JM4 is a four-transmembrane protein binding to the CCR5 receptor

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Abstract The CC chemokine receptor 5 (CCR5) is a major co-receptor for human immunodeficiency virus (HIV) and CCR5 mutants lacking the carboxy (C)-terminus interfere with HIV infection. Therefore, we analysed the C-terminus of CCR5 and here describe Jena-Muenchen 4 (JM4), a novel CCR5-interacting protein. JM4 is membrane-associated, co-precipitates with CCR5, and is ubiquitously expressed. It shares about 62% sequence similarity with JWA and glutamate transporter-associated protein 3-18 (GTRAP3-18), a regulator of an amino acid transporter. JWA, like JM4, is a four-transmembrane protein, which binds to the CCR5 receptor. Furthermore, JM4, JWA, and GTRAP3-18 co-localise and heterodimerise indicating a functional relationship. JM4 co-localises with calnexin in the endoplasmic reticulum and with the mannose 6-phosphate receptor in the Golgi. JM4 and GTRAP3-18 harbor a Rab-acceptor motif, indicating a function in vesicle formation at the Golgi complex. In conclusion, we describe a CCR5-interacting protein, which is suggested to function in trafficking and membrane localisation of the receptor, possibly also other receptors or amino acid transporters.

Keywords: CCR5; JM4; Intracellular trafficking; Endoplasmic reticulum; Golgi

1. Introduction

The CC chemokine receptor 5 (CCR5) belongs to the superfamily of the G protein-coupled receptors (GPCRs), which are seven-transmembrane proteins. Chemokine receptors play a crucial role in the regulation of inflammatory processes. Most importantly, the chemokine receptors CCR5 and CXCR4 have been identified as the two major co-receptors for the human immunodeficiency virus type 1, HIV-1, which infects $CD4^+$ target cells [1–6]. CCR5 is expressed on memory T lymphocytes, macrophages, and dendritic cells and is mainly associated with transmission of viruses during primary infection [2], while CXCR4 seems to be important at later stages of the disease [7,8].

Mutations in the CCR5 receptor have been shown to alter cellular signalling and HIV infection. The key role of CCR5 in HIV pathogenesis is demonstrated by the fact that individuals homozygous for a 32-base pair deletion in the *CCR5* gene are almost completely protected from HIV pathogenesis [9]. This 32-base pair deletion results in a carboxy (C)-terminally severely truncated protein, termed CCR5Δ32. A second naturally occurring mutant receptor, termed CCR5-893(−), lacks its cytoplasmic C-terminus. Like CCR5Δ32, this truncated receptor is not efficiently expressed at the cell surface and interferes with HIV infection [10]. Evidence for the role of the cytoplasmic tail in receptor trafficking to the plasma membrane has recently been demonstrated for the CCR2 receptor isoforms [11]. Furthermore, the palmitoylation of the C-terminal tail of CCR5 has a significant influence on targeting the receptor to the plasma membrane, on signalling, internalisation, and intracellular trafficking [12–15].

Here, we identified a novel binding protein of CCR5, which has been designated as JM4, Jena-Muenchen 4, in the database (NCBI Protein Database = NCB Accession No. CAA06753), no function has been assigned to this protein so far. Sequence prediction suggested that JM4 is a four-transmembrane-spanning protein with a preferential localisation at the endoplasmic reticulum (ER). JM4 shares sequence similarity with human JWA and the rat homologue glutamate transporter-associated protein 3-18 (GTRAP3-18), which regulates glutamate uptake by interacting with the ten-transmembrane-spanning excitatory amino acid carrier 1 (EAAC1) [16]. We show that JWA, like JM4, binds to CCR5. Based on sequence, structure, and function similarities, JM4, JWA, and GTRAP3-18 are related and localise in the ER and vesicular structures. They associate with CCR5 and are suggested to function in trafficking and membrane localisation of the receptor.

2. Materials and methods

2.1. Plasmid construction

The C-terminus of the human CCR5, encoding amino acids 295–352, was excised from pBABE.CCR5 (AIDS Research and Reference Reagent Program) and subcloned into the *EcoR*I/*Sal*I sites of pGBT9pheS using a *EcoR*I–*Sfa*I linker. The pGBT9pheS is a derivative of the pGBT9 bait vector containing a Gal4–DNA binding domain (Clontech) that was modified to add positive selection properties (M. Buchert, unpublished data) [17].

Gene mutations were introduced by QuickChange site-directed mutagenesis according to the manufacturer's instructions (Stratagene).

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Abbreviations: CCR5, CC chemokine receptor 5; GPCR, G protein-coupled receptor; HIV-1, human immunodeficiency virus type 1; N, amino; C, carboxy; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; M6PR, mannose 6-phosphate receptor; GTRAP3-18, glutamate transporter-associated protein 3-18; EAAC1, excitatory amino acid carrier 1; ARF, ADP-ribosylation factor; ARL, ARF-like; PRA1, prenylated Rab-acceptor 1; HA, hemagglutinin; VSV, vesicular stomatitis virus; CDD, conserved domain database

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The gene encoding full-length CCR5 was excised from pcDNAI.CCR5 (AIDS Research and Reference Reagent Program) and inserted into the pcDNA3 vector (Invitrogen) using a *Hind*III–*Bam*HI linker including an amino (N)-terminal hemagglutinin (HA)-epitope to create pcDNA3–HA–CCR5. The JM4-encoding sequence was excised from the pACT.1 plasmid (JM4, amino acids 7–178) and subcloned into the *Bam*HI restriction site of the pcDNAI-based expression vector pSuper' Vesicular Stomatitis Virus, VSV (pS'VSV; C. Hovens, unpublished data). A VSV–JM4 linker was inserted in-frame with the 5′-VSV-sequence to obtain pS'VSV–JM4 that encodes the full-length VSV–JM4.

JM4 and JWA cDNAs were subcloned into a pcDNA3 vector containing two Myc-epitope encoding sequences (pcDNA3-2 × Myc; E. Haas, own unpublished data). JM4 and JWA were amplified with Access RT-PCR kit (Promega) using pS'VSV–JM4 and RNA extracts from human neuroblastoma cell line SHSY-5Y as templates, respectively. The PCR products were subcloned into a pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) and inserted in the pcDNA3-2 × Myc vector to create pcDNA3-2 × Myc–JM4 and -JWA.

The GTRAP3-18–HA expression plasmid was kindly received from J. Rothstein (Johns Hopkins University, Baltimore, USA) [16].

All linker and primer sequences can be obtained upon request.

2.2. Yeast two-hybrid techniques

Y153 yeast cells were transformed with the bait plasmid and a human pre-B cell cDNA library cloned in pACT as prey (kindly provided by S. Elledge, Harvard Medical School, Boston, USA) [18,19]. Transformants were screened for histidine prototrophy in the presence of 10 mM 3-aminotriazole (Sigma). Interaction was demonstrated by $lacZ^+$ transformants.

2.3. Northern blot analysis

A JM4-probe labelled with [α-^{32}P]-dCTP (Amersham Biosciences) was prepared by random priming of a JM4-fragment using RadPrime DNA labelling system (Invitrogen) as described in the manufacturer's protocol. A human 12-lane multiple tissue Northern blot was used to analyse mRNA levels of JM4 according to the manufacturer's instructions (Clontech). The membrane was exposed to imaging plates, which were analysed with a PhosphorImager (Amersham Biosciences) using ImageQuant software.

The blot was stripped by boiling in 5 mM potassium phosphate buffer and reprobed with a β-actin probe to confirm equivalent amounts of RNA.

2.4. Cell culture and transfection

The human embryonic kidney-, HEK-, 293 and HeLa cell lines were grown in Dulbecco's modified eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Seratec). Cf2Th cells that stably express a codon-optimised, C-terminally epitope-tagged CCR5 (synCCR5-C9) were provided by J. Sodroski (Dana Farber Cancer Institute, Boston, USA) and maintained as described [20].

Cells were transfected using LipofectAMINE 2000 (Gibco) or a modified calcium phosphate transfection protocol [21].

HEK-293 cells transfected with HA–CCR5 encoding construct were selected for stable protein expression with 1 mg/ml G418 sulfate (Calbiochem) and sorted for CCR5 cell surface expression by fluorescence-activated cell sorting (FACS).

2.5. Cell lysis

For preparation of membrane and cytoplasmic cellular fractions, the cells were 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 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 homogenisation and centrifuged at 500 × g for 5 min to eliminate nuclei and debris. 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 (Beckman). The supernatant (cytoplasm) was adjusted to 100 mM NaCl and 0.5% Nonidet P-40. The membrane pellet was resolubilised in NETN buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 200 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, supplemented with inhibitors).

For native purification of CCR5, cells were lysed in solubilisation buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 7.5), 10% Glycerol, 0.5% Cymal-5 (Anatrace, Maumee, USA), 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).

2.6. 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 C20 (Santa Cruz); anti-HA antibodies: monoclonal mouse 12CA5 (Sigma), monoclonal rat 3F10 (Boehringer), polyclonal rabbit Y11 (Santa Cruz); anti-Myc antibodies: mouse monoclonal 9E1O (Roche), rabbit polyclonal A14 (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 antibody (Molecular Probes, Jackson ImmunoResearch Lab.); rabbit anti-calnexin antibody; rabbit anti-P300 mannose 6-phosphate receptor (M6PR) antibody [22].

2.7. Immunoprecipitation and Western blotting

Proteins were immunoprecipitated with antibody-precoupled sepharose-beads and eluted 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 according to their size on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences). Proteins were detected using specific antibodies and visualised with HRP-conjugated antibodies by enhanced chemiluminescence (Amersham Biosciences).

2.8. FACS analysis

HEK-293 cells expressing CCR5 wild-type were sorted in cold PBS supplemented with 1% FCS using the FITC-conjugated 2D7 antibody. Cell sorting was performed on a FACStar cell sorter (Becton Dickinson). Cells were analysed by flow cytometry using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

2.9. Confocal immunofluorescence microscopy

Cells were fixed with 3% paraformaldehyde for 15 min at 25 °C. Intracellular antigens were detected after permeabilisation with 0.5% Triton X-100 for 2.5 min at 25 °C. Cells were incubated with specific antibodies in PBS containing 5% new born calf serum (NCS; Invitrogen) for 60 min, followed by an incubation with a chromophore-conjugated secondary antibody for 60 min. Coverslips were mounted with Mowiol (Calbiochem) and images were collected on a Leica TCS4D confocal microscope (Leica Microsystems) and analysed using IMARIS bitplane software.

3. Results

3.1. Identification of the interaction of CCR5 with JM4

A Gal4-dependent yeast two-hybrid screen was performed with the C-terminus of the CCR5, amino acids 295–352, using a human B-cell cDNA library and resulted in the identification of an interacting protein. The corresponding cDNA was sequenced and identified in the database as JM4, lacking six N-terminal amino acid residues. JM4 did not bind to a deletion mutant of the CCR5 C-terminus (amino acids 295–320; data not shown).

3.2. JM4 expression profile

A human multiple tissue Northern blot was hybridised with a [^{32}P]-labelled JM4-specific probe. Analysis of JM4 mRNA