The HIV-1 gp120 inhibits the binding of adenosine deaminase to CD26 by a mechanism modulated by CD4 and CXCR4 expression

Julià Blanco[a,c,*], Agustín Valenzuela[b], Carolina Herrera[b], Carmen Lluís[b], Ara G. Hovanessian[a], Rafael Franco[b]

[a] *Unité de Virologie et d'Immunologie Cellulaire, ERS 572 CNRS, Institut Pasteur, 28, rue Dr. Roux, 75724 Paris Cedex 15, France*
[b] *Departament de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Martí i Franqués 1, 08028 Barcelona, Spain*
[c] *Fundació IrsiCaixa, Retrovirology Laboratory, Hospital Germans Trias i Pujol, 08916 Badalona, Spain*

Received 16 February 2000; received in revised form 13 June 2000

Edited by Hans-Dieter Klenk

Abstract HIV-1 external envelope glycoprotein gp120 inhibits adenosine deaminase (ADA) binding to its cell surface receptor in lymphocytes, CD26, by a mechanism that does not require the gp120–CD4 interaction. To further characterize this mechanism, we studied ADA binding to murine clones stably expressing human CD26 and/or human CD4, and transiently expressing human CXCR4. In this heterologous model, we show that both recombinant gp120 and viral particles from the X4 HIV-1 isolate IIIB inhibited the binding of ADA to wild-type or catalytically inactive forms of CD26. In cells lacking human CXCR4 expression, this gp120-mediated inhibition of ADA binding to human CD26 was completely dependent on the expression of human CD4. In contrast, when cells were transfected with human CXCR4 the inhibitory effect of gp120 was significantly enhanced and was not blocked by anti-CD4 antibodies. These data suggest that the interaction of gp120 with CD4 or CXCR4 is required for efficient inhibition of ADA binding to CD26, although in the presence of CXCR4 the interaction of gp120 with CD4 may be dispensable.

Key words: ADA; CD26; gp120; Chemokine receptors

1. Introduction

CD26 is a 105–110 kDa well-characterized multifunctional cell surface protein [1], known to show an intrinsic dipeptidyl peptidase (DPP IV) activity [2] and to be the receptor of the 43 kDa ecto-enzyme adenosine deaminase (ADA) [3,4]. In peripheral blood T lymphocytes, CD26 expression is highly regulated and enhanced upon T-cell activation [5]. CD26 is able to deliver intracellular signals, probably by means of its physical association with the membrane-linked tyrosine phosphatase CD45 [6]. This signaling capacity allows anti-CD26 mAbs or the natural occurring CD26 ligand, ADA, to have a costimulatory effect in TCR-mediated T-cell activation [7,8]. The expression of CD26 has been controversially correlated to HIV infection [9–12]. However, the ability of CD26 to cleave and modify the activity of HIV-1-inhibitory chemokines, such as the CXCR4 ligand SDF-1 [14] has highlighted the role of DPP IV activity of CD26 in HIV infection [13]. Moreover, we have reported that HIV-1 envelope protein gp120 inhibits the interaction between ADA and CD26 on the surface of human cells [15]. This inhibitory effect is independent of HIV infection and does not require the expression of the HIV receptor, the CD4 molecule [15].

The HIV-induced inhibition of ADA binding to CD26 suggests the existence of a functional interaction between cell surface-expressed CD26 and HIV-1 gp120. However, the mechanisms of this interaction, which are mediated by the C3 region of gp120 [15], remain unclear. The aim of this paper was to further characterize the interaction of gp120 with CD26. For this purpose, the gp120-induced inhibition of ADA binding to human CD26 was studied in murine cells expressing human CD26, CD4 and CXCR4. The role of the peptidase activity of CD26 was also investigated by using cells expressing the S630A mutant form of human CD26, which lacks DPP IV activity. Our results show that both, soluble gp120 and HIV-1 particles are able to inhibit the binding of ADA to human CD26 irrespective of DPP IV activity of CD26. Inhibition was completely dependent on CD4 expression in CXCR4$^-$ cells. The transfection of CXCR4 enhances the inhibitor effect of gp120 and makes the gp120–CD4 interaction dispensable, thus suggesting that gp120-mediated ADA binding inhibition is also CXCR4-dependent. The reduced ability of ADA to bind to CD26 in the presence of gp120 may affect T-cell function during HIV infection and may explain the correlation found between increased levels of serum ADA and the evolution of AIDS in HIV-1-infected individuals [16,17].

2. Materials and methods

2.1. Cells and antibodies

Murine clones expressing human CD26 were obtained as described [18]. Clones were selected by their expression of human CD26. Clones having lost human CD4 expression were used as control clones in different experiments.

Monoclonal antibodies TA5.9-CC1–4C8 directed against the ADA binding site in CD26 [4], and CB-T4 directed against the gp120 binding site in CD4 were kindly provided by Dr. E. Bossman, Eurogenetics, Tessenderlo and have been previously characterized [15,19].

2.2. Analysis of expression of cell surface proteins

Human CD26, human CD4 and human CXCR4 expression in different murine clones were determined by FACS analysis using the anti-human CD26 PE-labeled mAb Tal (Coulter), the anti-human CD4 FITC-labeled mAb OKT4 (Ortho), and the PE-labeled anti-CXCR4 mAb 12G5 (Pharmingen), respectively. Incubations were performed in FACS buffer (1% bovine serum albumin and 0.01% sodium azide in PBS) at 4°C for 30 min. After incubations, cells were washed

*Corresponding author. Fax: (34)-934653968.
E-mail: jblanco@ns.hugtip.scs.es

Abbreviations: ADA, adenosine deaminase; DPP IV, dipeptidyl peptidase IV

PII: S0014-5793(00)01751-8

twice, fixed in FACS buffer containing 1% formaldehyde and analyzed in a FACS scan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using the Lysis II software. Cell surface expression of CD26 was also monitored by determining the cleavage of GP-pNA (Glycylprolyl-*p*-nitroanilide, Sigma) as described previously, in order to confirm the phenotype of both forms of CD26 [18].

2.3. HIV-1 gp120, peptides and viral preparations

Recombinant HIV-1 envelope glycoprotein gp120 from the X4 isolate IIIB (construct deposited by Dr. I. Jones) and peptides covering the entire sequence of this protein [15] were kindly provided by the Medical Research Council AIDS Directed Program Reagent Project (Potters Bar, UK).

For the preparation of an HIV-1 stock, CEM cells were infected with a high dose of HIV-1 Lai (1 h, 37°C), washed and cultured for 3–4 days. Culture supernatant ($500\times g$, 10 min) was then recovered and stored at −135°C. Prior to use, thawed viral preparations were filtered through 0.22 μm pore size filters and centrifuged $50\,000\times g$ for 1 h at 4°C to eliminate free ADA that could interfere in the binding assays. Pellets were resuspended in fresh RPMI medium, usually in a third of the original volume.

2.4. Labeling of bovine ADA

Bovine ADA (Sigma Type VIII) was either iodinated by using the Bolton-Hunter reagent (DuPont, NEN) as described [18], or labeled with FITC. Briefly, ADA was dialyzed against labeling buffer (50 mM H_3BO_3, 200 mM NaCl, pH 9.2) at 4°C, and treated with fluorescein isothiocyanate (FITC, 5 mg/ml in DMSO) in a ratio of 0.1 mg of FITC per mg of protein. After 2 h at room temperature, unbound fluorochrome was separated by gel filtration using a Sephadex G-25 column.

2.5. FITC-labeled ADA binding

Cells (4×10^6) were washed twice with PBS and were incubated successively in the absence or the presence of anti-CD4 mAb CB-T4 (1 μg/ml, 15 min at 37°C), gp120 (100 mM, 15 min at 37°C) and FITC-labeled ADA (100 mM, 30 min at 37°C). The control for binding specificity was performed by preincubating cells with unlabeled ADA (4 μM, 15 min at 37°C) before addition of labeled ADA (100 nM). After incubation, cells were washed twice in ice-cold PBS and fixed in 2% paraformaldehyde, 60 mM sucrose in PBS, for 15 min at room temperature. Then, cells were washed twice with 20 mM glycine in PBS (buffer A) and incubated with the same buffer containing 1% BSA and 0.05% NaN3 before flow cytometry analysis in a EPICS Profile flow cytometer (Coulter; Hialeah, FL, USA). The parameters used to select cell populations for analysis were forward and side light scatter.

2.6. ^{125}I-labeled ADA binding

Cells were washed twice and resuspended at 1×10^7 cells/ml. Aliquots of 0.5×10^6 cells were preincubated (15 min, 37°C) in the presence or the absence of binding inhibitors (unlabeled ADA, mAb TA5.9, mAb CB-T4, gp120) and then incubated with 15 nM ^{125}I-labeled ADA in a final volume of 100 μl. After incubation (1 h, 37°C), cells were washed twice in cold PBS and lysed in 100 μl E buffer (20 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 100 U/ml aprotinin, 0.5% Triton X-100 and 7 mM 2-mercaptoethanol, pH 7.6). Lysates were centrifuged ($10\,000\times g$ for 5 min at 4°C) to eliminate nuclei, and supernatants were diluted with one volume of SDS–PAGE sample buffer [20]. Following SDS–PAGE, gels were fixed, dried and radioactivity associated with the 43 kDa ^{125}I-labeled ADA bands were quantified in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). In experiments designed to calculate K_d values, the binding of increasing concentrations of ^{125}I-labeled ADA (from 1 to 100 nM) was studied. Binding data were fitted using a non-linear regression program [18].

2.7. Transfection

The CXCR4 and CCR5 expression vectors pCDNAI.Fusin and pCDNAI.CCR5 were obtained from Dr. N. Landau through the HIH AIDS Research and Reference Reagent Program. W12 cells (4×10^6) were transiently transfected with 2 μg of plasmid using the Superfect Reagent from Qiagen (Hilden, Germany) as recommended by the manufacturer. Cells were grown for 40 h, before evaluation of CXCR4 and CCR5 expression by flow cytometry using 12G5 and 2D7 antibodies (Pharmingen).

3. Results

3.1. ^{125}I-labeled ADA binding to murine cells

Murine cells are an useful model to study the ADA binding to human CD26 since ^{125}I-labeled ADA does not compete with endogenous murine ADA, which is not able to bind to human CD26 [18]. We selected several murine clones stably transfected with human CD4 and CD26. Most of the selected clones expressing human CD26 also expressed human CD4, as in the case of the clone W12, transfected with wild-type human CD26, or in the clone M9, transfected with the S630A-mutated form of human CD26 (Fig. 1). Some clones, which lost human CD4 expression during selection and further culturing (Fig. 1, Clone M7) were used as CD4 negative control cells. To evaluate the presence of a functional ADA binding site on CD26-transfected cells, we studied the affinity of ^{125}I-labeled ADA binding to these cells and, for comparison, to CD26-expressing human cell lines. The K_d values found in all cells assayed were similar (Table 1) irrespective of the level of CD26 expression. Consistent with the specificity of the ADA–CD26 interaction under these experimental conditions, binding was inhibited to a similar extent in the presence of the mAb TA5.9, directed against the ADA binding site in human CD26, or in the presence of an excess of unlabeled ADA (Fig.

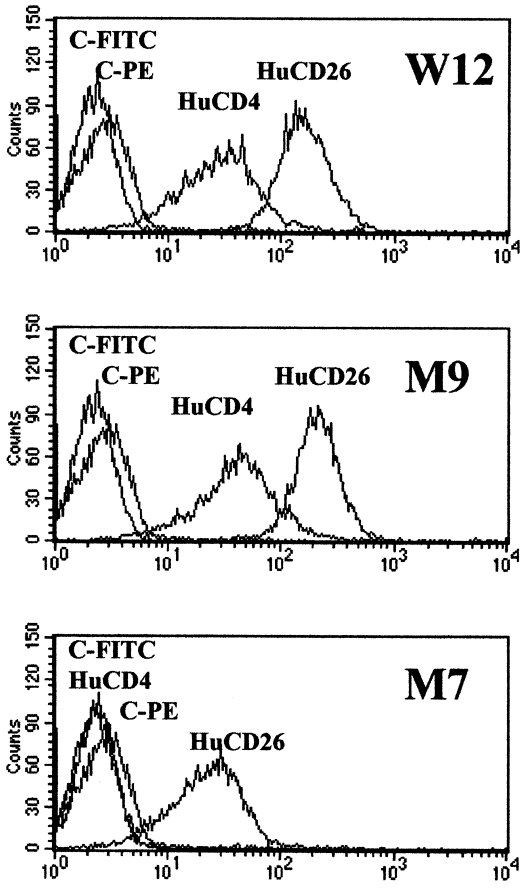

Fig. 1. Cell surface expression of human CD4 and human CD26 in different murine clones used. The expression of human CD4 and human CD26 was assayed by FACS analysis in the wild-type CD26-expressing clone W12 and the S630A CD26-expressing clones, M9 and M7. Antibodies used were: PE-labeled mAb Tal specific for human CD26, FITC-labeled OKT4 specific for human CD4, and control labeled antibodies (peaks C-PE and C-FITC).

2). Moreover no specific binding was found in murine cells lacking human CD26 expression (Table 1). These results confirm that in this experimental model the interaction ADA–CD26 is highly specific and is independent of the peptidase activity of CD26.

3.2. gp120-induced inhibition of ^{125}I-labeled ADA binding to human CD26 in murine cells expressing human CD4

We studied the effect of soluble gp120 on the binding of ^{125}I-labeled ADA to the wild-type CD26-expressing clone W12 and to the clone M9, which expresses the S360A mutated form of human CD26. Preincubation of these cells in the presence of 100 nM of gp120 resulted in a consistent and significant inhibition (40 and 61% in W12 and M9 cells, respectively) of the binding of ADA to human CD26 (Fig. 2). The effect was dose-dependent (data not shown), and the degree of inhibition was slightly lower but comparable to that observed in several human cell lines [15]. To map the region of gp120 implicated in the inhibition of ADA binding to CD26 in the heterologous system, a collection of overlapping peptides that cover the entire sequence of gp120 IIIB was used (for sequences see Valenzuela et al., 1997) [15]. In the two clones tested (W12 and M9), only peptides corresponding to the third constant domain of gp120 (C3 region) were able to significantly inhibit the binding of ADA to CD26 (Fig. 2). These peptides are p31 (HCNISRAKWNNTLKQIDSKL) and p33 (REQFGNNKTIIFKQSSGGDPE), which correspond to the residues 336–355 and 356–375 of gp120 IIIB, respectively. These results confirm that gp120-induced ADA binding inhibition is similar in murine and human cells.

3.3. The effect of CD4 expression on gp120-induced ADA binding inhibition

We next evaluated the requirement of human CD4 in gp120-induced ADA binding inhibition. Two different approaches were used, the first being the study of the effect of gp120 on the binding of ^{125}I-labeled ADA to CD26 expressed in the M7 murine clone, which lacks human CD4 expression. As shown in Fig. 3A, gp120 did not induce any significant decrease in ADA binding to CD26 expressed in the human CD4 negative clone M7. This clone expresses low levels of CD26 that could affect the inhibition of ADA binding to CD26. Therefore, we confirmed the requirement for a gp120–CD4 interaction by assaying the effect of gp120 and

Table 1
The binding of ^{125}I-labeled ADA to different CD26-expressing cells: effect of HIV-1 IIIB gp120

Cell line	ADA binding affinity K_d (nM)[a]
Human cells	
Jurkat (CD26^{low}/CD4$^+$ T-cell line)	15 ± 5
Jurkat (wild-type CD26-transfected, clone #11)	11 ± 3
Jurkat (S630A CD26-transfected, clone D28)	9 ± 4
SKW 6.4(CD26$^+$, CD4$^-$ B-cell line)	10 ± 1
Murine cells	
T6 (human CD26$^-$)	No binding
W12 (wild-type CD26-transfected)	12 ± 2
M9 (S630A CD26-transfected)	11 ± 4
M7 (S630A CD26-transfected)	10 ± 5

[a]The binding of increasing concentrations of ^{125}I-labeled ADA (1–50 nM) to different cells was studied. K_d values were calculated by non-linear regression of binding data as described [18]. Values ± S.D. given by the program.

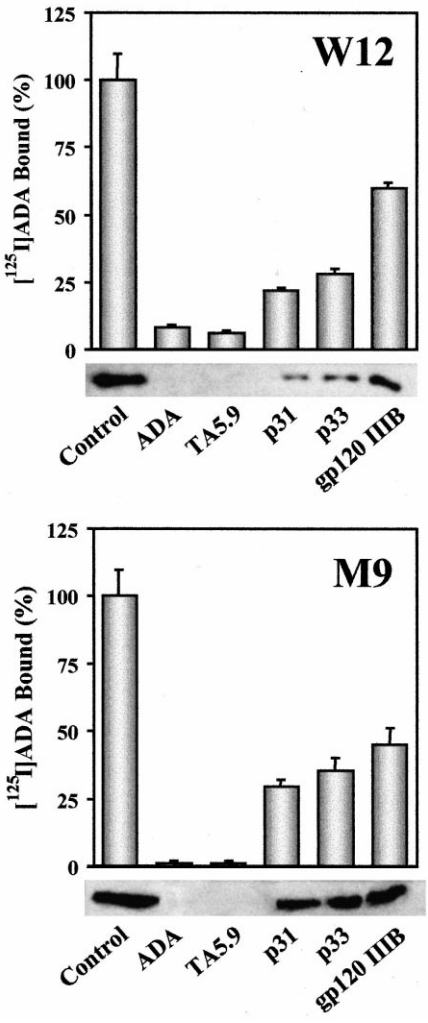

Fig. 2. The inhibition of ^{125}I-labeled ADA binding to human CD26 by gp120 is independent of DPP IV activity of CD26 and is mediated by the C3 region of gp120. The effect of either, 100 nM gp120, or 60 nM of peptides p31 and p33 (corresponding to the C3 region of gp120, see text for details), on the binding of 15 nM ^{125}I-labeled ADA to W12 cells, expressing wild-type human CD26 (upper panel), and M9 cells, expressing S630A human CD26 devoid of DPP IV activity (lower panel), was assayed by SDS–PAGE as described in Section 2. Unlabeled ADA (4 μM) and the mAb TA5.9 (1 μg/ml) were used as controls for specific binding. Results of a typical experiment are presented with a section of the autoradiogram showing the 43 kDa labeled band corresponding to cell-bound ^{125}I-labeled ADA. The histogram quantifies the associated radioactivity (mean ± S.E.M. from triplicate samples).

HIV-1 particles in W12 cells preincubated with an anti-human CD4 mAb (Fig. 3B). The addition of the antibody CB-T4 alone did not modify the binding of ^{125}I-labeled ADA to CD26, which was inhibited by the addition of 100 nM of gp120 and a concentrated preparation of viral particles in the absence of the antibody. However, in the presence of the anti-CD4 mAb CB-T4, the inhibitory effect of both, soluble gp120 and viral particles, was completely abolished (Fig. 3B). This result contrasts with what happens in human cells, in which the gp120–CD4 interaction is not required [15]. Therefore, it seems that the inhibition of the binding of ^{125}I-labeled ADA to CD26 by gp120 is dependent on the interaction of gp120 with human CD4. Moreover, murine clones

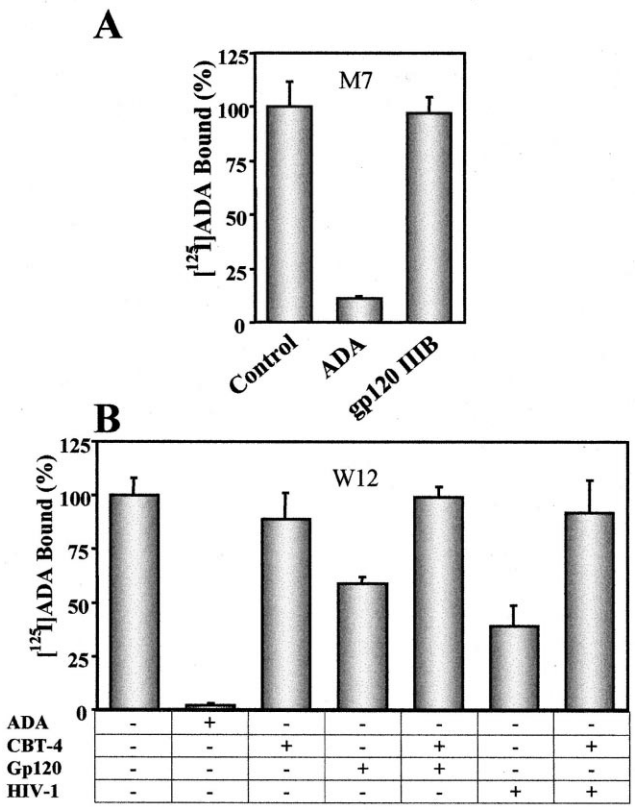

Fig. 3. The HIV-induced inhibition of ^{125}I-labeled ADA binding to human CD26 in murine cells requires gp120–CD4 interaction. A: The effect of 100 nM gp120 on the binding of 15 nM ^{125}I-labeled ADA to M9 cells (expressing human CD4) and M7 cells (lacking human CD4) was studied as described in the legend of Fig. 2. Unlabeled ADA was used as a control for specific binding. B: The effect of anti-CD4 mAb CB-T4 on gp120- and HIV-1 particle-induced ^{125}I-labeled ADA binding inhibition. The binding of 15 nM ^{125}I-labeled ADA to W12 cells was studied in the presence or the absence of 100 nM of gp120 or a concentrated preparation of HIV-1 Lai particles. In both cases, cells were preincubated (15 min, 37°C) with or without the mAb CB-T4 (1 μg/ml). Results of a typical experiment are presented (mean ± S.E.M. from triplicate samples).

seem to lack molecules, other than CD4 able to interact with gp120 to allow a subsequent interaction with CD26.

3.4. The effect of CXCR4 expression on gp120-induced ADA binding inhibition

The main candidate to mediate the inhibitory effect of gp120 on ADA binding is the chemokine receptor CXCR4. We found that murine clones lack murine CXCR4 expression as assessed by FACS analysis and RT-PCR studies (data not shown). Therefore, we transiently transfected the W12 murine clone with human CXCR4 to evaluate the role of this receptor on the gp120-induced inhibition of ADA binding to CD26. In these transiently transfected cells the binding of ADA was studied by FACS analysis using FITC-labeled bovine ADA. As shown in Fig. 4, transfection of human CXCR4 significantly enhanced the inhibitory effect of gp120. Inhibition was 91% in CXCR4 expressing cells (Fig. 4D) compared to 32% in control W12 cells (Fig. 4B). The effect was also studied in the presence of the anti-CD4 mAb CB-T4. In control cells lacking human CXCR4, the antibody completely reverted the effect of gp120 (Fig. 4C), whereas in CXCR4-expressing cells a 72% inhibition of ADA binding was still observed when the interaction of gp120 with CD4 was blocked (Fig. 4D). This effect appeared to be specific for CXCR4 expression since gp120-induced inhibition of ADA binding to CD26 in CCR5-transfected W12 cells was 39% (Fig. 4E), a value closely similar to that observed in control cells. Thus, the expression of CXCR4 specifically enhances the inhibitory effect of gp120 on ADA binding to CD26 and makes the interaction of gp120 with CD4 dispensable, as it happens in human SKW6.4 cells [15], which are CXCR4$^+$/CD4$^-$ (not shown).

4. Discussion

We have previously described the inhibition of ADA binding to CD26 by gp120 and viral particles [15]. This inhibition was observed in human CD4$^+$ and CD4$^-$ cells, thus appearing to be independent of the interaction of HIV-1 gp120 with CD4 and suggesting a direct interaction between gp120 and the ADA binding site of CD26. However, the data presented here in a heterologous model, showing the lack of effect of gp120 on ADA binding to CD26 in CD4$^-$/CD26$^+$ murine cells rule out this possibility, and suggest that gp120 should interact with other cell surface component(s) prior to its functional interaction with CD26.

Several cell surface components have been shown to interact with external glycoprotein gp120 from R4 HIV-1 isolates, such as CD4 [21], CXCR4 [22], galactosyl ceramides [23], the highly charged heparan sulfate proteoglycans [24], and cell-surface nucleolin [25]. However, it seems clear that gp120 binding induces the formation on the target cell surface of a complex in which, at least gp120, CD4 and CXCR4 associate [26,27]. Therefore, it was relevant to investigate the role of CXCR4 in the gp120-mediated inhibition of ADA binding to CD26. As a first approach, we studied the expression of CXCR4 in the human CD4$^-$ B-cell line SKW6.4, which is sensitive to the gp120-mediated inhibition of the binding of ADA to CD26 [15]. We observed that this cell line does express CXCR4. However, the activation of this receptor by SDF-1 modified the expression of CD26, and then the binding of ADA to CD26 (C.H., data in preparation). As an alternative approach, we evaluated the role of CXCR4 in murine cells transiently expressing this receptor. Although in murine CXCR4$^-$ cells, the expression of human CD4 is necessary for the inhibitory effect of gp120 on ADA binding, we show that the expression of human CXCR4 clearly makes CD4 dispensable. This suggests that CXCR4 is sufficient to allow a functional gp120–CD26 interaction. This is in our opinion the reason why in human CXCR4$^+$ cells the blockade of CD4 has a low effect on the inhibitory ability of gp120. The requirement of CXCR4 for a functional gp120–CD26 interaction could also explain the inhibitory effect of gp120 on the binding of ADA to the CD4$^-$/CXCR4$^+$ human B-cell line SKW6.4 [15], and the lack of interaction between soluble CD26 and recombinant soluble gp120 [10,28].

The third constant domain gp120 (C3 region) seems to be responsible for ADA binding inhibition in murine cells (Fig. 2), as we reported for human cell lines [15]. Although the interpretation of these data should be made with caution due to the use of synthetic peptides, some specificity for this effect can be assumed, due to the fact that a complete collection of peptides covering the gp120 sequence was used [15]. The C3 region of gp120 seems to be hidden in both, the recombinant oligomeric gp160 precursor protein and the

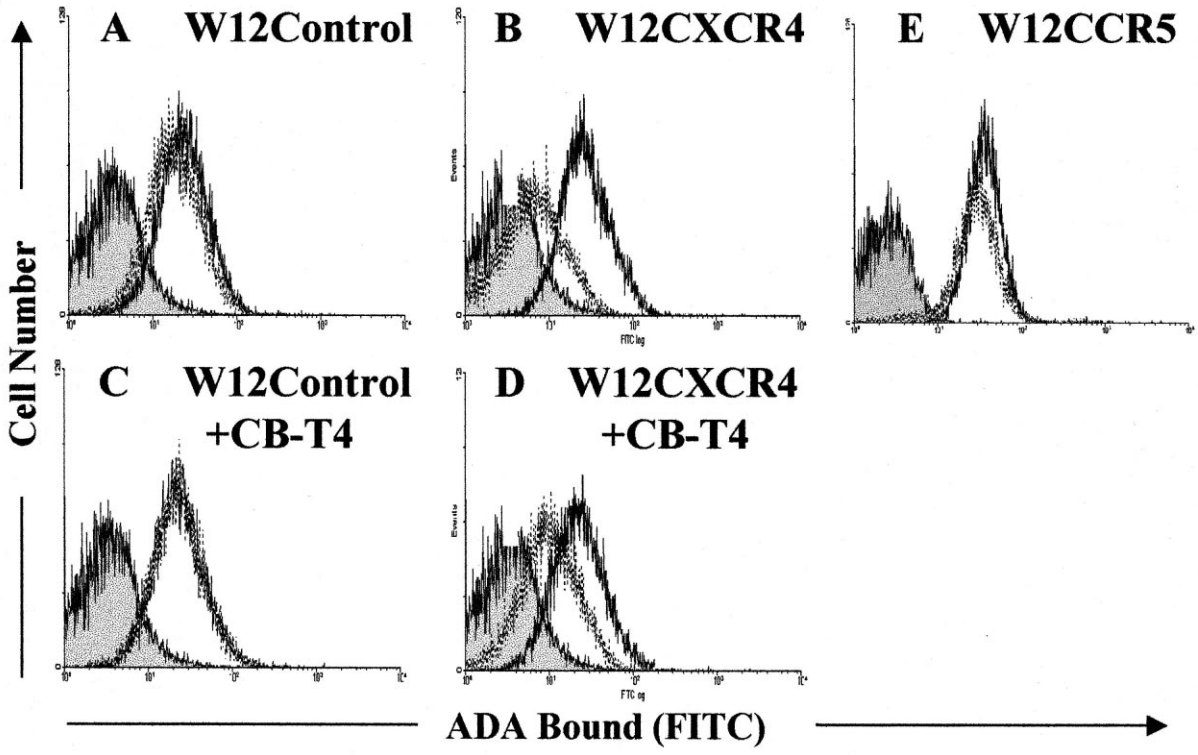

Fig. 4. The expression of CXCR4 enhances gp120-induced ADA binding inhibition. The binding of 100 nM FITC-labeled ADA (open peaks, solid line) and the effect of 100 nM of gp120 (open peaks, dashed line) was studied in W12 cells (A and C) and W12 cells transfected to express the chemokine receptor CXCR4 (B and D) in the absence (A and B) or presence (C and D) of the anti-CD4 mAb CB-T4. The binding of ADA to CD26 and the effect of gp120 was also studied in W12 cells transfected to express the chemokine receptor CCR5 (E). In each panel gray peaks correspond to the fluorescence obtained in cells preincubated with an excess of unlabeled ADA to evaluate specific FITC-labeled ADA binding. The figure shows a representative experiment of the three performed.

monomeric gp120 [29]. Moreover, recently reported data on the crystal structure of gp120 confirms that these sequences are not exposed on the outer surface of the protein [30]. In the context of gp120 interaction with the cell surface, if the C3 region is indeed responsible for ADA binding inhibition, the interaction of gp120 with CD4 and/or CXCR4 would contribute to unmask this hidden region and allow inhibition of ADA binding to CD26. Consistent with this hypothesis, transfection of CXCR4, but not CCR5 increased the inhibitory effect of gp120 on the binding of ADA to CD26 (Fig. 4).

The modification on the distribution of cell surface molecules after binding of gp120 to target cells has been reported by several authors. Concerning CD4 and CXCR4, the binding of gp120 induces an actin-dependent association of both receptors [26], that can be also observed in immunoprecipitation assays [27]. In addition to CD4 and CXCR4, gp120 induces cocapping of several cell surface molecules, CD26, CD45 and CD3 among others [31]. CD45RO regulates the CD4-associated tyrosine kinase $p56^{lck}$ and associates the CD26 [32]. Furthermore, the function and the anti-HIV activity of several chemokines, such as the CXCR4 agonist SDF-1α, are modulated by the peptidase activity of CD26 [14]. Given the functional relationships between these proteins (CD26, CD4 and CXCR4) on the cell surface, the presence of CD26 near these CD4–CXCR4 complexes is an attractive possibility.

Cell-surface ADA has a protective effect against locally high concentrations of adenosine [33]. Moreover, it has been reported that the blockade of ADA binding to CD26 by anti-ADA antibodies impairs T-cell proliferation [8]. Thus, the inhibition of ADA binding to CD26 exerted by gp120 might alter early events of T-cell activation, in which the ADA–CD26 interaction seems to be involved [34]. On the other hand, the results described here may explain, at least in part, the increased levels of serum ADA observed in HIV infected individuals [16]. The effect of gp120 on the ADA–CD26 module is not limited to $CD4^+$ cells, but it also occurs in CXCR4-expressing cells. In this regard, nocious effects associated with a gp120–CXCR4 interaction have been reported in neurons and $CD8^+$ T-cells [35,36]. The role of the ADA–CD26 module in these effects will merit attention.

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