We provide convergent and multiple evidence for a CD26/CXCR4 interaction. Thus, CD26 codistributes with CXCR4, and both coimmunoprecipitate from membranes of T (CD4+) and B (CD4−) cell lines. Upon induction with stromal cell-derived factor 1α (SDF-1α), CD26 is coimmunostained with CXCR4. CXCR4-mediated down-regulation of CD26 is not induced by antagonists or human immunodeficiency virus (HIV)-1 gp120. SDF-1α-mediated down-regulation of CD26 is not blocked by pertussis toxin but does not occur in cells expressing mutant CXCR4 receptors unable to internalize. Codistribution and cointernalization also occurs in peripheral blood lymphocytes. Since CD26 is a cell surface endopeptidase that has the capacity to cleave SDF-1α, the CXCR4/CD26 complex is likely a functional unit in which CD26 may directly modulate SDF-1α-induced chemotaxis and antiviral capacity. CD26 anchors adenosine deaminase (ADA) to the lymphocyte cell surface, and this interaction is blocked by HIV-1 gp120. Here we demonstrate that gp120 interacts with CD26 and that gp120-mediated disruption of ADA/CD26 interaction is a consequence of a first interaction of gp120 with a domain different from the ADA binding site. SDF-1α and gp120 induce the appearance of pseudopodia in which CD26 and CXCR4 colocalize and in which ADA is not present. The physical association of CXCR4 and CD26, direct or part of a supramolecular structure, suggests a role on the function of the immune system and the pathophysiology of HIV infection.

CD26 is a widely distributed lymphocyte activation antigen that displays dipeptidyl peptidase IV activity in its extracellular domain. The role of CD26 in HIV infection has been controversial. Its postulated role as coreceptor for HIV entry (1) is the natural ligand of CXCR4. gp120-induced redistribution of CD26 to the surface of lymphocytes is inhibited by HIV-1 particles and by the viral envelope glycoprotein gp120, with IC50 values in the nanomolar range. This inhibition is mediated by the C3 region of the gp120 protein and occurs in human T and B cell lines by a mechanism modulated by CD4 and CXCR4 expression (15, 16).

Given the functional relationship between dipeptidyl peptidase IV activity of CD26 and CXCR4, and the role of CXCR4 in gp120-mediated inhibition of ADA binding to CD26, we have studied the distribution of these proteins on the surface of human lymphocytes. In this paper we present evidence of a colocalization, coimmunoprecipitation, and modulation of CD26 and CXCR4. On the other hand, there is a redistribution of these cell surface proteins by the action of gp120 or SDF-1α, which is the natural ligand of CXCR4. gp120-mediated redistribution of CD26 and CXCR4 also affects cell surface ADA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fluorescein isothiocyanate (FITC), paraformaldehyde, and bovine serum albumin were purchased from Sigma. Bovine ADA (type VIII, Sigma) was filtered through Sephadex G-25. SDF-1α was purchased from R & D Systems (Minneapolis, MN). Glycine and electrophoresis reagents were obtained from Roche Molecular Biochemicals (Barcelona, Spain). The recombinant HIV-1 envelope glycoprotein gp120 from viral isolate IIIB (construct deposited by Dr. Jones) was produced in the baculovirus expression system and was kindly provided by the Medical Research Council AIDS Directed Program Reagent Project (Potters Bar, United Kingdom (UK)). The recombinant gp120 was conjugated to FITC was from Neosystem (Strasbourg, France). Sephadex G-25 fine grade columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Deionized water further purified. This paper is available on line at http://www.jbc.org
**CD26 and CXCR4 colocalization.** Jurkat J32 (A) or SKW6.4 (B) cells (4 × 10^6) were incubated 30 min at 37 °C in PBS buffer in the absence (−) or in the presence (+ gp120) of 15 μg/ml gp120. Cells were fixed and stained with 100 μg/ml FITC-conjugated Ta1 anti-CD26 (in green) and 15 μg/ml TRITC-conjugated 12G5 anti-CXCR4 (in red), as indicated under “Experimental Procedures.” Bar, 10 μm.

**Immunoprecipitation and Western Blots—**Cell extracts and membrane cell membranes were obtained as described by Ciruela et al. (22). Cell membrane was solubilized (22) and immunoprecipitated with anti-CD26 1F7 mAb or anti-CD8 mAb covalently coupled to a protein A matrix as described elsewhere (23) and incubated overnight. Each sample was washed and resuspended with 0.1 ml of SDS-polyacrylamide gel electrophoresis nonreducing sample buffer (0.125% Triton X-100, 6.8%, SDS, 20% v/v glycerol, 0.02% bromphenol blue). The immune complexes were dissociated by heating to 37 °C for 15 min and resolved by SDS-polyacrylamide gel electrophoresis in 7.5% gels (24). Immunoblotting was performed using anti-CD26 1F7 antibody (1/3,200) or anti-CXCR4 sc-6190 antibody (2 μg/ml) and anti-mouse IgG-peroxidase (Dako A/S, Glostrup, Denmark) or anti-goat IgG-peroxidase (Roche Molecular Biochemicals GmbH, Barcelona, Spain), respectively. The polyvinyl difluoride membrane (Immobilon-P, Millipore) was incubated in equal volumes of Supersignal chemiluminescent substrates 1 and 2 (Pierce). The detection reagent was drained off, and the filters were placed in contact with a film (Hyperfilm ECL), which was developed by chemiluminescence.

**Protein Determination—**Protein was quantified by the bicinchoninic acid method (Pierce) as described by Sorensen and Brodbeck (25) and using bovine serum albumin as standard.

**RESULTS**

**Colocalization of CXCR4 and CD26 in CD4^+ and CD4^-Cell Lines—**To analyze the possible codistribution of CXCR4, the coreceptor for T-tropic HIV-1, and CD26, two cell lines were selected: the Jurkat J32 CD4^- T cell line and the Epstein-Barr transformed B cell line SKW6.4, which does not express the main HIV receptor CD4. To establish a possible correlation between this codistribution and the well characterized binding of the viral glycoprotein gp120 to the CXCR4 chemokine receptor (26, 27), the degree of colocalization between CD26 and CXCR4 on the surface of Jurkat J32 and SKW6.4 cells was determined in the absence and in the presence of gp120. Jurkat J32 cells, fixed and labeled with FITC-conjugated anti-CD26 treated, if necessary, with several anti-CD26 monoclonal antibodies, ADA, gp120IIIB, SDF-1α P2G, or phorbol esters at 37 °C; and washed with PBS or with PBS containing 10 mM glycine, pH 2, for the samples treated with SDF-1α or P2G (21). For cells other than U373, fixation, permeabilization, and staining were done as described elsewhere (20). For fixation U273 cells were washed in PBS and treated with 1% paraformaldehyde solution in PBS at 20 °C for 5 min. For confocal microscopy analysis a Leica TCS 4D confocal scanning laser microscope was used to eliminate Leitz DMIRBE microscope (Leica Lasertecnica GmbH, Heidelberg, Germany) was used. The colocalization analysis was made by means of the Multicolor software (version 2.0; Leica Lasertecnica GmbH). Flow cytometry analysis was done with an EPICS Profile flow cytometer (Beckman, Hialeah, FL). The parameters used to select cell populations for analysis were forward and side light scatter.

**Comodulation of CXCR4 and CD26 in Lymphocytes**

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Ta1 and TRITC-conjugated anti-CXCR4 12G5, showed (Fig. 1A) a marked colocalization (in yellow) of the two proteins. Preincubation of cells with gp120 for 30 min at 37 °C prior to fixation and staining induces the formation of pseudopodia with a redistribution on cell surface CXCR4 and CD26 that are coclustered in these pseudopodia. This morphological change is reversible, with a maximal formation of pseudopodia at 30 min of treatment with gp120, and a disappearance at 2 h after the addition of the viral glycoprotein.

Staining of the CD4+ SKW6.4 cell line with FITC-conjugated anti-CD26 Ta1 and TRITC-conjugated anti-CXCR4 12G5 showed a high degree of colocalization between CD26 and CXCR4 (Fig. 1B). However, pretreatment with gp120 before fixation and labeling did not induce any morphological redistribution of these molecules on the cell surface.

CXCR4 Coimmunoprecipitates with CD26—The high degree of colocalization between CD26 and CXCR4 on the Jurkat J32 and SKW6.4 cell surface, and the coclustering of these proteins in the pseudopodia induced by gp120 in T cells, suggest a molecular interaction between both molecules. To test this possibility, coimmunoprecipitation experiments were performed with solubilized Jurkat J32 and SKW6.4 cells membranes. Immunoprecipitation was carried out with the 1F7 anti-CD26 mAb, and the Western blot of immunoprecipitates revealed a band of 110 kDa that corresponds to the CD26 when the Western blot was developed with anti-CD26 1F7 antibody (Fig. 2A) and two bands of 90 and 50 kDa when the immunoblot was developed with anti-CXCR4 sc-6190 (Fig. 2B). These two bands correspond to the CXCR4 receptor as it has been described previously (26, 27). These results and those shown in Fig. 1 indicate that an interaction CD26/CXCR4 occurs on the surface of Jurkat J32 and SKW6.4 cells.

Modulation of the CD26/CXCR4 Module by SDF-1α, by an Antagonist and by Pertussis Toxin—It has been fully described that SDF-1α, the physiological CXCR4 ligand, induces a rapid down-regulation of its receptor (17, 28–31). The incubation of Jurkat J32 or SKW6.4 cells with 400 nM SDF-1α induced the endocytosis of CXCR4 and also of CD26 as stated by immunocytochemistry and confocal microscopy analysis (Fig. 3). This process, which was evident at 30 min, could be already detected

![Fig. 3. SDF-1α-induced internalization of CXCR4/CD26 in cell lines.](http://www.jbc.org/)

![Fig. 4. Cointernalization of CXCR4/CD26 in peripheral blood lymphocytes and in Jurkat cells.](http://www.jbc.org/)
5 min after the addition of the ligand (data not shown). These results show that the modulation of the CD26-CXCR4 complex by chemokines takes place in cells irrespective of CD4 expression or gp120-induced pseudopodia.

Like gp120 (see Fig. 1), in Jurkat J32 cells, SDF-1α also induced the formation of pseudopodia after 30 min of incubation (Fig. 3A; 30 min), although the number of cells presenting pseudopodia was lower. In some cells the pseudopodia induced by the SDF-1α were less labeled with fluorescent antibodies than the rest of the cell and, therefore, it seems that CXCR4 and CD26 are internalized with different kinetics in the pseudopodia than in the rest of the cell. By analyzing permeabilized cells it is shown that CD26 and CXCR4 colocalize inside Jurkat J32 and SKW6.4 cells (Fig. 3) after 10 min of SDF-1α-induced internalization. At a longer period of incubation with SDF-1α (1 h), the two proteins do not codistribute inside the cell (Fig. 3), since they are localized in different endocytic vesicles, CXCR4 being more homogeneously distributed than CD26. In Jurkat J32 cells, cointernalization was also promoted by the phorbol ester PMA but did not occur in the presence of the antagonist of CXCR4, P2G (Fig. 4).

A colocalization between CXCR4 and CD26 was also evident on the cell surface of peripheral blood lymphocytes (Fig. 4). The membrane expression of both CD26 and CXCR4 in primary lymphocytes is down-modulated by SDF-1α, but not modified by the antagonist P2G. Therefore, CD26 and CXCR4 behave in primary cells as in the lymphocytic cell lines. To confirm the specificity of the effect of SDF-1α upon regulation of CD26 and CXCR4 expression, ligand-induced internalization was assayed in human U373 cells stably transfected with cDNAs encoding for full size CXCR4 (U373-CXCR4 cells) or for a version of the receptor lacking the COOH-terminal tail (U373-CXRC4Acyl cells). Parental U373 cells express endogenous CD26, and therefore, cointernalization studies are possible using single transfectants. Again, in cells expressing full size CXCR4, CD26/CXCR4 colocalization was found when cells were treated with SDF-1α or PMA. In contrast, neither CD26 nor CXRC4 were internalized in cells expressing the short version of the chemokine receptor (Fig. 5). Cointernalization was inhibited by treatment with sucrose or acetic acid, thus indicating that it is mediated by coated vesicles (Fig. 6), which is in agreement with the data reported by Amara et al. (17). Interestingly, blockade of G, operation by pertussis toxin at either 150 ng/ml (Fig. 6) or 5 µg/ml (not shown) did not affect the SDF-1α-mediated cointernalization of CD26 and CXCR4.

Effect of gp120 upon the Redistribution of ADA on the Cell Surface—As it had been previously reported (15), gp120 inhibits the binding of 125I-labeled ADA to CD26 in human lymphocytes and T cell lines. To characterize better this effect, experiments of confocal microscopy were performed using FITC-conjugated ADA. As shown in Fig. 7A, the interaction of exogenous ADA with cells (Fig. 7A) decreases in the presence of gp120 (Fig. 7B). The gp120-induced inhibition of ADA binding to CD26 was also detected by flow cytometry (Fig. 7C). The displacement of ADA bound to CD26 by gp120 occurs even for endogenous ADA. Thus, when nonfixed SKW6.4 cells were labeled with TRITC-conjugated anti-ADA (Fig. 7D) a decrease of fluorescence was observed by the pretreatment with gp120 (Fig. 7E), indicating that gp120 is able to disrupt the ADA/CD26 interaction on the cell surface. Similar results were observed with Jurkat J32 cells in the presence of an excess of anti-CD4 antibody (result not shown). We also investigated whether in the gp120-induced pseudopodia the CD26 would be free of cell surface ADA. In experiments in which Jurkat J32 cells were labeled with FITC-conjugated anti-ADA and TRITC-conjugated 12G5, a good staining of cell surface ADA and CXCR4 was observed (Fig. 8A) with a notable degree of colocalization (in yellow). This is consistent with the above described high degree of colocalization between the CXCR4 and the cell surface ADA-binding protein, CD26. In fact, the pretreatment of Jurkat J32 cells with gp120 induced the formation of pseudopodia in which no labeling of ADA could be detected by immunocytochemical assays (Fig. 8B). The inhibition of exogenous ADA binding and the release of endogenous ADA...
bound to CD26 by gp120 suggests that gp120 recognizes the ADA binding site of CD26. Interestingly enough, staining of fixed SKW6.4 cells with FITC-conjugated gp120IIIB and with TRITC-conjugated anti-ADA led to a very high colocalization (in yellow) of the two proteins (Fig. 9A). However, when the same experiment was performed using cells that were fixed after incubation with the reagents (see “Experimental Procedures”), a poor colocalization was found (Fig. 9B). Thus, the interaction CD26/gp120 occurs through an epitope different from the ADA binding site in CD26, and a subsequent conformational change in gp120 is required to block the ADA binding site of CD26.

gp120 Blocks the Binding of Specific Antibodies to CD26—To test the above described hypothesis, binding of FITC-conjugated gp120IIIB was done with fixed SKW6.4 cells preincubated with several anti-CD26 mAbs raised against different epitopes. Preincubation was performed at 4 °C to prevent internalization of cell surface molecules. Bound FITC-gp120IIIB was quantified by flow cytometry (Fig. 10). Two anti-CD26 mAbs, 202.36 and 4H12, were able to decrease the binding of gp120. This decrease could not be detected with other anti-CD26 antibodies as Ta1 or TA5.9, which is directed against the ADA binding site (not shown), or with anti-CD4 Leu-3a mAb used as an irrelevant antibody for these CD4+ cells. Moreover, the preincubation of SKW6.4 cells with gp120IIIB (Fig. 11) diminished the staining obtained using the anti-CD26 antibodies 202.36 and 4H12, but not the staining obtained using Ta1. With the human T cell line Jurkat J32, similar results were obtained when the gp120 binding to CD4 was blocked with an anti-CD4 mAb (data not shown). All these results suggest that there is a direct interaction between gp120 and CD26 and that the gp120 binding domain in CD26 is distinct from the ADA binding site.

**DISCUSSION**

In this paper we demonstrate for the first time that CD26 and CXCR4 interact in primary lymphocytes from human blood and also in T and B cell lines. The CXCR4 natural ligand SDF-1α has the conserved NH₂-X-Pro sequence (where X is any amino acid) at the NH₂ terminus, which is a consensus sequence for substrates of dipeptidyl peptidase IV activity of CD26. It has been demonstrated that SDF-1α can be processed by CD26 (4, 9, 11, 32). Therefore the CD26-CXCR4 complex is likely a functional unit in which the expression of CD26 on the cell surface may modulate SDF-1α-induced chemotaxis. Since SDF-1α is a natural antiviral agent (33, 34), the expression of CD26 can also modulate the activity of this substrate of CXCR4 against T-tropic strains of HIV.

The fact that SDF-1α induces the cointernalization of CXCR4 and CD26 in all cells tested (Figs. 3 and 4) is also relevant. The SDF-1α-induced internalization of the CD26/CXCR4 module was not CD4-dependent, as we could detect that the level of expression of both molecules on the cell surface decreased with SDF-1α incubation in T, but also in B, cells. The specificity and physiological relevance of CD26/CXCR4 cointernalization was demonstrated by the lack of internalization in the presence of the antagonist P2G in primary lymphocytes and in cell lines. The cells expressing a chemokine receptor lacking the COOH-terminal part were also a suitable model to assess the relevance of the interaction. In fact, the lack of the cytoplasmic tail, which is known to prevent SDF-1α–induced down-regulation of CXCR4, also prevents internalization of CD26. These results indicate that treatment of lymphocytes with chemokines or agents that activate protein kinase C leads to the simultaneous internalization of CXCR4 and CD26, probably via the same endocytic pathway, as we have demonstrated.
for other interacting cell surface proteins (35). SDF-1α-induced internalization of CXCR4 is mediated by coated vesicles (17). According to this, the blockade of the cointernalization by sucrose and by acetic acid further suggests that endocytosis of CXCR4 and CD26 follows the same endocytic pathway mediated by coated vesicles. Interestingly, after a long period of chemokine treatment, the codistribution of the two proteins inside the cell is lost. At 1 h of treatment CXCR4 is found homogeneously distributed near the plasma membrane (Fig. 3), which would fit with the rapid recycling reported for this chemokine receptor. In contrast, internalized CD26 is clustered in intracellular vesicles, thus confirming that the traffic of the two proteins after the cointernalization follows different routes. As reported previously, ligand-induced internalization of CXCR4 is not mediated by Gi proteins (17). The assays performed in the presence of pertussis toxin indicate that blocking of G-protein-mediated signaling does not prevent the cointernalization. This interesting finding is evidence for a G protein-independent signaling pathway that regulates the SDF-1α-induced simultaneous internalization of the two proteins. To our knowledge this is the second example of ligand-induced cointernalization of a receptor and the enzyme that inactivates the ligand for the receptor (35). In these models of interacting proteins, the existence of membrane-bound and soluble forms of degrading enzymes is relevant. Thus, in addition to membrane-bound enzyme, soluble forms of CD26 are able to inactivate SDF-1α (4, 7). While the soluble enzyme could regulate circulating concentrations of SDF-1, the cell-bound enzyme should be responsible for the control of local changes in ligand concentration. Cointernalization of CD26 with CXCR4 might represent a second mechanism of control of CXCR4 signaling. Consistent with the results shown here, it has been reported that peripheral blood lymphocytes migrating toward SDF-1α show low expression of CXCR4 and CD26. This finding can be a consequence of a higher response to SDF-1α from CD26low cells, but it could reflect a SDF-1α-induced cointernalization of CD26 and CXCR4 (see Fig. 3).

Iyengar et al. (36) have described that in peripheral blood mononuclear cells the addition of gp120 cause cocapping of CD4 and CXCR4 with subsequent pseudopodium formation. On the other hand, Feito et al. (37) described that gp120 from the syncytium-induced HIV-1 strain increased the capping of CD4 with some proteins, CD26 included. We here demonstrate that CD26-CXCR4 complexes are coregulated into gp120-induced pseudopodia. Taken together, these results suggest that the viral envelope glycoprotein gp120 induces the formation in T lymphocytes of pseudopodia in which the main virus receptor, the CD4, the coreceptor CXCR4, and the CD26 coexist.

Although the importance of CD4 and chemokine receptors in the process of HIV entry has been extensively described, the role of CD26 in this process is still not well understood. Previous results of our laboratory (15) described the inhibition of the binding of 125I-labeled ADA to the CD26 by HIV-1 envelope gp120 and viral particles, suggesting that gp120 recognizes the ADA binding site of CD26. gp120 blocks binding of ADA to CD26 in a CXCR4-dependent manner (16). It is possible that gp120 displaces ADA binding to CD26 in CD4-negative cells because of the presence of CXCR4. These results support the hypothesis (see Ref. 16) that gp120 has to bind to CXCR4 prior to its interaction with CD26 and the disruption of the ADA/CD26 interaction. In this report we confirm the gp120-induced inhibition of ADA binding to CD26, but we also demonstrate that gp120 provokes the release of endogenous ADA bound to CD26, and this happens without changes in the expression of cell surface CD26 (Fig. 11). These data would suggest that gp120 and ADA share a common binding site in CD26. As shown in Fig. 9B, when the FITC-conjugated gp120 incubation was done using nonfixed cells, the viral glycoprotein could produce some membrane protein rearrangements displacing cell surface ADA from CD26. However, when cells were fixed before the incubation with gp120, a marked colocalization between the viral glycoprotein and the cell surface ADA was observed (Fig. 9A). It should be noted that fixation avoids any cell membrane protein mobility or redistribution. These results led to hypothesize the existence of a gp120 binding domain in CD26 distinct from the ADA binding site. This indicates that
the gp120-mediated inability of ADA to bind to CD26 would be a consequence of a first interaction of gp120 with a distinct epitope in CD26. We detected two anti-CD26 mAb, 4H12 and 202.36p, raised against a different epitope from ADA binding center that decrease the binding degree of gp120. Both antibodies are, also, less effective in labeling CD26 when cells are

**Fig. 10.** Anti-CD26 antibodies-induced inhibition of gp120 binding. SKW6.4 cells (4 × 10⁶) were incubated in the absence (continuous line) or presence (broken line) of 202.36p, 4H12, Ta1, or anti-CD4 Leu3A mAb in PBS buffer for 45 min at 4 °C. Subsequently, cells were fixed and labeled with 15 μg/ml FITC-conjugated gp120 IIIB for flow cytometry analysis as described under “Experimental Procedures.” The solid peak represents unspecific binding.

**Fig. 11.** gp120-induced inhibition of CD26 labeling. SKW6.4 (4 × 10⁶) cells were untreated (continuous line) or treated (broken line) with 15 μg/ml gp120 IIIB for 45 min at 37 °C. Cells were then fixed and stained with FITC-conjugated antibodies 202.36p, 4H12, Ta1, or anti-CD4 Leu3A mAb and analyzed by flow cytometry (see “Experimental Procedures”). The solid peak represents unspecific binding.
incubated with the viral glycoprotein gp120. These results correlate with the hypothesis of an interaction of gp120 with CD26 at an epitope different from the ADA binding domain, and they also indicate a recognition of cell surface CD26 by the viral envelop protein gp120.

Comodulation of CXCR4 and CD26 by SDF-1α, gp120-induced formation of pseudopodia in which ADA is not present, and the role of ADA in the development and function of the immune system (38) suggest that the ADA and the role of ADA in the development and function of the immune system (38) suggest that the ADA-CD26 complex is important for the functionality of human lymphocytes.

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