religation of the vector. The so-lost α-catenin stop-codon is substituted by a vector stop-codon downstream of the α-catenin encoding sequence. This results in a Flag-α-catenin N-terminus extended by 26 vector-encoded amino acids (aa 1-611+26).

All linker sequences can be obtained upon request. The described truncation and substitution mutants were generated using site-directed mutagenesis according to the manufacturer's instructions (Stratagene). DNA was sequenced and oligonucleotides synthesized at Microsynth.

Yeast two-hybrid techniques

Y153 yeast cells were transformed with the bait construct using a standard yeast transformation protocol and tested for self-transactivation before screening a human peripheral lymphocyte (early pre-B cell) cDNA library in prey plasmid pACT (provided

by S. Elledge, University of Texas, USA) (137, 138). Yeast transformants were screened for histidine prototrophy in the presence of 10 mM 3-aminotriazole (Sigma). Interaction was demonstrated by $lacZ^+$ transformants from which prey plasmids were recovered using the Fast DNA Kit (BIO101). Plasmid DNA was purified using Geneclean beads (BIO101), amplified in *E.coli* DH5α (GibcoBRL) and extracted (Qiagen). Positive prey plasmids were re-tested in yeast by co-transformation with the original bait plasmid or control plasmid and subsequently sequenced.

For β-galactoside filter assay yeast colonies were grown for 2-3 days at 30 °C on agar plates (per liter: 1.45 g east N_2-base, 5.25 g NH_4SO_4, 20 g glucose, 10 g succinate, 2.8 g KOH (for plates, 8.5 g KOH and 20 g bacto agar) and 2 g of drop-out mix containing all amino acids except those for selection). Colonies were transferred to a Duralon-UV membrane (Stratagene), the membrane was immersed in liquid nitrogen and thawed two times, then put on top of a 3MM filter paper (Whatman No.3) saturated with sodium-phosphate buffer (100 mM) containing 0.5 mg/ml X-gal (dissolved in DMSO). Coloration of clones was indicative for interaction.

A non-lethal β-galactosidase plate assay was used as described elsewhere (139) with modifications. Transformed yeast colonies were resuspended in 10μl of TE buffer and grown for 24 h at 30 °C on a fresh agar plate. After incubation for one day, plates were flooded with chloroform for 8 min. The chloroform was decanted. The plates were inverted and allowed to dry then overlaid with 1 % low melting agarose containing 1 mg/ml X-gal in TBE buffer and incubated at room temperature.

Cell culture and transfection

The human embryonic kidney (HEK)-293 cell line was grown in Dulbecco's modified eagle's medium (DMEM; Gibco) supplemented with 10 % fetal calf serum (FCS; Seratec). G418 sulfate (1 mg/ml; Calbiochem) was added to the medium of HEK-293 cells stably expressing HA-CCR5 or synCCR5 for selection and maintenance. Cf2Th cells stably expressing a codon-optimized and C-terminal epitope-tagged CCR5 (synCCR5-C9) were provided by J. Sodroski and maintained as described (136).

The PM1 cell line (140) was distributed by the NIH AIDS Research and Reference Reagent Program and grown in RPMI-1640 medium (GIBCO) with 10 % FCS.

Cells were transfected using LipofectAMINE 2000 according to the manufacturer's instructions (Gibco) or using a modified calcium phosphate transfection protocol as described (141).

Cell lysis and Western analysis

All steps were performed at 4 °C. Before lysis cells were washed in phosphate buffered saline (PBS; Gibco). After *in vivo* labeling cells were lysed in NP-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 % Nonidet P-40, 0.2 % EDTA, 1 mM DTT, phosphatase / protease inhibitors (25 mM β glycerol-phosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine, 10 μM pepstatin, trasylol (500 Kalikrein inactivator units), 5 μg/ml leupeptin). Immunocomplexes were resuspended in RIPA buffer (20 mM Tris-HCl (pH 7.5), 135 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 0.5 % DOC, 10 % Glycerol, 2 mM EDTA, 1 mM DTT, phosphatase / protease inhibitors).

For purification of codon-optimized CCR5, cells were lysed in solubilization buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10 % Glycerol, 1 % Cymal-5, phosphatase / protease inhibitors).

PM1 cells were lysed in modified NP-40 with 150 mM NaCl and 1 % Nonidet P-40.

Cell lysates were cleared by centrifugation and protein concentrations of the supernatants were determined using the Protein Assay Kit (Bio-Rad).

Proteins were immunoprecipitated with 10 μl of sepharose beads precoupled with antibody. After precipitation the sepharose beads were washed 4 times with lysis buffer, proteins denatured and reduced in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 10 % glycerol, 2 % SDS, 0.1 % bromphenol blue, 50 μl/ml β-mercaptoethanol) for 5 min at 95 °C or 60 min at 55 °C. Proteins were separated by SDS-PAGE according to their molecular weight, transferred to nitrocellulose membranes (Amersham Pharmacia Biotech), detected using specific antibodies and visualized with HRP-conjugated secondary antibodies by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Antibodies

The following antibodies were used for immuno-precipitaion and -blotting: anti-α-catenin antibody: goat polyclonal C-19 and blocking peptide (both Santa Cruz); anti-

CCR5 antibodies: mouse monoclonal 2D7 (AIDS Research and Reference Reagent program) and goat polyclonal C-20 (Santa Cruz); anti-CXCR4 antibody: mouse monoclonal 12G5 (BD Pharmingen); anti-HA: mouse monoclonal 12CA5 (Sigma); anti-C9 antibody: mouse monoclonal 1D4 (Cell Culture Center); HRP-conjugated anti-mouse, anti-rabbit (Amersham Biosciences), anti-goat (Santa Cruz).

In vivo labeling and re-precipiation

Cells were incubated for 1 h in DMEM medium containing 10 % phosphate-free FCS, subsequently proteins were *in vivo* labeled with [^{32}P]-orthophosphate for 4 h. Cells were lysed in NP-40 lysis buffer and proteins immunoprecipitated from cleared lysates using first antibodies, either anti-HA or anti-Flag (1st IP). These first immunocomplexes were washed and 1/5 prepared for Western blot analysis. The remainder (4/5) was disassociated for 5 min at 95 °C in re-precipitation buffer, RIPA buffer was added in excess and divided in parts of 1/3 and 2/3 from which proteins were again immunoprecipitated (2nd IP). After SDS-PAGE and Western blotting, proteins were visualized by autoradiography using a PhosphorImager (Molecular Dynamics).

3. Results

The C-terminus of CCR5 interacts with α-catenin in yeast

The C-terminus of the CCR5 receptor, encompassing amino acids 295-352 was screened for interacting proteins in a GAL4-dependent yeast two-hybrid system using a human B-cell cDNA library (137). Out of 108 positive cDNAs, false positive clones were excluded by retransformation. Of twenty sequenced cDNAs two were further analyzed. Sequencing of cDNA from clone 30 identified an open reading frame of the C-terminus of α-catenin. The interaction of the C-termini of CCR5 and α-catenin was reproduced and further analyzed with CCR5-mutants as depicted (Fig. 1, right) in yeast. Site-directed mutagenesis was used to introduce stop-codons after amino acids S336, F320 or L308 of the receptor C-terminus. In addition the C-terminal leucine of CCR5 was substituted with proline (P352) to destroy a putative PDZ-domain binding motif. The receptor C-terminus and the mutants thereof were tested for interaction with the C-terminus of α-catenin (clone 30) in a non-lethal β-galactosidase plate assay for yeast (Fig. 1, left). The CCR5

wild-type (CCR5 ct), the very C-terminal substitution (P352) and one deletion mutant (S336) led to the formation of blue colonies (+), indicative of the interaction of the two protein domains. Further truncation of the receptor C-terminus abolished interaction with the C-terminus of α-catenin in yeast. The specificity of the interaction is indicated by the formation of white colonies after cotransformation of clone 30 cDNA with empty vector control.

The full-length proteins CCR5 and α-catenin interact in mammlian cells after overexpression

The interaction of the C-termini of CCR5 and α-catenin identified in yeast was confirmed in mammalian cells employing full-length proteins. HEK-293 that stably express functional HA-epitope tagged CCR5 receptor (unpublished data) were cotransfected with Flag-epitope tagged α-catenin. Proteins were labeled *in vivo* with [^{32}P]-orthophosphate and proteins immunoprecipitated from cleared cell lysates. Direct analysis of anti-HA immunocomplexes gave a background too strong for direct detection of coprecipitated proteins. Therefore, a re-immunoprecipitation from the disassociated first immunocomplexes was performed as described in Fig. 2. Proteins were size-separated by SDS-PAGE and [^{32}P]-labeled proteins were detected by autoradiography (Fig. 2). The *in vivo* labeled α-catenin was detectable after a 1st anti-Flag immunoprecipitation (1/5) at the predicted size (lane 1). The association of Flag-α-catenin with HA-CCR5 was shown by re-precipitation of α-catenin from anti-CCR5 immunocomplexes (lane 4). No bands of similar size were evident after immunoprecipitation from 1/5 of the lysate (lane 2) or after re-precipitation from 1st anti-HA disassociated precipitates using anti-HA antibodies (lane 3).

Association of CCR5 with α-catenin and mutants thereof in mammalian cells

The interaction was further confirmed and analyzed with plasmids encoding N-terminally Flag-epitope tagged α-catenin and two mutants thereof, as depicted in Fig. 3A. The N-terminal mutant (nt) comprises the amino acids 1 – 611. The C-terminal mutant (ct) contains amino acids 624 - 906.

Flag-α-catenin or mutants were separately transfected in Cf2Th cells (canine thymocytes) that stably express codon-optimized, C-terminal tagged CCR5 (synCCR5-C9) (136). The association of catenins with CCR5 was monitored by immunoprecipitation of CCR5 followed by an anti-Flag immunoblot. Binding of α-catenin to the CCR5 receptor was observed (lane 1). Deletion of the C-terminus or N-terminus of α-catenin did not abrogate the association with CCR5 (lane 2 and 3, respectively). Expression of α-catenin and mutants were detected in the direct cell lysates (DL) (Fig. 3B, lanes 4 - 6).

Endogenous interaction of α-catenin with the chemokine- and HIV-1 co-receptors CCR5 and CXCR4

To demonstrate the biological relevance, the interaction between the endogenous proteins of α-catenin and CCR5 was analyzed. First, interaction of α-catenin with codon-optimized synCCR5 stably expressed in HEK-293 cells (CCR5) was examined (Fig. 4A). Interaction of endogous α-catenin with exogenous CCR5 was detectable in HEK-293 cells that overexpessed the receptor (Fig. 4A, lane 2), but not in HEK-293 cells not expressing CCR5 (Fig. 4A, lane 1).

Interaction with endogenous CCR5 and CXCR4 proteins was shown in PM1 T lymphocytes after immunoprecipitation of the receptor proteins (Fig. 4B, lane 3 and 4, respectively).

The Western blot using an anti-α-catenin polyclonal antibody shows two bands at the approximately size of α-catenin. The upper band was shown to be specific for α-catenin since this band diminished in intensity after inclusion of a blocking peptide in competition immunoprecipitation studies, whereas the intensity of the lower band was not affected (compare Fig. 4A, lanes 3/4 with 5/6, and Fig. 4B, lanes 5 with 6).

4. Discussion

In this study α-catenin was identified as a new binding partner of the human chemokine- and HIV-1 co-receptor CCR5 by yeast two-hybrid analysis. The binding is mediated through the C-termini of both proteins. The interaction was confirmed by isolation of protein complexes by coimmunoprecipitation at ex- and endogenous protein level.

Furthermore, the endogenous interaction of α-catenin with CXCR4, the second major HIV-1 coreceptor was shown.

The cytoplasmic catenin proteins were initially identified to be complexed with the cellular adhesion plasma membrane molecule cadherin. Alpha-catenin is thought to act as a connector that anchors and stabilizes the cadherin complex at the plasma membrane, linking it to the cytoskeletal actin bundle (98, 99). Alpha-catenin is an integral part of the cellular cytoskeleton and could be involved in anchoring receptors at the plasma membrane, physically connecting them to actin filaments, which constitute the cytoskeletal backbone. Chemokine receptor activation can modulate the cytoskeleton by induction of F-actin polymerization (142). HIV-1 infection is blocked by cytochalasin D, which disrupts the actin-dependent concentration of receptors required for viral entry (81). In agreement with these findings resulted the treatment of peripheral blood lymphocytes with cytochalasin D in a reduced membrane fusion with cells expressing HIV-1 envelope protein that utilize CXCR4 or CCR5 receptors (143) and HIV-1 infection was shown to be promoted by CCR5 receptor cooperation (144).

Alpha-catein might not only be involved in anchoring or clustering of chemokine receptors, but could also direct the receptor assembly within distinct membrane environments. Actin-dependent receptor colocalization and clustering might provide a setting where coreceptors are in close proximitiy, thereby favoring HIV-1 binding and entry. The HIV-1 receptors CD4 and CCR5 and CXCR4 were shown to accumulate and colocalize in protruding membrane structures (145, 146). These are likely to be microvilli or membrane ruffles, which are supported by actin-polymerization at the cell surface. Other studies reported actin-dependant association of CD4 and CCR5 in the plasma membrane (81, 147). Furthermore, a constitutive association of the CD4-CCR5 receptors was detected by biochemical analyses (148, 149). Homo-/hetero-oligomerization of receptors affected CCR5-mediated signal transduction (38) and activation of CD4 resulted in altered CCR5 signaling due to cross-talk (83, 150). The signals induced by HIV-1 binding to the receptor complex do not necessarily reflect signaling induced by natural receptor ligands, but could represent unique signaling pathways. Since receptor distribution is affected and regulated by the cellular cytoskeleton our data suggest that α-

catenin also affects receptor signaling, thereby possibly affecting viral entry, integration, viral replication, or HIV-1 pathogenesis.

The chemokine receptors CCR5 and CXCR5 both constitutively associate with the motor protein nonmuscle myosin heavy chain-IIA (NMMHC-IIA) at the leading edge of migrating T lymphocytes (151). In addition, the colocalization of actin with the receptor C-termini was found in accordance with other studies reporting the association of actin with heptahelical receptors (152). This interaction might be involved in cellular rearrangement and cell motility after chemokine receptor activation since the cellular function of NMMHC-IIA has been connected with regulation of cell shape and formation of focal adhesions in HeLa cells (153).

In conclusion, the interaction of α-catenin with CCR5 and CXCR4 might enable accumulation of receptor proteins in distinct micro-environments at the plasma membrane, in which specific regulatory proteins can be centered. This might facilitate viral entry, receptor oligomerization and signaling or influence cellular migration. The cytoskeleton might be part of a mechanism for the directional transport of Golgi vesicles or (clustered) receptors to the plasma membrane, but might also be involved in the organization of cell surface localization of receptor molecules. Not only the process of transport or functional plasma membrane expression, but also internalization and subsequent intracellular vesicle-associated trafficking might thereby be affected.

Acknowledgements

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Figure legends

Fig. 1. The C-terminus of CCR5 interacts with the C-terminus of α-catenin in a yeast two-hybrid screen.

(A) Interaction of the C-termini of α-catenin and CCR5 was tested and analyzed in the yeast strain Y153. Plasmids pGBT9pheS.CCR5ct, that encodes DNA binding domain fused to CCR5 C-terminus (ct; aa 295-352) and pACT.catenin encoding DNA activation domain fused to the C-terminus of α-catenin (aa 799-906). Interaction of the fusion proteins mediated by CCR5 and α-catenin results in transcription of the *lacZ* gene and blue colony formation in a β-galactosidase assay (indicated by +). The interaction of α-catenin was further tested with different CCR5 C-terminal deletion mutants as depicted. Numbers indicated correspond to amino acid positions of CCR5.

Fig. 2. Interaction of α-catenin and CCR5 full-length proteins after overexpression in mammalian cells.

A scheme describing the experimental procedure is shown to the left. HEK-293 cells were transiently cotransfected with plasmids encoding Flag-epitope tagged α-catenin and HA-epitope tagged CCR5. The *in vivo* [^{32}P]-labeled proteins were immmunoprecipitated (1st IP) and 4/5 of this was used for separate re-immunoprecipitations (splitted in 1/3 and 2/3). All immunocomplexes were size-separated by SDS-PAGE and proteins detected by autoradiography (right).

Protein size is indicated in kilo Dalton (kDa). IP, immunoprecipitation

Fig. 3. CCR5 interacts with α-catenin and mutants thereof.

(A) A schematic representation of the α-catenin protein. The domains that are involved in diverse protein-protein interactions are outlined, the very C-terminal amino acids 799-906 were identified to interact with the CCR5 C-terminus in yeast in this study. Numbers correspond to amino acid positions. Alpha-catenin full-length (fl) and the N-terminal (nt) and C-terminal (ct) mutants used to analyze the interaction with CCR5 are schematically depicted. These α-catenin proteins are N-terminally tagged with a Flag-epitope sequence (light box). Numbers correspond to amino acid positions.

(B) To analyze interaction of proteins by coimmunoprecipitation studies, HEK-293 cells were transfected with codon-optimized, C9-epitope tagged CCR5 (synCCR5-C9) (136). The Flag-α-catenin proteins were cotransfected separately as indicated. Cells were lysed 36 h post transfection and immunoprecipitations of Flag-α-catenins (lanes 1-3) or synCCR5-C9 (lanes 4-6) were carried out using antibodies directed against the C9-epitope tag (anti-1D4 antibody) or against the Flag-epitope tag, respectively, as indicated. Immunocomplexes were size-separated by 12.5 % SDS-PAGE, transferred to a nitrocellulose membrane and α-catenin and mutants thereof were detected using the anti-Flag antibody.

Protein sizes are indicated in kilo Dalton (kDa). IP, immunoprecipitation; α-catenins: full-length (fl); N-terminus (nt); C-terminus (ct)

Fig. 4. Endogenous interaction of α-catenin with the chemokine receptors CCR5 and CXCR4.

(A) HEK-293 cells (control) and those stably expressing codon-optimized CCR5 (synCCR5) were lysed and proteins were immunoprecipitated using an anti-CCR5 antibody (2D7) or an antibody directed against α-catenin (C-19). The specificity of immunoprecipitation was tested by peptide block. Immunoprecipitates were analyzed by Western blot using the anti α-catenin antibody (C-19).

(B) Interaction of endogenous α-catenin with endogenous CCR5 and CXCR4 was analyzed in T lymphocytes (PM1 cell line). Cells were lysed and proteins immunoprecipitated with antibodies as indicated. Peptide block was used to determine specificity of α-catenin precipitation. Immunocomplexes were size-separated by SDS-PAGE and analyzed in a Western immunoblot using the anti α-catenin antibody.

Protein sizes are indicated in kilo Dalton (kDa). DL, direct lysate; IP, immunoprecipitation

Figure 1

Figure 2

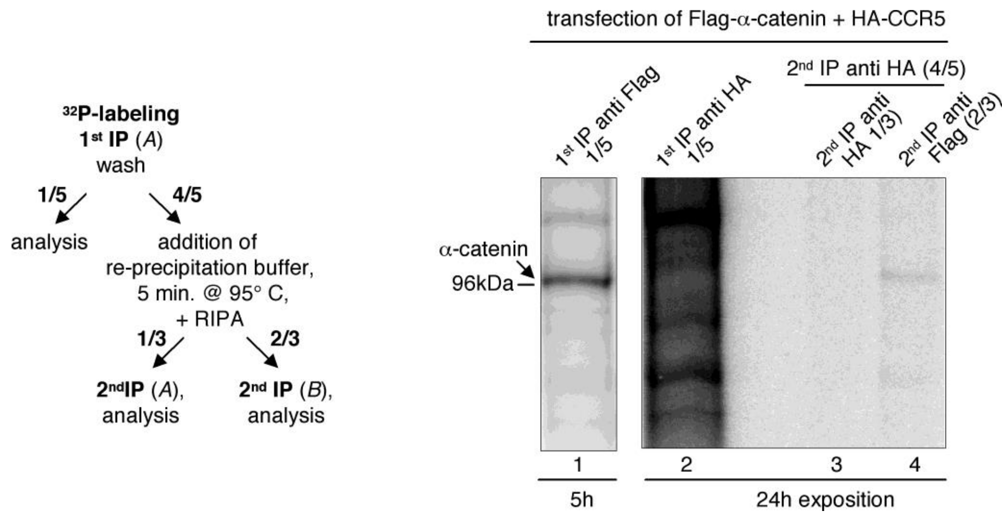
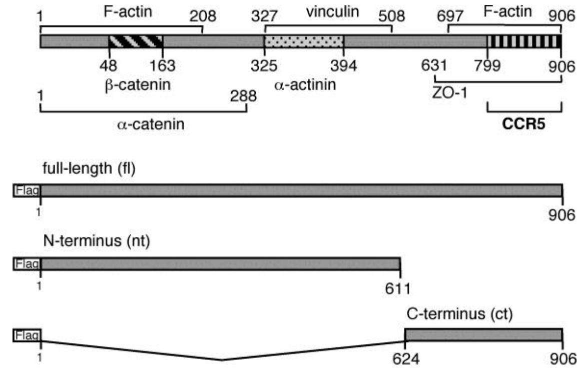


Figure 3

A



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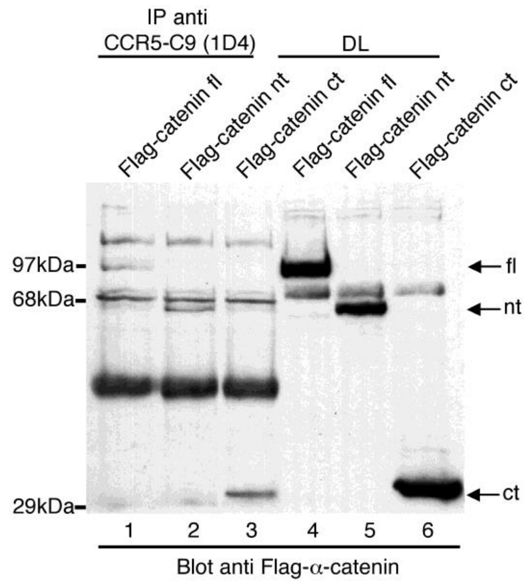
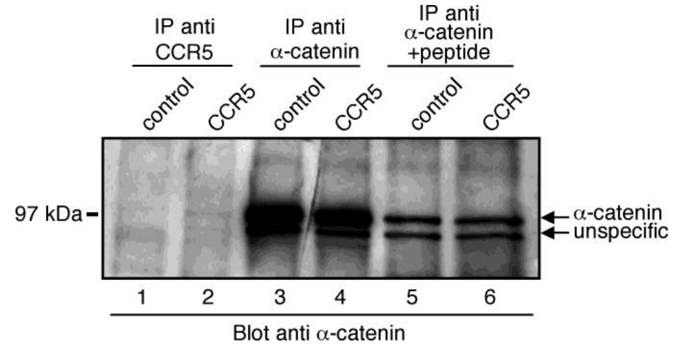


Figure 4

A



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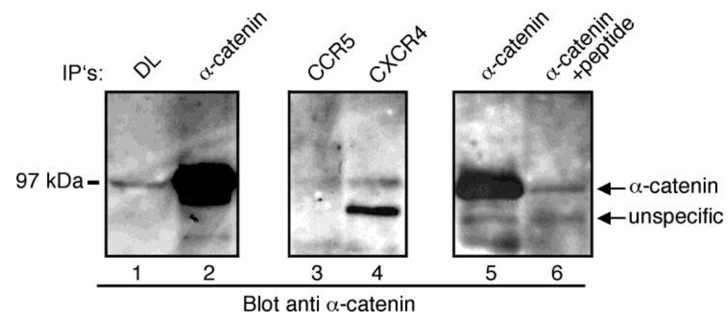

JM4 belongs to a new protein family that interacts with the chemokine- and HIV-1 co-receptor CCR5

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Abbreviations: CCR5, CC chemokine receptor 5; GPCR, G protein-coupled receptor; HIV-1, human immunodeficiency virus 1; GRK, G protein-coupled receptor kinase; TM, transmembrane

Summary

The human CC chemokine receptor 5 (CCR5) belongs to the heptahelical superfamily of G protein-coupled receptors and plays an important role in immune response modulation and human immunodeficiency virus (HIV-1) entry and pathogenesis. Using the cytoplasmic C-terminus of CCR5 as bait in a yeast two-hybrid screen we identified JM4 as a novel interacting protein. JM4 is a potential membrane-associated protein with four transmembrane spanning regions. Detailed analyses of the CCR5-JM4 complex formation in eukaryotic cells revealed that the association of JM4 with CCR5 comprises the cytoplasmic C-terminus of CCR5 and possibly additional site(s). We further show that JM4 is closely related four-transmembrane proteins such as human JWA or its rat homologue GTRAP3-18 known as a regulator of the glutamate transporter EAAC1. JM4, JWA, and GTRAP3-18 can form hetero-oligomers. Intriguingly, JWA also associates with CCR5 equivalent to JM4. Based on these observations, we hypothesize that JM4, JWA and GTRAP3-18 constitute a new family of proteins involved in the regulation of transmembrane receptors.

Introduction

The chemokine receptor CCR5 belongs to the superfamily of seven transmembrane-spanning G protein-coupled receptors (GPCRs). Chemokine receptors play a crucial role in the regulation of inflammatory processes. Agonist binding ultimately leads to activation and recruitment of cells to sites of inflammation (26). Furthermore, the chemokine receptors CCR5 and CXCR4 have been identified as coreceptors for human immunodeficiency virus (HIV-1) entry into $CD4^+$ target cells (55-60). CCR5 is expressed on memory T lymphocytes, macrophages, and dendritic cells and is mainly associated with the disease transmission of primary viruses (59), while CXCR4 seems to be important in later stages of the disease (63, 64).

Mutations in the CCR5 receptor have been shown to alter cellular signaling, HIV-1 entry or disease progression. Several natural mutants of CCR5 have been described (71, 154). The key role of CCR5 in HIV-1 pathogenesis is demonstrated by the fact that individuals, homozygous for a 32-base pair deletion in the receptors coding region, are almost completely resistant to HIV-1 infection and are long-term non-progressors (65). This

deletion results in a severely truncated protein that is not displayed at the plasma membrane. It does not result in any pathological phenotype. The mutant CCR5-893(-) receptor lacks its entire cytoplasmic C-terminus. This mutant also interferes with HIV-1 infection since the receptor is not efficiently trafficked to the cell surface (72). Evidence for the role of the cytoplasmic tail in receptor trafficking has recently been shown (73). Palmitoylation of the C-terminal tail of CCR5 has a significant influence on targeting the receptor to the plasma membrane, for signaling, internalization and intracellular trafficking (75, 77).

The regulation of seven transmembrane (7 TM) receptors is very complex and involves activation, dimerization, signaling, desensitization, internalization, and trafficking. Despite of some redundancy among different receptors and interacting proteins these processes are tightly and specifically regulated (2). Agonist-induced stimulation of GPCRs activates a complex network of intracellular signaling pathways. This leads to phosphorylation of GPCRs at their C-terminus by second messenger-dependent protein kinases and specific G protein-coupled receptor kinases (GRKs) (7). This phosphorylation event has been shown to be crucial for the desensitization of many GPCRs and promotes the binding of regulatory proteins, the most prominent being β-arrestin, to the C-terminus of CCR5 (12). This, in turn, sterically inhibits a further association of trimeric G proteins to the receptor, leading to uncoupling and internalization of the receptor. Multiple signal transduction cascades have been reported to be triggered by agonist-occupation of CCR5 (26). These events are thought to regulate cytoskeleton reorganization and subsequent polarization, migration, cell survival, differentiation, and activation of the cell. Multiple cellular signaling pathways can be activated during engagement of HIV-1 envelope with CD4 and the chemokine receptor (83). These may affect the susceptibility of the host cell to virus entry and infection in primary cells (134, 155). Identification of factors interacting with the C-terminus of CCR5 can contribute to further unravel the precise mechanisms responsible for efficient regulation of the receptor and might offer a possibility to interfere with HIV-1 infection. We identified novel binding partners of the C-terminus of CCR5 using a yeast two-hybrid system. One of these proteins is termed JM4 in the database and to date has not been described in the literature. Based on sequence, structure-based and biological properties,

homologous proteins from different species were identified. The JM4-related protein JWA is listed in the database with no known function, it interacts with similar efficiency as JM4 to CCR5. While this work was in progress the rat homologue to JWA has been described, GTRAP3-18, which binds to and regulates the amino acid carrier EAAC1 (122). These proteins constitute a novel family with four putative transmembrane regions that associate with cell surface receptors and potentially regulate their biological function.

Experimental procedures

Plasmid construction

The C-terminus of the human chemokine receptor was excised from pBABE.CCR5 (AIDS Research and Reference Reagent program) via restriction sites *SfaN*I and *Sal*I and subcloned into the *EcoR*I / *Sal*I sites of the yeast vector pGBT9pheS using a *EcoR*I-*Sfa*I linker. The pGBT9pheS is a derivative of pGBT9 (Clontech) that was modified to produce a Gal4-DNA binding domain containing bait vector with positive selection properties (M. Buchert, unpublished data) (135). Point mutations in the CCR5 C-terminus were introduced at three specific sites to produce stop codons after amino acids (aa) 336, 320 and 308. Furthermore, the very C-terminal leucine (aa 352) was substituted with a proline.

To construct a mammalian expression vector for expression of full-length CCR5, the gene was exerted from pcDNAI.CCR5 (AIDS Research and Reference Reagent program) and inserted into *Hind*III / *Xba*I restriction sites of pcDNA3 (Invitrogen) using a *Hind*III-*BamH*I linker to include an HA-epitope to create pcDNA3-HA-CCR5, which encodes a N-terminal HA-tagged CCR5.

The plasmid DNA encoding the codon-optimized, C-terminal tagged CCR5 (synCCR5-C9) was received from J. Sodroski (Dana-Farber Cancer Institute, USA) (136). The C-terminal epitope-tag (C9) was removed by mutagenesis to generate a codon-optimized plasmid encoding wild-type CCR5 (synCCR5).

The plasmid pACT.1 (JM4) was mutagenized to produce mutants Δ139, Δ93, and Δ44, that are C-terminally truncated from positions 139, 93, and 44, respectively. To subclone pACT.1 (clone obtained from the yeast two-hybrid screen encoding aa 7 to 178 of JM4) into a mammalian expression vector, the plasmid DNA was digested with *Bgl*II and

inserted into *BamH*I restriction site of pSuper'VSV (pS'VSV; C. Hovens, unpublished data). To obtain pS'VSV-JM4 encoding full-length VSV-JM4, the plasmid DNA was digested with *Xho*I and *BspE*I and a VSV-JM4 linker was inserted in-frame with the 5'-VSV-sequence. Plasmid pS'VSV-JM4 was mutagenized to produce C-terminal truncation mutants Δ154 and Δ139.

To subclone JM4 and JWA cDNAs into a pcDNA3 vector encoding two myc-epitope sequences (pcDNA3-2xmyc; E. Haas, unpublished data) the coding region for JM4 was amplified by PCR using pS'VSV-JM4 DNA as template. The coding sequence for JWA was amplified using the Access RT-PCR kit (Promega) according to the manufacturer's protocols using RNA extracts prepared from the human neuroblastoma cell line SHSY-5Y as template. The PCR products were subcloned into a pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen), subsequently excised by *Xba*I restriction and inserted into an *Xba*I restriction site downstream the myc-epitope sequences of the pcDNA3-2xmyc vector to create pcDNA3-2xmyc-JM4 and -JWA.

The GTRAP3-18-HA expression plasmid was kindly received from J. Rothstein (Johns Hopkins Medical Institutions, USA).

All linker and primer sequences can be obtained upon request. All described truncation and substitution mutants were generated using site-directed mutagenesis according to the manufacturer's instructions (Stratagene) and sequenced by automated DNA sequencing (Microsynth) to confirm successful cloning. Oligonucleotides were synthesized at Microsynth.

Yeast two-hybrid screen

Y153 yeast cells were transformed with the bait construct using a standard yeast transformation protocol and tested for self-transactivation before screening a human peripheral lymphocyte (early pre-B cell) cDNA library in prey plasmid pACT (provided by S. Elledge, University of Texas, USA) (137, 138). Approximately 500,000 yeast transformants were screened for histidine prototrophy in the presence of 10 mM 3-aminotriazole (Sigma), and positive colonies were scored for β-galactosidase activity by β-galactosidase filter assay. Prey plasmids were isolated from yeast using the Fast DNA Kit (BIO101) and further purified using Geneclean beads (BIO101), transformed into

competent *E.coli* DH5α (GibcoBRL) and amplified in *E.coli* to extract plasmid DNA (Qiagen). The selected prey plasmids were then re-tested by one-on-one transformations into yeast strain Y153 together with the original bait plasmid or control plasmid. Prey plasmids from positive yeast colonies were sequenced by automated DNA sequencing (Microsynth GmbH).

β-galactoside filter assay

The yeast colonies were grown on agar plates (per liter: 1.45 g east N_2-base, 5.25 g NH_4SO_4, 20 g glucose, 10 g succinate, 2.8 g KOH (for plates: 8.5 g KOH and 20 g bacto agar) and 2 g of drop-out mix containing all amino acids except those for selection). After 2-3 days at 30 °C, a Duralon-UV membrane (Stratagene) was put on top of grown colonies, removed after 1 min and twice immersed in liquid nitrogen and thawed. The membrane was then put on top of a 3MM filter paper (Whatman No.3) saturated with sodium-phosphate buffer (100 mM) containing 0.5 mg/ml X-gal (dissolved in DMSO). Coloration of positive clones was observed after 30 min up to 12 hours depending on the individual clone.

Non-lethal β-galactosidase plate assay for yeast

To better visualize blue yeast colonies, a non-lethal β-galactosidase plate assay was used according to the method described by Duttweiler (139) with some modifications. Colonies were grown as described above, picked, suspended in 10 μl of TE buffer and spotted onto a fresh agar plate. After incubation at 30 °C for one day, plates were flooded with chloroform for 8 min. The chloroform was decanted, and the plate inverted and allowed to dry on a rack for 5 min. The plate was overlaid with 1 % low melting agarose containing 1 mg/ml X-gal in TBE buffer. After the agarose had hardened, the plate was inverted and incubated at room temperature for up to 12 hours. The plates were photographed using a color film.

Northern Blot analysis

A [α-^{32}P]-dCTP (Amersham Pharmacia Biotech) labeled probe was prepared by random priming of a 0.95-kb fragment of JM4 using RadPrime DNA labeling system (Invitrogen)

as described in the manufacturer's protocols. A human 12-lane multiple tissue Northern blot (MTN; Clontech) was used for analysis of distribution of JM4 expression in human tissues. The membrane was prehybridized in ExpressHyb hybridization solution (Clontech) 68 °C for 30 min and hybridized with the probe in ExpressHyb solution 68 °C overnight. The MTN blot was washed three times for 15 min in 2xSSC containing 0.05 % SDS at room temperature and twice in 0.1xSSC containing 0.1 % SDS at 50 °C for 30 min. The blot was then exposed to imaging plates. Plates were read with a PhosphorImager (Amersham Biosciences) and analyzed using ImageQuant software.
Blots were stripped by boiling in 5 mM potassium phosphate and reprobed with a β-actin probe to confirm that RNA samples were present in equivalent amounts.

Cell culture and transfection

The human embryonic kidney (HEK)-293 cell line was grown in Dulbecco’s modified eagle’s medium (DMEM; Gibco) supplemented with 10 % fetal calf serum (FCS; Seratec). Cf2Th cells stably expressing a codon-optimized and C-terminal epitope-tagged CCR5 (synCCR5-C9) were provided by J. Sodroski and maintained as described (136).
Cells were transfected using LipofectAMINE 2000 according to the manufacturer’s instructions (Gibco) or using a modified calcium phosphate transfection protocol as described (141).
HEK-293 cells transfected with HA-CCR5 or syn-CCR5 encoding constructs were selected for stable protein expression with 1 mg/ml G418 sulfate (Calbiochem) and sorted for homogenous CCR5 cell surface expression by fluorescence-activated cell sorting (FACS).

Cell lysis

For preparation of membrane and cytoplasm, cells were washed with phosphate buffered saline (PBS; Gibco) and harvested in hypotonic lysis buffer (HLB; 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.2 mM EDTA, 1 mM DTT) supplemented with phosphatase / protease inhibitors (25 mM β glycerol-phosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine, 10 µM pepstatin, trasylol (500 Kalikrein ® inactivator units), 5 µg/ml leupeptin). Cell lysates were prepared by Dounce homogenization, subjecting the

cell suspension in HLB to 50 strokes of the tight pestle. To eliminate nuclei and debris, lysates were centrifuged at 500 g for 5 min. The supernatant was subjected to ultracentrifugation at 65,000 rpm for 60 min using the TLA-100.2 fixed angle rotor in Optima TL-100 ultracentrifuge (Beckmann). The supernatant was adjusted to 100 mM NaCl and 0.5 % Nonidet P-40. The membrane pellet was washed twice with HLP and resolubilized in NETN buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM EDTA, 0.5 % Nonidet P-40, 1 mM DTT, supplemented with inhibitors).

Cell lysis for native purification of CCR5 was performed as described elsewhere (136). Briefly, cells were washed with PBS and lysed in solubilization buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10 % Glycerol, 0.5 % Cymal-5 (Anatrace), supplemented with inhibitors).

All lysis steps were performed at 4 °C, cell lysates were cleared by centrifugation and protein concentrations of the supernatants were determined using the Protein Assay Kit (Bio-Rad).

Antibodies

The following antibodies were used: anti-VSV antibody: mouse monoclonal P5D4 (Sigma); anti-CCR5 antibodies: mouse monoclonal 2D7 (AIDS Research and Reference Reagent program), fluorescein isothyocyanate (FITC)-conjugated 2D7 (BD Pharmingen), goat polyclonal C-20 (Santa Cruz); anti-HA antibodies: monoclonal mouse 12CA5 (Sigma), monoclonal rat 3F10 (Boehringer), polyclonal rabbit Y-11 (Santa Cruz); anti-myc antibodies: mouse monoclonal 9E1O (Roche), rabbit polyclonal A-14 (Santa Cruz); HRP-conjugated antibodies: anti-mouse, anti-rabbit (Amersham Biosciences), anti-goat (Santa Cruz); FITC-conjugated anti-rabbit antibody, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse and anti-rat antibodies (Molecular probes, Jackson ImmunoResearch Lab.).

Immunoprecipitation and Western blotting

Proteins were immunoprecipitated for 4 hours with 10 µl of antibody-precoupled sepharose beads overnight. Afer immunoprecipitation sepharose beads were washed 4 times with lysis buffer and an equal volume of SDS sample buffer (62.5 mM Tris-HCl

(pH 6.8), 10 % glycerol, 2 % SDS, 0.1 % bromphenol blue, 50 µl/ml β-mercaptoethanol) was added to the pelleted beads. After incubation of 5 min at 95 °C or 60 min at 55 °C, proteins were size-separated in 12.5 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). Proteins were detected using specific antibodies and visualized with HRP-conjugated antibodies by enhanced chemiluminescence (Amersham Pharmacia Biotech).

FACS analysis

HEK-293 cells expressing the CCR5 variants were sorted and analyzed for receptor cell surface expression by FACS analysis. For sorting, $5x10^5$ cells were resuspended in cold PBS supplemented with 1 % FCS (PBS^{FACS}) and incubated with FITC-conjugated 2D7 antibody (BD Pharmingen) directed against the extracellular N-terminus of CCR5 for 60 min at 4 °C. Cells were washed with and resuspended in cold PBS^{FACS}. Cell sorting was performed on a FACStar cell sorter (Becton Dickinson). For analysis of cells stably expressing the CCR5 variants PBS^{FACS} was supplemented with 0.02 % sodium azide, 10^5 cells/sample were treated as described above and analyzed by flow cytometry using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

Confocal immunofluorescence microscopy

Cells were seeded on coverslips prior to transfection. For immunofluorescence detection, cells were rinsed with PBS and fixed with 3 % paraformaldehyde for 15 min at 25 °C. To detect intracellular antigens, cells were permeabilized with 0.5 % Triton X-100 in PBS at 25 °C for 2.5 min. To analyze cell surface expression of CCR5, cells were not permeabilized. After rinsing with PBS containing 5 % new born calf serum (NCS; Gibco) (PBS/NCS) cells were incubated with specific antibody in PBS/NCS for 60 min at 4 °C. Cells were rinsed with PBS/NCS followed by chromophore-tagged secondary antibody incubation for 60 min at 4 °C. After rinsing with PBS/NCS the coverslips were mounted with Mowiol (Calbiochem) and images were collected on a Leica TCS4D confocal microscope (Leica Microsystems) and analyzed using IMARIS bitplane software.

Results

Identification and characterization of the interaction of CCR5 with JM4 in yeast

In an attempt to identify proteins that directly interact with the cytoplasmic C-terminus of CCR5, a Gal4-dependent yeast two-hybrid screen was performed (137). Screening of a human B-cell cDNA library with the C-terminus of CCR5 (aa 295-352) yielded 108 positive cDNA clones. After exclusion of false positive clones by re-transformation, twenty potential candidates were sequenced, two of which were further analyzed. One clone, pACT.1, encoded the JM4 protein except for the six N-terminal amino acid residues.

The interaction was easily confirmed in yeast. To map the JM4-binding regions of the C-terminus of CCR5, three deletion mutants and one substitution mutant were generated (Fig. 1A). Stop-codons were introduced by site-directed mutagenesis after residues S336, F320 and L308. To destroy a putative PDZ-binding domain the C-terminal leucine of CCR5, which is a characteristic for ligands of PDZ domains, was substituted with proline (P352). Fig. 1A shows the interaction of the C-terminus of CCR5 (CCR5 CT) and JM4, resulting in formation of blue yeast colonies. The substitution of the very C-terminal leucine to proline or deletion of the C-terminal 16 amino acids did not affect the interaction and led to the formation of blue colonies, as seen with the wild-type. Shorter truncation mutants of the receptor C-terminus did not interact with JM4 in yeast. The empty vector control resulted in formation of white colonies, indicating the specificity of the interaction of JM4 with the carboxyl tail of CCR5 in yeast.

Furthermore, sequentially deleted JM4 proteins were tested for their association with the C-terminus of CCR5 (aa 295-352) in the yeast-two hybrid system (Fig. 1B). Again, we introduced stop-codons resulting in C-terminal deletion mutants of JM4 (Δ139, Δ93 and Δ44; Fig. 1B, right). All mutant forms of JM4 were no longer able to bind to the C-terminus of CCR5. Taken together, these data indicate a specific interaction of CCR5 and JM4 in yeast, suggesting that the binding domains reside in the C-termini of the proteins.

JM4 is a putative four-transmembrane protein and ubiquitously expressesd

The predicted amino acid sequence encoded by the JM4 cDNA is 178 amino acids long. Transmembrane prediction using the TMHMM program (v. 2.0) (123) and

hydrophobicity plots suggested four potential transmembrane helices, indicated by the dark shaded regions in Fig. 2A. Blast searches of both the full nucleotide- and predicted amino acid sequences did not reveal any domains associated with a biological function. To further characterize JM4, a human multiple tissue Northern blot was hybridized with a [^{32}P]-labeled JM4-specific probe. Northern blot analysis of JM4 expression in twelve human tissues revealed one single transcript of about 1.2-kb (Fig. 2B, top). Among the twelve adult tissues the JM4 transcript is expressed nearly ubiquitously. For loading control the presence of a 2.0-kb band is confirmed with a human β-actin control probe, a 1.8-kb actin isoform is found in heart and skeletal muscle (Fig. 2B, bottom).

JM4 is membrane-associated and coimmunoprecipitates with CCR5

We next examined the subcellular localization of JM4 and confirmed its interaction with CCR5 in mammalian cells. Cf2Th cells stably expressing a codon-optimized CCR5 receptor (136) were transfected with VSV-epitope tagged JM4 encoding plasmid or empty vector control. Proteins were immunoprecipitated from membrane (Mem) and cytoplasmic (Cyt) fractions prepared by ultracentrifugation and detected by Western blot analysis. As shown in Fig. 3, JM4 is associated with the membrane fraction (lanes 2 and 6) and was not detected within the cytoplasmic fraction (lanes 4 and 8). These findings further support the prediction that JM4 harbors transmembrane domains (compare Fig. 2A). The interaction of JM4 and the C-terminus of CCR5, as identified in yeast, was confirmed with the full-length receptor in mammalian cells. A complex of CCR5 and JM4 is shown by coimmunoprecipitation of JM4 with CCR5 in the membrane fraction (lane 10). CCR5 appears as a doublet, which is most likely due to glycosylation of the receptor.

CCR5 and C-terminal deletion mutants bind the JM4 protein

To further analyze the interaction of CCR5 and JM4 we established HEK-293 cell lines stably expressing different HA-epitope tagged CCR5 receptor molecules. The expressed CCR5 wild-type protein and mutant forms, truncated for 32 or 44 amino acids in their cytoplasmic C-terminal domain (.320 or .308, respectively) are depicted schematically in Fig. 4A. They correspond to the C-terminal constructs used in yeast (compare Fig. 1A).

These stable cell lines were analyzed by flow cytometry using a FITC-conjugated antibody, which recognizes the extracellular N-terminus of CCR5. HA-CCR5 and HA-CCR5.320 were detected at the cell surface, as indicated by the shift of the bold line to the right (Fig. 4B). Expression of HA-CCR5.308 receptor, that lacks the full cytoplasmic C-terminus, was not detectable at the plasma membrane. Similar results have been observed previously for the naturally occurring gene product CCR5-893(-), composed of 308 amino acids, thus lacking the entire cytoplasmic C-terminal tail (72). The specificity of the anti-CCR5 antibody binding is demonstrated by staining of the cells transfected with empty vector as control (pcDNA3) or incubation of the cells with a FITC-conjugated control mouse IgG_{2a} (thin line).

The subcellular localization of the CCR5 receptors was determined by immunofluorescence confocal microscopy (Fig. 4C). HEK-293 cells stably expressing CCR5 were immunostained with mouse anti-HA antibody followed by FITC-labeled anti-mouse IgG. Again, wild-type HA-CCR5 and HA-CCR5.320 were readily expressed at the plasma membrane. In contrast, HA-CCR5.308 was only detectable intracellularly after permeabilization of the cells. HEK-293 cells transfected with the vector pcDNA3 served as a control for the specificity of the anti-CCR5 immunostaining.

We next examined whether a complex consisting of JM4 and wild-type or mutant CCR5 could be detected. HEK-293 cells, stably expressing receptor molecules, were transfected with VSV-JM4 or empty vector control. After immunoprecipitation of CCR5 we used the anti-VSV antibody to detect coprecipitated JM4 in the immunoblot (Fig. 4D). JM4 interacted with the wild-type HA-CCR5, the deletion mutant HA-CCR5.320 and HA-CCR5.308, a mutant deleted for the full cytoplasmic C-terminus. In yeast, employing only the C-terminus of CCR5, the interaction was mediated by the very C-terminus of CCR5 only (Fig. 1A). In HEK-293 cells deletion of the C-treminus of wild-type CCR5 did not abrogate the binding of JM4. This suggests a second binding site for JM4 on CCR5. Furthermore, the interaction was not dependent on the cell surface expression.

JM4 and C-terminal deletion mutants thereof coimmunoprecipitate with CCR5

To further characterize the interaction of CCR5 with JM4 the C-terminus of JM4 was deleted. Fig. 5A schematically outlines the JM4 protein with an N-terminal VSV-epitope

tag and its four potential transmembrane domains. Premature stop-codons were introduced using PCR-directed mutagenesis, resulting in truncation of JM4 after residue 154 or 139 (Δ154 or Δ139, respectively), indicated by black arrowheads. JM4 or mutants were separately transfected in HEK-293 cells stably expressing codon-optimized CCR5. Interaction of JM4 proteins was analyzed after immunoprecipitation of CCR5 in an immunoblot with an antibody directed against the VSV-tag of JM4 (Fig. 5B). The wild-type CCR5 receptor coimmunoprecipitated JM4, with less efficiency Δ154, and with even lower efficiency Δ139 (lanes 6, 7, and 8, respectively).

JM4 forms homo-multimers

Analyzing JM4 by immunoblotting we detected signals corresponding to oligomeric forms of JM4. In order to test for multimerization of JM4, HEK-293 cells were transiently transfected with plasmids encoding VSV-JM4, deletion mutants Δ154, Δ139, or empty vector control. After immunoprecipitation of JM4, we detected the monomeric form of JM4, as well as multimeric complexes of JM4 in an immunoblot (Fig. 6A). Multimerization of JM4 did not depend on an intact C-terminus since both deletion mutants multimerized.

In order to directly show complex formation, JM4-encoding constructs with either VSV- or myc-epitope at the N-terminus or empty vector control plasmids were coexpressed in HEK-293 cells (Fig. 6B). After immunoprecipitation using an anti-VSV antibody, myc-VSV was detected in an anti-myc immunoblot (lane 4). Not only monomers of JM4, but also dimers and trimers of JM4 coprecipitated. Similarily, VSV-JM4 was detected by Western blotting after precipitation of myc-JM4 (data not shown). The formation of dimers and trimers is still apparent after SDS-PAGE analysis, indicating that the complex formation is of high stringency.

JM4 is member of a four-transmembrane protein family forming homo-/hetero-oligomers

Sequence analyses at the nucleotide- or amino acid level did not suggest any known biological function connected with JM4. A database search for related proteins identified several proteins throughout different species, all of which share sequence homologies with JM4, are of similar size, and display four potential transmembrane helices in a

pattern similar to JM4. An amino acid alignment is shown in Fig. 7A. The structural homology of JM4 (human) and GTRAP3-18 (rat) is reflected in the transmembrane plots for JM4 and GTRAP3-18 shown below. While this study was ongoing, the GTRAP3-18 protein from rat was identified and a biological function was described (122). The protein binds to and inhibits the cell membrane glutamate transporter EAAC1. GTRAP3-18 and its human homologue JWA share 92 % sequence identity at amino acid level. The *JWA* gene is suggested to be transcriptional regulated by vitamin A and proposed to be associated with the cytoskeleton (NCBI Protein Database = NCB Accession # AAC64360). JM4 shows 42 % identity and additional 21 % similarity with JWA at the amino acid level. Since the human JM4, JWA and the rat GTRAP3-18 share sequence and structure-based homologies we tested whether these proteins also localize to similar sites within the cell. After expression of VSV-JM4, myc-JWA, and GTRAP3-18-HA in HeLa cells, these were analyzed using the respective antibodies directed against the tagged proteins. The proteins localized to similar defined structures within the cell as shown by indirect confocal laser scanning microscopy (Fig. 7B). The localization of these proteins suggests an association with the cytoskeleton or endoplasmic reticulum. Similar results were obtained in COS cells (data not shown).

Since JM4, JWA and GTRAP3-18 are distributed similarly within the cell, we asked whether they might have also functional similarities. This was tested for by colocalization and hetero-dimer formation by immunofluorescence and coimmunoprecipitation studies. As shown before, JM4 tends to form homomultimers (Fig. 6). We coexpressed pairs of proteins, consisting of JM4 and JWA, JM4 and GTRAP3-18, or JWA and GTRAP3-18 in HeLa cells or HEK-293 cells. The colocalization of the protein pairs is indicated by the yellow colors in the overlay of red and green colors from the fluorescence-labeled secondary antibodies (Fig.7C). We then examined the ability of these proteins to form hetero-complexes. Proteins were expressed pairwise in HEK-293 cells and immunoprecipitated from cellular lysates with anitbodies recognizing either the VSV-, myc-, or HA-epitope tag fused to the proteins. Coprecipitated proteins were detected by Western blotting (Fig.7D). Indeed, each one of the proteins was capable of interacting with the other two proteins. The top panel shows, that JWA and GTRAP3-18 copreciptated with JM4 (lane 3 and 6, respectively). After precipitation of JWA (middle

panel), JM4 (lane 9) or GTRAP3-18 (lane 12) were detected in the corresponding immunoblot. Immunoprecipitation of GTRAP3-18 resulted in coprecipitation of JM4 (lane 15) or JWA (lane 18). These data indicate that JM4, JWA, and GTRAP3-18 are not only structurally related but might share biological functions, as they are located to the same structures within the cell and can physically interact with each other.

CCR5 can bind to JM4 and JWA

Heterodimers might be of relevance for recruiting and/or clustering of different cellular proteins. Since JM4, JWA and GTRAP3-18 can homo- and hetero-multimerize, we examined whether JWA, the human homologue of rat GTRAP3-18, also resembles JM4 in its ability to bind to the CCR5 receptor. Therefore, HEK-293 cells stably expressing the codon-optimized CCR5 receptor were transfected with VSV-JM4 or myc-JWA. After cell lysis, immunoprecipitation with an antibody directed against the N-terminus of CCR5 was performed. An anti-VSV or anti-myc antibody was used to detect coprecipitated JM4 or JWA, respectively (Fig. 8). Indeed, JM4 (lane 4) and JWA (lane 8) were both able to bind to the receptor, further indicating the close homology of these proteins.

Discussion

The biological actions of chemokine receptors are regulated at various levels, through interaction with diverse adaptor and scaffold proteins, plasma membrane expression and ligand binding, internalization, recycling, or degradation (3). The C-terminal tail of CCR5 has been shown to affect signal transduction, as well as trafficking of the receptor. The aim of this study was to identify CCR5-interacting proteins that might lead to a better understanding of the mechanisms responsible for the modulation of chemokine- and HIV-1 co-receptor function. We identified JM4 as a new interaction partner of CCR5. Sequence, structural, and biochemical analyses of JM4 led to the characterization of a family of integral membrane proteins with similar biological properties, interacting with and possibly regulating plasma membrane proteins.

Databases from NCBI (LocusLink) mapped *JM4* to chromosome Xp11.23, the gene contains 3 exons. Several proteins similar to JM4 proteins were found in different species

by sequence analysis using standard protein-protein BLAST (blastp) (121). At the amino acid level JM4 exhibits 42 % and 41 % sequence identity to JWA (human) and GTRAP3-18 (rat), respectively.

The human JWA protein has not been functionally characterized, but shares 90 % sequence identity with the murine Aip-5 (ADP-ribosylation-like factor 6 (ARL6) interacting protein-5). Aip-5 was found to interact with ARL6 in a yeast two-hybrid screen (124). The ADP-ribosylation factor (ARF) group of proteins is subdivided into ARF and ARF-like (ARL) molecules and ARF6 has recently been shown to enhance endocytosis of the heptahelical, G protein-coupled β_2-adrenergic receptor (β_2-AR) through its interaction with β-arrestin (17). Since JM4 closely resembles JWA and Aip-5, we tested whether JM4 could be a scaffold/adaptor protein like β-arrestin, possibly linking ARL6 and CCR5. We performed coimmunoprecipitation and pull-down experiments of JM4 and ARL6 in a GTP-dependent manner, but could not detect an interaction in these overexpression systems (data not shown).

GTRAP3-18 binds to the ten-transmembrane glutamate transporter EAAC1 and modulates the transporter activity, as it decreases the affinity of EAAC1 to glutamate, possibly by inducing allosteric changes (122, 156). The C-terminal truncated EAAC1 protein was readily expressed at the plasma membrane, but transporter function was no longer regulated by GTRAP3-18, as the deletion abolished the interaction. Given the homology of GTRAP3-18 and JM4, we tested different C-terminal truncation mutations of CCR5 for interaction with JM4. In yeast, employing only the carboxyl tail of CCR5 and deletion mutants thereof, truncation abolished the interaction However, in HEK-293 cells similar deletions of the full-length receptor molecule did not affect the interaction, which suggests additional CCR5-intramolecular binding site(s) for JM4. This is in accordance with recent data showing that besides the C-terminus of CCR5 an additional site within the second intracellular loop of the chemokine receptor mediates β-arrestin binding (12). This sequence motif (Asp-Arg-Tyr), which is conserved throughout the GPCR family, is involved in G protein activation after agonist occupation of the receptor and in secondary β-arrestin binding (7, 80, 157). Therefore, this sequence might additionally be involved in mediating JM4-binding, which will be tested for.

Chemokine stimulation of CCR5 did not alter binding of JM4 (data not shown). A constitutive association of the proteins is suggested by the fact that JM4 interacted with a CCR5 mutant that was not expressed at the cell surface. Deletion of the complete C-terminus of CCR5 (CCR5.308) resulted in the cytoplasmic retention of the receptor shown by FACS and immunofluorescence analyses (Fig. 4B, C). This is in accordance with the impaired cell surface expression of the natural CCR5-893(-) mutant (72). In conflict, CCR5 truncations after amino acid 307 (158) or 308 (80) did not abolish cell surface expresssion nor did they interfere with HIV-1 entry in two other studies. The latter study described reduced cell surface expression level of truncated CCR5 in HEK-293 cells, when compared to wild-type receptor. Plasma membrane expression seemed to vary in different cells, which might be attributable to and emphasizes the importance of the cellular background and/or receptor overexpression.

We identified the binding site for CCR5 in the C-terminal region of JM4, as deletion mutants were no longer able to interact with the C-terminus of CCR5 in yeast. Similar deletion mutants of JM4 still bound with less efficiency to full-length CCR5 in HEK-293 cells. This binding can be explained by the fact that JM4 wild-type and deletion mutants are able multimerize. Endogenous JM4, interacting with CCR5, could therefore complex mutant JM4 with the receptor. Northern blot analysis proved endogenous expression of JM4 in HEK-293 (data not shown).

Hydrophobicity and secondary structure-based analyses of JM4, JWA and GTRAP3-18 suggested four conserved transmembrane domains in all three proteins. Furthermore, we detected colocalisation by imunofluorescence analysis at distinct cellular structures, resembling endoplasmic reticulum. The four transmembrane domains are not necessarily traversing the plasma membrane but might be associated with membrane domains of intracellular organelles or vesicles. The nature of these structures can be further characterized using ER- or cytoskeleton-specific marker proteins. JM4 and JWA are predicted to be in the ER (NCBI AceView), JWA is in the database proposed to be cytokeleton associated.

As described here for JM4, GTRAP3-18 was reported to form homomultimers (122). Biochemical analyses of JM4, JWA and GTRAP3-18 showed not only homo-, but also hetero-multimers. This finding supports the biological resemblance of members of the

protein family. The functional replaceability of the proteins is furthermore emphasized by the fact that JWA, like JM4, interacted with CCR5.

Our studies identified novel CCR5-interacting proteins, JM4 and JWA, and led to the characterization of a family of integral membrane proteins that associate with plasma membrane proteins. GPCRs have been shown to elicit many different receptor-specific properties regarding trafficking, desensitization, internalization, and activation of various signaling cascades, even independent of G proteins. These are regulated by diverse proteins and are furthermore affected by the cellular context. Identification of JM4 and its biological homologues will also help to elucidate the biological function of other members that belong to the same new protein family. This might serve as a basis to gain further insights into mechanisms regarding regulation of plasma membrane proteins, as already suggested for GTRAP3-18.

Acknowledgements

We thank D. Meyer for the help with plasmid construction, G. Radziwill and M. Schwemmle for helpful discussions, M. Hoechli, EMZ, University Zurich, Switzerland, for assistance concerning confocal microscopy, and E. Niederer from the Institut für Biomedizinische Technik (ETH und University Zürich, Switzerland) for help with FACS sorting. The distribution of cells stably expressing the synCCR5-C9 and the corresponding expression plasmid by Joseph Sodroski and the distribution of the GTRAP3-18-HA expression plasmid by Jeffrey D. Rothstein are gratefully acknowledged. The lymphocyte (early pre-B cell) cDNA library was kindly provided by Stephen J. Elledge. This work was partly funded by the James B. Pendleton Charitable Trust.

Figure legends

Fig. 1. Complex formation of JM4 and CCR5 depends on intact C-termini of both proteins in the yeast-two hybrid assay.

(A) The yeast strain Y153 was cotransformed with plasmids pGBT9pheS.CCR5ct, encoding the C-terminus (CT) of CCR5 (aa 295-352, fused to the Gal4-DNA binding domain) and pACT.JM4 (aa 6-178, fused to the Gal4-DNA activation domain). Interaction of the proteins results in transcription of the *lacZ* gene, which can be monitored by β-galactosidase assay resulting in blue colony formation (indicated by +). JM4 was further tested with a panel of CCR5 C-terminal deletion mutants as depicted on the left. Numbers indicated correspond to amino acid positions.

(B) Complex formation of the C-terminus of CCR5 with JM4 and deletion mutants in the yeast-two hybrid assay. The tested JM4 constructs are depicted to the left. Specific interaction results in blue color of the yeast colonies.

Fig. 2. Sequence characteristics and human tissue distribution of JM4.

(A) The nucleotide- and deduced amino acid sequences of JM4 (NCBI Protein Database = NCB Accession # CAA06753). Potential transmembrane (TM) helices were detected by the program TMHMM (v. 2.0) (123) and are indicated in the sequence in grey. (B) Tissue distribution of JM4. A human multiple tissue Northern blot (Clontech) was hybridized with a [^{32}P]-labeled probe derived from human JM4 (top), stripped and reprobed with a β-actin probe (bottom).

Fig. 3. Interaction of CCR5 with JM4 in mammalian cells.

Cf2Th cells stably expressing a codon-optimized CCR5 with a C-terminal epitope tag (synCCR5-C9) (136) were cotransfected with plasmids encoding N-terminal VSV-epitope tagged JM4 (VSV-JM4) or empty plasmid control. Cells were lysed 36h post transfection. Membrane (Mem) and cytoplasmic (Cyt) fractions were prepared by ultracentrifugation. Immunoprecipitations of VSV-JM4 and CCR5 were carried out using antibodies directed against the VSV-tag of JM4 and against the N-terminus of CCR5, respectively. Proteins were size-separated by 12.5 % SDS-PAGE, transferred to a

nitrocellulose membrane and proteins detected using the anti-VSV antibody and the goat anti-CCR5 antibody directed against the C-terminus of CCR5 as indicated.
DL, direct lysate; IP, immunoprecipitation

Fig. 4. Interaction of CCR5 wild-type and C-terminal deletion mutants with JM4.

(A) A schematic representation of the CCR5 receptor proteins stably expressed in human embryoinc kidney (HEK)-293 cells. Shown are the N-terminal hemagglutinin (HA)-epitope tagged wild-type CCR5 (wt) and two receptors deleted by 32 or 44 amino acids from their C-terminus (.320 or .308, respectively). Dark shaded regions correspond to the seven transmembrane domains of CCR5.

(B) HEK-293 cells were transfected separately with HA-CCR5-encoding plasmids or empty vector control, then selected for stable protein expression in the presence of G418. Stable cell lines were analyzed by FACS using a fluorescein isothyocyanate (FITC)-conjugated mouse anti-CCR5 antibody (bold line) or FITC-conjugated control IgG_{2a} (thin line) (y-axis: cell count, x-axis: fluorescence intensity).

(C) To analyze the cell surface expression of CCR5 on selected cell lines, cells were fixed and immunostained with antibodies directed against the HA-epitope, followed by incubation with FITC-conjugated anti-mouse antibody. Note, that HA-CCR.308 expression was detected intracellulary only with permeabilization of the cells prior to incubation with antibodies (bottom).

(D) HEK-293 cells stably expressing HA-CCR5, HA-CCR5.320 or HA-CCR5.308 were transfected with plasmids encoding VSV-JM4 or empty vector control as indicated. Cells were lysed and HA-tagged CCR5 was immunoprecipitated using the anti-HA antibody. Proteins were size size-separated by 12.5 % SDS-PAGE, transferred to a nitrocellulose membrane, and coprecipitated VSV-JM4 was detected using the anti-VSV antibody.
IP, immunoprecipitation; l.c., light chain

Fig. 5. Complex formation of CCR5 with JM4 wild-type and deletion mutants in mammalian cells.

(A) Schematical representation of the VSV-tagged JM4 construct with mutations indicated by black arrowheads used for expression in mammalian cells. Premature stop-

codons were introduced, encoding proteins that are C-terminual truncated (JM4Δ154 and JM4Δ139). JM4Δ139 corresponds to the protein expressed in yeast (Fig. 1B). Shaded regions indicate predicted transmembranbe domains. Numbers correspond to positions of amino acids.

(B) HEK-293 were transfected with codon-optimized CCR5 (synCCR5), selected for stable expression with G418 and subsequently sorted for homogenous CCR5 cell surface expression by FACS analysis (data not shown). VSV-JM4-encoding plasmids were transfected as indiacted. After cell lysis CCR5 was precipitated using a mouse antibody recognizing the N-terminus of CCR5. Proteins were size-separated by 12.5 % SDS-PAGE, transferred to a nitrocellulose membrane and coprecipitated VSV-JM4 was detected using the anti-VSV antibody.

DL, direct lysate; IP, immunoprecipitation; l.c., light chain

Fig. 6. JM4 forms homo-multimers.

(A) After expression of VSV-epitope tagged JM4, JM4Δ154 or JM4Δ139 in HEK-293 cells, cellular lysates were incubated with an antibody directed against the VSV-epitope. Immunoprecipitates were analyzed by Western blotting using the anti-VSV antibody. Dimers and trimers are indicated.

(B) HEK-293 cells were transfected with VSV-JM4, myc-JM4 and empty vector control plasmids as indicated. Cell lysates were immunoprecipitated using the anti-VSV antibodies. Proteins were size-separated by 12.5 % SDS-PAGE, transferred to a nitrocellulose membrane and detected using the mouse anti-myc antibody 9E10.

IP, immunoprecipitation; DL, direct lysate; α, anti; l.c., light chain; h.c., heavy chain

Fig. 7. JWA and GTRAP3-18 share sequence homologies with JM4, colocalize within the cell and interact with each other.

(A) Alignment of JM4 and homologous proteins from different species using MacVector™ 7.1. The shaded regions indicate conserved amino acid residues, the grey bars the four predicted transmembrane (TM) helices of JM4 (compare Fig. 2A). The structural homology of JM4 (human) and GTRAP3-18 (rat) is reflected in transmembrane

plots shown below (y-axis: TM probability, x-axis: number of amino acid). Potential transmembrane helices of JM4 and JWA are shown in red (TMHMM (v. 2.0)) (123).

(B) HeLa cells were transfected with plasmids encoding VSV-tagged JM4, myc-tagged JWA, or HA-tagged GTRAP3-18 (rat). To analyze localization of the proteins, cells were immunostained using the anti-VSV, rabbit anti-myc, or rabbit anti-HA antibodies, respectively, followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated (red) anti-mouse or FITC-conjugated (green) anti-rabbit antibodies. Colocalization is indicated by yellow color in the merged pictures.

(C) HeLa cells were transfected as indicated (top: VSV-JM4 and myc-JWA; middle: VSV-JM4 and GTRAP3-18-HA; bottom: GTRAP3-18-HA and myc-JWA). For immunodetection appropiate antibodies were used (top: anti-VSV, rabbit anti-myc; middle: anti-VSV, rabbit anti-HA; bottom: rat anti-HA, rabbit anti-myc) followed by incubation with FITC-anti-rabbit and TRITC-anti-mouse or -rat antibodies.

(D) HEK-293 cells were transfected with plasmids encoding VSV-JM4, myc-JWA, GTRAP3-18-HA or empty vector control as indicated. Subsequently cells were lysed and immunoprecipitates were prepared using either anti-VSV, rat anti-myc or mouse anti-HA antibodies. Proteins were size-separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with anti-VSV, rat anti-myc or rabbit anti-HA antibodies as indicated.

IP, immunoprecipitation; DL, direct lysate; α, anti)

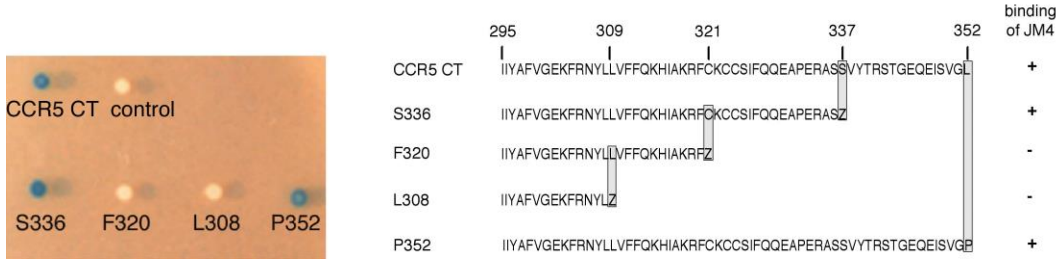
Fig. 8. Interaction of CCR5 with JM4 and JWA

HEK-293 cells stably expressing synCCR5 (as shown in Fig. 5B) were transfected with VSV-JM4 or myc-JWA expressing plasmids. After cell lysis CCR5 was precipitated using a mouse anti-CCR5 antibody. Proteins were size-separated by 12.5 % SDS-PAGE, transferred to a nitrocellulose membrane. VSV-JM4 and myc-JWA were detected using the anti-VSV and rabbit anti-myc antibodies, respectively.

DL, direct lysate; IP, immunoprecipitation; l.c., light chain

Figure 1

A



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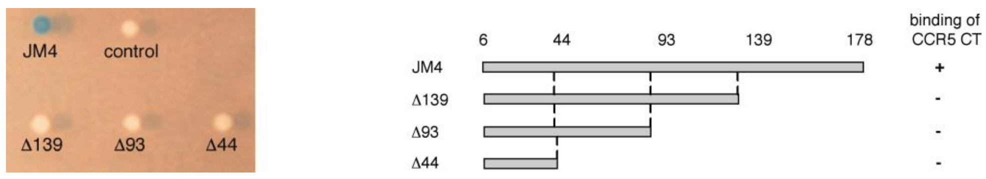

Figure 2

A

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1/1                                         31/11
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 M   S   E   V   R   L   P   P   L   R   A   L   D   D   F   V   L   G   S   A
61/21                                       91/31
cgt ctg gcg gct ccg gat cca tgc gac ccg cag cga tgg tgc cac cgc gtc atc aac aac
 R   L   A   A   P   D   P   C   D   P   Q   R   W   C   H   R   V   I   N   N
121/41                                      151/51
ctc ctc tac tac caa acc aac tac ctt ctc tgc ttc ggc atc ggc ctc gct ctc gcc ggg
 L   L   Y   Y   Q   T   N   Y   L   L   C   F   G   I   G   L   A   L   A   G
181/61                                      211/71
tac gtg cgg cca ctt cat acg ctc ctg agc gcg ctg gta gtg gcg gtg gcc ctc ggc gtg
 Y   V   R   P   L   H   T   L   L   S   A   L   V   V   A   V   A   L   G   V
241/81                                      271/91
ctg gtg tgg gca gct gag acc cgc gca gct gtg cgc cgc tgc cgc cgc agc cac cct gca
 L   V   W   A   A   E   T   R   A   A   V   R   R   C   R   R   S   H   P   A
301/101                                     331/111
gcc tgc ctg gcc gca gtg ctt gcc gtc ggc ctc ctg gtg ctc tgg gtc gcg ggc ggc gct
 A   C   L   A   A   V   L   A   V   G   L   L   V   L   W   V   A   G   G   A
361/121                                     391/131
tgc acc ttc ctg ttc agc atc gcc ggg ccg gtg ctt ctg atc ctg gtg cac gcc tcg ttg
 C   T   F   L   F   S   I   A   G   P   V   L   L   I   L   V   H   A   S   L
421/141                                     451/151
cgc ctg cgc aac ctt aag aac aag att gag aac aag atc gag agc att ggt ctc aag cgg
 R   L   R   N   L   K   N   K   I   E   N   K   I   E   S   I   G   L   K   R
481/161                                     511/171
acg cca atg ggc ctg cta cta gag gca ctg gga caa gag cag gag gct gga tcc tag
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B

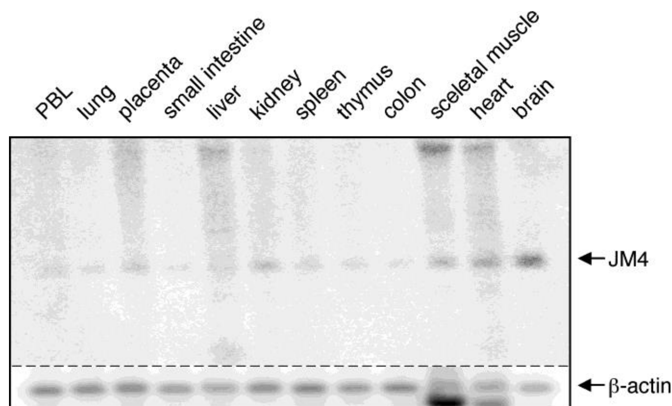

Figure 3

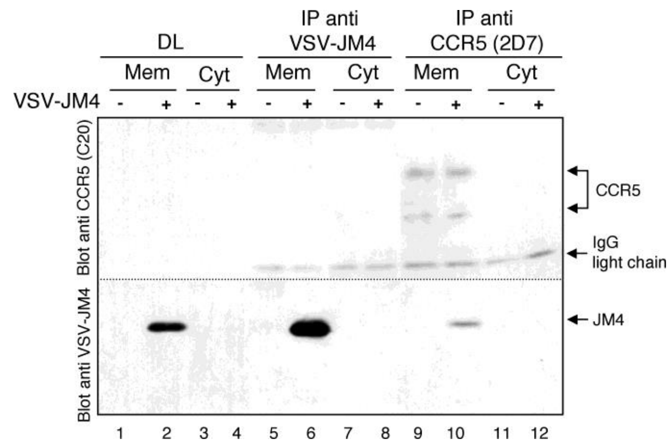

Figure 4

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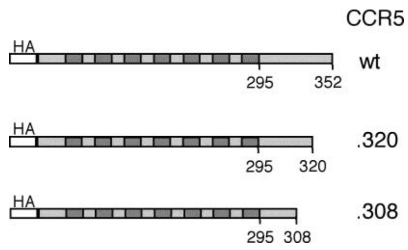

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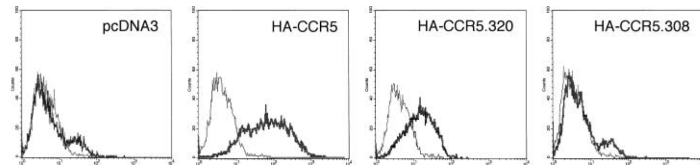

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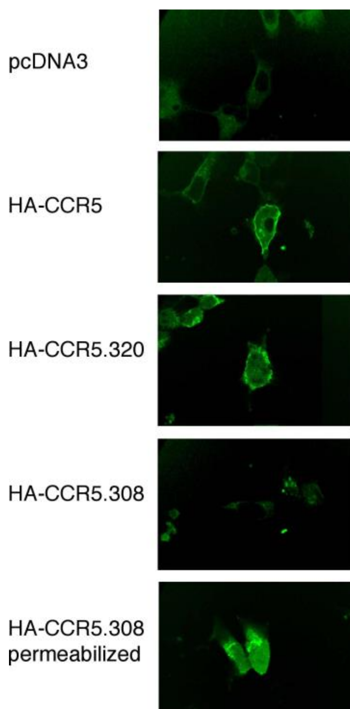

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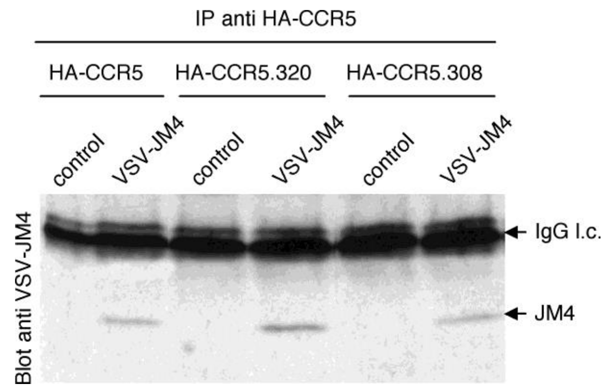

Figure 5

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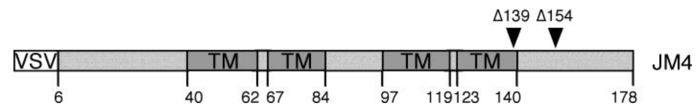

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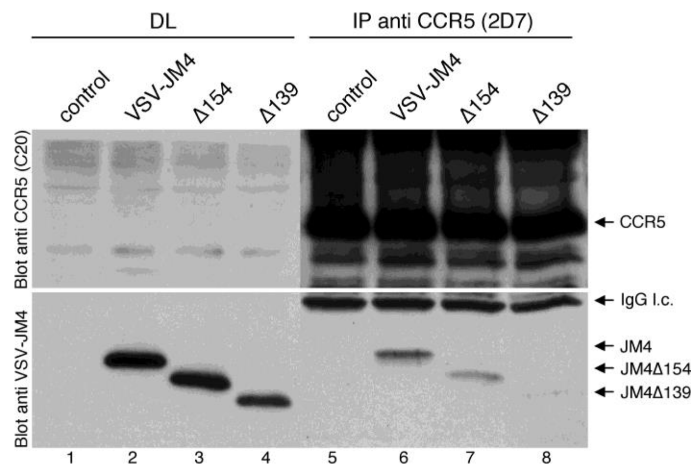

Figure 6

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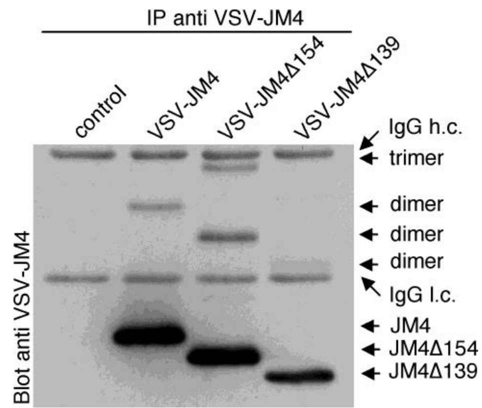

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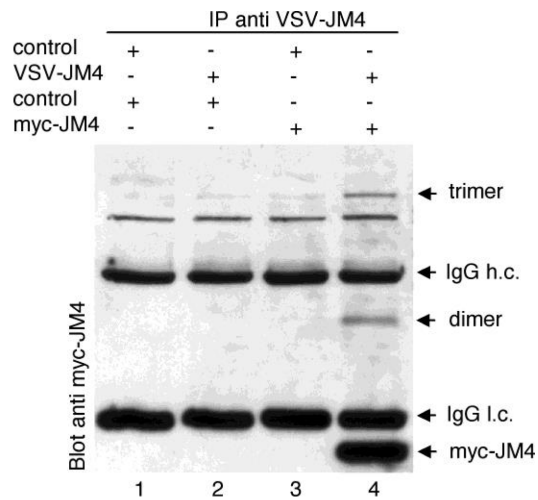

Figure 7

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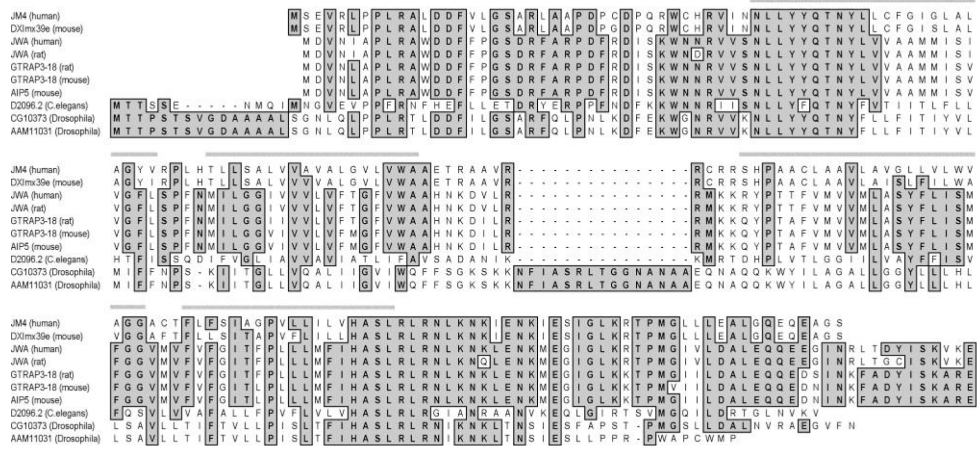

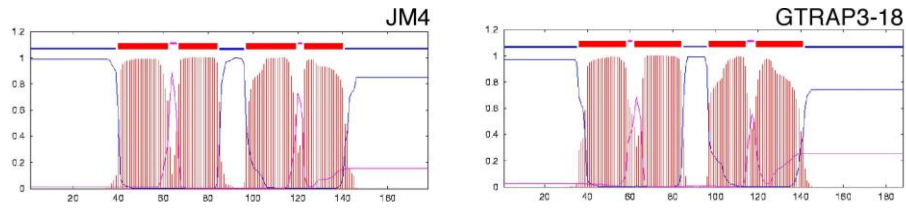

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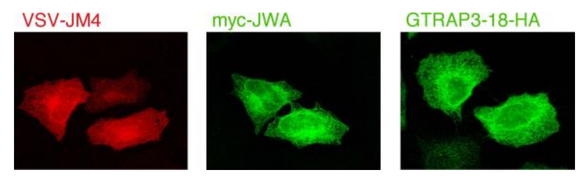

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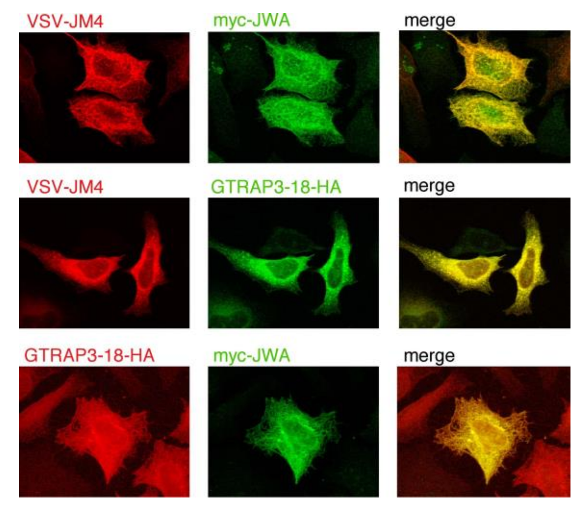

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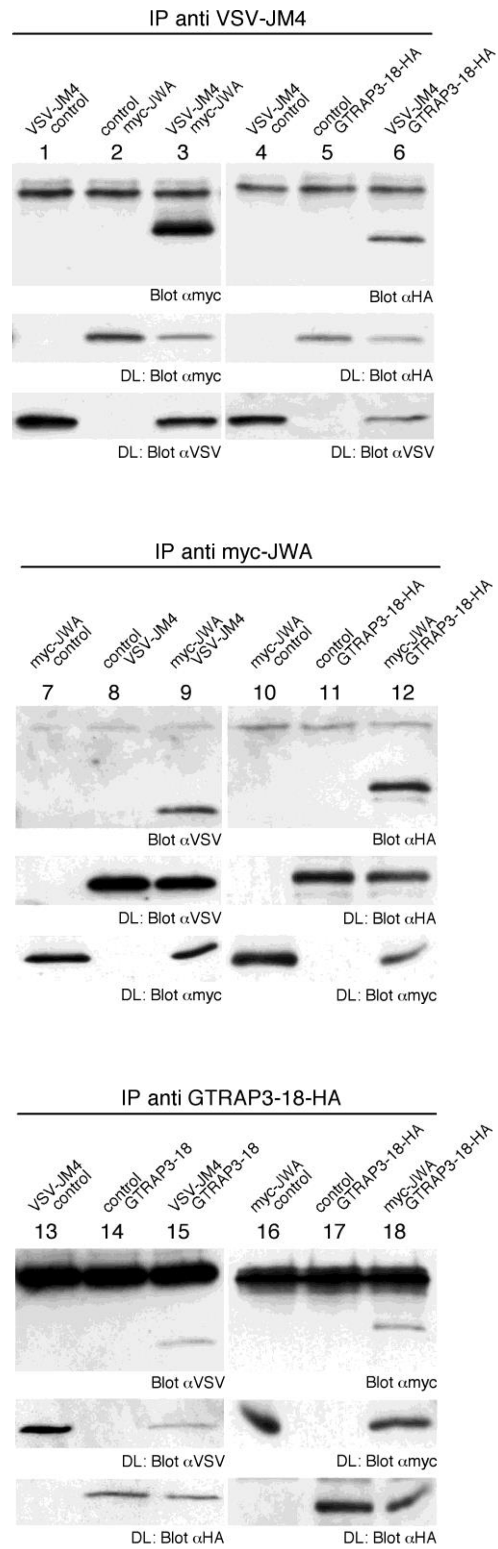
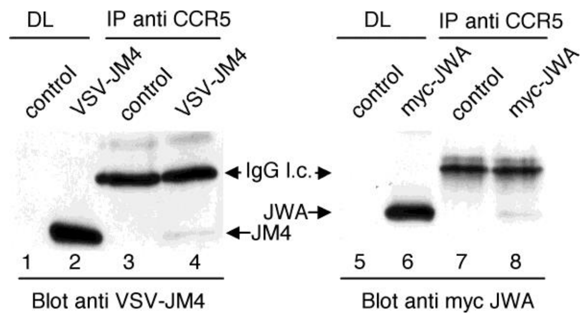


Figure 8



2.3 Influence of α-catenin and JM4 on CCR5-mediated HIV-1 entry

CCR5 is most prominent as HIV-1 coreceptor and plays an important role in primary infection and viral pathogenesis. We identified two proteins, JM4 and α-catenin, to interact with this receptor. In further studies we want to analyze the effect of these proteins on HIV-1 entry and replication.

2.3.1 Effect of wild-type and mutant JM4 expression on HIV-1 infection in HEK-293 cells

JM4 shares sequence and structural similarities with GTRAP3-18 (in detail described in chapter 2.2). During the course of these studies the glutamate transporter EAAC1-associated protein (GTRAP3-18) was identified to interact with the ten-transmembrane soanning excitatory amino acid carrier 1 (EAAC1) (122). It was shown that this interaction regulates the transporter function. Overexpression or upregulation of GTRAP3-18 led to inhibition of EAAC1-mediated glutamate transport. Binding of GTRAP3-18 resulted in a decreased affinity of the transporter to its substrate, possibly explained by GTRAP3-18-dependent modulation of the EAAC1 conformation.

Agonist binding to CCR5 was shown to induce a conformational change of the receptor molecule (159). Given the close similarity of GTRAP3-18 and JM4 we anticipated JM4 to regulate CCR5 receptor function in a similar fashion. We analyzed the effect of JM4 overexpression on HIV-1 infection. In a first attempt we used the HEK-293 cell system (Fig.10). Cells were transiently transfected with plasmids encoding the HIV-1 coreceptors CCR5 and CD4 to render the cells susceptible to infection. JM4 was coexpressed to analyze its effect on HIV-1 infection. Besides the JM4 wild-type protein we included a mutant JM4 protein (JM4.mut). A single amino acid in the C-terminal "SIGL" sequence of JM4 wild-type was substituted resulting in a glycine to arginine substitution at amino acid position 157 by site-directed mutagenesis. This motif was mutated, since the "SIGL" sequence resembles the conserved "GLGF"-motif of PDZ domains and may be involved in a PDZ-like interaction of JM4 with the C-terminal leucine of the CCR5 receptor. The JM4 mutant should therefore no longer bind to CCR5 and could titrate out endogenous JM4 due to its ability to form multimers.

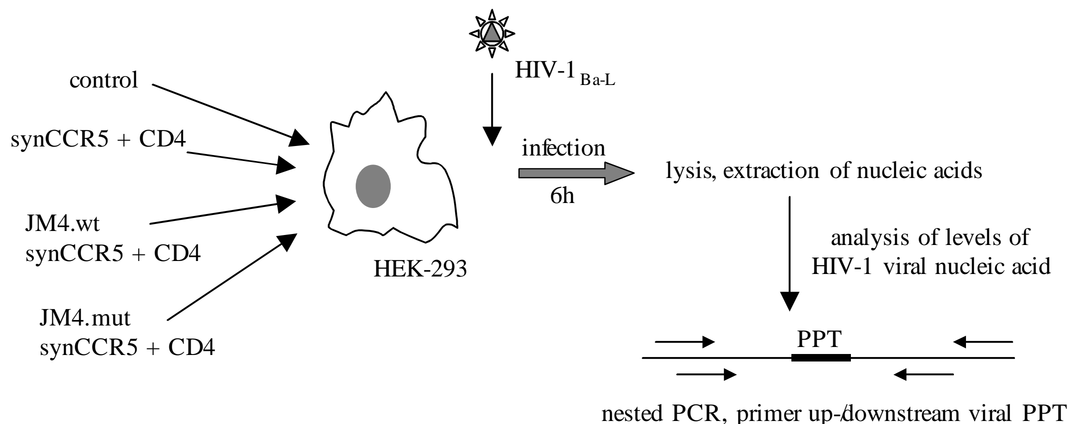


Fig. 10. The cellular system to analyze the effect of JM4 on HIV-1 infection is depicted. HEK-293 cells were cotransfected with CCR5-, CD4-, and JM4-encoding plasmids as indicated. Empty vector was used as a control. After protein expression the cells were challenged with virus HIV-1_{Ba-L} (virus dilutions of 1:10 and 1:100) for six hours, subsequently lysed and the nucleic acids were extracted. The level of HIV-1 infection was monitored by analysis of viral DNA using nested PCR. As a positive control, DNA extracts from persistently infected PM1 cells were used. Abbreviations used are: syn, codon-optimized for mammalian expression; HEK, human embryonic kidney; PPT, polypurine tract; PCR, polymerase chain reaction.

After expression of the proteins the cells were challenged with two concentrations of the virus strain HIV-1_{Ba-L}. This strain utilizes CCR5 as coreceptor for cellular entry (160). After infection the cells were washed extensively to remove excessive virus and the nucleic acids were extracted from the lysed cells (Qiagen). The level of viral DNA reflects the efficiency of virus entry and was analyzed by nested polymerase chain reaction (PCR) using specific primers up- and downstream of the highly conserved viral polypurine tract (PPT). The result of this experiment is shown in Fig. 11. As expected, efficient infection required expression of both receptors, CCR5 and CD4 (lanes 5/6). The coexpression of JM4 wild-type did not affect HIV-1 infection (lanes 7/8). Yet, expression of mutant JM4 protein interfered with infection, since less viral nucleic acid was detectable (lanes 9/10) when compared with control or JM4 encoding plasmids transfected cells. Overall, the level of infection correlated with the dilution of virus, arguing for quantitative detection of HIV-1 specific signals. No HIV-1 PPT-specific PCR products were detectable in the PCR-Mastermix, in extracts of uninfected control cells, and cells transfected with empty vector control (lanes 1, 2 and 3/4, respectively). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control showed no variations of nucleic acids in the various lysates. The PCR was repeated with the same nucleic acid extracts to confirm the results (lower panel).

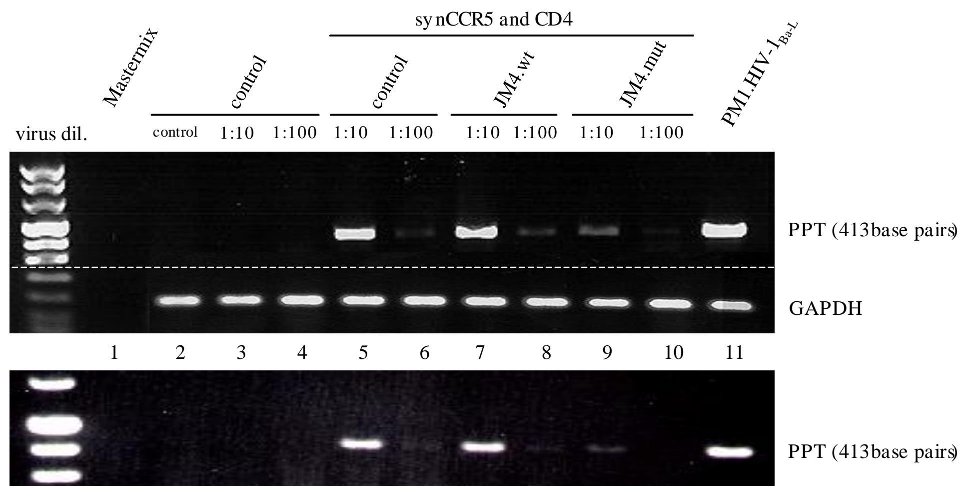


Fig. 11. Analyis of the influence of JM4 and a JM4 mutant on HIV-1 infection. As described in Fig. 10, HEK-293 cells were used to analyze HIV-1 infection . The levels of HIV-1_{Ba-L} viral nucleic acids after infection were analyzed by nested PCR using primers up- and downstream of the highly conserved PPT situated in the *nef* gene. For positive control PCR lysates of PM1 cells infected with HIV-1_{Ba-L} were included (lane 11). PCR products generated with primers for GAPDH reflect that the amount of the nucleic acids in each sample is constant. To control these PCR-results the reaction was repeated (lower panel). Abbreviations used are: PPT, polypurine tract; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

This experiment shows that a mutant of JM4 was able to interfere specifically with CCR5-mediated HIV-1 infection. However, we were not able to reproduce this result in similar settings, possibly due to the fact, that three proteins needed to be coexpressed to physiological levels. Furthermore, a quantitative evaluation of viral entry efficiency by PCR was difficult to establish and to reproduce.

2.3.2 Effect of JM4, α-Catenin, and mutants on HIV-1 infection in HIV-1 indicator cell lines

Another alternative to the HIV-1 infection using natural HIV-1 host cells and a possibilty to quantify HIV-1 infection is the use of the indicator cell lines derived from HeLa cells, P4.2 and P5 cells (161). HeLa cells, which express CXCR4 endogenously were stably transfected with the HIV-1 receptor CD4 (P4.2, second generation of P4 cells). The P5 cells express furthermore the CCR5 receptor, rendering them susceptible to X4 and R5 virus strains (162, 163). R5 virus strains depend on CCR5 as a coreceptor whereas X4 virus strains utilize CXCR4 as coreceptor. Infection of these cell lines ultimately leads to expression of the viral Tat protein (transactivator of transcription), which, in turn, will

initiate LTR-dependent transcription of the *lacZ* reporter gene, which is linked to a viral LTR promoter (Fig. 12).

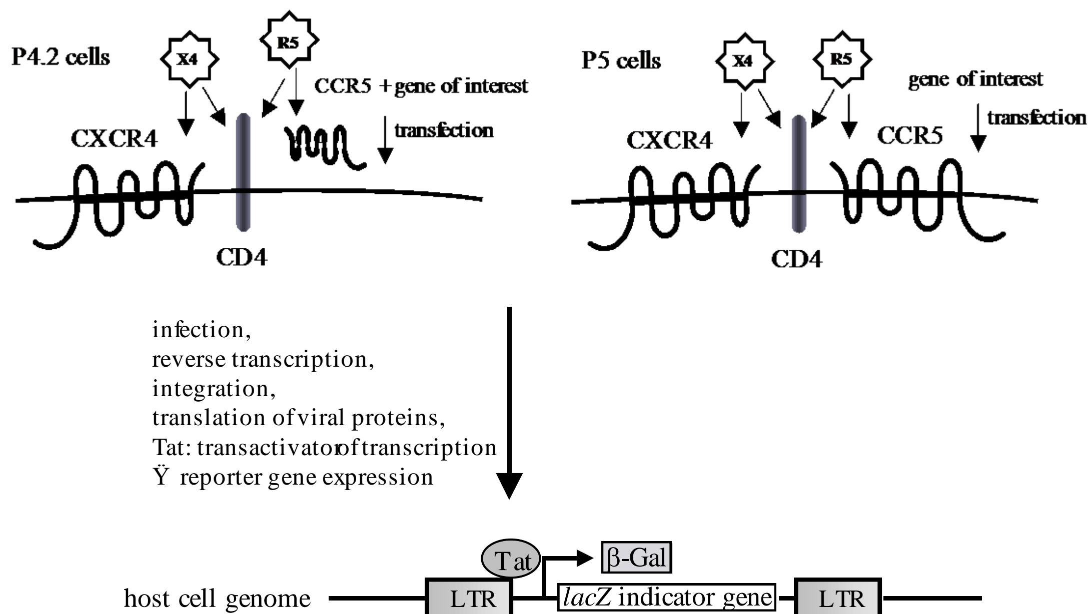


Fig. 12. Indicator cell lines P4.2 and P5 used to quantify HIV-1 infection. P4.2 and P5 differ in the expression of CCR5. For infection of P4.2 cells with R5 virus, the CCR5 receptor is coexpressed with the gene of interest prior to infection. Infection leads to expression of the viral Tat protein, which activates LTR-dependent transcription of an indicator gene, e.g. *lacZ*.

HIV-1 infection can be determined quantitatively with ELISA against β-galactosidase (β-Gal) expressed in infected cells. We used P4.2 as well as P5 cells to monitor the influence of JM4 or α-catenin on HIV-1 infection of R5 virus. To render P4.2 cells susceptible for infection with R5 viruses we cotransfected plasmids encoding CCR5 together with the gene of interest. The benfit of this approach is that only cells coexpressing JM4 or α-catenin were susceptible to CCR5-mediated HIV-1 infection due to cotransfection. A drawback was the lack of stable reproducibility since coexpression of two proteins is required.

2.3.2.1 Infection of P5 HIV-1 indicator cell lines

Prior to infection, the P5 cells were transfected with plasmids encoding JM4 or α-catenin. We included the C-terminal deletion mutants of JM4 (JM4Δ154 and JM4Δ139, chapter) lacking the potential binding site of JM4 with CCR5 (as described in chapter 2.2). To

evaluate the effect α-catenin in addition to overexpression, we expressed only the C-terminal domain of the protein, which still interacts with CCR5, but lacks the N-terminal domain required for association with the cytoskeleton. This mutant could therefore resemble a dominant-negative mutant, blocking the binding of functional endogenous α-catenin to the receptor. After expression of the proteins, the cells were challenged with HIV-$1_{Ba\text{-}L}$. Subsequently virus was replaced with fresh cell growth medium and the cells incubated for 48h to allow the virus to reverse transcribe its genome, integrate into the host cell genome and express viral proteins. The viral protein Tat (transactivator of transcription) in turn transactivates LTR-dependent transcription of the indicator gene *lacZ*, resulting in β-Gal expression. Cells were lysed and β-Gal expression quantified by coloriometric detection of β-Gal activity using ELISA according to the manufacture's instructions (Roche). The results are presented in Fig. 13. Infection of the cells led to quantitative expression of β-Gal, less infection assay resulted in less expression of β-Gal. Cells expressing JM4, α-catenin, or mutants did not show a significant difference in susceptibility to HIV-1 infection when compared with vector control (v.c.) transfected cells. The mock-uninfected cells reflect background level of β-Gal expression.

Since transfection efficiency in these experiments was about 50 % using FuGENE reagent, according to the manufacturer's protocol (Roche), we cannot prevent infection of cells not expressing the genes of interest in this cellular system. HIV-1 does not yield high titers of infectious virus, which makes it impossible to infect sufficient amount of cells to rule out the possibility that the virus "bypasses" transfected cells, only infecting non-transfected cells. Therefore, a possible influence of JM4, α-catenin, and mutants thereof might not be detectable in this cellular system.

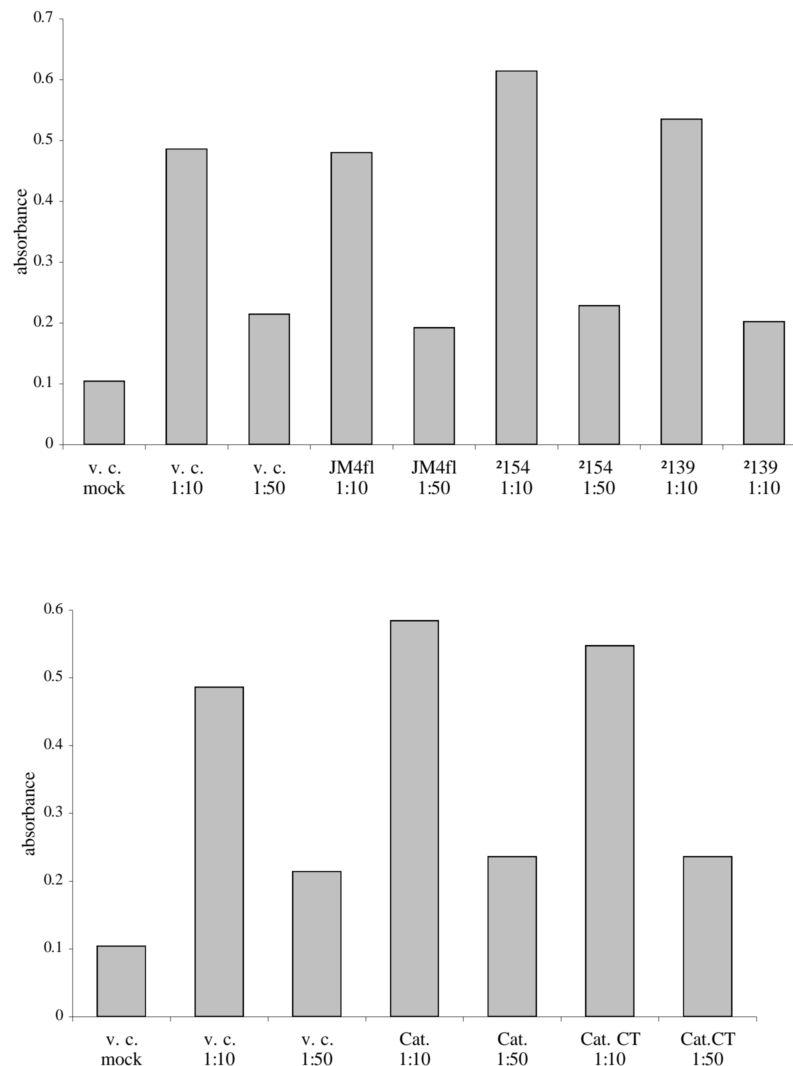


Fig. 13. To evaluate the effect of JM4, α-catenin, or mutants on HIV-1 infection the indicator cells P5 were transfected as indicated. After protein expression the cells were challenged with virus (dilution 1:10) for 4 hours. The amount of HIV-1 infection-dependent *lacZ* gene expression (compare to Fig. 12) was quantitatively measured as β-galactosidase enzyme activity using a colorimetric assay. The absorbance [A_{405}] is indicated on the y-axis of the chart. The upper chart corresponds to cells expressing JM4 or mutants, the lower one to expression of α-catenin or mutants. Abbreviations used are: v.c., vector control; fl, full-length; Cat., α-catenin; CT, C-terminus.

2.3.2.3 Infection of P4.2 HIV-1 indicator cell lines

The P4.2 cells do not express a receptor, which mediates R5 virus strain entry. Therefore, these cells were used to render only those cells susceptible to virus infection, which expressed the gene of interest. This was accomplished by cotransfection of the CCR5 receptor with JM4, α-catenin, or mutants prior to challenge with virus. After protein

expression, cells were challenged with HIV-1_{Ba-L}. Two days after infection the β-Gal expression was measured using a β-Gal ELISA kit. The results are depicted in Fig. 14.

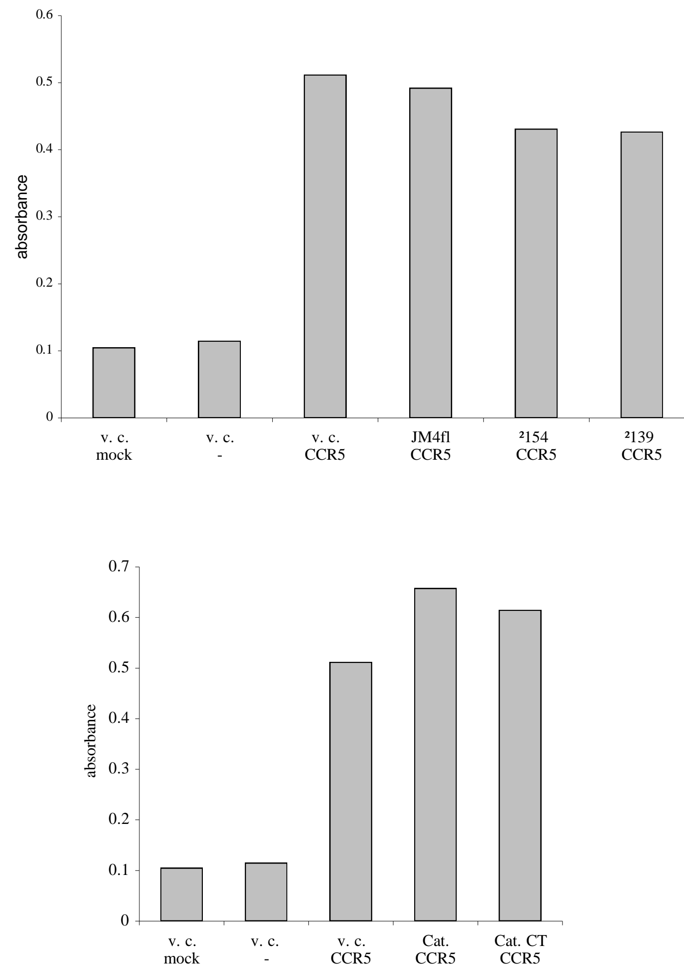


Fig. 14. The P4.2 cells were used to determine the influence of JM4, α-catenin, and mutants on HIV-1 infection in indicator cell lines. Cells were transfected for expression of proteins as indicated. Celles were incubated with HIV-1_{Ba-L} (virus diluton 1:10) for 4 hours. The quantity of β-galactosidase expression (y-axis) correlates with efficiency of HIV-1 infection (compare to Fig. 14). The upper chart corresponds to cells expressing JM4 or mutants, the lower one to expression of α-catenin or mutants. Abbreviations used are: v.c., vector control; (-), no CCR5 expression; fl, full-length; Cat., α-catenin; CT, C-terminus.

Again, β-Gal background expression levels were determined in mock-uninfected cells. Expression of CCR5 was required for HIV-1_{Ba-L} infection, since virus challenge of cells lacking CCR5 expression (-) did not lead to HIV1-dependent expression of β-Gal. No effect of JM4, α-catenin, and mutants was detected on HIV-1 infection in this setting. This reflects the possible importance of the expression ratios of endogenous and

exogenous proteins involved in the process of HIV-1 infection. We believe that the natural host cell environment, which includes different components and compositions of cellular factors and proteins, is crucial for analysis of the impact of CCR5-interacting proteins on HIV-1 infection.

2.4 The interaction of CCR5 and JM4 is not mediated by a classical PDZ-domain

As described the C-terminus of CCR5 is involved in multiple receptor properties as signal transduction, trafficking and HIV-1 infection. We wanted to identify novel CCR5-interacting proteins that might be involved in the regulation of receptor function. The very C-terminal leucine of the receptor constitutes a potential binding site for PDZ proteins. PDZ-domain containing proteins are known to bind and regulate diverse transmembrane proteins. Since the C-terminus embodies a promising target for such proteins we used the cytoplasmic tail of CCR5 as bait in a yeast two-hybrid assay. We screened a human B cell cDNA library and identified two novel interacting proteins, JM4 and α-catenin (in detail described in chapters 2.1 and 2.2).

PDZ-domain-containing proteins have a hydrophobic pocket through which they bind other proteins. The sequence motif GLGF within this hydrophobic pocket interacts with the very C-terminal motif of target proteins, thus mediating interaction and clustering of proteins (27, 28). Sequence analysis of JM4 revealed a motif that might resemble the GLGF sequence of many other PDZ-proteins. This consensus-sequence motif SIGL (aa 155-158) is conserved in JM4-related proteins throughout different species, which indicates a high degree of conservation.

A JM4-deletion mutant, that lacks this region (JM4Δ139, chapter 2.2) led to abrogation of the interaction with the C-terminus of CCR5 in a yeast-two hybrid assay.

To analyze whether JM4, to date an uncharacterized protein, interacts with the wild-type CCR5 in a PDZ-like manner, a mutation was introduced in the JM4 protein. The glycine within the conserved SIGL-motif was changed to arginine (SIRL mutant). Furthermore, we substituted the very C-terminal leucine (L) of wild-type CCR5 with proline (P). All mutations were introduced by site-directed mutagenesis to generate expression plasmids

encoding CCR5 and JM4 mutant proteins. All proteins were tested for interaction by coimmunoprecipitation and subsequent Western blot analysis.

VSV-epitope tagged JM4 wild-type (wt) or mutant (mut) proteins were expressed in HEK-293 cells or HEK-293 cells stably expressing the CCR5 receptor. After cell lysis we performed immunoprecipitation with antibodies directed either against VSV-epitope (lanes 1-4) or CCR5 (lanes 5-8). Immunocomplexes were then analyzed for coprecipitated proteins in a Western immunoblot.

As shown in Fig. 15, change of the JM4 SIGL-motif to SIRL did not significantly alter the interaction with CCR5. After immunoprecipitation of the VSV-epitope tagged JM4 wild-type or mutant the CCR5 receptor was detectable in the immunocomplex (lanes 3 and 4). In addition, JM4 wild-type or mutant were both detected in the immunoblot after immunoprecipitation of the receptor (lanes 7 and 8). Therefore, substitution of SIGL to SIRL in JM4 did not affect the association of JM4 to the receptor.

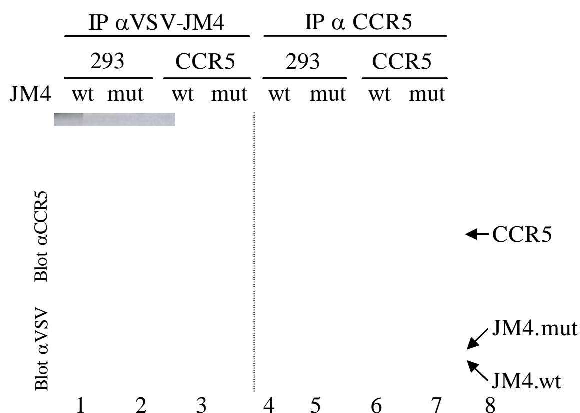


Fig. 15. Interaction of CCR5 with wild-type or mutant JM4. HEK-293 cells and HEK-293 cells stably expressing codon-optimized CCR5 were transfected with plasmids encoding VSV-JM4 wild-type (wt) or mutant (mut). Cells were lysed and proteins were immunoprecipitated using anti (α) VSV or CCR5 antibidies as indicated. After immunoprecipitation of JM5.wt or JM4.mut, CCR5 can be detected in the immunocomplex (lanes 3 and 4, respectively). Similarly, JM4.wt or JM4.mut coprecipitate with CCR5 (lanes 7 and 8, respectively).

The mutant JM4 migrates slightly higher in the SDS-polyacrylamide gel. This is most likely due to a conformational change of the protein.

Furthermore, JM4 was tested for interaction with a mutant CCR5 receptor, in which the very C-terminal leucine was substituted with proline, termed CCR5.L/P (Fig. 16). HEK-293 cells or HEK-293 cells stably expressing wild-type or mutant receptor were transfected with plasmids encoding VSV-epitope tagged JM4. After protein expression the cells were lysed and the protein interactions were analyzed by coimmunoprecipitation and subsequent Western blot analysis.

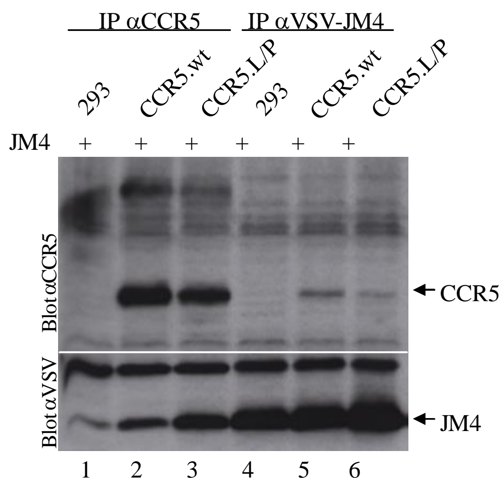


Fig. 16. Interaction of JM4 with wild-type or mutant CCR5. HEK-293 cells and HEK-293 cells stably expressing codon-optimized CCR5 wild-type (wt) or a receptor with C-terminal substitution of leucine with proline (L/P) were transfected with VSV-epitope tagged JM4. Cells were lysed and proteins were immunoprecipitated as indicated. JM4 co-precipitates with CCR5.wt and CCR5.L/P (lanes 2 and 3, respectively). Furthermore, CCR5.wt and .L/P can be detected in the immunoblot after immunoprecipitation of VSV-JM4 (lanes 5 and 6, respectively).

When compared with CCR5 wild-type the mutant CCR5.L/P exhibited a rduced ability to interact with JM4. This is shown in Fig. 16 by coprecipitation of JM4 with CCR5 or CCR5.L/P (lanes 2 and 3, respectively) and coprecipitation of the receptor molecules with JM4 (lanes 5 and 6, respectively).

The substitutions of single amino acids in JM4 or CCR5 resulted only in some reduced CCR5-JM4 interaction. Therefore, we decided to partly delete potential PDZ-domain interaction sites within the C-termini of CCR5 and JM4 and tested for interaction. These analyses are described in chapter 2.2. Even though the deletion of a potential PDZ-domain on JM4 and a putative binding site on CCR5 did not completely abolish the association of JM4 and CCR5, we cannot conclude that these proteins interact in a "classical" PDZ-dependent manner.

The interaction was mediated by the very C-terminal domain of CCR5 in yeast, whereas in mammalian cells deletion of the cytoplasmic domain of CCR5 did not abrogate the complex formation with JM4. This suggests a second intracellular binding or docking site for JM4 on CCR5, which has been shown for other CCR5-interacting proteins, e.g. β-arrestin. Different interaction domains might be involved in diverse intermediate steps of association, thereby regulating the interaction. The overall CCR5-JM4 association might not be influenced by a C-terminal deletion due to the second binding site and the interaction between the CCR5 C-terminus and JM4 might partly be mediated by a PDZ-like domain. Furthermore, the substitution of the very C-terminal leucine with proline might not be sufficient to interfere with a PDZ-dependent interaction.

2.5 Regulation of JM4 expression by retinoic acid?

The JM4 protein shares 42 % identity and an additional 21 % similarity with human JWA protein. JWA has been deposited in the database as a novel differentially displayed vitamin A responsive gene that is cytoskeleton related (NCBI Protein Database = NCB Accession # AAC64360). However, so far no biological role has been described for the protein. During the course of our study the rat homologue of JWA was identified in a yeast-two hybrid screen performed by the group of Dr. Rothstein (122). This protein was shown to bind the excitatory amino acid carrier 1 (EAAC1), therefore termed glutamate transporter EAAC1-associated protein, GTRAP3-18. Overexpression of GTRAP3-18 results in negative regulation of the EAAC1-mediated glutamate transport by decreasing the transporters affinity to the amino acid. A second study showed similar results for the mouse GTRAP3-18 protein (156). As suggested for JWA, the rat GTRAP3-18 protein expression was shown to be upregulated by RA (122).

Retinoic acid (RA) is member of the retinoids, which are derived from vitamin A (retinol). RA has been shown to be involved in the regulation of cellular processes, such as cell division, differentiation, development and others (164-166). RA is obtained from the blood, where it circulates as retinol bound to retinol-binding protein. Intracellulary retinol is rapidly metabolized to RA. There are two isomers of RA, namely all-*trans*-RA and 9-*cis*-RA. These enter the nucleus to regulate gene activity by binding to retiniod receptors, which act as transcription. At least six retinoid receptors are known, they are subdivided into two subfamilies: the RA receptors (RARs) α, β and γ, and the retinoid X receptors (RXRs) α, β and γ (167). The 9-*cis*-RA serves as a ligand for the RARs and RXRs, whereas all-*trans*-RA only activates RARs (168). The RARs and RXRs are members of the steroid/thyroid hormone superfamily. The ligand-occupied receptors can bind to hormone responsive elements (HREs) or retinoic acid responsive elements (RAREs) within the regulatory region of target genes (168). The agonist binding furthermore induces a conformational change, which in turn facilitates the association of co-activators or -repressors involved in gene regulation (169, 170).

The close similarities of JM4, JWA and its rat homologue GTRAP3-18 at amino acid level, structure, cellular distribution, and localization (compare to chapter 2.2) suggest that RA might have been a useful tool to upregulate endogenous JM4 expression in order

to analyze its effect on CCR5 receptor function in cells naturally expressing the receptor. We tested the effect of RA on JM4 expression in different cell lines - HEK-293 (human embryonic kidney), HeLa (cervix adenocarcinoma), SH-SY5Y (neuroblastoma), PM1 (T lymphocytes). Cells were grown in medium containing all-*trans*-RA. Since no antibody for the detection of JM4 protein was available we analyzed the level of JM4 transcripts by Northern blot. Similar amounts of sample RNA were size-separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then hybridized and subsequently exposed to imaging plates. Plates were read with a PhosphorImager (Amersham Biosciences) and analyzed using ImageQuant software.
Blots were stripped and reprobed with a β-actin probe to confirm that RNA samples were present in equivalent amounts. A representative result for the influence of RA on JM4 transcription is shown in Fig. 17 with SH-SY5Y cells.

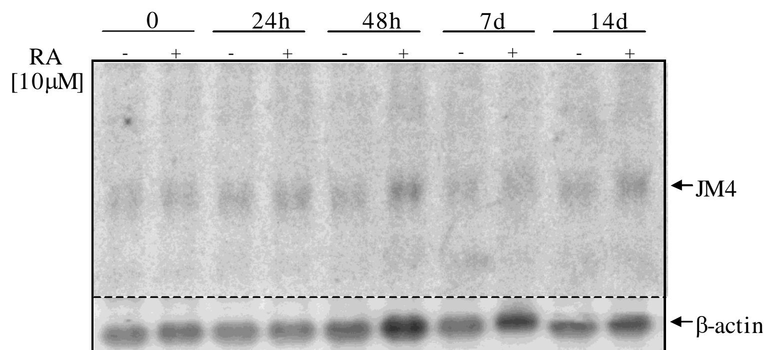


Fig. 17. Influence of retinoic acid (RA) on transcription of JM4 in SH-SY5Y cells. The cells were grown for the indicated time in medium containing either 10 μM RA (Sigma, solubilized in DMSO) or carrier as control. The medium containing RA was changed every 24h. Nucleic acids were extracted using TRI REAGENT according to manufacturer's protocol (Sigma) and mRNA level of JM4 was analyzed by Northern blot with a [α-^{32}P]-dCTP labeled probe, which was prepared by random priming of a 0.95-kb fragment of JM4 using RadPrime DNA labeling system (Invitrogen) as described in the manufacturer's protocols. After analysis of JM4 levels the blot was stripped and reprobed with a β-actin probe to compare loading in each lane.

Retinoic acid did not induce an increase of JM4 transcripts in SH-SY5Y cells. We performed similar experiments in other cells as mentioned above, but did not see an effect of RA on JM4 expression.
Just recently a follow-up on the initial study on GTRAP-18 was published (171). It was reported that the RA used in the initial study for upregulation of GTRAP3-18 was complexed with methyl-β-cyclodextrin (MeβCD). This macrocyclic polysaccharide is

commonly used to enhance solubility of hydrophobic compounds such as RA. The authors could show that rather MeβCD than RA was accountable for the upregulatory effect. This finding is in accordance with our results, where RA did not affect the transcript of JM4.

2.6 Influence of JM4 on CCR5 chemokine receptor function

Chemokines are regulatory molecules that play a pivotal role in the activation and recruitment of cells of the immune system, e.g. the maturation of leukocytes, trafficking and homing of lymphocytes and the development of lymphoid tissues (43). The binding of chemokines to members of the G protein-coupled receptor family triggers diverse signaling pathways as described above (chapter 1.1). Our aim was to analyze the effect of the VSV-JM4 protein on chemokine receptor function of CCR5. Therefore, we employed HEK-293 cells to study the CCR5-mediated cellular actin polymerization and internalization of the receptor.

2.6.1 Does JM4 influence receptor-mediated actin polymerisation?

Activation of chemokine receptors leads to diverse cellular responses, which are mediated in part by heterotrimeric G proteins that activate a variety of downstream effectors, e.g. members of the Rho subfamily of small GTPases Rho, Rac, and Cdc42. These proteins have emerged as key regulators for assembly and organization of the actin cytoskeleton, which mediates a variety of essential biological functions as it forms the structural framework around which cell shape and polarity are defined. The dynamic structures enable cells to migrate and to divide (172-174).

The initial polymerization of globular G-actin to filamentous F-actin is required for chemotaxis (175, 176). This polymerization leads to the formation of actin filaments immediately adjacent to the plasma membrane at the tip of the leading edge of the cell. The polymerized actin fibers force the membrane outwards and the so-formed filopodia and lamellipodia adhere to the extracellular matrix, enabling the cell to migrate along a chemokine gradient (177).

The influence of VSV-JM4 on CCR5-mediated cellular actin polymerization was assessed as one read-out for CCR5 receptor activation in HEK-293 cells. Cells stably

expressing codon-optimized CCR5 (synCCR5) were transfected with plasmids encoding VSV-epitope tagged JM4 or control plasmid using Fugene (Roche) according to manufacturer's protocol. One day after transfection the cells were starved over night in culture medium without fetal calf serum (starve medium). Cells were then detached from the culture plates with 2 mM EDTA in phosphate buffered saline (PBS), washed with and resuspended in cold starve medium. For receptor activation cells were incubated with 10 nM of the chemokine CCL5. Actin polymerization can be detected with FITC-conjugated phalloidin, which binds to and stabilizes polymeric F-actin. The reaction was stopped at different time points, the cells stained and washed extensively to remove excessive non-bound phalloidin. The results are shown in Fig. 18.

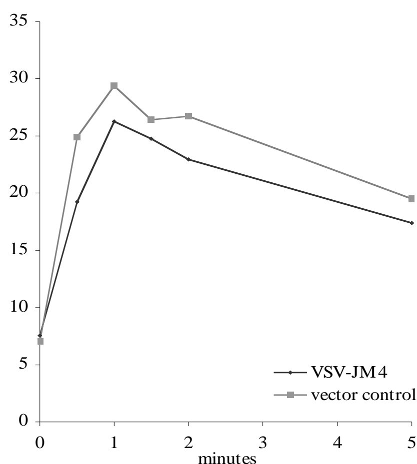


Fig. 18. Analysis of the influence of VSV-JM4 on CCR5 mediated actin polymerization. HEK-293 cells stably expressing codon-optimized CCR5 were transfected with VSV-JM4-encoding plasmid or control. Cells were stimulated with the chemokine CCL5 and reaction was stopped with fix-stain-solution (3.6 % paraformaldehyde, 0.1 μg/μl L-αlysophosphatidylolcholin, 8.25 μg/ml FITC-conjugated phalloidin) after indicated time points (x-axis). Cells were then fixed and stained for 30 min at 4 °C with FITC-conjugated phalloidin to detect polymerized F-actin. The mean fluorescence intensity shown on the y-axis represents the amount of cellular actin polymerization and was quantified by FACS analysis.

The mean fluorescence intensity (MFI, y-axis) reflects the quantity of phalloidin associated with F-actin at a given time point (x-axis) and was measured by FACS analysis. As expected, agonist binding to CCR5 induces rapid polymerization of cellular actin and peaks about 1 min. after addition of the chemokine to CCR5-expressing cells. The transient increase of polymeric F-actin is due to the rapid desensitization of the receptor following its activation.

No significant effect of VSV-JM4 on chemokine activation of CCR5 can be detected in this experimental setting. The overexpression of VSV-JM4 did not significantly alter the extent of actin polymerization hence it might not have a biological effect on ligand-

stimulated CCR5 activation in this biological context. However, these experiments were carried out employing HEK-293 cells stably overexpressing the CCR5 receptor. Therefore, we cannot rule out the possibility that ectopic expression of both proteins might not reflect the appropriate cellular background to analyze the effect of VSV-JM4 on CCR5-mediated actin polymerization. Furthermore, differences in cellular complements of GRK, β-arrestin, or other regulatory proteins could affect receptor activation and cellular responsiveness (7).

2.6.2 Does JM4 regulate CCR5 receptor internalization?

Internalization, also termed endocytosis or sequestration of chemokine receptors is essential to determine the duration of leukocyte activation and migration. After agonist-induced activation and subsequent signal transduction, chemokine receptors are being desensitized (see also chapter 1). In a first step G protein-coupled receptors (GPCRs) need to be phosphorylated by second messenger-regulated kinases (PKA and PKC) and by G protein-coupled receptor kinases (GRKs) leading to "uncoupling" of the receptor from G proteins (7-10). During the course of homologous desensitization, the agonist-occupied receptor is then being phosphorylated by associated GRKs at its C-terminus (178). Heterologous desensitization results from phosphorylation of a non-activated receptor by a kinase activated by a different signaling cascade (179). Phosphorylation within the cytoplasmic C-terminal domain creates high-affinity binding sites for arrestins, which are thought to regulate signal transduction as well as internalization of many heptahelical receptors (14, 15). The clathrin-coated vesicle pathway engaged in the internalization of receptors is directed by β-arrestin. Plasma membrane coated pits consist of the trimeric protein Clathrin, which is recruited to the membrane by the tetrameric adaptor protein-2 (AP-2) (180). AP-2 has been shown to interact with β-arrestin, constituting a functional complex upon receptor stimulation, which is required for internalization (181, 182). The association of AP-2 with the membrane is dependent on membrane phosphoinositides, an integral membrane protein synaptotagmin and specific sorting signals in the cytoplasmic tail of the receptor. Clathrin-coated pit formation requires binding of AP180 to both AP-2 and Clathrin. Formation and detachment of the invaginated coated pit has been shown to require various additional proteins (183).

Receptor internalization might be part of the desensitization process or, as well, serve for resensitization of the receptor, since the intracellular receptor trafficking to endosomal compartments can result in dephosphorylation and recycling to the cell surface (184, 185) or its degradation.

Analysis of receptor internalization was performed in HEK-293 after cotransfection of plasmids encoding codon-optimized synCCRR5 with VSV-epitope tagged JM4 or control. After protein expression cells were challenged with 10 nM of the chemokine CCL5 or carrier as control. Subsequently cells were fixed and incubated with a FITC-conjugated anti-CCR5 antibody raised against the extracellular N-terminus to quantify cell surface expression by FACS analysis (Fig. 19).

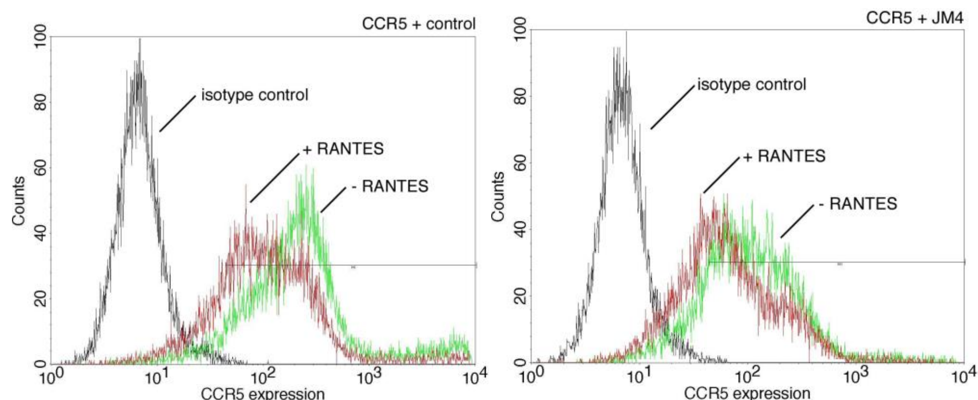


Fig. 19. Quantification of cell surface expression of CCR5 after VSV-JM4 coexpression. HEK-293 cells were transfected with plasmids encoding CCR5 and empty vector control or VSV-JM4. The level of CCR5 expression at the plasma membrane (x-axis) after addition of chemokine (red line) or carrier control (green line) for 30 min at 37 °C was analyzed using a FITC-conjugated anti-CCR5 antibody by FACS analysis. FITC-conjugated control IgG_{2a} (black line) reflects unspecific antibody binding.

The FACS analysis shown to the left was performed with cells expressing CCR5, whereas VSV-JM4 was coexpressed with the receptor in the analysis shown to the right. The level of CCR5 expression at the plasma membrane is shown by fluorescence intensities on the x-axis. Red lines reflect the cells that were incubated with the chemokine in contrast to non-stimulated cells (green lines). To control the specificity of antibody binding a FITC-conjugated control mouse IgG_{2a} was used (black line).

The internalization of CCR5 after chemokine stimulation is illustrated by the shift to the left of the red line when compared with the green one, indicating that less CCR5 receptor is detectable at the cell surface. Comparison of the mean fluorescence intensities (MFI)

showed that 35 % of receptors expressed at the plasma membrane were internalized after chemokine stimulation of cells expressing VSV-JM4 (MFI: 127.3 vs. 196.3), whereas in cells not overexpressing VSV-JM4 (empty plasmid control) 55 % of the CCR5 receptor was internalized (MFI: 267.4 vs. 600). This result indicates a potential influence of JM4 on receptor internalization. Notably, the overall level of CCR5 cell surface expression was reduced by 67 % in cells coexpressing VSV-JM4 when compared with cells expressing no exogenous JM4 (MFI: 196.3 vs. 600). This effect of VSV-JM4 on CCR5 cell surface expression was not seen when stably expressed in HEK-293 cells (data not shown). At this point we cannot say whether the diminished internalization of CCR5 is due to a JM4-regulatory function. It might as well be accountable to the overall reduced cell surface expression of the receptor which we noted after transient coexpression of VSV-JM4. As mentioned above the protein overexpression in HEK-293 clearly does not reflect the natural cellular background of the CCR5 receptor. No physiological levels of CCR5 are expressed and in addition cellular factors involved in the complex regulation of chemokine receptors might be missing. This strongly suggests the study of chemokine receptor biology in endogenous cell systems.

2.7 Generation of anti-JM4 antibodies

Two proteins, JM4 and α-catenin were identified as new interacting proteins of the chemokine- and HIV-1 co-receptor CCR5 in the yeast two-hybrid system. We confirmed interaction of CCR5 with JM4 and α-catenin in human embryonic kidney (HEK)-293 cells after protein overexpression. Endogenous α-catenin was shown to interact with overexpressed CCR5 in HEK-293 cells and with endogenous CCR5 and CXCR4 in the T lymphocyte derived cell line PM1. The chemokine receptor CXCR4 is a member of the heptahelical G protein-coupled receptor family and, like CCR5, was identified to be a HIV-1 coreceptor (55).

JM4 is not described in the literature and no biological function has been assigned to the protein. In order to show the interaction of endogenous proteins and characterize JM4 we raised antibodies directed against the human JM4 in mice.

JM4 encoding sequences were cloned into the pGEX-6P-2 plasmid (Amersham Biosciences) to express a recombinant GST- JM4 fusion protein in bacteria. The full-

length GST-JM4 turned out to be toxic in bacteria, possibly due to the four transmembarne-spanning domains of JM4. Therefore, only the sequences encoding either the 42 N-terminal or the 42 C-terminal amino acids were introduced in the pGEX-6P-2 plasmid. Expression of GST-JM4.NT and GST-JM4.CT was weak and did not yield large amounts of recombinant protein even after employing different solubilization and purification protocols. Best results were achieved using the following protocol.

The plasmids encoding pGEX-6P-2-JM4.NT or .CT were transformed into the bacteria BL21-CodonPlus strain (Stratagene) by heat shock as described in the manufacturer's protocol and grown on LB-agar plates (10 g/l Trypose, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar). Bacteria cultures were grown from a single colony in LB-medium containing antibiotics (ampicillin 100 μg/ml, tetracycline 20 μg/ml, chloramphenicol 100 μg/ml) at 37 °C. The overnight culture was diluted 1:10 and bacteria were grown at 25 °C to an OD_{595} of 0.6-0.8 when protein expression was induced by addition of 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). Bacteria were grown for another 7h, pelleted by centrifugation and resuspended in ice-cold lysis buffer (30 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 % Glycerol, 0.1 % Triton X-100, 1 mM DTT, 1 mM EDTA) complemeted with protease inhibitors (Complete, Roche). All following steps were carried out on ice or at 4 °C. Cells were lysed by sonication (7 cycles for 30'', output 5, constant duty cycle) and cell debris removed by centrifugation. Recombinant proteins were pull-downed from cleared lysates with glutathione sepharose (Sigma), the beads washed extensively with lysis buffer and proteins eluted by addition of 10 mM gluthation at a pH 8.0. Expression and yield of proteins were analyzed after purification by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 20).

The recombinant purified protein was eluted in a total of 500 μl from which an aliquot of 5 μl was loaded on the gel. The fusion proteins GST-JM4.NT and .CT (lanes 2 and 3, respectively) are detectable at higher molecular weight than GST itself (lane 1). The bands of smaller molecular weight indicate that the proteins are partly being cleaved and degraded by proteases. A bovine serum albumin (BSA) protein standard was used to estimate the amount of protein.

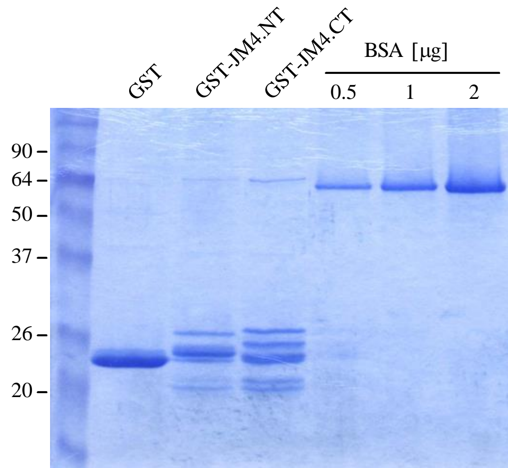


Fig. 20. Analysis of recombinant GST-JM4.NT and .CT purified after expression in bacteria. GST is shown as control. 5 μl of total 500μl of purified recombinant GST-JM4.NT and .CT were analyzed by SDS-PAGE. Partial degradation of the recombinant proteins can be detected. The protein standard BSA serves to estimate the amount of protein. Standard protein molecular weight marker (BenchMark prestained protein ladder, Invitrogen) is shown to the left, molecular weight is indicated in kilo-Dalton (kDa). GST is about 25 kDA of size, the N- and C-terminal fragments of JM4 are about 5kDa of size.

In order to raise antibodies directed against JM4, B57/Bl6 mice were actively immunized in a DNA-prime / protein-boost scheme. First mice were injected with 100 μg of JM4-encoding plasmid intramuscularly, followed by intraperitoneal injection of approximately 1 mg of recombinant GST-JM4.NT or .CT after 14 days. This immunization scheme was repeated every 14 days. After the third administration of protein, blood serum samples were taken. The blood serum samples were tested for their ability to detect JM4 in an immunoblot. A representative Western blot with serum taken from a mouse immunized with plasmid DNA encoding JM4 and recombinant GST-JM4.NT protein is shown (Fig. 21).

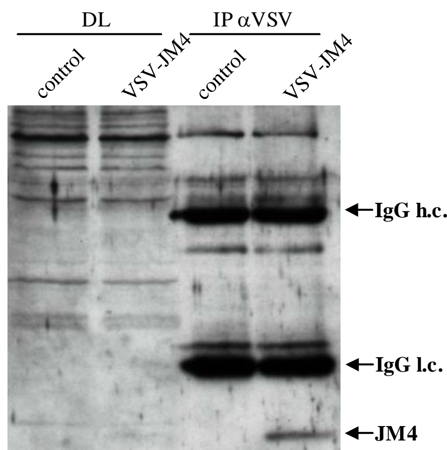


Fig. 21. HEK-293 cells were transiently transfected with plasmids encoding VSV-JM4 or empty vector control as indicated. Cell lysates were immunoprecipitated using the anti-VSV antibodies. Proteins were seperated by 12.5 % SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were detected in an immunoblot using blood serum sample from a mouse immunized with JM4-encoding DNA and recominant GST-JM4.NT protein. IP, immunoprecipitation; DL, direct lysate; α, anti; l.c., light chain; h.c., heavy chain.

HEK-293 cells were transfected with VSV-JM4 or empty vector control. After expression cells were lysed in solubilization buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10 % Glycerol, 0.5 % Cymal-5; supplemented with phosphatase / protease inhibitors (25

mM β glycerol-phosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine, 10 μM pepstatin, trasylol (500 Kalikrein inactivator units), 5 μg/ml leupeptin)). Proteins were precipitated from cleared lysates using an anti-VSV antibody, separated according to their size on 12.5 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). Proteins were detected using blood serum sample (1:500) and visualized with HRP-conjugated antibodies by enhanced chemiluminescence (Amersham Pharmacia Biotech). Indeeed overexpressed JM4 was detected by anti-JM4 antibodies present in blood of immunized mouse in direct cell lysates (DL) and after immunoprecipitation (IP) of VSV-JM4. Mice are still healthy and alive. We will analyze the interaction of endogenous JM5 and CCR5. Furthermore, protein levels - and localization - can be tested in different cells and compared with expression (levels) of CCR5. Since immunization of mice with the GST-JM4 fusion proteins proved to induce an effective anti-JM4 antibody production one might consider large scale production of recombinant proteins which can be used for immunization of rabbits to receive larger amounts of antibody directed against JM4.

3. Discussion and outlook

3.1 CCR5 as a target to interfere with HIV-1 infection

Recent approaches have identified new G protein-coupled receptors (GPCRs) interactions that provide new mechanisms of receptor function and regulation. Chemokine receptors are members of the family of GPCRs. They are major players in mediating diverse biological responses and a lot of attention was drawn to this large family of receptors since CCR5 and CXCR4 were identified as coreceptors required for HIV-1 entry in 1996 (55-60). Naturally occurring mutants of CCR5 have been shown to interfere with HIV-1 infection. The *CCR5Δ32* gene encodes a protein lacking the last three transmembrane segments of the receptor (65, 66). Individuals homozygous for this gene are highly protected against HIV-1 infection. Another mutant, the CCR5-893(-) receptor was found to be deleted for its entire cytoplasmic C-terminus (72). Cell surface expression of these truncated receptor proteins was shown to be substantially impaired. The lack of functional coreceptor cell surface expression interferes with HIV-1 infection. Whatsoever, the lack of functional CCR5 expression does not result in any known pathological phenotype. Therefore, the receptor represents a valuable target to interfere with HIV-1 infection.

The actions of chemokine receptors are regulated at various levels, through their direct interaction with a variety of adaptor / scaffold proteins, as well as at the level of cell surface expression coherent with ligand binding, de- and resensitization, internalization, recycling, or degradation and cross-talk of receptors (2, 3, 52, 186).

The identification of chemokine- or GPCR-interacting proteins has been shown to facilitate the study and unravel diverse biological properties of these heptahelical molecules. Members of the G protein-coupled receptor kinases (GRKs), arrestins, PDZ- and non-PDZ-scaffolds are involved in the regulation and are as well regulated by various other proteins (4, 187).

The aim of this study was to identify proteins that are interacting with the chemokine- and HIV-1 coreceptor CCR5. The C-terminus of CCR5 has been shown to play an important role in receptor-mediated signal transduction, cellular activation, endocytosis, and intracellular receptor trafficking. Furthermore, the very C-terminal leucine of CCR5 constitutes a potential binding site for PDZ proteins, which are known to bind and

regulate various transmembrane receptors. Therefore, we chose to employ the cytoplasmic C-terminus of CCR5 in a yeast two-hybrid screen. Interacting proteins should be analyzed for their potential role in receptor-regulation or characterized for their influence on receptor-mediated cellular responses.

3.2 CCR5 and α-catenin

In this study we identified the cytoskelatal protein α-catenin as a novel interacting partner of the chemokine- and HIV-1 coreceptor CCR5.

Using the yeast two-hybrid system, the C-terminal amino acids 799-906 of α-catenin were found to interact with the C-terminal tail of CCR5 (aa 295-352). Deletions within this region of CCR5 did abrogate the interaction with the C-terminus of α-catenin in yeast, which indicates the specificity of the interaction.

The interaction was certified in mammalian cells employing full-length proteins of CCR5 and α-catenin. We were able to show association of both proteins after overexpression by coimmunoprecipitation studies. Furthermore, the interaction of endogenous α-catenin with ex - and endogenous CCR5 chemokine receptor was shown in HEK-293 cells and T lymphocytes, respectively. As described before α-catenin is involved in the organization of the actin-based cytoskeleton and links several proteins of this network. Therefore, we tested the effect of α-catenin on CCR5 receptor function. Besides overexpression, the knock-out or knock-down of the protein and the identification of dominant-negative mutants would facilitate this study. Such mutants would compete out binding of endogenous α-catenin to CCR5, but not be connected to a biological function due to deletion of other functional domains. Mutants of α-catenin were cloned, one encompassing the C-terminal domain identified to interact with CCR5 but otherwise deleted for interaction sites within the N-terminus (α-catenin ct). Another mutant, termed α-catenin nt, was deleted for the C-terminal domain that was identified to mediate CCR5-binding in yeast.

The capacity of these mutants to interact with full-length CCR5 was analyzed in coimmunoprecipitation studies. Both mutants were able to bind to CCR5 after overexpression in mammalian cells, even though α-catenin nt is deleted for the initially

identified CCR5-binding domain. In these experiments we used full-length CCR5 receptor in contrast to yeast, where only the C-terminus of CCR5 was employed. The full-length receptor might comprise, next to its C-terminus, an additional binding site for α-catenin nt. A further α-catenin-intramolecular binding site for the receptor within the N-terminal domain of α-catenin could also explain association of α-catenin nt with the receptor. In addition α-catenin has been shown to form homomultimers in solution (101). It is therefore conceivable that endogenous proteins link the mutant α-catenin to the receptor by multimerization.

Both mutants and the full-length protein, fused to green fluorescent protein (GFP) were overexpressed in HeLa cells and analyzed for their distribution and effect on the cytoskeleton network by confocal laser scanning microscopy (data not shown). The GFP-α-catenin nt aggregated in cellular compartments, possibly due to unproper folding or modifications, resulting in retention in the Golgi network or endoplasmic reticulum. Expression of the GFP-α-catenin ct led to deformed, misshaped, ruffled-like cell morphology, demonstrating the role of α-catenin in the organization of the cellular cytokeleton network and cell morphology. Overexpression of α-catenin ct resulted in distortion of the cytoskeleton. The GFP-α-catenin wild-type expression pattern resembled, as expected, cytoskeletal structures, which excludes an influence of GFP on protein distribution.

Analyses concerning localization of the CCR5 - α-catenin complex and whether α-catenin associates constitutively with CCR5 or if this interaction is regulated by e.g. stimulation of CCR5 might help to characterize the nature and function of the interaction.

The actin-based cellular cytoskeleton has been shown to affect HIV-1 infection (81). Cytochalasin D specifically impairs F-actin polymerization in the cytoskeleton and is used to inhibt membrane ruffling (188). Pre-treatment of cells with cytochalasin D interfered with HIV-1 infection, indicating the dependence on certain cellular cytoskeletal structures for this event. Treatment of peripheral blood lymphocytes (PBLs) with methyl-β-cyclodextrin (MeβCD), a lipid raft inhibitor that extracts cholesterol from membranes, or cytochalasin D reduced susceptibility of the cells to membrane fusion with cells expressing the HIV-1 envelope protein gp120 (143).

Clustering is a process not well understood and receptor-clusters have been observed in small *trans*-Golgi vesicles, where they might be organized shortly after protein synthesis and before insertion in the plasma membrane. Immunogold labeled CCR5, CXCR4 or CD4 receptors were found to cluster separately but closely apposed at the plasma membrane (145). They localized to specific microdomains, so-called microvilli, which are supported by actin-polymerization at the cell surface. Receptor molecules were shown to accumulate and colocalize in protruding membrane structures (146), likely to be microvilli or membrane ruffles, which are characterized by the presence of actin and as well as the cytoskeletal linker protein erzin. Others have described that colocalization or association of receptors in lipid raft domains is required or at least supportive for HIV-1 infection (143, 147, 149, 189, 190) and cooperation of CCR5 molecules was shown to be required for HIV-1 infection (144). In contrast, one study could not detect a requirement of HIV-1 coreceptor localization in lipid membrane rafts for viral infection (191). Whatsoever, in the latter study cholesterol was still found to modulate HIV-1 entry process independently of its ability to promote raft formation. Discrepancies might be explained by differences of the cell type used in the studies, which affects membrane raft constitution and biochemical properties of receptors. Cholesterol and lipid raft domains were described to be important for maintenance of CCR5 receptor conformation and influence chemokine binding capacities and function of the receptor (192).

The accumulation of receptor proteins in distinct compartments and membrane domains might facilitate viral entry, receptor oligomerization, or influence other receptor functions, e.g. through establishment of a cellular microenvironment, in which specific regulatory proteins can be centered. The cytoskeleton might be part of a mechanism for the directional transport of Golgi vesicles or (clustered) receptors to the plasma membrane, but might also be involved in the organization of cell surface localization of receptor molecules. Not only the process of transport or functional plasma membrane expression, but also internalization and subsequent intracellular vesicle-associated trafficking might thereby be affected.

Actin-dependent receptor colocalization and clustering might provide a setting with coreceptors in close proximitiy, therby favouring HIV-1 binding and entry. Homo- or hetero-oligomerization has been shown to influence CCR5-mediated signal transduction

(38). CD4 and CCR5 have been shown to associate in the membrane, which depends on actin (81, 147). Furthermore, a constitutive association of the two receptor molecules was detected by biochemical analyses (148, 149) leading to cross-talk and resulting in altered CCR5-signaling after activation of CD4 (83, 150). Signals induced by HIV-1 binding to the receptor complex do not necessarily reflect signaling induced by natural ligands, but could represent unique signaling pathways contributing to viral entry, integration, viral replication, and HIV-1 pathogenesis.

The chemokine receptors CCR5 and CXCR4 both constitutively associate with the motor protein nonmuscle myosin heavy chain-IIA (NMMHC-IIA) at the leading edge of migrating T lymphocytes (151). In addition, the colocalization of actin to the receptor C-termini was found in accordance with other studies reporting the association of actin with heptahelical receptors (152). This interaction might be involved in cellular rearrangement and cell motility after chemokine receptor activation since the cellular function of NMMHC-IIA has been connected with regulation of cell shape and formation of focal adhesions in HeLa cells (153).

Recent studies suggest that GPCRs are segregated within distinct microdomains of the plasma membrane before internalization and that the actin-based cytoskeleton plays a specific role in endocytic sorting after internalization of heptahelical receptors (193, 194). As described in chapter 2.3.2, α-catenin and a potential dominant-negative mutant (α-catenin ct) were tested for their effect on viral infection employing HIV-1 indicator cell lines. No effect on viral entry after overexpression was detectable in this cellular system. As described above cellular milieu and subcellular membrane domains evidently have an influence on chemokine receptor properties and most certainly differ between different cell types, thereby affecting the CCR5-mediated biological outcome. The analysis of HIV-1 infection, and of course other CCR5 receptor functions, therefore requires to perform studies in cells susceptible to HIV-1 infection, which reflect the natural complement and composition of cellular factors, proteins and membrane domains. Furthermore, the cell surface density of CCR5 can determine postentry efficiency of HIV-1 infection (195). Natural host cells will be utilized to study the impact of overexpression of CCR5-interacting protein and mutants thereof.

The recent identification of small interfering RNAs (siRNAs) has made it possible to study gene function by mammalian genetic approaches (196-199). RNA interference (RNAi) describes the silencing of target genes by homologous double-stranded RNA (dsRNA) (Fig. 22).

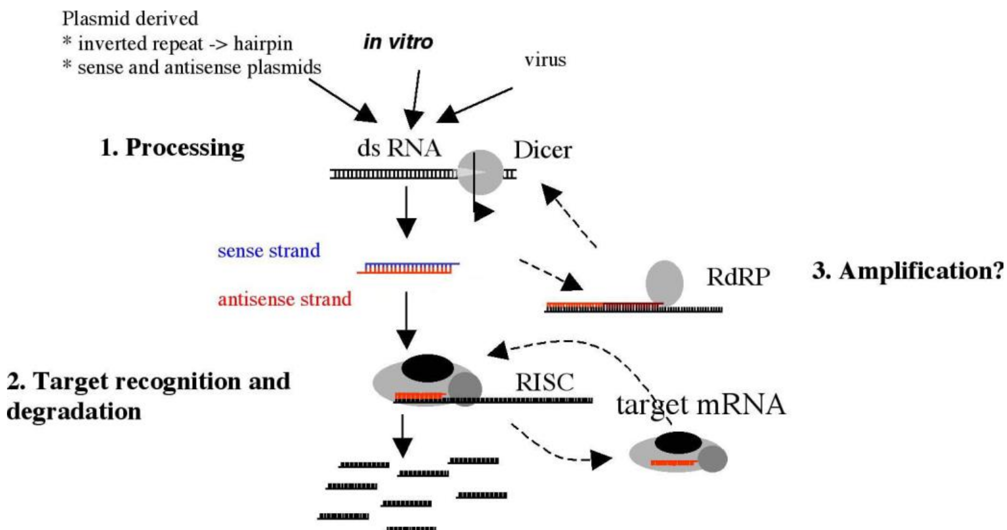


Fig. 22. Schematic diagram of the RNA interference (RNAi) mechanism used to knock-out/-down genes of interest. The action of RNAi is described in detail in the text.

The mechanism is highly conserved among species ranging from primitive eukaryotes to mammals. RNAi is a multistep process, in which dsRNA is first cut into 21-23 nt dsRNAs, so called siRNAs by the RNAseIII-like enzyme DICER. This enzyme has also been connected with the processing of small temporal RNAs (stRNA), which regulate developmental timing. The siRNAs associate with a multicomponent nuclease, the “RNA induced silencing complex” (RISC), inducing target mRNA destruction.

The viral delivery of specific siRNAs can be utilized to stably suppress expression of the target gene (200). The construction of retroviral vectors encoding siRNAs, which target α-catenin can be employed as a tool to suppress the protein expression. An advantage of this approach is that a total knock-out of α-catenin would render non-viable cells, due to the destruction of the cytoskeleton. Therefore, a cellular knock-down could be used to study the effect on CCR5. The efficiency of suppression and effect on CCR5 receptor trafficking, compartmentalization and function, e.g. CCR5-dependent HIV-1 entry can be analyzed.

3.3 CCR5 and the JM4 protein family

We have identified a novel protein, termed JM4 that interacts with CCR5. To date no biological function has been described for JM4. We anticipate that the interaction has a biological function on chemokine receptor-mediated cellular processes as signaling or retrovirus uptake.

As already described and discussed in chapter 2.2 in detail, the sequence, structural, and biochemical analyses suggested JM4 to belong to a small family of tetrahelical proteins, members are conserved throughout different species. JM4 shares 42 % identity and in addition 21 % similarity with JWA, also no function has been assigned to this protein. We could show that CCR5 binds not only JM4 but with similar efficiency JWA. While this work was in progress the rat homologue of JWA, designated GTRAP3-18 with 95 % homology was identified. It was shown to interact with the 10-transmembrane excitatory amino acid carrier EAAC1 in rat brain (122). The function of GTRAP3-18 is to regulate EAAC1-mediated glutamate transport.

JM4, JWA and GTRAP3-18 are hydrophobic proteins with four potential transmembrane helices. They do not only share sequence and structure-based homologies but also colocalize to similar defined structures within the cell as shown by confocal laser scanning microscopy. The localization of these proteins suggests an association with the cytoskeleton or endoplasmic reticulum.

The human JWA protein has not been characterized. It shares 90 % sequence identity with the murine Aip-5 (ADP-ribosylation-like factor 6 (ARL6) interacting protein-5). Aip-5 interacted with ARL6, a member of the ARF-family, in a yeast two-hybrid analysis (124). ARFs seem to be involved in vesicle formation, in intracellular traffic, and associate with membranes on the secretory and endocytic compartment (125). The ARF6 protein has recently been shown to associate with β-arrestin and enhance endocytosis of the heptahelical, G protein-coupled β_2-adrenergic receptor (β_2-AR) (17). JM4 closely resembles JWA and Aip-5. Therefore, we tested whether JM4 could bind ARL6 and CCR5. We performed GTP-dependent interaction studies in overexpression systems. No association was detected after coimmunoprecipitation of JM4 with ARL6 or after pull-down experiments using recombinant radioactive labeled proteins. Nevertheless we do not rule out an association of JM4 and ARL6. Two putative phosphorylation sites of JM4

(NetPhos 2.0) (201) might be involved in regulation of this interaction. In addition further – cellular - factors or phosphorylation events might be crucial for interaction of the two proteins. This remains to be elucidated.

As described for GTRAP3-18, we could show that the JM4 proteins can form multimers, which might be important for their biological function, e.g. acting as an adaptor or scaffold protein (122). Not only homo-, but also hetero-multimers of JM4, JWA, and GTRAP3-18 were detected, which possibly are involved in recruiting and/or clustering different cellular receptors via these binding proteins.

It was shown by the group o fProf. Rothstein that binding of GTRAP3-18 to the C-terminus of EAAC1 altered glutamate transport by lowering the transporters affinity to its substrate, possibly by inducing a conformational change of the receptor due to binding. Therefore, overexpression of GTRAP3-18 interfered with EAAC1-mediated glutamate uptake (122). In an ongoing collaboration with the group of Prof. Rothstein it was shown that JM4, similar to GTRAP3-18, inhibited EAAC1- and EAAT2-mediated transport of glutamate

We have tested the influence of JM4 overexpression on CCR5-mediated HIV-1 infection or actin polymerization and analyzed receptor internalization. To render cells susceptible to HIV-1 infection, HEK-293 and HeLa-derived HIV-1 indicator cell lines were transfected, either transiently or stably with plasmids encoding receptor proteins. The HIV-1 indicator cell lines were also used for the study of α-catenin as described before. Actin polymerization and internalization were studied in HEK-293 cells after overexpression of CCR5 and JM4.

The fact that up to three proteins need to be coexpressed and the receptor(s) possibly even to physiological levels are a drawback of this system and might explain why no biological effects of JM4 on CCR5 were detectable or reproducible. Furthermore, it is shown that the cell type with its different components and expression levels of cellular factors and proteins has a significant impact on CCR5 function. The experiments might be more promising when carried out in natural host cells of HIV-1 or cells expressing endogenous CCR5 receptor.

The approaches using overexpression of proteins to elicit biological function of JM4 and JWA were chosen in analogy to the EAAC1 - GTRAP3-18 setting, where end- and

exogenous overexpression of the latter protein resulted in inhibition of EAAC1-mediated transporter function. No obvious effect of JM4 on CCR5 receptor function was detectable after overexpression.

As already discussed in the context of the studies employing α-catenin, the choice of cell type can influence outcome of receptor action and analyses. Studies with cells expressing endogenous receptor therefore reflect much better the natural situation of the CCR5 –JM4 interaction.

Endogenous GTRAP3-18 expression was described to be upregulated by treatment of cells with retinoic acid (RA) (122). To conduct experiments with natural HIV-1 host cells, which express endogenous CCR5, we analyzed the effect of RA on expression levels of JM4 in PM1 T-lymphocytes susceptible to HIV-1 infection. No elevation of JM4 mRNA level was detectable even after 14 days of treatment. Parallel tests were carried out in neuronal SH-SY5Y, HEK-293, and HeLa cells. Neither one of these cell lines reacted to RA by upregulating JM4.

Very recently it was reported that the described upregulation of GTRAP3-18 was not attributable to RA but rather to methyl-β-cyclodextrin (MeβCD) (171). MeβCD, a macrocyclic polysaccharide is commonly used to enhance the solubility of hydrophobic compounds such as RA. This finding is in accordance with our results, where RA did not affect the transcript level of JM4. MeβCD is a lipid raft inhibitor that extracts cholesterol from membranes, which leads to loss of compartmentalization of the molecules located in the membrane thereby affecting activity of membrane receptor proteins (202). As described above cholesterol is involved in maintaining the lipid order of rafts, influences receptor conformation, and has an inhibitory effect on chemokine binding to CCR5 (192). This actually rules out the usage of the MeβCD agent for upregulation of JM4 for the study of receptor proteins.

The RNAi technology, described in chapter 3.2, could serve as a tool to downregulate JM4 expression level. Given the close similarity and probable redundancy of JM4 and JWA, these knock-down studies should include siRNAs targeting both proteins, JM4 and JWA. We introduced three independent sequences that target JM4 and three that target JWA in both, the pSUPER (203) and in the pRETRO-SUPER vector (200). These are being used to stably express siRNAs, either after transfection or retroviral transduction of

the vectors in target cells. We will test the efficiency of downregulation by immunofluorescence, FACS analysis, Northern blot, and Wstern blot using the anti-JM4 antibody we generated as described in chapter 2.7.

Other experimental settings could encompass the analysis of dominant-negative mutants of JM4. Deletion mutants of JM4, which bind to the CCR5 receptor but cannot form homo-/hetero-multimers would block binding of endogenous wild-type JM4 or JWA to the receptor. These analyses will not only help to elucidate the biological role of JM4 in respect to CCR5. Moreover the identification of such mutants might in analogy be supportive for the study JWA and its homologue GTRAP3-18.

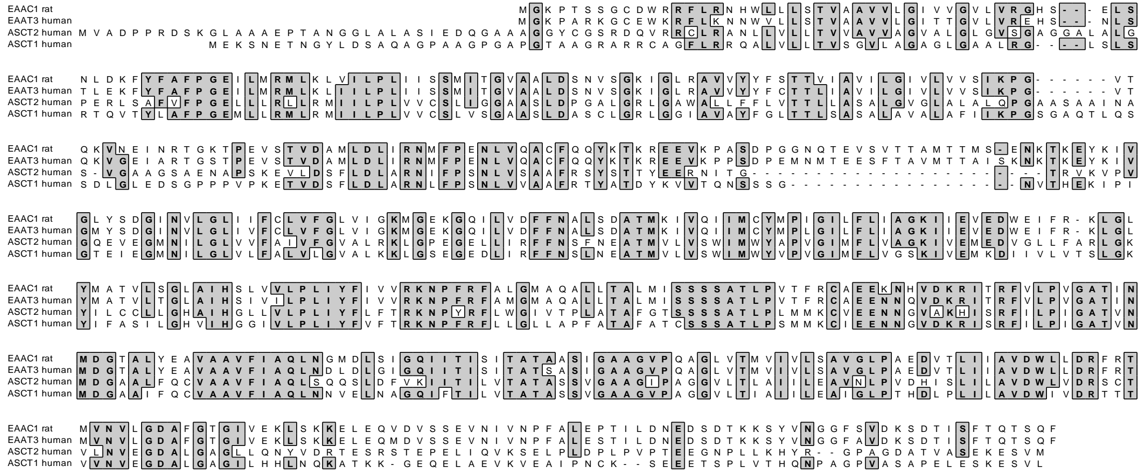
Retroviruses bind to diverse transmembrane spanning amino acid transporters, e.g. ecotropic mouse leukemia virus binds to a specific 14 transmembrane spanning cationic amino acid transporter (CAT). GTRAP3-18 binds and negatively regulates the excitatory amino acid carrier 1 (EAAC1), a ten-transmembrane spanning glutamate transporter. Sequence and structure-based analyses showed similarities of EAAC1 and the human sodium-dependent neutral amino acid transporters type 1 (ASCT1) (Fig. 23). The cell surface molecules ASCT1/2 have been identified as cellular receptors for the human endogenous retrovirus type D (HERV-W) (126). Additionally, ASCT2 is a common cell surface receptor for viruses including the feline virus RD114, the baboon endogenous retrovirus BaEV, type D simian retroviruses, and avian reticulendotheliosis viruses (127, 128).

A potential interference of JM4 with retroviral entry in these virus systems appears now very likely. Pseudotyped viruses carrying an indicator gene could be used for infection studies after overexpression of JM4 and mutants thereof or after knock-out or -down of JM4 in viral host cells. JM4 and also JWA, which we have cloned from neuronal cells, could be tested for their influence. A collaboration with Prof. Weiss, University College London, UK, on the effect of JM4 on receptor-mediated viral entry has been initiated.

The CCR5 receptor binds JM4 and JWA, indicating similar or shared biological actions of these proteins. The JWA homologue GTRAP3-18 is recognized to interfere with the EAAC1-mediated glutamate transport. EAAC1-related proteins as ASCT2 are involved in cellular entry of various viruses. Therefore, it can be anticipated to analyze the JM4, JWA, and GTRAP3-18 proteins in this context (Fig. 24). These studies could serve as a

proof of principle for the regulation of cell surface molecules by members of the characterized family of four-transmembrane proteins.

A.



B.

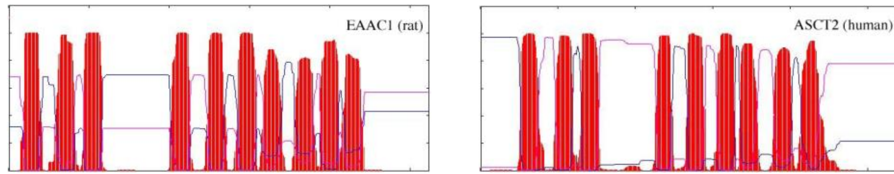


Fig. 23. Comparison of amino acid transporters by sequence and structure analyses. A. Sequence alignments of glutamate transporter EAAC1 (rat), its human homologue EAAT3 and human amino acid transporter ASCT1 and 2. EAAC1 interacts with GTRAP3-18, a protein resembling JM4. ASCT2 has been identified as a cellular receptor for various viruses including the feline virus RD114, Baboon endogenous retrovirus, type D simian retroviruses, and avian reticulendotheliosis viruses. Alignment of the four amino acid transporters shows sequence homologies B. Predictions about structure–function relationship of amino acid transporter molecules EAAC1 (rat) and ASCT2 (human) indicate a similar transmembrane spanning topology. Predicted transmembrane domains are indicated by red shaded area.

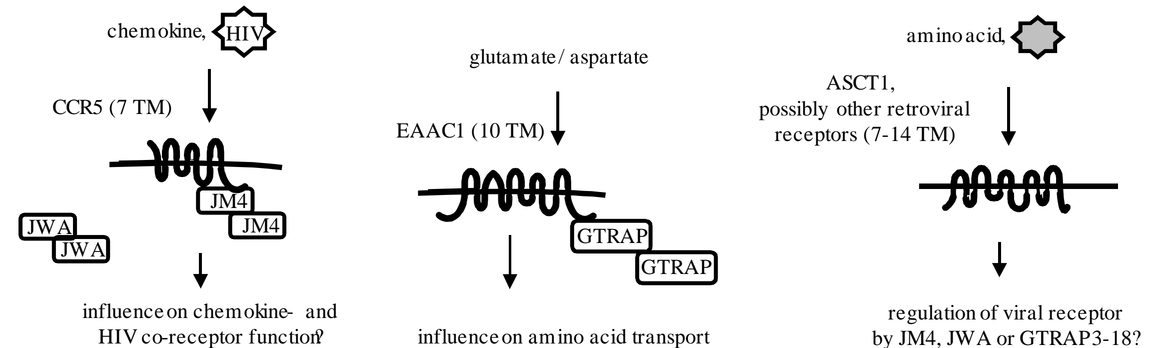


Fig. 24. Schematical depiction of the cellular contexts JM4, JWA and GTRAP3-18 are – possibly – involved in and might influence.

As suggested in the study on the EAAC1 transporter, GTRAP3-18 and as well JM4 and JWA could elicit their biological influence at the plasma membrane by inducing conformational changes of cell surface proteins. These changes can alter binding properties or affinity to extracellular agonists, they might influence activation status, signal transduction or interaction with intracellular proteins of regulatory function.

JWA has been proposed to be associated with the cytoskeleton (NCBI Protein Database = NCB Accession # AAC64360). A biological function of JM4, JWA and GTRAP3-18 as discussed for α-catenin is conceivable. JM4-similar proteins might be involved in scaffolding and clustering receptor/transporter molecules at the plasma membrane. An intracellular role of JM4, JWA and GTRAP3-18 might encompass post-translational sorting, clustering, and trafficking to control cell surface expression. Furthermore, endocytosis and subsequent targeting to specialized intracellular compartments and membrane domains might be regulated or directed and could influence the fate of receptors.

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Publications

1. K. Moelling, K. Schad, M. Bosse, S. Zimmermann, and M. Schweneker. 2002. Regulation of Raf-Akt Cross-talk. *J Biol Chem* 277, 34:31099.
2. K. Ladell, J. Heinrich, M. Schweneker, and K. Moelling. 2003. A combination of plasmid DNAs encoding murine fetal liver kinase 1 extracellular domain, murine interleukin-12, and murine interferon-gamma inducible protein-10 leads to tumor regression and survival in melanoma-bearing mice. *J Mol Med* 81, 4:271.
3. G. Burkhard, E. Haas, T. Fritzius, M. Bosse, M. Schwemmle, S. Zimmermann, J. Heinrich, M. Schweneker, and K. Moelling. 2003. Akt-Interacting Protein with WD Repeats and FYVE Domain Targets Akt to GLUT4 Vesicles. *Manuscript submitted.*

4. M. Schweneker, and K. Moelling. 2003. The chemokine- and HIV-1 co-receptors CCR5 and CXCR4 interact with α-Catenin, a component of the cellular cytoskeleton. *Manuscript submitted.*
5. M. Schweneker, A. Bachmann, S. Zimmermann, and K. Moelling. 2003. JM4 belongs to a new protein family that interacts with the chemokine- and HIV-1 co-receptor CCR5. *Manuscript submitted.*

Patents

European Patent Application #1207202
"Nucleic acid molecules encoding a protein interacting with the chemokine receptor CCR5 or other chemokine receptor family members"
Karin Moelling, Marc Schweneker

Grant applications

The James B. Pendleton Charitable Trust (2000)
"Interaction partners of HIV-1 coreceptors involved in signalling"
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The James B. Pendleton Charitable Trust (2001)
"Interaction partners of HIV-1 coreceptors involved in signaling"
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The James B. Pendleton Charitable Trust (2002).
"A new family of proteins interacting with transmembrane receptors which serve for amino acid or retroviral uptake"
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