Role of *N*-glycans and SDF-1α on the coassociation of CD4 with CXCR4 at the plasma membrane of monocytic cells and blood lymphocytes

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Abstract CXCR4 is a coreceptor, along with CD4, for human immunodeficiency virus type 1 (HIV-1). Trimolecular complexes between HIV-1 glycoprotein (gp)120, CD4 and CXCR4 constitute a prerequisite for HIV entry. We studied whether CD4 is associated with CXCR4 on CD4+ CXCR4+ cells. Using the conformation-dependent anti-CXCR4 mAb 12G5, CD4 was coimmunoprecipitated with CXCR4 from the membrane of U937 cells which support HIV-1_{LAI} efficient infection, and from that of peripheral blood lymphocytes (PBL). CD4 association with CXCR4 increased upon PBL coculture for 5 days with autologous monocytes, decreased upon treatment of the cells or the CD4-CXCR4 complex with either *N*-glycanase or stromal cell derived factor-1α (SDF-1α) and was abolished by incubation of the cells with both, *N*-glycanase and SDF-1α. This indicates that glycans are partly involved in CD4 association with CXCR4 and may partly explain the inhibitory effect of SDF-1α on HIV infection.

Key words: CD4; CXCR4; Glycan; SDF-1α

1. Introduction

CD4 is human immunodeficiency virus type 1 (HIV-1) primary receptor [1]. However, HIV-1 tropism has been accounted for by usage of CCR5 or CXCR4 [2–10]. CCR5-using strains (R5) are present throughout the course of infection; CXCR4-using viruses (X4) [9] develop late in disease [10]. RANTES (regulated on activation normal T cells expressed and secreted), macrophage inflammatory protein (MIP)-1α and MIP-1β inhibit R5 HIV infection of cells expressing CCR5 and CD4 [6,11–16], while stromal cell derived factor-1 (SDF-1), a CXCR4-specific ligand, inhibits X4 strain entry [17]. We have demonstrated that stromal cell derived factor-1α (SDF-1α) interacts with glycosaminoglycans (GAGs), and also with mannan or mannose [18,19] and that HIV envelope glycoproteins (gp) have carbohydrate binding properties [20–28]. Therefore, glycans and GAGs could play a role in HIV-1 infection and in the physiology of SDF-1α. Certain subclones (minus clones) of the U937 cell line do not support infection and fusion mediated by X4 HIV-1 although the CD4 and CXCR4 concentrations at their surfaces are similar to those of clones susceptible to HIV-1 entry (plus clones) [29]. Trimolecular complexes between X4 HIV gp120, CD4 and CXCR4 constitute a prerequisite for HIV entry into CD4+ CXCR4+ cells [30]. A low level of spontaneous coassociation of CD4 and CXCR4 occurred in U937 cells; this coassociation was observed in a lesser extent in the minus clones than in the plus ones [31]. Moreover, a gp120 induced inefficient coimmunoprecipitation of CD4 and CXCR4 was observed in the minus clones but not in the plus ones [31]. The fact that SDF-1α binding to U937 cells was inhibited not only by anti-CXCR4 antibodies but also by soluble CD4, has suggested an interaction between SDF-1α and CD4 [19]. Moreover, CD4 and CXCR4 are *N*-linked glycosylated molecules [32,33]. This study was designed to further examine the association of CD4 with CXCR4 which occurs at the membrane of U937 cells, susceptible to HIV-1 entry and to determine whether such association can also be observed at the membrane of peripheral blood lymphocytes (PBL). The possible role of glycans, SDF-1α or PBL stimulation by coculture with autologous monocytes [34] on such association was also investigated.

2. Materials and methods

2.1. SDF-1α labeling

Synthetic SDF-1α (a gift from F. Baleux, Institut Pasteur, Paris, France), recombinant SDF-1α (Pepro Tech EC LTD, London, UK) or RANTES (synthetic, a gift from C. Vita, CEA, Saclay, France) (all at 5 μg) were radiolabeled as previously described [18,19]. Specific activities were 0.4 MBq/μg. Similar data were obtained with both SDF-1α.

2.2. Cells

HIV-1_{LAI}-susceptible monocytic U937 and lymphoid CEM cells [35] were cultured as previously described [19]. Ficoll-Hypaque-separated blood mononuclear cells (PBMC) from cytapheresis of healthy volunteers (Seine-Saint-Denis Blood Bank, France) were cultured as previously reported [15,16,36]. Non-adherent cells were then removed by several washes; they represent more than 98% T- or B-CD3+ or CD19+ lymphocytes (PBL), as assessed by labeling with FITC-conjugated mAb anti-CD3 and anti-CD19 mAb (Becton Dickinson Immunocytometry, Mountain View, CA, USA), followed by flow cytometry analysis with a FACScan (Becton Dickinson). These PBL were cultured for 1 (PBL-1d) or 5 days (PBL-5d) in the presence of adherent autologous monocytes. The cells (5×10^5 in 100

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Abbreviations: PBL, peripheral blood lymphocytes; SDF-1α, stromal cell derived factor-1α; RANTES, regulated on activation normal T cells expressed and secreted; MIP, macrophage inflammatory protein; GAGs, glycosaminoglycans; gp, glycoprotein; 12G5, anti-CXCR4 mAb 12G5; G19, anti-CXCR4 polyclonal antibodies G19

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μl PBS, 0.05% BSA, 0.05% sodium azide) (PBS-BSA-A) were incubated for 30 min at 4°C with 2.5 μg anti-CCR5 mAb 2D7, anti-CXCR4 mAb 12G5 (12G5) or their isotype IgG2a (all from Pharmingen, San Diego, CA, USA); 12G5 recognizes a conformational epitope in ECL2 [37] and inhibits HIV-1_{LAI} mediated cell fusion [35]. Cells were then incubated for 30 min at 4°C in 100 μl of FITC-labeled anti-mouse immunoglobulin goat antibodies or FITC-anti-mouse IgG2a antibodies (1/20; Beckman-Coulter or Pharmingen, Paris, France). Alternatively, cells were incubated for 30 min at 4°C in PBS-BSA-A with 1 μg FITC-CD4 (clone 13B.8.2) or its isotype (1 μg; IgG1-FITC) (both from Beckman-Coulter), then fixed in 1% paraformaldehyde (Sigma-Aldrich) in PBS and analyzed by flow cytometry. In all these experiments, the non-specific binding of the antibodies to Fc receptors was determined by the use of the respective isotypes.

2.3. Cell membranes

Membranes, obtained as previously described [19] were submitted to SDS–PAGE (12% polyacrylamide) under non-reducing conditions, then electroblotted onto Immobilon strips (Millipore, Paris, France) [18,19]. In some experiments, strips were incubated for 5–18 h at 20°C or 37°C with native or heat-inactivated $1–2\times10^6$ cpm ^{125}I-SDF-1α (8×10^{-9} M) (10 min at 100°C in the presence of 1% SDS, 5% β-mercaptoethanol from Sigma-Aldrich) and exposed at −20°C for 18–72 h. Alternatively, strips were incubated with anti-CD4 Q4120 mAb (Sigma-Aldrich; a mAb that blocks the binding of gp120 to CD4), 12G5 or anti-CXCR4 polyclonal antibodies G19 (G19) (Peprotech EC LTD, London, UK; a goat antibody raised against a peptide mimicking [37] the first extracellular domain of CXCR4) or the isotypes (all at 0.2 μg/ml; Pharmingen or Peprotech) and stained with HRP-labeled horse anti-mouse antibody followed by ECL photoluminescence detection (Amersham, Great Chalfont, UK).

2.4. Coimmunoprecipitation of CD4 with CXCR4. Effect of N-glycanase or SDF-1α

The cells (2×10^8) in 200–500 μl PBS were incubated for 2 h at 4°C with or without 12G5, its isotype or with native SDF-1α (all at 2 μg). This cell concentration was used in order to enhance the amount of CXCR4 recovered after immunoprecipitation with 12G5. Cell viability was >99%, as assessed by trypan blue exclusion dye. Moreover, the analysis by flow cytometry of these highly concentrated cells after their labeling by anti-CXCR4 and anti-CD4 mAbs did not differ from that observed in the presence of more diluted cells. In some experiments, the cells were treated for 4 h at 37°C with 10 mU *N*-glycanase (peptide *N*-glycosidase F from *Flavobacterium meningosepticum*; Glyko Nocato, CA, USA). Cells were then incubated either with Abs or with 10–50 nM FITC-labeled concanavalin A (ConA; Sigma-Aldrich), then analyzed by flow cytometry or lysed by incubation for 30 min on ice, in 20 mM Tris/HCl buffer, pH 8.2, 150 mM NaCl, 1% v/v Brij 97, supplemented with protease inhibitors (all from Sigma-Aldrich). Lysates were cleared by centrifugation and incubated for 18 h at 4°C with 100 μl Sepharose-protein G beads (Pharmacia, Paris, France). Beads were washed in solubilization buffer and resuspended in 250–500 μl of 2× Laemmli buffer, heated for 10 min at 100°C in order to release bound components, and analyzed after electroblotting by incubation with anti-CXCR4, anti-CD4 Abs, anti-CCR1 mAb (clone: 53504.111; R&D Systems) or the isotypes. Alternatively, lysates were incubated with the beads conjugated as previously described [18,19] with 12G5, goat anti-SDF-1 Abs or the isotypes (R&D Systems). Beads were then washed and incubated in some experiments with *N*-glycanase (10 mU for 24 h at 37°C).

3. Results and discussion

3.1. Electroblotted CXCR4 binds to SDF-1α and 12G5

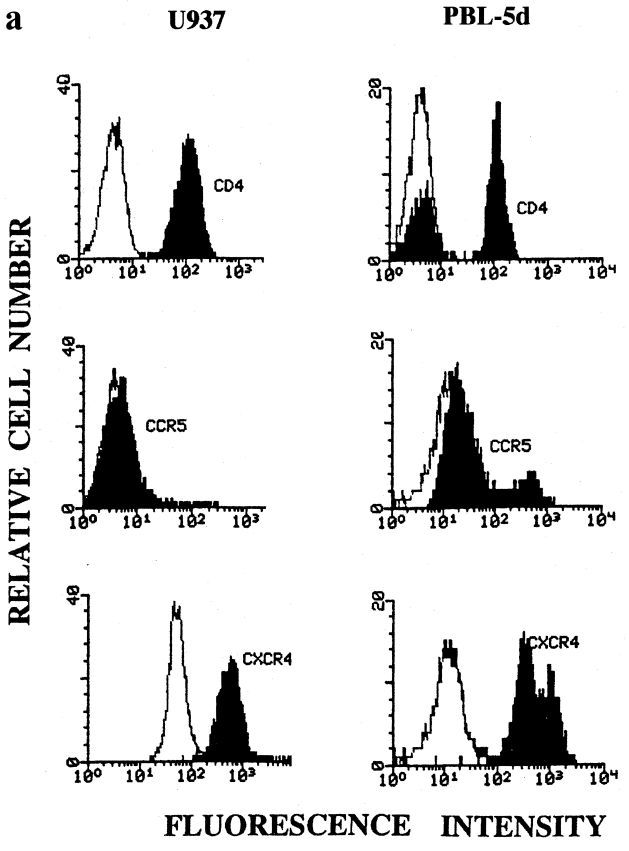
U937 and CEM cells expressed, as expected CD4 and CXCR4. 50–60% PBL-1d or PBL-5d expressed CD4; all the lymphocytes expressed CXCR4 (Fig. 1a and data not shown). We then observed that CXCR4 from electroblotted membranes of U937 and CEM cells bound its natural ligand, SDF-1α and a conformation-dependent mAb, 12G5: native ^{125}I-SDF-1α, G19 and 12G5 bound to diffuse or double bands of 48–55 kDa and 98–100 kDa and slightly to bands of higher molecular weight (about 150 kDa) (Fig. 1b and c). The isotypes, anti-CCR1 mAb, heat-treated ^{125}I-SDF-1α or ^{125}I-RANTES, the latter used as negative control (as no RANTES-specific receptor has been previously detected on these cells [38]), exerted no binding (Fig. 1b and c). Moreover, double or simple bands of 48–52 kDa and 96–105 kDa, which bound to native ^{125}I-SDF-1α and G19 but neither to heat-denaturated ^{125}I-SDF-1α nor to the isotypes, were observed with electroblotted cell lysates from PBL-5d (Fig. 1d). This indicates that several CXCR4 isoforms, interacting with SDF-1α and the conformation-dependent 12G5, are observed on electroblotted cell membranes or cell lysates from CXCR4+ cell lines or primary cells, and that under these conditions, the native conformation of CXCR4 is preserved.

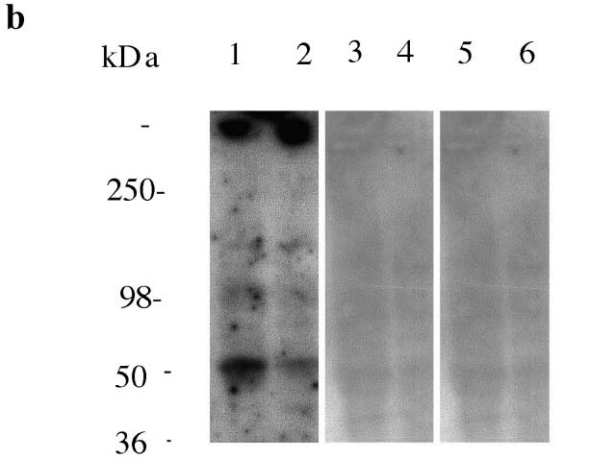
3.2. Coassociation of CD4 with CXCR4: role of cell stimulation and N-glycans

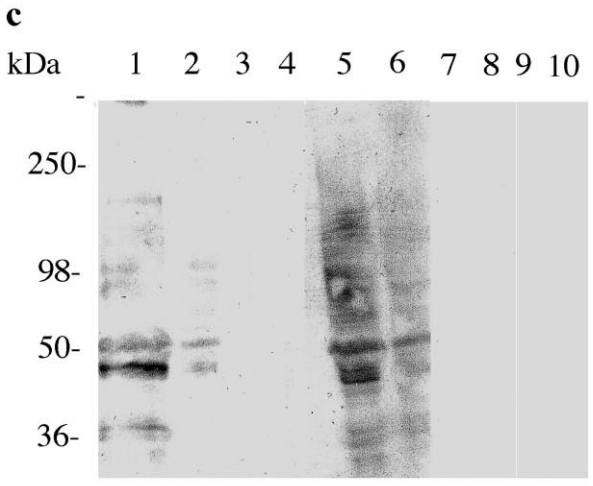
Proteins of 150 kDa and 48 kDa were immunoprecipitated by 12G5 from the plasma membranes of U937 cells and were immunoreactive with this mAb, but not with the isotype (Fig. 2a). Moreover, 150 kDa and 55 kDa proteins from the 12G5 interacting material were immunoreactive with anti-CD4 mAb Q4120 but not with the isotype (Fig. 2a). When the same procedure was applied to PBL-1d and PBL-5d, a 48 kDa protein, immunoreactive with G19 and 12G5, but neither with their isotypes, nor with anti-CCR1 mAb, was detected. Moreover, proteins of 36 kDa and 42 kDa, immunoreactive with 12G5 but not with its isotype, were also noticed (Fig. 2b). A 55 kDa protein was characterized as CD4 by its immunoreactivity with anti-CD4 mAb Q4120, but not with its isotype. However, the amount of this protein was substantially decreased in the 12G5 interacting material from PBL-1d as compared to PBL-5d (Fig. 2b). No CXCR4 or CD4 proteins were detected when PBL or U937 cells were incubated with the isotype instead of 12G5 (data not shown). These data indicate the occurrence of CD4-CXCR4 complexes at the plasma membrane of U937 cells and PBL-5d, which increase during PBL coculture with autologous monocytes.

When lysates from U937 cells were freshly prepared and then incubated with 12G5-coated beads, we observed that diffuse 48–58 kDa and 100–150 kDa proteins from the 12G5 interacting material were immunoreactive with this mAb, but not with its isotype. Moreover, 55 kDa and 100 kDa proteins from this material were immunoreactive with anti-CD4 mAb Q4120, but not with its isotype (Fig. 3). Therefore, CD4-CXCR4 complexes are detected in these lysates.

Treatment of U937 cells with *N*-glycanase did not modify their labeling with anti-CXCR4 and anti-CD4 Abs and on contrary decreased their labeling by FITC-labeled ConA, which indicates the enzyme efficiency (Table 1). However, when the treated cells were lysed and incubated with 12G5-coated beads, the diffuse 100–150 kDa proteins described above, which are immunoreactive with 12G5 and the 100 kDa proteins, immunoreactive with anti-CD4 mAb Q4120, were no more observed. Therefore, the CD4-CXCR4 complex characterized by a molecular weight of 100–150 kDa, disappeared after *N*-glycanase treatment of U937 cells, which suggests that *N*-glycans are involved in its formation. Moreover, after this enzyme treatment, a shift from the diffuse 48–58 kDa band, immunoreactive with 12G5, toward a sharp 48 kDa band, occurred (Fig. 3). Therefore, the diffuse migration of the 48–58 kDa proteins, described above, may be related







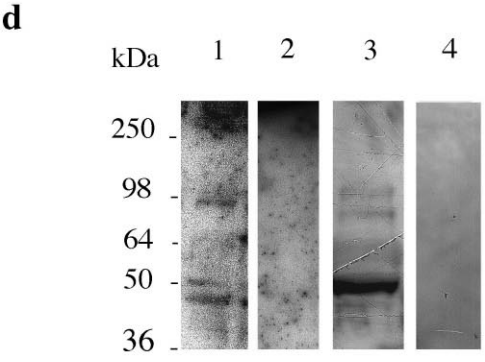


Fig. 1. CXCR4 expression of U937 cells, CEM cells or PBL-5d. a: U937 cells or PBL-5d were labeled with anti-CD4 (by direct immunofluorescence assay) anti-CCR5 mAb 2D7 or 12G5 (by indirect immunofluorescence assay) (black peaks) or with the isotypes (white peaks). b: Membranes from U937 (lanes 1, 3, 5) or CEM cells (lanes 2, 4, 6) were electroblotted and incubated with native (lanes 1, 2) or heat-denaturated ^{125}I-SDF-1α (lanes 3, 4) or native ^{125}I-RANTES (lanes 5, 6). c: Membranes from U937 (lanes 1, 3, 5, 7, 9) or CEM cells (lanes 2, 4, 6, 8, 10) were electroblotted and incubated with 12G5 (lanes 1, 2), G19 (lanes 5, 6), the isotypes (lanes 3, 4, 7, 8) or anti-CCR1 mAb (lanes 9, 10). d: Lysates from PBL-5d were electroblotted and incubated with native (lane 1) or heat-denaturated ^{125}I-SDF-1α (lane 2), G19 (lane 3) or the isotype (lane 4). Data are representative of three to five independent experiments.

with their heterogeneous glycosylation (Fig. 3). Nevertheless, the fact that the 55 kDa protein, immunoreactive with anti-CD4 mAb Q4120, was still detected in the 12G5 interacting material, even after *N*-glycanase treatment of the U937 cells (Fig. 3), indicates that some coassociation of CD4 with CXCR4 still occurs. Similar data were observed when the U937 cells were first incubated with 12G5, then lysed before the incubation of the CD4-CXCR4-12G5-coated beads with *N*-glycanase (data not shown). Moreover, in these latter conditions, the amount of the 55 kDa protein, immunoreactive with anti-CD4 mAb Q4120, and obtained from PBL-5d, was substantially decreased (Fig. 2b). The fact that no CD4 or CXCR4 molecules were detected in the electroblotted supernatants from the *N*-glycanase-treated cells or beads rules out that these molecules have been eluted in the medium during the enzyme treatment (data not shown). Taken together, our data suggest that *N*-linked glycans are partly involved in CD4 association with CXCR4.

Table 1
U937 cell membrane expression of CD4 and CXCR4 before or after treatment by *N*-glycanase

Antibodies used	Mean fluorescence intensity (units)	
	−*N*-glycanase	+*N*-glycanase
IgG2a+FITC-anti-mouse (Fab′)2	7	5.5
12G5+FITC-anti-mouse (Fab′)2	75	64.5
FITC-IgG1	3.5	4
FITC-anti-CD4 mAb (clone 13B.8.23)	28	25
FITC-ConA	50	30

'FITC' indicates labeling by FITC. The results are representative of three independent experiments.

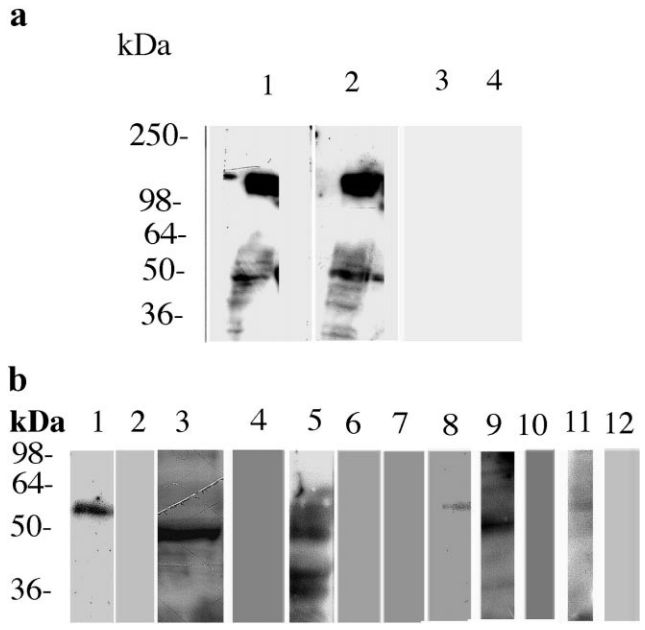


Fig. 2. CD4-CXCR4 complex at the membrane of U937 cells and PBL. U937 cells (a) PBL-5d or PBL-1d (b) were incubated with 12G5 and lysed. Lysates were incubated with Sepharose-protein G beads. The retained material from the U937 cells was electroblotted and incubated with (a) 12G5 (lane 1), anti-CD4 mAb Q4120 (lane 2), murine IgG2a (lane 3), murine IgG1 (lane 4). In b, the specifically retained material from PBL-5d was electroblotted and incubated with anti-CD4 mAb Q4120 (lane 1), murine IgG1 (lane2), G19 (lane 3), goat IgG (lane 4), 12G5 (lane 5), murine IgG2a (lane 6), or anti-CCR1 mAb (lane 7). In some experiments, the proteins bound to the matrix were incubated with *N*-glycanase before electroblotting and incubation with anti-CD4 mAb Q4120 (lane 8). The 12G5 interacting material from PBL-1d was electroblotted and incubated with 12G5 (lane 9), murine IgG2a (lane 10), anti-CD4 mAb Q4120 (lane 11), murine IgG1 (lane 12). Data are representative of three independent experiments.

3.3. Effect of SDF-1α on CD4 association with CXCR4

While SDF-1α did not modify CD4 membrane expression of the U937 cells, it induced a decrease in their labeling with 12G5, which was related to an internalization of the receptor and to a competition between the chemokine and the mAb

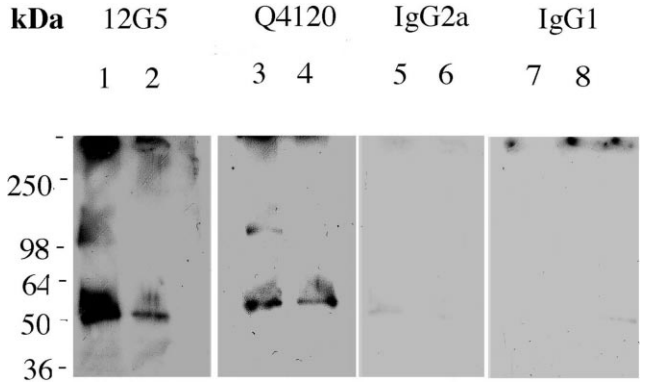


Fig. 3. Effect of *N*-glycanase on the CD4-CXCR4 complex from U937 cells. U937 cells were incubated for 4 h at 37°C in medium supplemented (lanes 2, 4, 6, 8) or not (lanes 1, 3, 5, 7) with *N*-glycanase, and lysed. CXCR4 and CXCR4-associated molecules were immunoprecipitated on 12G5-coated beads, and electroblotted. Strips were incubated with 12G5 (lanes 1, 2), anti-CD4 mAb Q4120 (lanes 3, 4), murine IgG2a (lanes 5, 6), or murine IgG1 (lanes 7, 8). Results are representative of three independent experiments.

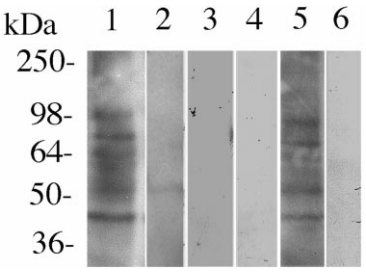


Fig. 4. Effect of SDF-1α on the CD4-CXCR4 complex from U937 cells. SDF-1α (250 nM) was incubated with U937 cells. Cells were lysed; the SDF-1α-bound components were immunoprecipitated with anti-SDF-1α antibody-coated beads, and electroblotted. Strips were incubated with 12G5 (lane 1), anti-CD4 mAb Q4120 (lane 2), murine IgG2a (lane 3), or IgG1 (lane 4). The same as above, but SDF-1α was incubated with *N*-glycanase-treated U937 cells. Strip was incubated with 12G5 (lane 5) or anti-CD4 mAb Q4120 (lane 6). Results are representative of three independent experiments.

[19,35] (Table 2). When the U937 cells were lysed after their incubation with SDF-1α and the SDF-1α-bound components immobilized on anti-SDF-1 Abs-coated beads, proteins of 100, 70, 63, 55 and 48 kDa, immunoreactive with 12G5, which may represent several different CXCR4 conformational states, were observed. No binding of the isotype was detected (Fig. 4). However, a slight binding of 55 kDa protein to anti-CD4 mAb Q4120 was observed (Fig. 4). In some experiments, a slight binding of 100 kDa proteins to anti-CD4 mAb Q4120 was also observed (data not shown). Nevertheless, after *N*-glycanase treatment of the cells, a shift of the 100 and 70 kDa bands, immunoreactive with 12G5, toward 98 and 68 kDa bands and a decrease of the intensity of the 48 kDa band, immunoreactive with this antibody, were observed (Fig. 4). These data further indicate enzyme efficiency. Moreover, no CD4 molecules were detected in the SDF-1α-bound components. Therefore, the CXCR4 molecules which have been bound to SDF-1α, are poorly associated with CD4 and *N*-glycanase treatment of the cells further abolishes the CD4-CXCR4 association.

4. Conclusion

While HIV-1 gp120 induces an association between CD4 and CXCR4, a low level of constitutive colocalization between CD4 and CXCR4 has been detected by confocal laser scanning microscopy on CXCR4 transfected cell lines [39]. Our data indicate that a coassociation of CD4 with CXCR4 occurs, in the absence of HIV gp120, in the plasma membrane of monocytic U937 cells, which support efficient HIV-1 infection [22] and in that of PBL, previously stimulated by

Table 2
U937 cell membrane expression of CD4 and CXCR4 in the absence or the presence of SDF-1α

Antibodies used	Mean fluorescence intensity (units)	
	−SDF-1α	+SDF-1α
IgG2a+FITC-anti-mouse (Fab′)2	2	5
12G5+FITC-anti-mouse (Fab′)2	45	16
FITC-IgG1	3	2
FITC-anti-CD4 mAb (clone 13B.8.23)	51	53

'FITC' indicates labeling by FITC. The results are representative of three independent experiments.

5 days coculture in the presence of adherent autologous monocytes. That this coimmunoprecipitation was only very slightly detected on PBL-1d suggests that it depends on cell stimulation. This is in agreement with Singer et al. [40] showing that CD4 and CXCR4 are clustered and closely apposed, but with lack of constitutive colocalization, on microvilli of human T cells, cultured in the absence of autologous monocytes. This coassociation, which partly depends on *N*-glycans and on the cell activation state, does not occur in a significant manner when the CXCR4 molecules are bound to SDF-1α. Such association, which seems to be a prerequisite for HIV entry into the cells, represents one possible target for anti-HIV therapy.

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