Differential regulation of CXCR4 and CCR5 endocytosis

Nathalie Signoret1, Mette M. Rosenkilde2, P. J. Klasse1, Thue W. Schwartz2, Michael H. Malim3, James A. Hoxie4 and Mark Marsh1,*

1Medical Research Council Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, UK
2Department of Pharmacology, The Panum Institute, Blegdamsvej 3, Copenhagen DK-2200, Denmark
3Howard Hughes Medical Institute and Departments of Microbiology and Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
4Department of Medicine, University of Pennsylvania, 415 Curie Blvd, Philadelphia, PA 19104, USA

*Author for correspondence (e-mail: m.marsh@ucl.ac.uk)

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SUMMARY

The chemokine receptors CCR5 and CXCR4 are major co-receptors/receptors for the CD4-dependent and CD4-independent entry of human and simian immunodeficiency viruses. The chemokines that bind and activate these receptors can inhibit the entry of viruses that use the respective co-receptor molecules. Chemokine-induced co-receptor internalisation is a significant component of the mechanism through which chemokines inhibit virus entry. CXCR4 internalisation is induced by the CXCR4 ligand stromal cell derived factor-1 (SDF-1), phorbol esters and, in T cells, cellular activation. Here we show that CXCR4 endocytosis can be mediated through either one of two distinct internalisation signals. A COOH-terminal serine rich domain is required for ligand- but not phorbol ester-induced CXCR4 internalisation. However, a Ser/Ile/Leu motif, similar to that required for the endocytosis of CD4 and the T cell receptor/CD3 complex, is required for phorbol ester-induced, but not ligand-induced, CXCR4 endocytosis. By contrast, CCR5 internalisation is induced by the β-chemokine RANTES but not by phorbol esters. CCR5 lacks the Ser/Ile-Leu sequence required for phorbol ester-induced uptake of CXCR4. Together these results indicate that distinct mechanisms can regulate CXCR4 and CCR5 endocytosis and trafficking.

Key words: CXCR4, CCR5, SDF-1α, RANTES, Phorbol ester, Internalisation motif

INTRODUCTION

Chemokine receptors are members of the seven transmembrane domain (7TM) G protein-coupled receptor superfamily (GPCRs) that mediate chemotactic activity in leukocytes but are expressed on a wide range of cell types (Murphy, 1994; Premack and Schall, 1996). Several chemokine receptors have been shown to act as co-receptors for the human and simian immunodeficiency viruses either independently of (Endres et al., 1996), or together with, CD4 (Berger, 1997; Moore et al., 1997). CCR5 has been implicated as the major co-receptor for SIV and M-tropic strains of HIV-1 (Alkhataib et al., 1996; Deng et al., 1996; Dragic et al., 1996) while CXCR4 permits entry of T-tropic and CD4-independent strains of HIV (Berson et al., 1996; Endres et al., 1996; Feng et al., 1996). Other chemokine receptors, such as CCR2b (Doranz et al., 1996) and CXCR3 (Choe et al., 1996; He et al., 1997) in addition to chemokine receptor-like orphan proteins such as STRL33/Bozo (Alkhataib et al., 1997; Deng et al., 1997), GRP-15/BOB (Deng et al., 1997; Farzan et al., 1997) and GRP1 (Farzan et al., 1997), can also function as co-receptors for M and/or T tropic HIV and SIV strains (Berger, 1997; Berger et al., 1998; Moore et al., 1997).

In several cases chemokines or chemokine antagonists have been shown to inhibit HIV entry (Bleul et al., 1996; Cocchi et al., 1995; Oberlin et al., 1996; Simmons et al., 1997). For CCR5, the β-chemokines RANTES, MIP-1α, MIP-1β and several RANTES antagonists inhibit infection by M-tropic HIV-1 strains (Arenzana-Seisdedos et al., 1996; Cocchi et al., 1995; Simmons et al., 1997), while stromal cell-derived factor 1 (SDF-1α and SDF-1β) and eotaxin can inhibit the entry of viruses that use CXCR4 and CCR3, respectively (Bleul et al., 1996; Choe et al., 1996; Oberlin et al., 1996). The mechanism(s) through which ligands and antagonists block virus infection has been unclear. One model is that the ligands block infection sterically by preventing interaction of the viral envelope protein (Env) gp120 with critical sites on the co-receptor molecule. An alternative view is that ligand-induced chemokine receptor internalisation would effectively remove the co-receptor from the cell surface (Wells et al., 1996). While these two modes of action are not mutually exclusive, two recent studies have suggested that chemokine-induced internalisation of CCR5 and CXCR4 can contribute to the mechanism of chemokine-mediated inhibition of virus entry (Amara et al., 1997; Signoret et al., 1997). Furthermore, a RANTES antagonist AOP-RANTES that is a particularly potent inhibitor of M-tropic strains of HIV-1 (Simmons et al., 1997), induces very efficient CCR5 down-modulation by
promoting receptor internalisation and blocking recycling (Mack et al., 1998).

The mechanisms regulating the endocytosis and trafficking of chemokine receptors are not well understood. For CXCR4, the SDF-1 receptor, ligand and phorbol ester treatment of cells induce rapid internalisation of cell surface receptors (Amara et al., 1997; Forster et al., 1998; Signoret et al., 1997). For T cells, stimulation through CD28, CD3 or CD2 can also down modulate cell surface CXCR4 (Jourdan et al., 1998) and may be the physiological counterpart of the pathway activated by phorbol esters. Internalisation, at least in the case of phorbol esters, occurs through coated pits and results in delivery of the receptor and ligand to endosomes, from where the receptor is able to recycle to the cell surface (Amara et al., 1997; Signoret et al., 1997). For CCR5, ligand also induces uptake into endosomes (Amara et al., 1997; Mack et al., 1998), most likely through coated vesicles (Mack et al., 1998). For this receptor, G-protein coupled receptor kinases (GRKs) and members of the family of β-arrestins can facilitate internalisation (Aramori et al., 1997). In our studies of CXCR4 we have shown that phorbol esters and SDF-1α induce internalisation through distinct biochemical mechanisms. Phorbol ester-induced uptake is inhibited by staurosporine and calphostin C suggesting a role for PKC, while ligand-induced uptake is not (Signoret et al., 1997). As phorbol ester-induced uptake of CD4 and the T cell antigen receptor (TcR) is known to involve phosphorylation-dependent endocytosis signals that are recognised by the AP2 clathrin adaptor complex (Dietrich et al., 1997; C. Pitcher, and M. Marsh, unpublished), we suggested that CXCR4 might be capable of interacting with both the AP2 complex and with β-arrestin through distinct signalling motifs (Signoret et al., 1997).

Previous studies have indicated that deletion of the carboxy terminal domain of CXCR4 abrogates both SDF-1 and phorbol ester-induced receptor internalisation (Amara et al., 1997; Haribaba et al., 1997; Signoret et al., 1997), suggesting that sequences required for endocytosis might reside within this domain. Comparison of the COOH-terminal sequences from the established chemokine receptors indicates that although these cytoplasmic domains have some features in common, there is also significant variability. In this paper we show: (1) that in contrast to CXCR4, CCR5 is not down modulated by treatment of cells with phorbol esters, (2) we identify a phorbol ester-induced internalisation signal in CXCR4 similar to signals previously identified in CD4 and the CD3γ subunit of the TcR, and (3) we demonstrate that mutations in this signal abrogate CXCR4 responses to phorbol ester but not to ligand. Together these results demonstrate that the cell surface expression of CXCR4 and CCR5 can be modulated through distinct biochemical mechanisms. Furthermore, the results suggest that CXCR4 can interact with distinct sets of adaptor proteins that affect endocytosis.

**MATERIALS AND METHODS**

**Reagents**

Tissue culture reagents were from Gibco Ltd (Paisley, UK), and other chemicals were from Sigma (Poole, UK), unless otherwise indicated. Tissue culture plastic was from Nunc, and radioactive reagents were from Amersham International plc (Little Chalfont, UK). Recombinant stromal cell-derived factor 1α (SDF-1α) was expressed in and purified from *Escherichia coli* (provided by Dr Mike Luther, Glaxo Wellcome Inc, RTP, NC, USA). This SDF-1α had an additional N-terminal methionine residue that was not removed following synthesis and purification; however its properties were indistinguishable from those of a chemically synthesised SDF-1α (Crump et al., 1997; Signoret et al., 1997).

**Antibodies**

Anti-human CXCR4 monoclonal antibodies 44702 and 44716 and anti-human CCR5 mAbs 45517, 45523, 45529, 45531 and 45533 were kindly provided by Dr Monica Tsang (R&D Systems, Minneapolis Minnesota; USA). The anti-CD4 antibody, OKT4, was purchased from Ortho Diagnostics Systems Inc. (Raritan, New Jersey). The anti-CXCR4 mAb 12G5 (IgG2a) and the anti-CD4 mAb Q4120 (IgG1) were described previously (Endres et al., 1996; Healey et al., 1990). 12G5 was 125I-labelled using Bolton and Hunter reagent (Amersham International plc) as described (Signoret et al., 1997). Specific activities of 300–400 Ci/mmol were obtained for different iodinations. Radioidotinated proteins, diluted in PBS containing 0.25% gelatin and 0.02% NaN3 and stored in small aliquots at −20°C, were stable for up to 4 months.

**Cells**

Retrovirus mediated gene transfer was used to stably express the CCR5 chemokine receptor in CEM-SS cells (CEMss/CCR5) such that they were rendered susceptible to HIV-1 variants. To achieve this, a 100 mm-diameter culture of 293T cells was transiently transfected with 10 μg each of the vectors LP-M/CCR5, pHIT/G and pHIT60 using calcium phosphate as described (Crump et al., 1997; Signoret et al., 1997). Virus supernatant was then harvested at 24 hours and used to infect a parental CEM-SS cell. A stably transduced cell population CEMss/CCR5 was selected and maintained in complete RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and 0.2 μg/ml puromycin.

Mink Mv-1-Lu cells and Mv-1-Lu cells stably expressing human CD4 (Mv-1-Lu-CD4) were obtained from the MRC AIDS Reagents Programme (NIBSC, Potters Bar, UK) and maintained in DMEM containing 10% FCS, 2% penicillin and 0.1 mg/ml streptomycin (PenStrep), with or without 1 mg/ml G418 as required. Mv-1-Lu cells were transfected by electroporation with wild-type or mutated human CXCR4 cDNAs (see below) inserted in the expression vector pTEJ8 (Johansen et al., 1990). Stable transfectants were selected in the presence of 1 mg/ml G418 and colonies were screened for cell surface expression by indirect immunofluorescence using the anti-CXCR4 mAb 12G5 as previously described (Signoret et al., 1997). Similarly, Mv-1-Lu-CD4 cells were transfected with pTEJ8 vectors containing human CXCR4 or CCR5 cDNAs, together with the plasmid pBABE-hyg encoding the hygromycin B resistance gene (Morgenstern and Land, 1990). Colonies were selected in medium containing 1 mg/ml G418 and 500 μg/ml hygromycin B and screened for CXCR4 or CCR5 expression by immunofluorescence using the 12G5 mAb or a mix of anti-CCR5 mAbs (45517, 45523 and 45531), respectively.

COS-7 cells were cultured in DMEM F12 containing 5% FCS, 2 mM glutamine and PenStrep. Cells were transiently transfected using calcium phosphate as previously described (Kledal et al., 1997).

**Mutagenesis**

Truncated CXCR4 molecules were generated using human CXCR4 constructs in which stop codons were inserted at codon positions 322, 330 or 341 by site directed mutagenesis. Point mutations were made in the CXCR4 cDNA in order to replace specific amino acids (S324 and S325, I328 and L329) with alanine residues. The mutations were introduced by the polymerase chain reaction-mediated overlap extension technique, as previously described (Rosenkilde et al., 1994). All constructs were cloned into pTEJ8 vector and sequenced.

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Flow cytometry/FACS analysis
Adherent cells were detached in PBS containing 10 mM EDTA and resuspended in culture medium. After centrifugation (2,000 rpm, 5 minutes), the cells were resuspended in PBS containing 0.1% bovine serum albumin (BSA) and 1% formaldehyde and left to fix overnight at 4°C. After two washes in wash buffer (WB; PBS containing 0.1% BSA and 0.05% azide), cells were labelled with primary antibody in WB for 1 hour at room temperature. Subsequently, the cells were stained in WB with a FITC-conjugated goat anti-mouse antibody (1/100; Pierce and Warriner [UK] Ltd, Chester, UK) for a further hour. Cells were analysed using a Becton Dickinson FACsCalibur flow cytometer (Becton-Dickinson UK Ltd, Oxford, UK).

Phorbol ester induced receptor down modulation on CEMss/CCR5 cells
CEMss/CCR5 cells (2×10^5) were collected and washed twice in binding medium (BM: RPMI-1640 without bicarbonate, containing 0.2% BSA, 10 mM HEPES, and adjusted to pH 7.4) at room temperature. Cells were resuspended in 7 ml of BM and one aliquot of 1 ml (3×10^5 cells) was put directly on ice in cold BM for time point t=0. The rest of the cells were centrifuged (1,500 rpm, 5 minutes) and resuspended in 6 ml of prewarmed BM containing 100 ng/ml phorbol 12-myristate 13-acetate (PMA). At each time point a 1 ml aliquot (3×10^5 cells) was transferred into a 15 ml tube containing 5 ml of cold BM and kept on ice. When the last sample was collected, cells were pelleted by centrifugation (1,500 rpm, 5 minutes), and washed twice in cold WB before fixation for FACS analysis. For each time point aliquots of 2.5×10^5 cells were placed in the well of a 96-well plate, labelled for either CD4, CXCR4 or CCR5 and analysed by flow cytometry.

Immunofluorescence microscopy
Mv-1-Lu-CD4 CXCR4 and CCR5 cells were grown on coverslips for 2 days to a final density of 1-2×10^5 cells. The distribution of CXCR4 and CCR5 was examined on adherent cells incubated for 5 minutes in cold BM adjusted to pH 3.0 to remove the surface-bound ligand as previously described (Signoret et al., 1997). Endocytosis assays on adherent cells were performed essentially as described (Pelchen-Matthews et al., 1989, 1991; Signoret et al., 1997). Briefly, adherent cells were seeded in 16 mm wells in 24-well plates and grown for 2 days to a final density of 1-2×10^5 cells per well. The cells were cooled on ice, washed with BM and incubated for 2 hours at 4°C with 300 μl of 0.5 nM 125I-12G5 antibody in BM. Subsequently, the cells were washed in BM to remove free antibody, and then warmed by addition of 1 ml 37°C BM with or without PMA.

RESULTS

**CXCR4 but not CCR5 is internalised after PMA treatment**

We previously demonstrated that the receptor for the CXC chemokine SDF-1α, CXCR4, is internalised from the cell surface and down modulated when cells are treated with phorbol esters (Signoret et al., 1997). To determine whether other chemokine receptors involved in mediating HIV entry are similarly regulated we investigated the cell surface expression of CCR5 on cells treated with phorbol esters or ligands. We first used transfected mink lung epithelial cells (Mv-1-Lu) in which we previously found CXCR4 to exhibit responses to phorbol esters and ligand similar to those seen in T cell lines (Signoret et al., 1997). Mv-1-Lu-CXCR4 cells, which stably express human CD4, were transfected with cDNAs encoding either human CXCR4 or human CCR5 and clones selected for stable cell surface expression of the chemokine receptors. Chemokine receptor expression was monitored by FACS analysis (not shown) and immunofluorescence (see below).

The distribution of CXCR4 and CCR5 was examined on permeabilised cells by immunofluorescence, either before or after treatment with phorbol ester or ligand (Fig. 1). We also monitored the distribution of CD4 on these cells. On untreated cells, CXCR4 and CCR5 were present mainly on the plasma...
membrane as indicated by the diffuse fluorescence illustrated in Fig. 1A and B, panels I. Similar staining was seen on intact cells confirming that the antigens were located primarily on the cell surface (not shown). When cells were stained for CD4, diffuse cell surface fluorescence and some intracellular punctate fluorescence was seen (Fig. 1A and B, CD4 panels I). This internal fluorescence likely corresponds to CD4 molecules located in the endocytic and exocytic pathways, as previously described (Marsh and Pelchen-Matthews, 1996). We then examined whether the distribution of the chemokine receptors was changed when cells were treated for 45 minutes at 37°C with ligand (SDF-1α for CXCR4 and RANTES for CCR5) or the phorbol ester PMA. As previously observed (Signoret et al., 1997), we found that SDF-1α and PMA induced the redistribution of cell surface CXCR4 to intracellular structures which appeared punctate by immunofluorescence and were often located in regions of the cell adjacent to the nucleus (Fig. 1A, CXCR4 panels II and IV). By contrast, the CC chemokine RANTES had no effect on CXCR4 distribution (Fig. 1A, CXCR4 panel III). Cell surface CD4 was redistributed into punctate intracellular structures on cells treated with phorbol ester (Fig. 1A, CD4 panel II). Neither SDF-1α nor RANTES appeared to affect the distribution of CD4 (not shown). When cells expressing CCR5 were examined, we found no detectable change in the cell surface staining on SDF-1α treated cells (Fig. 1B, CCR5 compare panels I and IV), whereas RANTES induced internalisation of the receptor into intracellular organelles (Fig. 1B, CCR5 panel III). Significantly, PMA had little effect on the distribution of CCR5 and did not appear to induce redistribution of the cell surface receptor (Fig. 1B, CCR5 panel II). The CCR5 expressing cells were, however, able to respond to phosphol ester as demonstrated by their ability to down modulate CD4 when treated with PMA (Fig. 1B, CD4 panel II). These observations indicated that although both CXCR4 and CCR5 were induced to internalise by their specific ligands, CCR5 does not undergo the phosphol ester-induced internalisation seen for CXCR4.

**Chemokine receptor modulation on T cells**

To determine whether CCR5 cell surface expression also fails
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We compared the properties of this receptor expressed on CEMss/CCR5 cells. These cells were derived from the CEMss T cell line after transfection with cDNA for human CCR5. CEMss cells constitutively express both CD4 and CXCR4. Following transfection and selection, CCR5 could also be detected on the surface of these cells by FACS (Fig. 2A). This allowed us to investigate the regulation of surface expression for the three markers simultaneously. After incubation of CEMss/CCR5 cells at 37°C in the presence of 100 ng/ml PMA for up to 2 hours, surface expression of CD4, CXCR4 and CCR5 was determined using antibodies against the specific receptors and flow cytometry (Fig. 2B). On cells treated with PMA we found that cell surface expression of both CD4 and CXCR4 declined with time and was reduced by 60-80% after 2 hours of treatment (Fig. 2B panel a). The kinetics of down modulation for CXCR4 on these cells was slower than that previously seen with SupT1 cells (Signoret et al., 1997). Nevertheless, down modulation was reproducibly detected using several different anti-CXCR4 mAbs. When CCR5 expression was monitored, we saw little change in cell surface expression over 2 hours using 5 different anti-CCR5 mAbs (Fig. 2B, panel b).

The observation that CCR5 and CD4 are internalised independently supports the notion that there is little constitutive interaction of these two molecules on the surfaces of cells in the absence of HIV (Signoret et al., 1997). Moreover, the trimolecular complexes of Env, CD4 and co-receptor that have been proposed to form during viral entry (Lapham et al., 1996; Trkola et al., 1996; Ugolini et al., 1997) are likely to be induced directly by the viral Env protein.

CXCR4 contains an S(x)nLL type motif

Chemokine receptors and related 7TM GPCRs have been shown to undergo endocytosis on binding their specific ligands (Amara et al., 1997; Aramori et al., 1997; Ferguson et al., 1996a; Haribabu et al., 1997; Mueller et al., 1997; Prado et al., 1996; Signoret et al., 1997; Solari et al., 1997; von Zastrow and Kolbika, 1992). Serine rich COOH-terminal domains of several 7TM GPCRs have been implicated in these events, though no distinct internalisation motifs has been identified. We previously demonstrated that SDF-1α and phorbol ester-induced internalisation of CXCR4 occurs through distinct biochemical mechanisms. Endocytosis induced through both reagents requires elements in the COOH-terminal domain of CXCR4, as deletion of the bulk of this domain abrogates both ligand and phorbol ester-induced uptake. However, phorbol ester, but not SDF-1α-induced internalisation is inhibited by staurosporin and calphostin C, implicating a role for protein kinase C only in phorbol ester-induced internalisation (Signoret et al., 1997).

We have previously shown that the cell surface expression of CD4 can be modulated by phorbol esters (Hoxie et al., 1988; Pelchen-Matthews et al., 1993). For CD4, endocytosis is dependent on a signal in the 37 amino acid cytoplasmic domain of the molecule that involves a pair of leucine residues (L413 and L414 in human CD4) and the phosphorylation of an adjacent serine residue (S408) (Pelchen-Matthews et al., 1993; C. Pitcher and M. Marsh, unpublished; Shin et al., 1991). Mutation of either L413, L414 or S408 blocks constitutive and phorbol ester-induced endocytosis of CD4. Phosphorylation of S408 activates the signal and leads to association of CD4 with...
The other three replace the indicated amino acids with alanine residues.

human CXCR4 cDNA by site directed mutagenesis. Three mutations introduced premature stop codons that truncate the cytoplasmic domain.

double line clear box. (B) Mutations in the cytoplasmic tail of CXCR4. Six mutations were introduced into the COOH-terminal domain of the amino acid residues for one or both subclasses of chemokine receptors. The di-leucine based motif specific of CXCR4 is highlighted by a recently defined as CXCR5 (Gunn et al., 1998). The black boxes highlight identical amino acids and the grey boxes conservative changes of receptors were aligned using the Clustal Method algorithm in the DNAStar Megalign package. In addition to defined CXC and CCR chemokine receptors, we included the receptor for the CXC chemokine B-lymphocyte chemoattractant (BLC), Burkitt’s lymphoma receptor 1 (BLR-1) recently defined as CXCR5 (Gunn et al., 1998). The black boxes highlight identical amino acids and the grey boxes conservative changes of amino acid residues for one or both subclasses of chemokine receptors. The di-leucine based motif specific of CXCR4 is highlighted by a double line clear box. (B) Mutations in the cytoplasmic tail of CXCR4. Six mutations were introduced into the COOH-terminal domain of the human CXCR4 cDNA by site directed mutagenesis. Three mutations introduced premature stop codons that truncate the cytoplasmic domain. The other three replace the indicated amino acids with alanine residues.

cloathin coated pits, most likely through interaction with the clathrin AP2 adaptor complex (C. Pitcher and M. Marsh, unpublished). A very similar S(X)_{a}L type signal in the γ subunit of the CD3 complex is also involved in the endocytosis of the T cell receptor (TCR)/CD3 complex and mediates association with AP2 (Dietrich et al., 1994, 1997). Inspection of the cytoplasmic sequences (loops 1-3 and the COOH-terminal domain) of the five human CXCR and nine distinct CC chemokine receptors characterised to date, indicates that CXCR4 contains a S(X)_{a}L-like sequence SSLKI{L in its COOH-terminal domain (Fig. 3A, NB, isoleucine can replace leucine in di-leucine sorting signals; Sandoval and Bakke, 1994). None of the other human chemokine receptors contains the serine rich domain. In addition we changed the S324 and S325 in the full length CXCR4 sequence, respectively. The Δ31 truncation removes the serine rich domain and the SSLKIL sequence, Δ23 retains the SSLKIL sequence but removes the serine rich domain, and Δ12 removes the bulk of the serine rich domain. In addition we changed the S324 and S325 in the full length CXCR4 sequence to alanines (S324A;S325A), and I328 and L329 to alanines (I328A;L329). A third mutant contained all four of these alanine substitutions (S324A;S325A;I328A;L329A). The mutants and wt CXCR4 cDNAs were transfected into Mv-1-Lu cells and stable clones derived by selection with G418. Resistant colonies were tested for complete replacement with the

**CXCR4 COOH-terminal domain mutants**

To examine the role of the SSLKIL sequence in both ligand and phorbol ester-induced internalisation of CXCR4 we generated truncation and site specific mutations in human CXCR4 (Fig. 3B). Stop codons were introduced in place of the codons for amino acids R322, S330 and S341 to generate truncation and site specific mutations in human CXCR4 (Fig. 3B). Stop codons were introduced in place of the codons for amino acids R322, S330 and S341 to generate molecules with COOH-terminal domains truncated by 31 (Δ31), 23 (Δ23) and 12 (Δ12) amino acids, respectively. The Δ31 truncation removes the serine rich domain and the SSLKIL sequence, Δ23 retains the SSLKIL sequence but removes the serine rich domain, and Δ12 removes the bulk of the serine rich domain. In addition we changed the S324 and S325 in the full length CXCR4 sequence to alanines (S324A;S325A), and I328 and L329 to alanines (I328A;L329). A third mutant contained all four of these alanine substitutions (S324A;S325A;I328A;L329A). The mutants and wt CXCR4 cDNAs were transfected into Mv-1-Lu cells and stable clones derived by selection with G418. Resistant colonies were tested for complete replacement with the
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12G5 mAb. All the mutants were expressed at levels comparable to that of the wt protein as indicated by FACS analysis (Fig. 4), and 125I-SDF-1α binding experiments indicated that as with other COOH-terminally truncated chemokine receptors (Arai et al., 1997; Ben-Baruch et al., 1995) the CXCR4 mutants bind SDF-1α with similar affinities (see below).

The SSKIL sequence is required for phorbol ester-induced endocytosis of CXCR4

We first verified that the mutations did not affect the constitutive endocytosis of the receptor using the anti-CXCR4 antibody 12G5, as previously described (Signoret et al., 1997). After cell surface labelling with 125I-12G5 mAb (0.5 nM) for 2 hours at 4°C, Mv-1-Lu CXCR4 transfectants were warmed to 37°C for periods of up to 60 minutes. At the indicated times duplicate sets of cells were placed on ice and the intracellular radioactivity determined using an acid stripping assay that removes only the cell surface counts. As observed previously (Signoret et al., 1997), we measured only low levels of constitutive endocytosis for the wt and all six CXCR4 mutants, with maximally 12% of the initial cell surface pool of receptor internal after 60 minutes at 37°C (Fig. 5A).

When similar experiments were performed with 100 ng/ml PMA included in the medium during the 37°C incubation we found that the rate and extent of endocytosis of Δ31 was similar to that of untreated cells (Fig. 5B). By comparison, the rate of uptake of the wt molecule was increased 7-8 fold (as measured over the first 5 minutes of uptake) and 70% of the initial cell surface pool was internal after 60 minutes of incubation at 37°C. The Δ23 and Δ12 mutants showed intermediate effects. Both showed an increase in the rate and extent of endocytosis in the presence of PMA, though neither construct was internalised as rapidly nor to the same extent as wt CXCR4. Of these two mutants, Δ12 was less impaired than the Δ23 mutant. Thus CXCR4 molecules containing the COOH-terminal SSKIL sequence exhibit phorbol ester-induced increases in endocytosis, although the magnitude of the response may be regulated by other elements in the COOH-terminal domain.

Analysis of the alanine mutations indicated that all three constructs had lost the ability to undergo phorbol ester-induced endocytosis (Fig. 5C). Taken together, the data support the notion that the SSKIL sequence is crucial for the phorbol ester-induced internalisation of CXCR4 and that the serine residues and the isoleucine/leucine pair are both essential components of the motif.

The SSKIL sequence is not required for SDF-1α-induced endocytosis of CXCR4

To determine whether the mutations described above influence the ability of CXCR4 to undergo chemokine-induced down modulation, cells were incubated in medium containing 125 nM SDF-1α at 37°C for periods up to 60 minutes. At the end of the incubation the cells were placed on ice, cell surface bound SDF-1α was eluted by washing the cells in low pH medium and the level of cell surface CXCR4 measured using 125I-12G5. In these experiments we found that truncation of the cytoplasmic domain of CXCR4 progressively abrogated the ability of the molecule to undergo ligand-induced down modulation. Removal of the COOH-terminal 12 and 23 amino acids (Δ12 and Δ23, respectively) reduced the response by 75 and 50%, respectively. As previously shown for a CXCR4ΔCyt construct lacking the bulk of the cytoplasmic domain (Signoret et al., 1997), the Δ31 construct showed no significant SDF-1α-induced down modulation (Fig. 6A). By contrast, all three of the alanine mutants showed essentially wt responses to SDF-1α (Fig. 6B).

Together the data show that the SSKIL sequence in CXCR4 is not required for SDF-1α-induced CXCR4 down modulation and is likely to explain the ability of this receptor to undergo phorbol ester-induced internalisation.

SDF-1α binding to wild-type and mutated CXCR4 molecules

SDF-1α-induced down modulation of the CXCR4 COOH-
terminal domain truncation mutants was reduced compared to wt CXCR4 (Fig. 6A). This most likely reflects the loss of crucial elements in the COOH terminus required for endocytosis. However, the mutations might also affect the ability of the molecule to bind SDF-1α. We therefore compared the binding properties of SDF-1α to the wt CXCR4 and all 6 COOH-terminal mutants. In initial experiments with Mv-1-Lu cells, we observed high affinity SDF-1α binding to these cells in the absence of transfected human CXCR4 molecules (not shown). This may reflect the presence of mink CXCR4 molecules (or another chemokine receptor able to bind SDF-1α) on these cells that cannot be detected with our anti-human CXCR4 antibodies. COS-7 cells have previously been used to assay CXCR4/SDF-1α interaction (Kledal et al., 1997). We therefore transiently expressed the wt and COOH-terminal CXCR4 mutants in these cells for competition binding analysis.

Fig. 7 shows the titration curves for 125I-SDF1α binding in the presence of increasing concentrations of unlabelled SDF-1α. The wt and mutant CXCR4 molecules exhibited very similar SDF-1α binding activity. IC₅₀, Hill coefficient and Bₘₐₓ values were calculated from four independent experiments and did not show significant differences between the wild type and mutant CXCR4 molecules (Table 1). These data indicate that SDF-1α binding affinity was not affected by the COOH-terminal mutations.

DISCUSSION

The role of chemokine receptors as the principal or co-receptors for HIV and SIV entry is now well established, with CCR5 and CXCR4 implicated as major chemokine receptors involved in virus infection in vivo (Berger, 1997; Berger et al., 1998; Moore et al., 1997). Cell entry of HIV and SIV is believed to occur by membrane fusion, induced through the
interaction of the viral envelope protein with CD4 and/or a chemokine receptor, at the surface of target cells. Endocytosis of virus is not known to play a role in this process (Madden et al., 1986; Orloff et al., 1991; Pelchen-Matthews et al., 1995).

Indeed, cytoplasmic domain deleted forms of CD4, CCR5 and CXCR4, which show significantly reduced endocytic properties, will function efficiently as virus receptors (Amara et al., 1997; Edinger et al., 1997; Madden et al., 1988; Orloff et al., 1991; Pelchen-Matthews et al., 1995; Signoret et al., 1997). Although receptor internalisation appears not to be required for virus entry, receptor down modulation from the cell surface may still influence virus infection. Ligand-induced internalisation has been described for a number of 7TM GPCRs including both CC and CXC chemokine receptors, the related C5a and fMLP receptors, the β2-adrenergic receptor, thrombin receptor and many others (Amara et al., 1997; Aramori et al., 1997; Ferguson et al., 1996a; Forster et al., 1998; Haribabu et al., 1997; Hoxie et al., 1993; Jourdan et al., 1998; Mueller et al., 1997; Prado et al., 1996; Signoret et al., 1997; Solari et al., 1997; von Zastrow and Kolbka, 1992) and has been proposed to play a role in both desensitisation and resensitisation (Ferguson et al., 1996a; Krueger et al., 1997). The CC chemokines MIP-1α, MIP-1β, RANTES, etoxatin, the CMV-encoded vMIP-II, and the CXC chemokine SDF-1, have been shown to inhibit CCR5, CCR3 or CXCR4 dependent HIV-1 infection (Bleul et al., 1996; Cocchi et al., 1995; Oberlin et al., 1996; Simmons et al., 1997). However, the mechanism(s) through which this inhibition occurs has not been established. Several recent studies have indicated that agonist-induced chemokine receptor internalisation may be a component of the mechanism of chemokine inhibition (Amara et al., 1997; Mack et al., 1998; Signoret et al., 1997).

The mechanisms through which chemokines induce endocytosis of their receptors are not well understood. Following on from studies of the β2-adrenergic receptor (β2-AR) (Ferguson et al., 1996b; Godiska et al., 1997; Goodman et al., 1996, 1997; Menard et al., 1997; Zhang et al., 1996), it has been shown that agonist-induced internalisation of CCR5 is enhanced by over-expression of G-protein receptor kinases (GRKs) and β-arrestins (Aramori et al., 1997), and that internalisation is likely to proceed through clathrin coated vesicles (Mack et al., 1998). Thus, ligand-binding is believed to initiate GRK-induced phosphorylation of cytoplasmic sequences of the receptor and increase the association of the receptor with β-arrestin. β-arrestin couples the receptor to clathrin and facilitates endocytosis via clathrin coated vesicles. For CXCR4, phorbol esters can induce receptor internalisation independently of ligand association (Amara et al., 1997; Forster et al., 1998; Haribabu et al., 1997; Lapham et al., 1996; Signoret et al., 1997). In T cell lines that constitutively express CXCR4, and in transfected Mv-1-Lu cells, phorbol ester treatment increases the rate of CXCR4 endocytosis from a basal rate of about 1% of the cell surface pool per minute to about 6-8% per minute (Signoret et al., 1997). Phorbol ester-induced CXCR4 internalisation occurs through clathrin coated pits (Signoret et al., 1997) and, in contrast to ligand-induced uptake, involves activation of PKC (Signoret et al., 1997). Although the physiological relevance of the phorbol ester-induced internalisation of CXCR4 is still to be established it likely reflects signalling events that activate PKC (Acres et al., 1986). Indeed recent studies have shown that stimulation of T cells and T cell clones by cross-linking cell surface CD28, CD2 and CD3 can induce CXCR4 down modulation (Jourdan et al., 1998).

Unlike ligand-induced down modulation, the rapid phorbol ester-induced increase in endocytosis seen with CXCR4 is not observed for all chemokine receptors. Here we show that the cell surface expression of CCR5 is not altered following exposure of cells to phorbol esters, indicating that there is no increase in endocytic activity and no consequent down modulation. The experiments were performed in transfected T cell lines expressing CCR5 together with CD4 and CXCR4, and in transfected Mv-1-Lu cells with and without CD4. In both cellular backgrounds CD4 and CXCR4 could be demonstrated to undergo PMA-induced down modulation but the CCR5 molecule was unaffected. We have previously studied the effects of phorbol esters on CD4 and found that for this molecule, phosphorylation of a specific serine residue (S408 in human CD4) in the cytoplasmic domain of the molecule, activates an endocytosis signal that leads to CD4 recruitment into clathrin coated pits and endocytosis (Marsh...
The endocytosis signal involves a pair of leucine residues four amino acids COOH-terminal to S408. We noted the presence of a similar sequence (SSLKIL) at position 324-329 in the COOH-terminal domain of human CXCR4 but not the other well characterised chemokine receptors (Fig. 3a, with the possible exception of CCR5; see above). Here we have shown that this motif is required for phorbol ester-induced endocytosis of CXCR4, but not for ligand-induced internalisation. Mutation of either the isoleucine/leucine pair and/or the serine pair to alanine completely abrogated the phorbol ester response without affecting SDF-1 induced uptake. In contrast, limited truncation (∆12) of the cytoplasmic domain of CXCR4 inhibited SDF-1 induced down modulation without significantly affecting the phorbol ester response.

The endocytosis signal in the cytoplasmic domain of CD4 is dependent on phosphorylation of serine 408 for activity. In the absence of phosphorylation CD4 has similar properties to a cytoplasmic domain-deleted form of the protein (Marsh and Pelchen-Matthews, 1996). For CXCR4, both phorbol ester and ligand have been found to induce phosphorylation of the receptor cytoplasmic COOH-terminal domain (Haribabu et al., 1997), though the precise residues have not been identified. Ligand-induced receptor phosphorylation has also been observed for CCR5 (Aramori et al., 1997). As with a number of other 7TM GPCRs the extreme COOH-terminal domain of the protein is rich in serine residues, many of which are potential targets for phosphorylation. Our mutational analysis, together with the previous observation that inhibitors of PKC activity block phorbol ester but not SDF-1α induced CXCR4 internalisation (Signoret et al., 1997), suggests that ligand and phorbol esters are likely to induce phosphorylation on distinct sites in the COOH-terminal domain of CXCR4. Crucially, phorbol esters are likely to induce phosphorylation of S324 and/or S325, with or without activity on other COOH-terminal residues. Phosphorylation of the COOH-terminus following SDF-1(α) treatment is likely to exclude S324 and S325.

The recruitment of β2-AR into coated pits has been shown to be enhanced through association with a β-arrestin, β-arrestin being able to couple the receptor to clathrin directly without the apparent involvement of clathrin adaptors complexes (Goodman et al., 1996, 1997). Data from studies with CCR5 suggest that chemokine receptors may undergo similar recruitment into clathrin coated pits (Aramori et al., 1997). The phosphorylated S(XX)LL endocytosis signal in CD4 is believed to promote interaction with the AP2 clathrin adaptor complex. A related sequence in the γ subunit of the TcR/CD3 complex has been found to interact with AP2 complexes and we similarly found that peptides encoding the CD4 internalisation signal associate with AP2 complexes (C. Pitcher and M. Marsh, unpublished). Thus it might be expected that the SSLKIL sequence in CXCR4 also provides a binding site for AP2 and would explain the observation that minor sequence changes that completely abolish the phorbol ester response (i.e. I328A/L329A) have no effect on ligand-induced internalisation. Together the observations suggest that CXCR4 has the capacity to interact with two distinct sets of endocytic adaptors, the AP2 complex and a β-arrestin, respectively, and that these interactions are likely to be regulated through phosphorylation of distinct sets of one or more serine residues in the COOH-terminal domain of the molecule.

Our results indicate that the cell surface expression and endocytosis of CXCR4 and CCR5 can be differentially regulated. Why CXCR4 alone of the chemokine receptors contains this S(XX)LL type signal is unclear. CCR5 is a non-essential protein that appears to function primarily in leukocyte chemotaxis (Liu et al., 1996; Samson et al., 1996). By contrast, in mice at least, CXCR4 is essential. It is widely expressed not only on leukocytes (Bleul et al., 1997; Forster et al., 1998) but also on neurons (Hesselgesser et al., 1997) and endothelial cells (Gupta et al., 1998; Volin et al., 1998). Moreover, SDF and CXCR4 have been implicated in vascular development, neuronal patterning, haematopoiesis as well as T cell migration and activation (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). The additional endocytosis signals may be required for the wider functional activities of CXCR4. Alternatively, the presence of a S(XX)LL motif on CXCR4, as well as CD4 and the TcR/CD3 complex (Dietrich et al., 1994, 1997; Marsh and Pelchen-Matthews, 1996; Shin et al., 1991), may allow the cell surface expression of these molecules to be co-ordinately regulated on T cells. Significantly, primary T cells and dendritic cells appear to have intracellular stores of CXCR4 that can be relocated to the cell surface under certain conditions (Jourdan et al., 1998; Zaitseva et al., 1997). Thus the trafficking of CXCR4 and perhaps other chemokine receptors is tightly controlled and is likely to depend on the regulation of multiple trafficking or sorting signals.

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REFERENCES


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