

Myosin IIA is involved in the endocytosis of CXCR4 induced by SDF-1 α

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Summary

Endocytosis of chemokine receptors regulates signal transduction initiated by chemokines, but the molecular mechanisms underlying this process are not fully defined. In this work, we assessed the involvement of the motor protein nonmuscle myosin heavy chain IIA (MIIA) in the endocytosis of CXCR4 induced by SDF-1 α (also known as CXCL12) in T lymphocytes. Overexpression of the C-terminal half of MIIA inhibited the ligand-induced endocytosis of CXCR4, but not that of transferrin receptor. Targeting MIIA either by silencing its expression with small interfering RNA (siRNA) or by blebbistatin treatment also inhibited endocytosis of CXCR4. Inhibition of endocytosis of CXCR4 by targeting endogenous MIIA resulted in an increased migration of T cells induced by

SDF-1 α , and in the inhibition of the HIV-1-Env anti-fusogenic activity of this chemokine. Co-immunoprecipitation and protein-protein binding studies demonstrated that MIIA interacts with both the cytoplasmic tail of CXCR4 and β -arrestin. Moreover, SDF-1 α promotes a rapid MIIA- β -arrestin dissociation. Our data reveal a novel role for MIIA in CXCR4 endocytosis, which involves its dynamic association with β -arrestin and highlights the role of endogenous MIIA as a regulator of CXCR4 internalization and, therefore, the onset of SDF-1 α signaling.

Key words: Chemokines, T cells, Signal transduction

Introduction

Chemokine receptors are seven-transmembrane G-protein-coupled receptors (GPCR) implicated in immune and inflammatory responses that regulate crucial processes, such as the activation and migration of leukocytes, development of immune cells (Rossi and Zlotnick, 2000; Rottman, 1999), and angiogenesis (Salcedo and Oppenheim, 2003). The binding of chemokines to their receptors triggers diverse signaling cascades, including activation of G proteins and the PI3-K, Jak/STAT, Rho-p160 ROCK and MAPK pathways (Ganju et al., 1998; Mellado et al., 1998; Vicente-Manzanares et al., 2002; Vicente-Manzanares et al., 1999; Vicente-Manzanares et al., 2003; Wu et al., 1993). The activation of these signaling pathways is often accompanied by the internalization of chemokine receptors and their trafficking back to the plasma membrane. This intracellular turnover determines the leukocyte responsiveness to chemokines (Fan et al., 2004).

CXCR4 is a chemokine receptor that plays an important role in homeostasis, cell migration, inflammation, B lymphocyte development and tumor metastasis (Müller et al., 2001; Proudfoot et al., 1999; Wells et al., 1998). It binds to SDF-1 α and is one of the most important co-receptors for HIV (Berger et al., 1999; Bleul et al., 1996a; Bleul et al., 1996b). It is well established that both SDF-1 α and phorbol esters promote a rapid endocytosis of CXCR4 (Signoret et al., 1997), but only the internalization mediated by SDF-1 α uses an arrestin-dependent pathway (Orsini et al., 1999). Nevertheless, the molecular mechanisms involved in CXCR4 internalization are

still poorly understood. It is known that ligand-stimulated chemokine receptor internalization occurs through clathrin-coated pits that fuse to early endosomes, in which chemokine receptor is dephosphorylated by protein phosphatase 2A, and delivered to either recycling endosomes or late endosomes/lysosomes for its degradation, as demonstrated for CXCR2 (Fan et al., 2004).

Several mechanisms for the regulation of CXCR4 internalization, both dependent and independent of its phosphorylation, have been described (Fernandis et al., 2002). The C-terminal domain of CXCR4, rich in serine/threonine residues, is crucial for receptor internalization, because these residues are phosphorylated by GPCR kinases (GRKs) (Haribabu et al., 1997). This promotes the binding of arrestins, which induces the dissociation of receptor from G proteins, thus switching off signaling pathways (Orsini et al., 1999). Arrestins also act as adaptors between the receptor and the endocytic machinery, which includes molecules such as clathrin and its adaptor AP-2. Furthermore, ubiquitylation is also involved in the endocytosis and fate of CXCR4 mediated by β -arrestin. In this regard, a degradation motif in CXCR4 has been identified, and it is also well established that the proteasome pathway plays a major role in its downmodulation (Fernandis et al., 2002; Marchese et al., 2003).

Nonmuscle myosin II is a molecular motor and a member of the myosin superfamily of proteins that comprise at least three distinct isoforms: MIIA, MIIB and MIIC (Simons et al., 1991). MIIA and MIIB are present in all cell types (Simons et

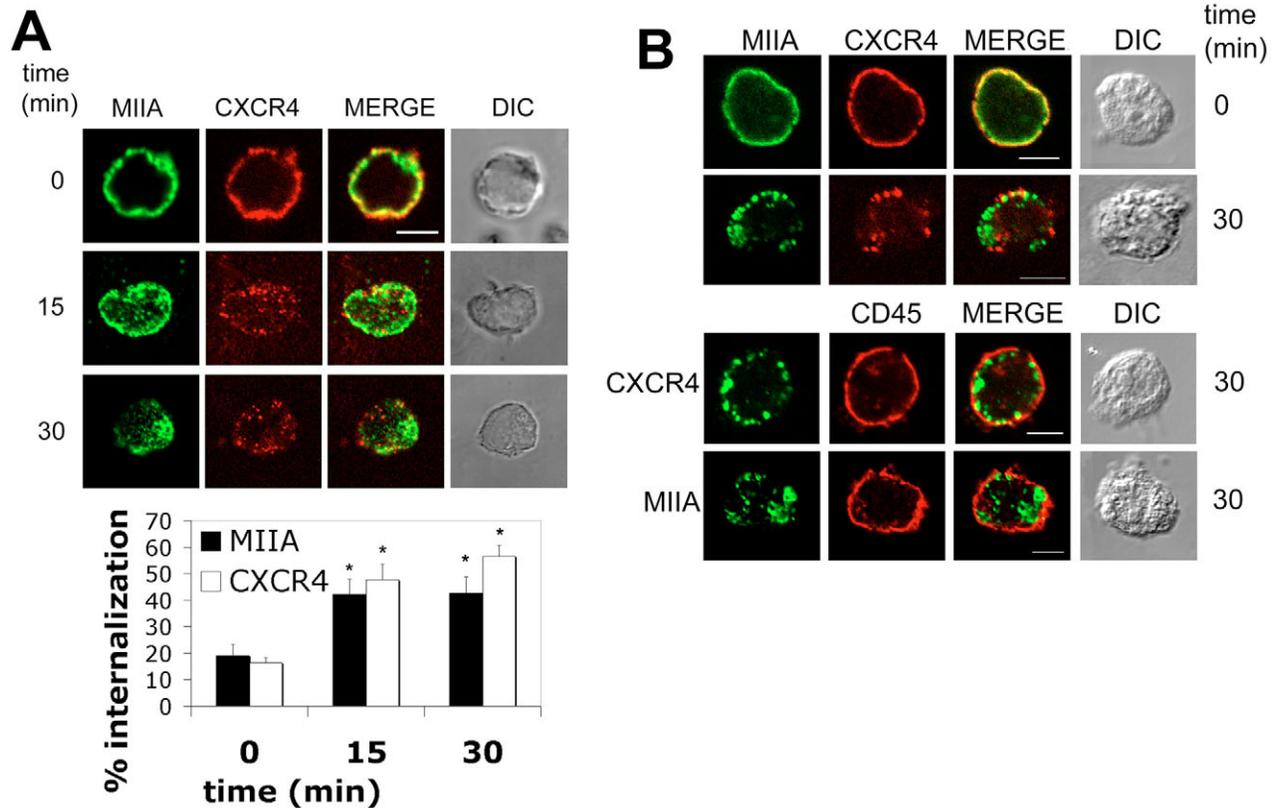


Fig. 1. Confocal microscopy analysis of SDF-1 α -induced endocytosis of MIIA and CXCR4. (A) Confocal analysis of MIIA- and CXCR4-staining in PBLs untreated or treated with 300 nM SDF-1 α for the indicated time periods. Quantification of the fluorescence signal of CXCR4 and MIIA in the cytosolic compartment versus the plasma membrane was performed on the complete 3D confocal stack for $n=10$ cells from two independent experiments, and represented as the mean percentage of internalization \pm s.d.; * $P<0.05$. (B) Confocal analysis of MIIA, CXCR4 and CD45 in J77 T cells untreated or treated with 300 nM SDF-1 α for the indicated periods of time. CD45 is depicted as a control of plasma membrane molecule that is not internalized in response to SDF-1 α . Bars, 5 μ m.

al., 1991), whereas the recently discovered MIIC is more abundant in human adult tissues than in fetal tissues (Golomb et al., 2004). The specific physiological roles of these isoforms are still far from clear. Some authors postulate that these myosins are functionally redundant, whereas other groups support separate and unique roles, based on their differences in subcellular localization, enzyme kinetics and velocity of movement inside the cell (Kimura et al., 1993; Maupin et al., 1994). Hence, there are some phenomena in which MIIA seems to be the major isoform implicated, such as the preservation of cell shape (Wei and Adelstein, 2000) and motility (Jacobelli et al., 2004), as well as cell membrane repair (Togo and Steinhardt, 2004), cellular adhesion (Wylie and Chantler, 2001) and neurite retraction (Wylie and Chantler, 2001).

The association of MIIA with the cytoplasmic tail of CXCR4 and CCR5 has been described previously (Rey et al., 2002). The MIIA isoform was found evenly distributed on the submembrane compartment of spherical non-stimulated lymphocytes, whereas it localized at the leading edge of migrating polarized lymphocytes in response to chemokines, colocalizing with the chemokine receptor. However, the functional relevance of these molecular complexes and the possible involvement of MIIA in intracellular signaling have not been determined. In this work, we show that MIIA

participates in the SDF-1 α -dependent endocytosis of CXCR4, and that MIIA interacts dynamically with β -arrestin, a key component of the CXCR4 internalization pathway.

Results

MIIA and CXCR4 are internalized in response to SDF-1 α in T lymphocytes

We assessed the possible role of the interaction between MIIA and CXCR4 in SDF-1 α -induced-CXCR4 internalization in T cells. Immunofluorescence experiments showed that CXCR4 and MIIA were located at the plasma membrane and in the submembranous compartment, respectively, of untreated PBLs and J77 cells (Fig. 1A and B, respectively) and Rey et al. (Rey et al., 2002). Interestingly, upon cell treatment with SDF-1 α , most of the signal of both CXCR4 and MIIA was detected in cytoplasmic vesicles already after 15 minutes (see confocal images and quantification in Fig. 1A). However, upon internalization, they barely colocalize (Fig. 1A,B). As a control of plasma membrane staining, the unrelated membrane receptor CD45, which remained at the cell surface, was used (Fig. 1B).

MIIA C-terminal tail domain prevents SDF-1 α -induced CXCR4 endocytosis in T lymphocytes

To assess the role of MIIA in the process of endogenous

CXCR4 internalization, a GFP fusion protein that contains the C-terminal fragment of MIIA (MIIA tail-GFP) was generated. This fragment lacks the actin-binding domain and yet colocalizes in transfected HeLa cells with endogenous MIIA (data not shown). A 30 kDa protein fragment containing the N-terminal fragment of MIIA fused to GFP (MIIA head-GFP) was used as negative control in some experiments.

J77 T cells transfected with MIIA tail-GFP showed a diminished endogenous CXCR4 endocytosis upon stimulation with SDF-1 α , as determined by quantitative flow cytometry analyses. This effect was not observed in cells transfected with GFP alone or with MIIA head-GFP (Fig. 2A). Kinetic experiments performed in J77 cells transfected with MIIA tail-GFP showed a marked inhibition of CXCR4 internalization at the different time points studied (Fig. 2C). By contrast, the internalization of endogenous CXCR4 induced by PMA was not affected by the expression of the C-terminal fragment of MIIA (Fig. 2A). Furthermore, MIIA tail-transfected J77 cells showed the same profile of transferrin receptor endocytosis as untransfected cells (Fig. 2B). These data indicate that MIIA is selectively involved in ligand-induced CXCR4 endocytosis, but not in PMA-induced CXCR4 internalization.

Interference with endogenous MIIA expression or function inhibits the ligand-induced endocytosis of CXCR4

To assess the involvement of endogenous MIIA in the endocytic process of CXCR4, different small interfering RNA (siRNA) oligonucleotides (MIIA1 and MIIA2) designed to inhibit MIIA expression were transfected into J77 cells, and SDF-1 α -mediated CXCR4 endocytosis was measured by flow cytometry. As determined by western blot, cell transfection with each one of the two siRNA sequences

produced a partial decrease (of approximately 30-50%) of MIIA, but not of MIIB expression, on J77 cells, with respect to an unrelated control sequence (Fig. 3A). Although the silencing of MIIA was partial, it resulted in statistically significant higher amounts of CXCR4 remaining on the cell surface after SDF-1 α -treatment, which suggests a block of CXCR4 endocytosis (Fig. 3B).

CXCR4 internalization experiments performed with blebbistatin, a specific inhibitor of nonmuscle myosins, further confirmed the implication of MIIA in the ligand-induced endocytosis of this receptor (Fig. 3C). By contrast, blebbistatin did not alter the endocytosis of the transferrin receptor (not shown). In addition, interference with endogenous MIIA expression did not seem to alter the formation of clathrin vesicles, because no apparent differences in the clathrin pattern were observed in cells transfected with the siRNA targeting MIIA and its negative control (Fig. 3D).

Targeting of MIIA increases migratory response to SDF-1 α in T cells and negatively affects the anti-HIV-1 fusogenic capacity of SDF-1 α

The functional relationship between MIIA and CXCR4 was then analyzed. We found that cells transfected with MIIA tail-GFP or siRNA MIIA, but not MIIA head-GFP, had a higher migratory response to SDF-1 α (Fig. 4A,B). These results suggest that the targeting of MIIA interferes with CXCR4 internalization and alters the function of this receptor. The anti-HIV-1 activity of SDF-1 α relies in its capacity to bind to CXCR4 – thereby impeding the interaction between the HIV-1-envelope and CXCR4 – and to internalize the chemokine receptor (Amara et al., 1997). Accordingly, in additional experiments we found that in cells transfected with MIIA tail, SDF-1 α had a diminished HIV-1-Env anti-fusogenic activity at

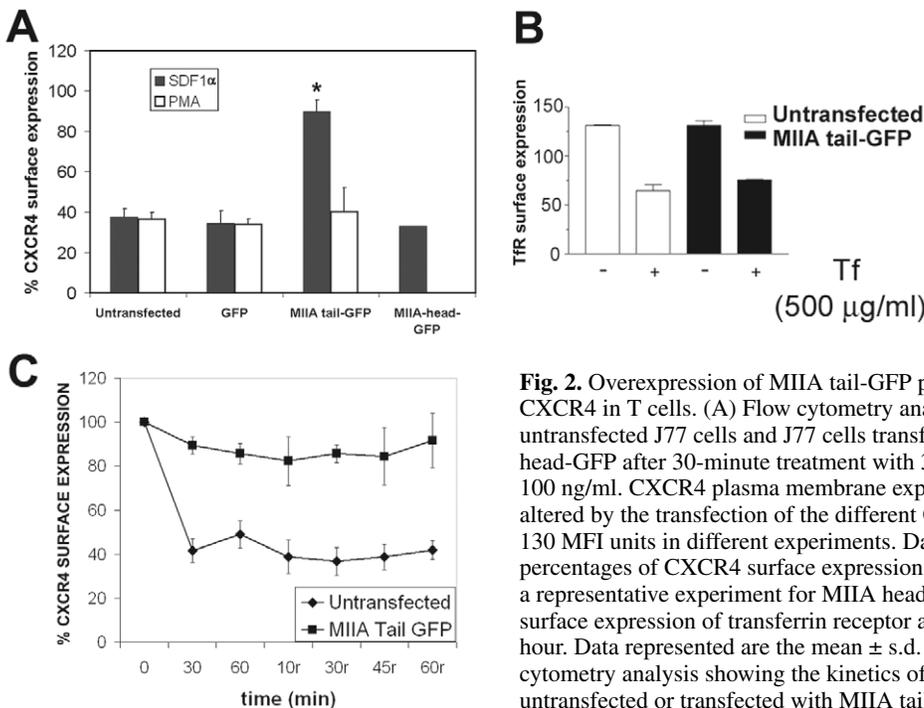


Fig. 2. Overexpression of MIIA tail-GFP prevents the ligand-induced endocytosis of CXCR4 in T cells. (A) Flow cytometry analysis of CXCR4 surface expression in untransfected J77 cells and J77 cells transfected with GFP, MIIA tail-GFP and MIIA head-GFP after 30-minute treatment with 300 nM SDF-1 α , or 1-hour treatment PMA 100 ng/ml. CXCR4 plasma membrane expression levels in untreated samples were not altered by the transfection of the different GFP constructs and were in the range of 100-130 MFI units in different experiments. Data are represented as the mean \pm s.d. of the percentages of CXCR4 surface expression of three independent experiments * P <0.05 or a representative experiment for MIIA head-GFP. (B) Flow cytometry analysis of cell surface expression of transferrin receptor after treatment with 500 μ g/ml transferrin for 1 hour. Data represented are the mean \pm s.d. of three independent experiments. (C) Flow cytometry analysis showing the kinetics of CXCR4 membrane expression in J77 cells untransfected or transfected with MIIA tail-GFP. Data represent the mean percentage of CXCR4 expression \pm s.d. with respect to the expression at 0 minutes of three independent experiments.

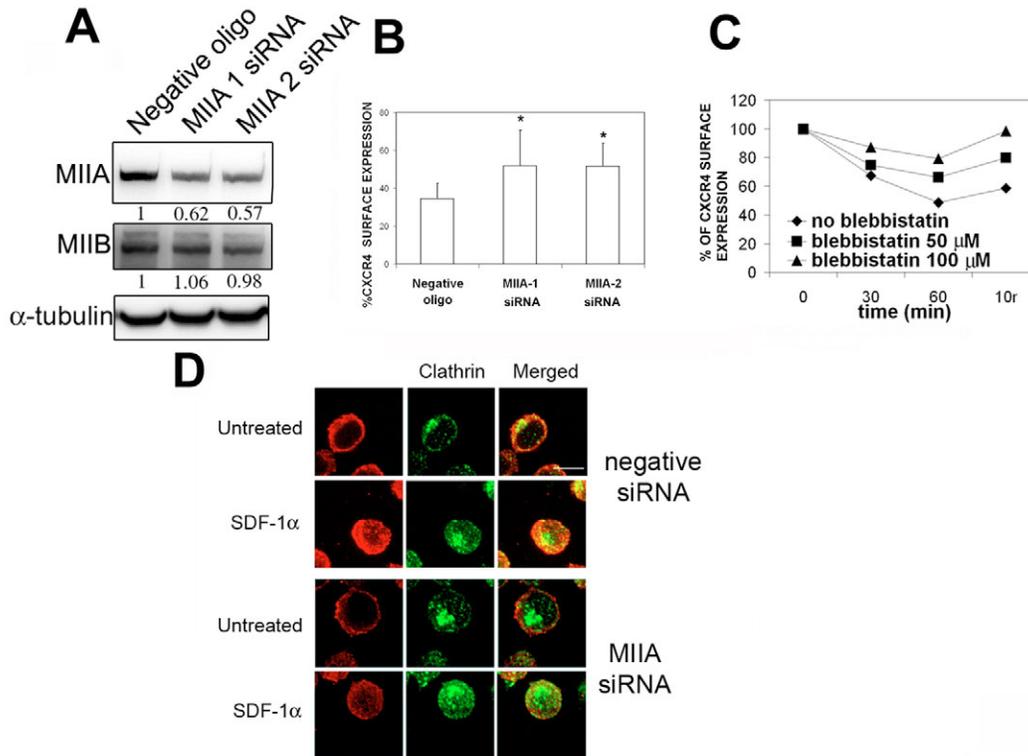


Fig. 3. Interference with MIIA expression or function inhibits the ligand-induced endocytosis of CXCR4. (A) Western blot analysis of J77 cells transfected with siRNA oligonucleotides and stained for MIIA, MIIB and tubulin. Numbers depict the quantification of MIIA and MIIB expression, corrected with tubulin loading and related to cells transfected with the negative control oligonucleotide. (B) Flow cytometry analysis of cell surface expression of CXCR4 in cells transfected with siRNA targeting MIIA after 30-minute treatment with 300 nM SDF-1 α . CXCR4 plasma membrane expression levels in untreated samples were not altered by the transfection of the different siRNA oligonucleotides and were in the range of 90-120 MFI units in the different experiments. Data represent the percentage of CXCR4 with respect to untreated cells (mean \pm s.d.) of six independent experiments * P <0.05. (C) Flow cytometry analysis showing cell surface expression of CXCR4 in cells pretreated for 30 minutes with 50 or 100 μ M blebbistatin and then exposed to 300 nM SDF-1 α at 37°C for 60 minutes. CXCR4 plasma membrane expression levels before SDF-1 α addition were not altered by blebbistatin treatment and ranged 80-100 MFI units in the different experiments. Data represent the percentage of CXCR4 expression with respect to that at 0 minutes in a representative experiment ($n=6$ experiments). (D) Confocal microscopy analysis of J77 cells transfected with siRNA targeting MIIA (MIIA1) and negative control siRNA. Cells were treated with 300 nM SDF-1 α at 37°C for 30 minutes when indicated, and stained for MIIA and clathrin. Bar, 5 μ m.

chemokine concentrations that mainly exert this effect by inducing CXCR4 internalization. Hence, an about twofold higher chemokine dose was required to block HIV-1-Env-mediated cell fusion (Fig. 4D and quantified in C).

Direct association of MIIA with CXCR4-tail and β -arrestin

To gain mechanistic insight on the functional role of MIIA in chemokine receptor endocytosis, we next addressed whether there is a direct association between CXCR4 and MIIA. For this purpose, [35 S]Met-MIIA was incubated with CXCR4-GST and control GST alone, showing a direct interaction (Fig. 5A). Since β -arrestin is a crucial element in chemokine-receptor endocytosis, the possible relationship among CXCR4, MIIA, and β -arrestin was explored. Interestingly, we found that [35 S]Met MIIA was able to associate in vitro to β -arrestin 1 and β -arrestin 2 (Fig. 5B). In addition, co-precipitation experiments showed that the association between the endogenous proteins also occurs in T cells. Interestingly, the treatment with SDF-1 α (300 nM) induced the dissociation of

the endogenous MIIA- β 1-arrestin complex in a time-dependent manner (Fig. 5C, panels a and b).

Discussion

Internalization of chemokine receptors upon engagement by their agonists is an essential feature of immune cells because this phenomenon acts as a regulatory loop of leukocyte chemotaxis and activation. Furthermore, the internalization of HIV co-receptors, such as CXCR4 and CCR5, is a potentially important mechanism by which endogenous ligands for these receptors may modulate HIV infection (Venkatesan et al., 2003). Nevertheless, mechanistic insight involved in chemokine receptor internalization and downstream events is still lacking. In this work, we report that the motor protein MIIA is involved in the ligand-induced endocytosis of endogenous CXCR4. Hence, the SDF-1-mediated endocytosis of CXCR4 is inhibited by overexpression of the MIIA tail, a protein fragment that lacks the actin-binding domain. The inhibition of CXCR4 endocytosis is also observed when cells are transfected with siRNA sequences for MIIA, or treated with

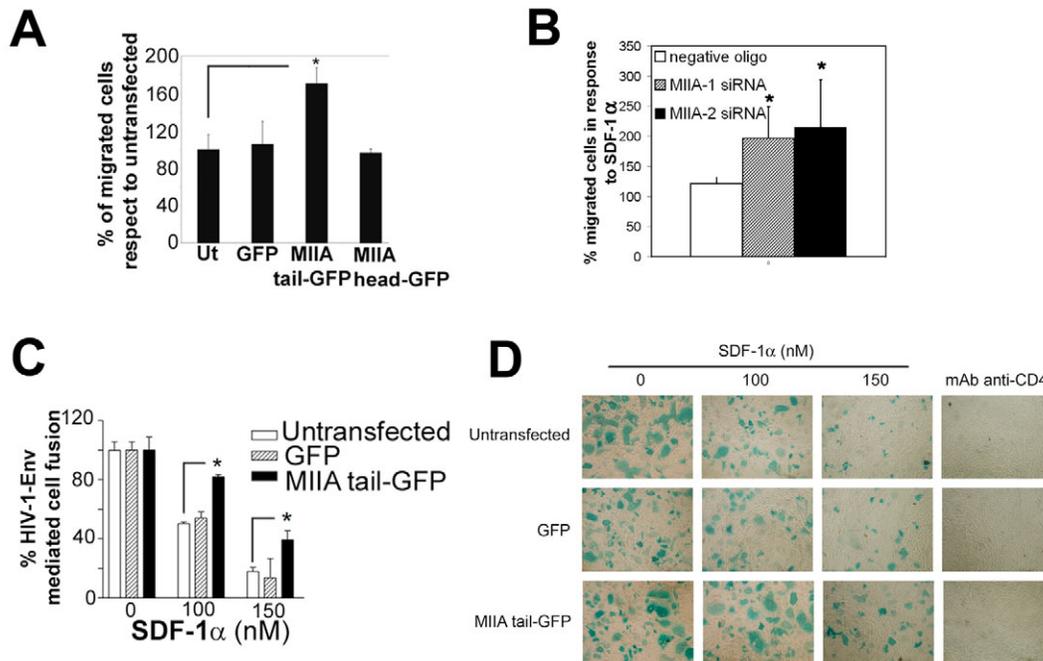


Fig. 4. Functional effects of MIIA targeting. (A) Increase in the migratory response of J77 cells transfected with MIIA tail-GFP to 100 nM SDF-1 α . Data represent the percentages of migrated cells in response to SDF-1 α , normalized with respect to untransfected (Ut) cells treated with SDF-1 α (mean \pm s.e.m.) of three independent experiments * P <0.05. (B) Increase in the migratory response of J77 cells transfected with siRNA oligonucleotides for MIIA (MIIA1 and MIIA2) to 100 nM SDF-1 α . Data represented are the percentages of migrated cells in response to SDF-1 α , normalized with respect to cells with no SDF-1 α -treatment (mean \pm s.d.) of three independent experiments * P <0.05. (C) Loss of the anti-HIV-1 fusogenic capacity of SDF-1 α in cells transfected with MIIA tail-GFP, in Env-HIV-mediated cell fusion experiments, by using MIIA tail-GFP-transfected HeLa cells, at non-saturating concentrations of the chemokine. The level of cell fusion was evaluated by quantitative measurement of chemiluminescence, using a kit that couples β -galactosidase and luciferase activity (β -gal reporter gene assay); results are shown as the mean \pm s.e.m. of three independent experiments * P <0.05. (D) Series of images of a representative experiment of those quantified in C, showing the block of Env-mediated cell-to-cell fusion by SDF-1 α at different concentrations by using a fusion model, based on HeLa cells transfected with MIIA tail-GFP. The number and size of syncytia formed were monitored by X-Gal staining. Images were acquired with a 10 \times objective.

a specific inhibitor of nonmuscle myosins. As expected, the dominant-negative mutant was able to almost completely abolish SDF-induced CXCR4 endocytosis, whereas the partial silencing of MIIA expression achieved with siRNA oligonucleotides had a quantitatively smaller effect. Nevertheless, this effect seems to be selective of CXCR4 and restricted to SDF-induced internalization, because overexpression of the MIIA tail does not affect the internalization of CXCR4 induced by PMA. In this regard, SDF (but not PMA) has been described to recruit arrestins to the plasma membrane (Orsini et al., 1999), indicating the usage by these two stimuli of different molecular mechanisms to induce CXCR4 internalization.

Several lines of evidence support that myosin adaptor molecules are involved in the internalization of cell-surface receptors, by linking clathrin-coated endocytic vesicles and the cortical actin network. In addition, myosins I, V, VI and VII have been directly implicated in membrane traffic (Allan, 1995; Buss et al., 2001a; Fan et al., 2004; Soldati, 2003; Tuxworth and Titus, 2000), myosin VI being the most studied (Buss et al., 2001a; Buss et al., 2001b; Buss et al., 2002; Franck et al., 2004; Hasson, 2003; Morris et al., 2002; Osterweil et al., 2005). However, to our best knowledge, there is no previous evidence of the implication of conventional myosins in

clathrin-mediated endocytic processes. Our data reveal that MIIA may function by coupling the membrane receptor to the molecular endocytic machinery, favoring the formation and uptake of CXCR4-bearing clathrin-coated endocytic vesicles. In contrast to other cell types, in lymphocytes transferrin receptor endocytosis is enhanced by exposure of the cell to its ligand, and is often used as a model of clathrin-mediated endocytosis (Batista et al., 2004; Das et al., 2004; Ponka and Lok, 1999). However, our data indicate that MIIA is not involved in the uptake of the transferrin receptor, suggesting that this molecular mechanism is specific for chemokine-receptor endocytosis. These data, together with the lack of inhibition of PMA-induced endocytosis, also rule out a general effect of MIIA targeting on actin cytoskeleton or clathrin-mediated endocytosis.

The role of the cytoskeleton in endocytic processes has not been fully addressed, and the involvement of F-actin remains controversial. In mammals, this issue has been studied by using drugs that disturb the actin cytoskeleton and, although the results are highly dependent on cell type and experimental procedures, it can be concluded that actin participates in many steps of the endocytic process. In this regard, a recent study underscores a key role for actin during clathrin-mediated endocytosis in mammalian cells (Yarar et al., 2005). During the

2003), CCR5 is partially included in lipid rafts and can undergo different endocytic processes – both clathrin-dependent and -independent (Venkatesan et al., 2003).

In sum, we postulate a new ligand-induced and β -arrestin-mediated endocytic pathway for CXCR4. This involves the motor protein MIIA and its interactions with the receptor and the β -arrestin. MIIA may thus act as an adapter molecule for CXCR4 and arrestin, allowing an alternative mode of interaction specific for the chemokine receptor, that links the endocytic molecular complex to actin cytoskeleton.

Materials and Methods

Cells

Cells of the human J77 T cell line, derived from a patient with acute leukaemia, were grown in RPMI 1640 with 10% FBS (Cambrex Bioscience Verviers, Belgium). Peripheral blood lymphocytes (PBLs) were isolated by Ficoll-Hypaque cushions (Sigma Chemical Co., St Louis, MO). HeLa P4.2 cells, stably transfected with human CD4 cDNA and an HIV-LTR-driven β -gal reporter gene (Pleskoff et al., 1997), and HeLa 243 cells, expressing Tat and Env HIV proteins (Pleskoff et al., 1997; Schwartz et al., 1994), were provided by M. Alizon (Hôpital Cochin, Paris, France).

Antibodies and reagents

Human recombinant SDF-1 α was from R&D (Minneapolis, MN), PMA from Sigma and blebbistatin (a specific inhibitor of the motor activity of nonmuscle myosins) from Calbiochem (Darmstadt, Germany). The anti-CD45 (D3/9) monoclonal antibody (mAb) was generated in our laboratory. The pan- β -arrestin polyclonal antibody (pAb) (against residues 172–268) was from F. Mayor Jr's lab (CBMSO, Madrid, Spain) and the mAb that recognizes β -arrestin 1 (against residues 38–44) was donated by L. Donoso (Thomas Jefferson University, Philadelphia, PA). The rabbit pAb that recognizes pan-arrestin (residues 384–397) used for binding experiments was purchased from Calbiochem. Rabbit anti-MIIA and anti-MIIB pAbs were from Covance (Princeton, NJ), biotinylated anti-CXCR4 mAb and the anti-clathrin mAb were from Becton-Dickinson Pharmingen (San Diego, CA) and Transduction Laboratories (San Diego, CA), respectively. Anti-transferrin receptor L5.1 mAb and transferrin were kind gifts from M. Alonso (CBM, Universidad Autónoma de Madrid, Madrid, Spain). Secondary Abs and phalloidin conjugated to Alexa Fluor-647 were from Molecular Probes and Becton-Dickinson Pharmingen. Human fibronectin was from Sigma, and the [³⁵S]Met and the Amplify reagent from Amersham (Uppsala, Sweden). Bovine recombinant β -arrestin 1 and arrestin 2 were provided by V. V. Gurevich (Vanderbilt University Medical Center, Nashville, TN).

Immunofluorescence staining

J77 cells or PBLs were allowed to adhere to coverslips coated with fibronectin (35 or 50 μ g/ml for J77 cells or PBLs, respectively), treated with 300 nM SDF-1 α at 37°C for the indicated periods of time and fixed with 3% paraformaldehyde. When indicated, cells were permeabilized with FACS lysing solution (Becton-Dickinson). Samples were analyzed in an inverted Leica TCS-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany) using a 63 \times oil immersion objective.

RNA interference

The siRNAs oligonucleotides used for RNA interference (RNAi) assays were all purchased from Eurogentec; the sequences are: MIIA1, 5'-GGCGACCUAU-GAGCGGAUG-3'; MIIA2, 5'-GGAGCGGAACACUGACCAG-3'. The siRNA used as a negative control in these experiments was also from Eurogentec. Oligonucleotides (20 μ M) were electroporated into J77 cells using a Gene Pulser II (Bio-Rad) at 450 V and 950 μ F. The expression of MIIA was determined by western blotting (WB), being the knocking down of MIIA maximal at 72 h.

Cell transfections

The N-terminal and C-terminal fragments of MIIA were subcloned into the pEGFP-C1 and pEGFP-N1 vectors, respectively (Clontech, Palo Alto, CA). The N-terminal fragment of MIIA was obtained by PCR with primers 5'-CCC GCGGCCATGGCACAGCAAGCTGCCC-3' (forward) and 5'-GCCTTACTATCAAAA-GCTTGAGGTTGGTGGTGAACCTC-3' (reverse), the C-terminal fragment with primers 5'-CCC GCGGCCATGCTCACAGAAGAGGAGGAG-3' (forward) and 5'-GCCTTACTATCAAAAAGCTTTTCGGCAGGTTTGGCCTC-3' (reverse). The N-terminal half of the molecule produced a 30-kDa fragment, corresponding to the beginning of the head domain of MIIA, which was fused to GFP (MIIA head-GFP). J77 cells were electroporated or nucleofected (Amaxa, Köln, Germany).

Endocytosis assays

J77 cells, transfected or not, were treated for 1 hour at 37°C, with 300 nM SDF-1 α , 100 ng/ml PMA or 500 μ g/ml transferrin, centrifuged and resuspended in 50 μ l acid wash buffer (150 mM glycine pH 2.3) for 3 minutes. Then, cells were fixed

with 2% paraformaldehyde. For kinetic assays, cells were treated with 300 nM SDF-1 α for 1 hour at 37°C, and then washed with PBS and allowed to recover in RPMI 10% FBS.

To determine receptor endocytosis, cells were stained for CXCR4 or transferrin receptor, and analyzed in a FACScalibur flow cytometer (Becton-Dickinson). The basal expression of CXCR4 remained unchanged when using the different transfection procedures, inhibitors or siRNA treatments, and were in the range of 80–130 MFI units in the different experiments. Two different methods were employed for measuring the ratio of internalized versus external CXCR4; flow cytometry, which measures plasma membrane expression in non-permeabilized cells and, confocal microscopy, in which the membrane or the submembranous compartment signal (quantified with Leica Confocal Software) is related to the total signal of the cell in the different optical slices.

Protein-protein binding assays

The MIIA cDNA plasmid was transcribed, translated and labeled with [³⁵S]Met within a TNT-coupled rabbit reticulocyte system (Promega, Madison, WI). For binding experiments, labeled MIIA was mixed with 1 μ g of recombinant β -arrestin 1 or β -arrestin 2 and immunoprecipitated with anti- β -arrestin pAb (384–397). For MIIA-CXCR4 binding experiments, [³⁵S]Met-labeled MIIA was mixed with the GST fusion protein containing the C-terminus of CXCR4, described previously (Rey et al., 2002). Proteins bound to beads were resolved by SDS-PAGE under reducing conditions, and analyzed by fluorography and autoradiography.

Co-precipitation assays

J77 cells (10⁷ per point) treated or not with SDF-1 α were lysed (1% TX-100 in PBS with protease inhibitors (Roche, Mannheim, Germany) for 20 minutes at 4°C. Cell lysates were incubated overnight at 4°C with 3 μ g of a rabbit pAb against the β -arrestin residues 172–268 previously bound to protein A-sepharose beads (Amersham). Sepharose pellets were washed with the lysis buffer and resuspended in Laemmli buffer. Samples were separated by SDS-PAGE and gels blotted against MIIA or β -arrestin using the monoclonal antibody against residues 38–44 of β -arrestin.

Migration assays

J77 cells (3 \times 10⁵), either untransfected or electroporated with GFP, MIIA tail-GFP or MIIA siRNA (MIIA1 and MIIA2) were resuspended in 100 μ l of medium with 0.2% BSA, and poured in the upper chamber of Transwell migration chambers (8 μ m pore, Costar). In the lower chamber, 600 μ l of medium with or without 100 nM SDF-1 α were added and cells were allowed to migrate for 3–4 hours at 37°C. The number of migrated cells was determined by flow cytometry.

HIV-1 Env-mediated cell-to-cell fusion experiments

Fusion experiments were performed as described previously (Schwartz et al., 1994). Briefly, HeLa 243 cells, lipotransfected with Megafectin 20 (Q-Bio Gene, Irvine, CA) were co-incubated with HeLa P4.2 cells in 96-well plates at a 1:1 ratio for 16 hours, washed and lysed, and the level of cell fusion was quantified by chemiluminescence, using a kit that couples both β -galactosidase and luciferase activity (β -gal reporter gene assay, Roche Diagnostics GmbH). When indicated, HeLa P4.2 cells were pre-incubated with SDF-1 α or with a neutralizing anti-CD4 mAb.

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