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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

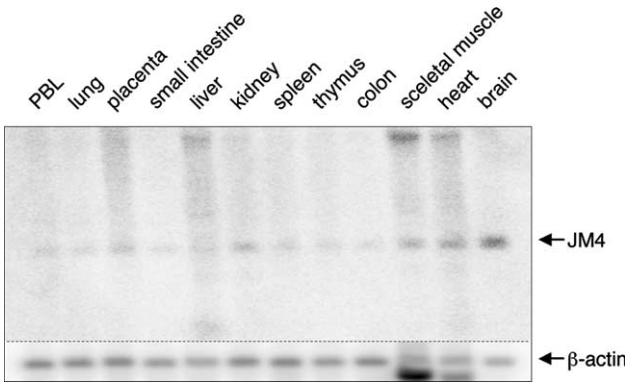

Fig. 1. JM4, a novel CCR5-interacting protein. Tissue distribution of JM4. A human multiple tissue Northern blot (Clontech) was hybridised with a [^{32}P]-labelled probe derived from human JM4 (top), stripped, and reprobed with a β-actin probe (bottom).

expression in 12 human tissues revealed one single transcript of about 1.2 kb (Fig. 1). Among the 12 adult tissues the JM4-transcript is expressed nearly ubiquitously. For loading control a β-actin probe was used (2.0 and 1.8 kb).

3.3. Association of JM4 with CCR5 and cellular membranes

To verify the interaction in mammalian cells, JM4 was expressed in HEK-293 cells selected and sorted for stable expression of HA–CCR5. Using these cells, we were able to demonstrate the specific interaction of JM4 with CCR5 by co-immunoprecipitation (Fig. 2A, upper part, lane 4).

We wanted to confirm the predicted membrane association of JM4 and therefore analysed its subcellular localisation by cellular fractionation. Canine thymocytes, Cf2Th cells, transfected with a codon-optimised CCR5 designed for enhanced gene expression and selected for stable receptor expression [20] were transfected with a plasmid encoding VSV–JM4 or empty vector control. Proteins were immunoprecipitated from membrane (Mem) and cytoplasmic (Cyt) fractions prepared by ultracentrifugation and JM4 and CCR5 detected by Western blot analysis. As shown in Fig. 2B, JM4 is associated with the membrane fraction, which contains plasma membranes and intracellular membranes (lane 2). This result supports the prediction that JM4, due to its transmembrane domains, is associated with cellular membranes. JM4 is not detected in the cellular cytoplasmic fraction (lane 4). The interaction of JM4 and the C-terminus of CCR5, as identified in yeast and verified with full-length proteins in HEK-293 cells, was further confirmed here in the Cf2Th mammalian cell line. A complex of CCR5 and JM4 is shown by co-immunoprecipitation of JM4 with CCR5 from the membrane fraction (lane 6). As described for Cf2Th cells and other cell lines, expression of a CCR5 protein with lower molecular mass was detectable in parallel with the mature CCR5 receptor (Fig. 2B) [20].

3.4. JM4-related proteins and cellular localisation

We further studied JM4 by sequence analyses. JM4 was so far uncharacterised and no biological function has been attributed to the protein. The full-length sequence of the JM4 protein is shown in Fig. 3A, top. The protein is predicted to be 178 amino acids in size. A transmembrane prediction using the TMHMM program (v. 2.0) [23] and hydrophobicity plots suggested that four-transmembrane-spanning helices. These

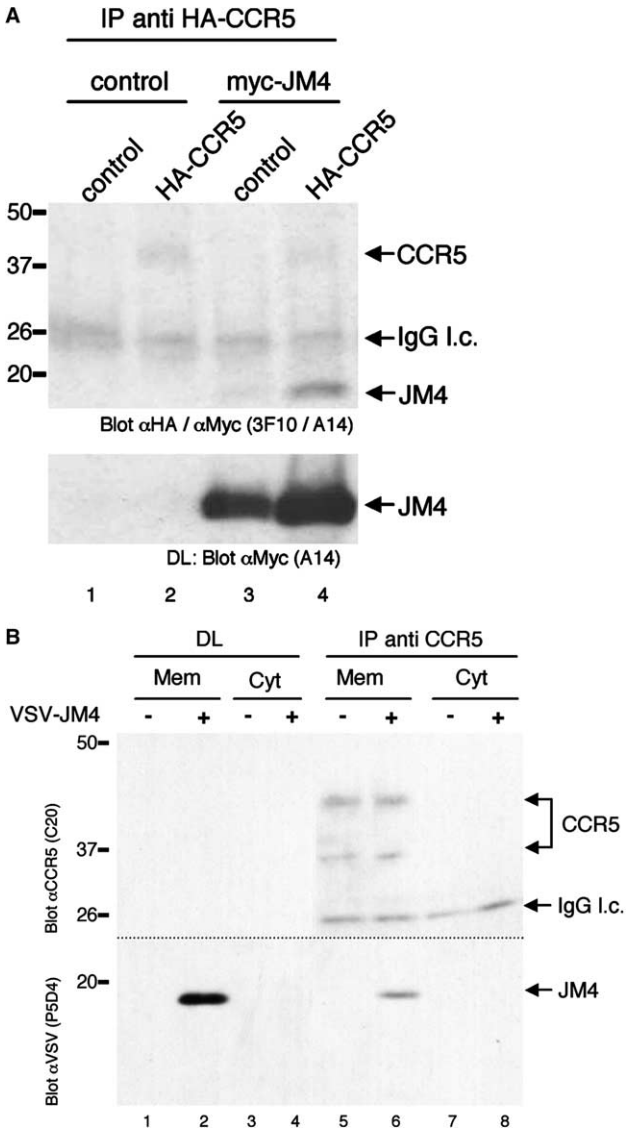

Fig. 2. Interaction of JM4 and CCR5. (A) Interaction of full-length CCR5 with JM4 in mammalian cells. HEK-293 cells or HEK-293 cells stably expressing HA–CCR5 were transfected with plasmids encoding Myc–JM4 or empty vector control as indicated. Cells were lysed and HA–CCR5 was immunoprecipitated using an anti-HA antibody (12CA5). Proteins were separated according to their size by 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. Protein expression and co-precipitation of Myc–JM4 with HA–CCR5 were analysed using anti-Myc and anti-HA antibodies as indicated. The result was reproducible in several independent experiments. Numbers on the left correspond to molecular weight marker sizes in kilodalton. DL, direct cell lysate; IP, immunoprecipitation; l.c., light chain; h.c. heavy chain. (B) Cf2Th cells stably expressing a codon-optimised CCR5 with a C-terminal epitope tag (synCCR5-C9) [20] were transfected with plasmids encoding VSV-epitope-tagged JM4 (+) or empty plasmid control (−). Membrane (Mem) and cytoplasmic (Cyt) fractions of cellular lysates were prepared by ultracentrifugation. Immunoprecipitation of synCCR5-C9 was carried out using an antibody directed against the second extracellular loop of CCR5 (2D7). Proteins were separated according to their size by 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. Proteins were detected using anti-VSV and anti-CCR5 antibodies as indicated.

are indicated by grey bars. A database search for related proteins identified several proteins throughout different species, which are aligned in Fig. 3A. All of these share sequence

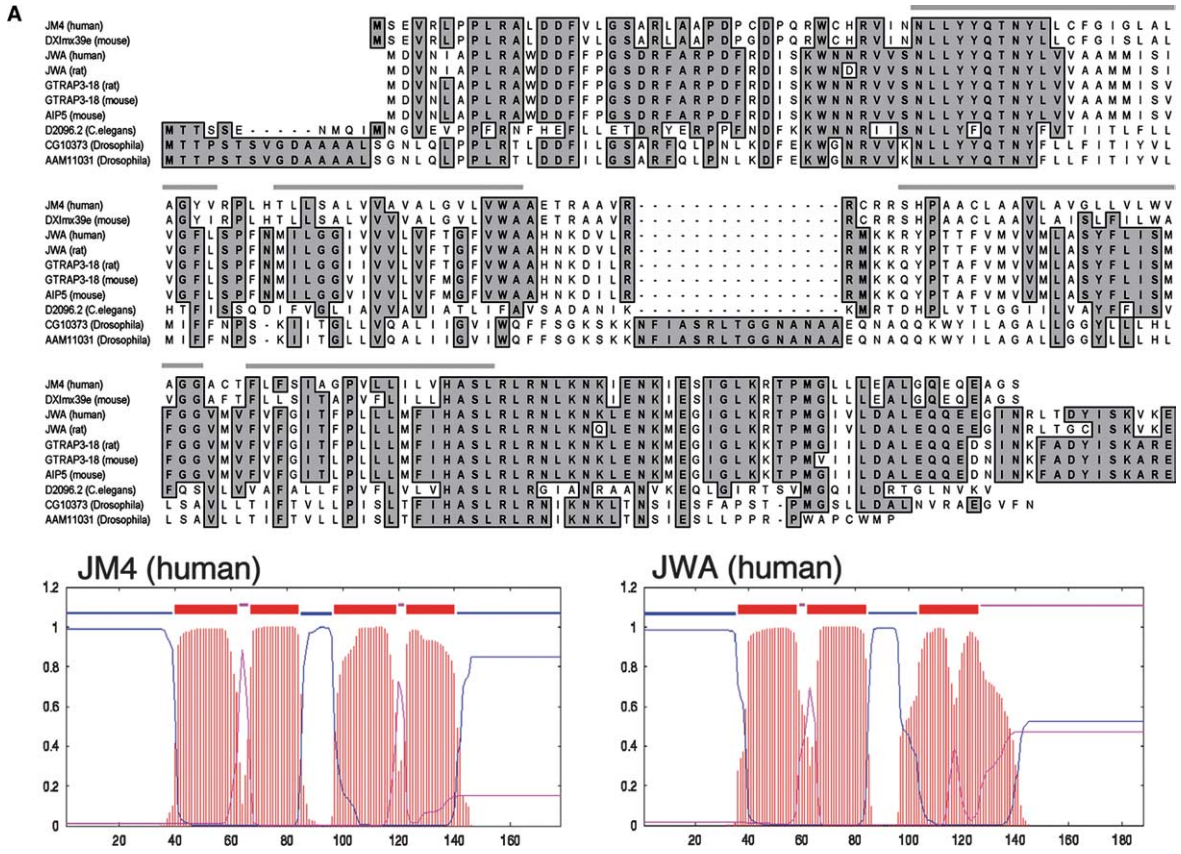

Fig. 3. JM4, JWA, and GTRAP3-18 share sequence and structure homologies and co-localise within the cell. (A) Alignment of JM4 and related proteins from different species using the program MacVector™ 7.1. The shaded regions indicate conserved amino acid residues and grey bars represent the four predicted transmembrane helices of JM4. The structural homology of JM4 (amino acids 1–178) and JWA (amino acids 1–188) is reflected in the transmembrane plots shown below (*y*-axis: transmembrane probability; *x*-axis: amino acid position). Potential transmembrane helices of JM4 and JWA are shown in red (TMHMM (v. 2.0)) [23]. (B) Co-localisation of JM4, JWA, and GTRAP3-18. HeLa cells were transfected with plasmids as indicated (top: VSV–JM4 and Myc–JWA; bottom: VSV–JM4 and GTRAP3-18–HA). For detection by immunofluorescence the appropriate antibodies were used (top: mouse anti-VSV, rabbit anti-Myc; bottom: mouse anti-VSV, rabbit anti-HA) followed by incubation with TRITC-conjugated (red) anti-mouse and FITC-conjugated (green) anti-rabbit antibodies. The yellow colour in the merged pictures indicates co-localisation of the proteins (right). (C) Cellular distribution of JM4. HeLa cells were transiently transfected with a vector encoding VSV–JM4. Cellular localisation of proteins was analysed by immunofluorescence using specific antibodies. Expression of JM4 is shown in red, cellular calnexin (marker of the endoplasmic reticulum) or M6PR (marker of *trans*-Golgi and endosomes) are visualised in green. Cellular co-localisation of JM4 with calnexin (top) or with M6PR (bottom) results in yellow colour in the merged pictures (right).

homologies with JM4, are of similar size, and display potential transmembrane helices in a pattern similar to JM4. Thus, the protein is conserved. The structural homology of JM4 and JWA is reflected in the transmembrane plots (Fig. 3A, bottom). GTRAP3-18, the rat homologue of JWA binds to and inhibits the function of the glutamate transporter EAAC1 [16]. GTRAP3-18 and its human homologue JWA share 92% sequence identity at the protein level. JM4 shows 42% sequence identity and an additional 21% protein sequence similarity with JWA.

Since the human proteins JM4 and JWA and its rat homologue GTRAP3-18 share sequence and structure homologies, we analysed their cellular localisation using confocal laser scanning microscopy. VSV–JM4, Myc–JWA, and GTRAP3-18–HA were expressed pairwise in HeLa cells and proteins were detected using the respective antibodies directed against the epitope tags and fluorescence-labelled secondary antibodies. The co-localisation of protein pairs is indicated by the yellow colour in the merged pictures (Fig. 3B).

We wanted to further define the cellular distribution of JM4. Prediction of protein sorting signals and localisation sites in amino acid sequences, PSORTII analysis, predicted a 56% probability for JM4-association with the ER and about 10% for each other compartment, for plasma membrane, Golgi, or mitochondrial or vacuolar localisation. Based on this prediction and the relationship of JM4 to proteins of the intracellular trafficking machinery, we analysed the intracellular localisation of JM4 in comparison with calnexin, which resides in the ER, and the M6PR, a marker of *trans*-Golgi and endosomes. Immunofluorescence analysis indicated that JM4 is co-localised with calnexin and M6PR in granular structures, (Fig. 3C, top and bottom, respectively). These results substantiate the presence of JM4 in the ER and *trans*-Golgi network (TGN). Similar results were obtained in COS cells (data not shown).

3.5. Relationship of JM4, GTRAP3-18, and JWA as demonstrated by heterodimerisation

We had noticed that JM4, like GTRAP3-18 [16], formed multimeric complexes. To test this, HEK-293 cells were transfected with JM4-encoding constructs, either VSV- or Myc-epitope-tagged at their N-termini, or empty vector control

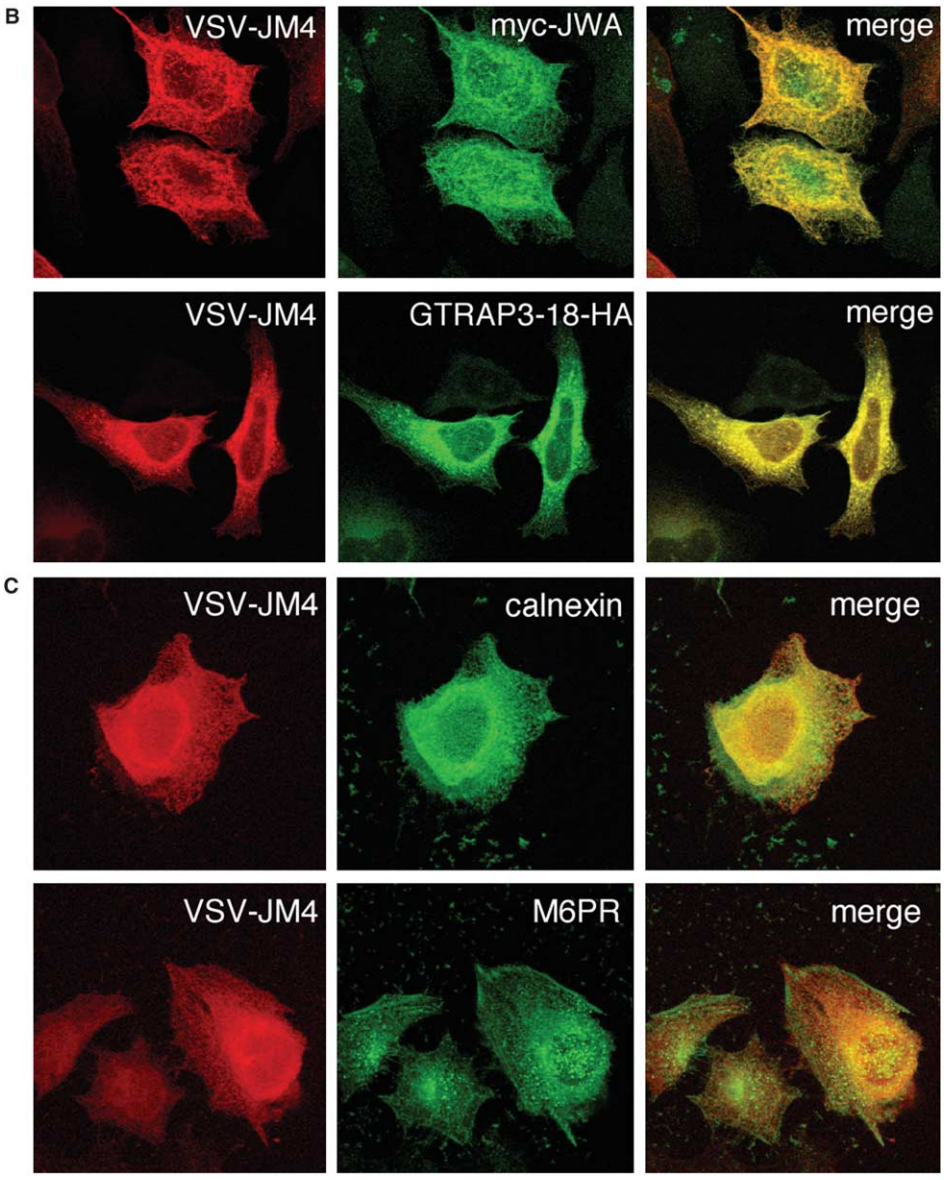

Fig. 3 (*continued*)

plasmids. Myc–JM4 was detected in an anti-Myc immunoblot after immunoprecipitation from cellular lysates using an anti-VSV antibody (Fig. 4A, lane 4). Not only monomers of JM4, but also dimers and trimers of JM4 were co-precipitated. Similarly, 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 heat-denaturation and non-native SDS–PAGE, indicating that the complex formation is of high stringency. JM4, JWA, and GTRAP3-18 form hetero-complexes as shown by pairwise expression of the proteins in HEK-293 cells and co-immunoprecipitation with the respective antibodies. Thus, JM4, JWA, and GTRAP3-18 are structurally related, located in the same structures within the cell (Fig. 3B), and can physically interact with each other (Fig. 4B).

JWA also resembles JM4 in its ability to bind to the CCR5 receptor. HEK-293 cells stably expressing the CCR5 receptor were transfected with plasmids encoding Myc–JM4 or Myc–JWA and used for co-immunoprecipitation studies. Indeed, JM4 and JWA were both able to bind to the CCR5 receptor (Fig. 4C, middle panel, lanes 4 and 6, respectively).

3.6. JM4 affects CCR5 protein levels

To analyse whether JM4 affects levels of CCR5 at the cell surface, HEK-293 cells were co-transfected with plasmids encoding HA–CCR5 and Myc–JM4 or control. Receptor levels at the plasma membrane were analysed by FACS. About 50% reduction of CCR5 protein at the plasma membrane was detected in cells overexpressing JM4 when compared to CCR5 control cells (Fig. 5A, grey and darker line, respectively). The effect was further analysed for protein levels of CCR5 in total cell lysates by Western Blot. HEK-293 cells were co-transfected with plasmids encoding synCCR5-C9 and JM4 or empty control plasmid. CCR5 was precipitated from whole cellular lysates using a conformation-dependent anti-CCR5 antibody (2D7) or an antibody directed against the C9-epitope tag fused to the C-terminus of CCR5 (1D4). CCR5 protein levels were

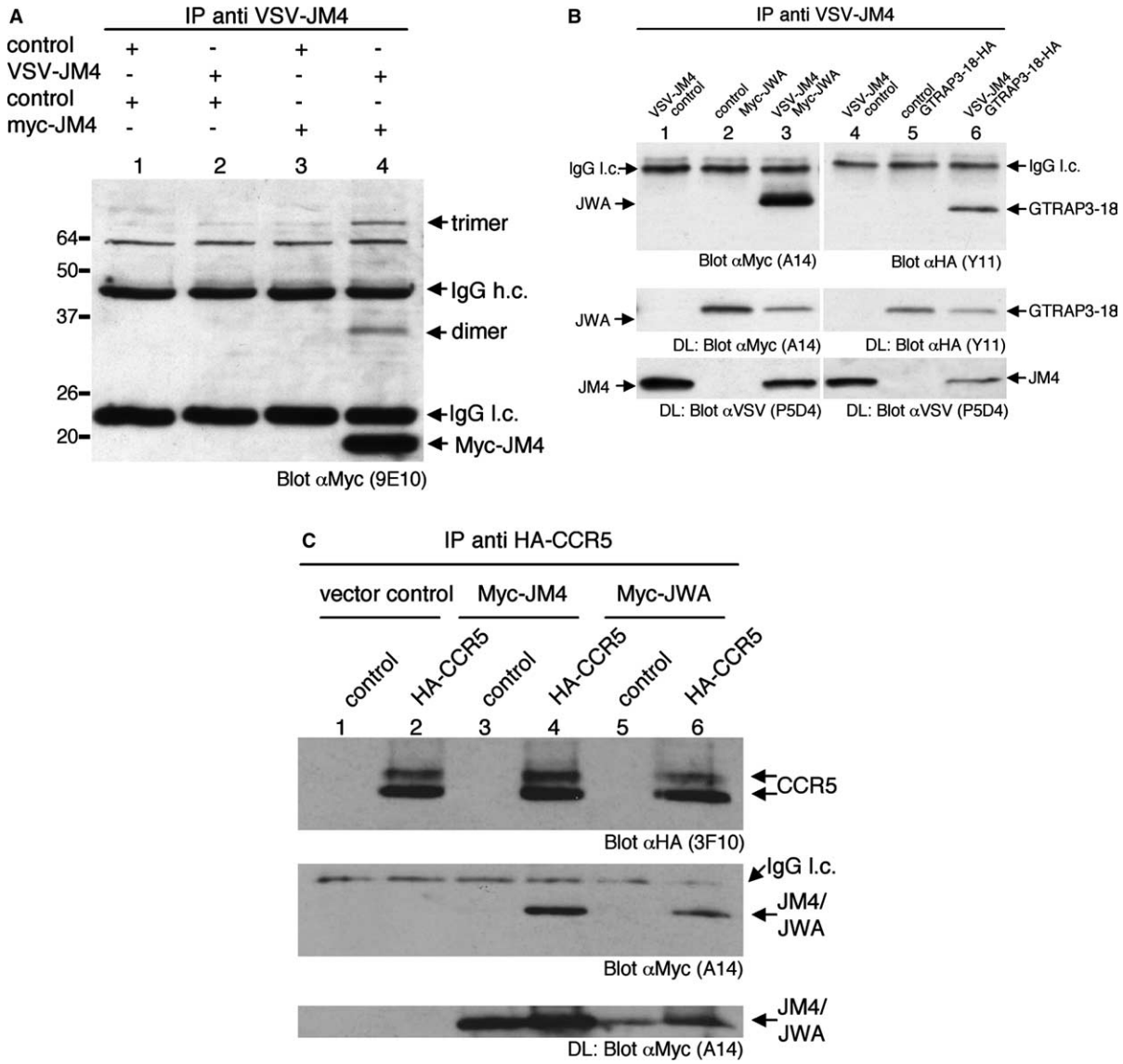

Fig. 4. (A) JM4 forms homo-multimers. HEK-293 cells were transfected with VSV–JM4, Myc–JM4, or empty vector control plasmids as indicated. Cell lysates were immunoprecipitated using an anti-VSV antibody (P5D4). Proteins were separated according to their size by 12.5% SDS–PAGE, transferred to a nitrocellulose membrane, and the co-precipitating Myc–JM4 detected using an anti-Myc antibody. Numbers on the left correspond to molecular weight marker sizes in kilodalton. (For abbreviations see Fig. 2.) (B) Hetero-multimerisation of JM4, JWA, and GTRAP3-18. 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 proteins immunoprecipitated using a mouse anti-VSV antibody (P5D4). Proteins were separated according to their size by SDS–PAGE and transferred to nitrocellulose membranes for immunoblotting with anti-VSV, anti-Myc, or anti-HA antibodies as indicated. (C) Interaction of CCR5 with JM4 and JWA. HEK-293 cells (control) or HEK-293 stably expressing HA–CCR5 (CCR5) were transfected with Myc–JM4, Myc–JWA, or empty vector control plasmids. After cell lysis, HA–CCR5 was precipitated using an anti-HA antibody (12CA5). Proteins were separated according to their size by 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. HA–CCR5, Myc–JM4, and Myc–JWA were detected using the anti-HA and anti-Myc antibodies, respectively. The results were confirmed in two independent experiments.

reduced in cells co-transfected with JM4 (Fig. 5B, lanes 5 and 6) when compared to cells co-transfected with empty plasmid control with some preferential reduction of the upper mature form of CCR5 (Fig. 5B, lanes 3 and 4).

4. Discussion

Here, we describe the JM4 protein, which interacts with the C-terminus of the CCR5 receptor. Moreover, the JM4-related protein JWA also associates with CCR5. Previously, it has been shown that the JM4-related rat protein GTRAP3-18 binds to the amino acid transporter EAAC1 [16]. Therefore, JM4, JWA, and GTRAP3-18 might comprise a family of proteins that interact not only with CCR5, but also with other GPCRs or transmembrane-spannning proteins. Other GPCR-interacting proteins such as β-arrestins or members of the family of GPCR-associated sorting proteins (GASP) have been shown to bind and regulate not only one specific but several GPCR proteins [24–26].

JM4 is ubiquitously expressed and computer-based data analyses predict that JM4 is a four-transmembrane-spanning

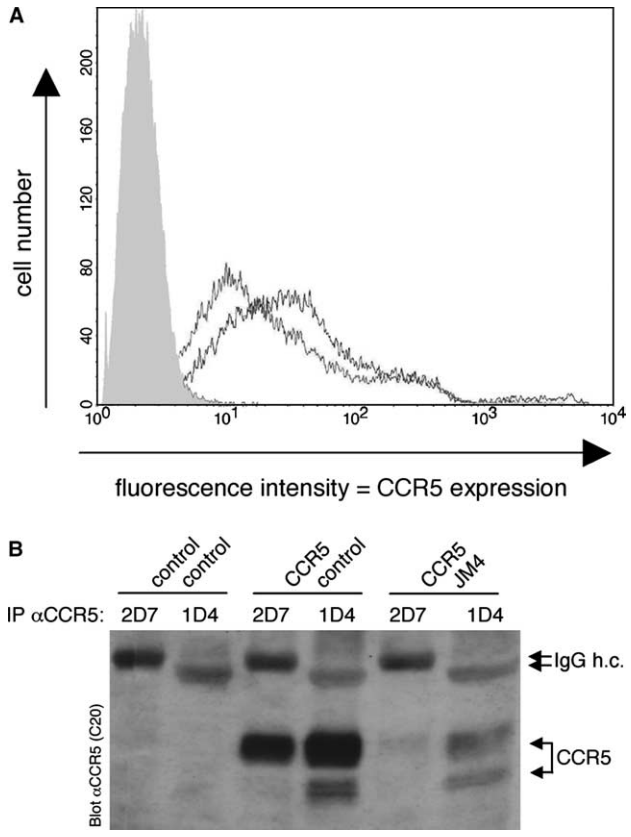

Fig. 5. Co-transfection of JM4 and CCR5 affects receptor protein levels. (A) CCR5 expression levels at the cell surface of HEK-293 cells co-transfected with plasmids encoding HA–CCR5 and Myc–JM4 (grey line) or HA–CCR5 and control plasmid (darker line) were determined by flow cytometry using the FITC-conjugated anti-CRR5 antibody 2D7. Staining with isotype control antibody is shown in shaded histogram. The results were reproducible in several experiments. (B) HEK-293 cells were transfected with plasmids encoding synCCR5-C9 and Myc–JM4 or empty vector control as indicated. Cells were lysed and CCR5 was immunoprecipitated using a conformation-dependent or -independent antibody (2D7 and 1D4, respectively) as indicated. Proteins were separated according to their size by SDS–PAGE and transferred to a nitrocellulose membrane for immunoblotting with anti-CCR5 antibody (C20). (For abbreviations see Fig. 2.)

protein, which is associated with the ER. Immunofluorescence analyses of JM4 and the related protein JWA show localisation of these proteins in a network-like structure, resembling the ER and the Golgi. In particular, JM4 co-localises with calnexin and M6PR. Calnexin is as an integral membrane component of the ER and acts as a molecular chaperone involved in quality control of newly synthesised proteins and protein sorting in the secretory pathway [27,28]. The second protein, M6PR, is located particularly in the TGN and endosomes and functions primarily in the transport of M6P-containing glycoproteins from the TGN to endosomes or lysosomes [29]. JM4 must interact with CCR5 at intracellular membranes during trafficking possibly when the receptor is trafficked from the ER, through the Golgi apparatus to the plasma membrane, because we did not detect JM4 at the plasma membrane by immunofluorescence analyses (Fig. 3B) as it has previously been reported for GTRP3-18 [20].

Sequence analyses suggested a relationship of JM4 with an interaction partner of an ADP-ribosylation factor (ARF) family member [30]. ARF and ARF-like (ARL) proteins belong to a large family of GTPases, some of which have been demonstrated to be involved in the formation of transport vesicles and intracellular traffic [31–33]. Analyses using the conserved domain database, CDD [34], showed significant similarities of JM4 with a family of proteins bearing a prenylated Rab-acceptor 1 (PRA1) motif. This family of proteins includes also GTRAP3-18. PRA1 regulates vesicle biogenesis at the Golgi [35] and like ARFs associates with membranous vesicles during assembly and transport [36]. Furthermore, PRA1 has just recently been shown to interact with a GPCR [37], underscoring its relationship with JM4.

No obvious cellular phenotype could be detected after down-regulation of JM4 using small interfering RNAs even though RNA interference clearly reduced JM4 at the mRNA and protein level (data not shown). Loss of JM4 function is compensated for by related proteins, such as JWA or other unknown proteins.

Considering the putative biological function of JM4 and JWA in intracellular transport, we analysed cell surface expression of CCR5 after overexpression of JM4 in a transient co-transfection system. Indeed, JM4 reduced CCR5 levels at the plasma membrane but also in cellular lysates, where especially the CCR5 form of higher molecular weight was affected (Fig. 5). This suggests that JM4 may be a guardian for incorrect balance of proteins, their folding or other parameters involved in transport and may induce proteasomal degradation. This needs to be further analysed.

JM4 affected not only expression and cell surface localisation of CCR5 but also of other membrane proteins such as CXCR4 or CD4, but not the cytoplasmic green fluorescence protein (data not shown). This has also been reported for parallel co-transfection of other membrane proteins [37].

In summary, we identified a novel CCR5-interacting protein JM4, which, based on structural and functional similarities to JWA and GTRAP3-18 and on computer-database-assisted predictions, is suggested to play a role during protein transport through the ER and Golgi to the plasma membrane. JM4 function may not be restricted to CCR5, but may affect also other transmembrane proteins, as suggested by its similarity to GTRAP3-18, a known regulator of the plasma membrane-associated glutamate transporter EAAC1.

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