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Solubilization of the Chemokine Receptor CXCR4

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The chemokine receptor CXCR4 was solubilized from the human T-cell line CEM by using the detergent *n*-dodecyl-*β*-maltoside (DDM) and cholesteryl hemisuccinate ester (CHS). Binding studies with ^{125}I-SDF-1*α* revealed a dissociation constant of 5.33 nM and a receptor density (B_{max}) of 2.68 pmol/mg in CEM membranes at 4°C. The affinity of solubilized CXCR4 for SDF-1*α* was identical to membrane-bound CXCR4. Binding of gp120 to solubilized CXCR4 was demonstrated by coprecipitation of gp120 with anti-CXCR4 antibodies. © 2000 Academic Press

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The chemokine SDF-1*α* and its receptor CXCR4 are involved in diverse biological processes, such as immunomodulation, organogenesis, and hematopoiesis (1–4). In 1996 CXCR4 was identified as the principal coreceptor for T-cell line adapted (X4) HIV-1 isolates, essential for the entry of these viruses into target cells (5). SDF-1 has been shown to block entry of X4-HIV-1 isolates (6, 7). CXCR4 is a member of the G-protein coupled receptor (GPCR) super-family with seven membrane-spanning domains (8). GPCR are targets for over 30% of pharmaceutical agents currently used in clinical practice (9, 10). Nonetheless, structural information on this large family of proteins is sparse. The dependence of the native conformation of these proteins on the hydrophobic, intra-membrane environment has complicated attempts to isolate these receptors. Irreversible protein denaturation may occur during solubilization of the cell membrane. Detergent solubilization is a crucial step for purification and biochemical, biophysical and structural characterization of a receptor molecule. Here we describe the detergent solubilization of conformationally relevant CXCR4 from CEM cells. The methods described herein contribute to the analysis of CXCR4 as a chemokine receptor as well as HIV-1 coreceptor.

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MATERIALS AND METHODS

Materials. Recombinant human SDF-1*α* was purchased from Peprotech (Rocky Hill, NJ). ^{125}I-SDF1*α* (specific activity 2200 Ci/mmol), sodium iodide-125 (17.4 Ci/mg) soluble CD4 (sCD4) and $gp120_{LAI}$ were from NEN Life Science Products (Boston, MA). DDM was purchased from Boehringer Mannheim (Indianapolis, IN) and Cymal-5 from Anatrace (Maumee, OH). Protease inhibitor cocktail, CHS and digitonin were obtained from Sigma (St. Louis, MO), CHAPS from Pierce (Rockford, IL) and goat polyclonal C-terminal anti-CXCR4 from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells. The human T-lymphocyte cell line CEM was obtained from the American Type Culture Collection, Rockeville, MD and was maintained in RPMI 1640 medium containing 10% FBS, 50 *μ*g per ml of penicillin and streptomycin and GHOST-CD4-CXCR4 and GHOST-CD4-CCR5 cell lines were kindly provided by Dr. Dan Littman. Cells were grown in DMEM containing 10% FBS, 4 mM glutamine, 50 *μ*g per ml of penicillin, streptomycin and hygromycin, 10 *μ*g per ml of gentamicin and 1 *μ*g per ml of puromycin.

Crude membrane preparation. Membranes were prepared as described previously for Swiss 3T3 cells (11). Briefly, cells were rinsed with phosphate-buffered saline (PBS). Cells were then resuspended in lysis buffer (50 mM HEPES, pH 7.4, 1 mM EGTA containing protease inhibitor cocktail) and homogenized with 40 strokes with a tight pestle in a Dounce homogenizer. Nuclei and unbroken cells were then pelleted by a low speed centrifugation (800*g* for 10 min at 4°C). The supernatant was centrifuged at 45,000*g* for 30 min at 4°C. The crude membrane pellet was washed once and then resuspended in above buffer with the aid of a Dounce homogenizer, quickly frozen in methanol-dry ice and stored at −80°C.

Binding experiments with membrane preparations. 15–20 *μ*g of CEM crude membrane protein were incubated with 0.5–0.6 nM of ^{125}I-SDF-1*α* in a buffer containing 20 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 150 mM NaCl and 1% BSA, in a volume of 100 *μ*l at 4°C for 3 h. Nonspecific binding was defined in the presence of 500 nM SDF-1*α*. Receptor bound radioligand was separated from unbound ligand by rapid filtration through Whatman GF/C filters, presoaked in 0.3% polyethylenimine. Filters were rinsed twice with 4 ml of ice-cold buffer (20 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 500 mM NaCl). Radioactivity was measured in a Packard Cobra II λ-counter. The data were analyzed with the program GraphPad Prism (Version 3.0).

Solubilization protocol. CEM membrane preparations were solubilized at a protein concentration of 4–4.5 mg/ml with 1% DDM (w/v) and 0.12% CHS (w/v) in 20 mM HEPES, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 10% glycerol (v/v) and protease inhibitor cocktail. The mix-

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ture was incubated at 4°C for 30 min in an end-over-end rotator and subjected to ultracentrifugation at 100,000*g* for 60 min.

Development of a binding assay for solubilized CXCR4. ^{125}I-SDF-1α binding to solubilized receptor was assayed in 20 mM HEPES, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 1% BSA, 150 mM NaCl and protease inhibitor cocktail, containing 75–90 μg of solubilized protein and 0.37–0.4 nM ^{125}I SDF-1α in a volume of 100 μl. Again, inclusion of NaCl in the assay buffer was essential for specific binding. The final detergent concentration was 0.3%. Nonspecific binding was determined by inclusion of 500 nM unlabeled ligand. After incubation for 3 h at 4°C receptor bound radioligand was recovered by rapid filtration through Whatman GF/F filters, presoaked in 1% polyethylenimine for 3 h, followed by one rapid wash with 4 ml of ice-cold 20 mM HEPES, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 500 mM NaCl, 0.1% BSA and 0.1% DDM. Radioactivity on the filters was determined as above.

Iodination of Gp120. Soluble $gp120_{LAI}$ was iodinated by using Iodobead (Pierce). A specific activity of 1200 Ci/mmol was obtained by using 10 μg of protein and 250 μCi of Na^{125}I. Na^{125}I was preincubated with one Iodobead in a volume of 150 μl in PBS for 5 min at 0°C and then transferred to an Eppendorf tube containing 10 μg of gp120. Radiolabeling was allowed to proceed for 5 min at 0°C. Iodinated gp120 was purified from free Na^{125}I by separation through a 1.5 ml Sephadex G25 column, pre-equilibrated in 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA and 1% BSA. Protein fractions were eluted in the void volume of the column. Specific activity was determined by trichloroacetic acid precipitation.

Immunoprecipitation of CXCR4. 150 μg of solubilized membrane protein was incubated with 6.4 nM ^{125}I-$gp120_{LAI}$ in 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1% BSA, containing protease inhibitor cocktail, in a volume of 200 μl at 4°C for 12 h. 100 nM sCD4 was included, since our naturally occurring clone of CEM cells lost CD4 expression (data not shown). 3 μg of goat polyclonal anti-CXCR4 antibody were then added. CXCR4 was precipitated with 20 μl of Protein G sepharose beads. The beads were washed three times with above buffer containing 0.15% DDM and pelleted. 25 μl of SDS-sample buffer was added to the beads, followed by resuspension and incubation for 5 min at 96°C. Samples were run on 10% SDS-polyacrylamide minigels, which were then visualized by autoradiography.

RESULTS

Binding Parameters of ^{125}I-SDF-1α to Membrane Preparations

Suspensions of crude membranes prepared from CEM cells were assayed for specific binding of radiolabeled SDF-1α. Specific binding, which amounted to 75–80% of total binding, required the inclusion of NaCl in the binding assay. To determine the affinity of SDF-1α for membrane-bound CXCR4, we generated full homologous competition curves (Fig. 1A). The slope (pseudo-Hill coefficient, n_H) was not different from unity (1.16 $\pm$ 0.1) at 4°C. Scatchard transformation of the competition curves (Fig. 1B) revealed a single binding site with a K_D of 5.33 $\pm$ 1.03 nM and a B_{max} of 2.68 $\pm$ 0.4 pmol/mg. Binding of ^{125}I-SDF-1α was specific for membranes from cells expressing CXCR4. We examined ^{125}I-SDF-1α binding to membranes prepared from two closely matched cell lines that differed only in transfection of coreceptor. 20 μg of either GHOST-CD4-CCR5 or GHOST-CD4-CXCR4 membranes were incubated with 0.33 nM ^{125}I-SDF-1α in a volume of 100 μl. GHOST cells had a low level of endogenous CXCR4 expression. At equilibrium, 6.7 pM ^{125}I-SDF-1α was bound to membranes from GHOST-CD4-CCR5 cells, and 61.6 pM from GHOST-CD4-CXCR4 cells.

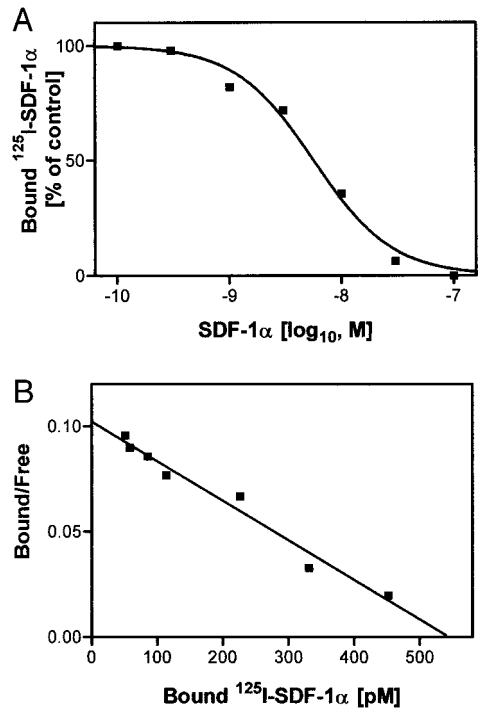

FIG. 1. (A) ^{125}I-SDF-1α binding to CEM membranes. 0.58 nM ^{125}I-SDF-1α was incubated with 0.2 mg/ml of membrane protein in the presence of increasing concentrations of SDF-1α. Specifically bound ^{125}I-SDF-1α at equilibrium was 50.8 pM. (B) Scatchard transformation of homologous competition data. K_D and B_{max} were obtained by linear regression analysis.

Solubilization and Binding Assay for Solubilized CXCR4

We tested several detergents, including CHAPS, digitonin, Cymal-5 and DDM/CHS for their ability to extract CXCR4 with functional binding properties for SDF-1α. Cymal-5 was recently used to solubilize the second principal HIV-1 coreceptor (12). Only DDM/CHS and to a lesser extent CHAPS and Cymal-5 were successful in releasing the binding sites in an active form (data not shown). We tested several methods, commonly used in binding studies with solubilized receptor molecules, to separate free from receptor-bound radioligand. Precipitation with polyethylenglycol (PEG) resulted in co-precipitation of ^{125}I-SDF-1α, a protein of 8 kDa and variations in ionic strength of the filtration-medium or PEG-content did not result in the preferential precipitation of the radioligand-receptor-complex (data not shown). We next tested a series of filters for their ability to retain ^{125}I-SDF-1α-solubilized CXCR4-complexes. DEAE ion exchange fil-

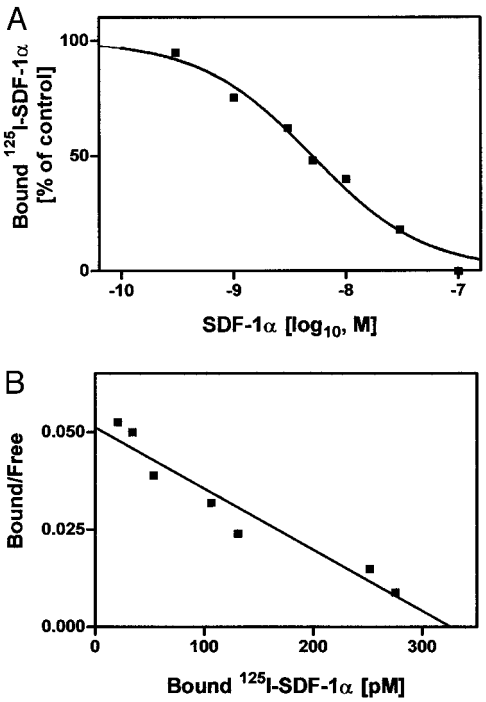

FIG. 2. (A) Equilibrium binding of ^{125}I-SDF-1α to solubilized membranes. 0.4 nM ^{125}I-SDF-1α were incubated with 0.87 mg/ml of solubilized membrane protein in the presence of the indicated concentrations of SDF-1α. Specifically bound ^{125}I-SDF-1α at equilibrium was 20.4 pM. (B) Scatchard transformation of competition data. K_D and B_{max} were obtained by linear regression analysis.

ters, which were used successfully for assaying binding of IL-8 to the solubilized erythrocyte chemokine receptor (13), retained unbound ^{125}I-SDF-1α (data not shown). Whatman GF/F filters, presoaked in polyethylenimine were useful in this assay. Equilibrium binding data of SDF-1α to solubilized CXCR4 are presented in Figs. 2A and 2B. Specific binding was 70–85% of total binding. A single class of binding sites was found in Scatchard analysis and the slope of the competition curve was not different from unity ($n_H = 0.92 \pm 0.05$). We calculated a dissociation constant of 5.7 ± 0.9 nM and a binding capacity of 365 ± 25 fmol/mg of protein. Detergent solubilization resulted in the extraction of 56–62% protein and 11–15% of active binding sites. The solubilized receptor remained stable at 4°C without loss of binding activity for at least one week.

Binding of ^{125}I-gp120$_{LAI}$ to Solubilized CXCR4

To examine whether solubilized CXCR4 was capable of binding HIV-gp120, co-precipitation experiments were conducted. Radiolabeled gp120 could be co-precipitated by a polyclonal anti-CXCR4 antibody, when incubated in the presence of sCD4. Excess of unlabeled gp120 or SDF-1α blocked the co-precipitation of ^{125}I-gp120 (Fig. 3).

DISCUSSION

In this report we show that the chemokine receptor CXCR4 can be successfully solubilized by the combination of the detergent DDM and the cholesteryl ester CHS. Detergent solubilization had no effect on the binding affinity of CXCR4 since the K_D values for membrane and solubilized receptors were practically identical. Our data are also in agreement with the affinity of CXCR4 for SDF-1α in CEM cells, with a reported K_D of 7.1 nM (14). Detergent solubilization is a crucial step in the molecular characterization of a receptor protein. A rich source in starting material is usually necessary for successful solubilization of a transmembrane-protein. We used the human T lymphocyte cell line CEM, which showed a high level of CXCR4 expression (2.68 pmol/mg). The detergent DDM and the cholesteryl ester CHS in a glycerol-containing buffer extracted 56–62% of protein and 11–15% of active SDF-1α binding sites. In our hands the solubilized chemokine receptor showed great stability at 4°C with no loss of binding activity for one week when kept in the glycerol-containing buffer with the appropriate protease inhibitors. Recently, it has been shown that the amino-terminal region of CXCR4 constitutes an important SDF-1 binding domain (15). However, it is not known whether SDF-1 interacts directly with this region or whether mutations in this region affect overall CXCR4 conformation. Detergent solubilization with the protocol we describe in this report did not affect the domains important for chemokine binding. Our data provide also evidence for an intact binding site for HIV-1-gp120, which has been shown to be a highly conformational structure, depending on the integrity of all extracellular domains (15). Coprecipitation of gp120 with anti-CXCR4 antibodies can only occur if the interaction between solubilized CXCR4 and gp120-CD4 is left intact. We used polyclonal C-terminal anti-CXCR4 antibodies because they do not interfere with binding of SDF-1 or gp120-CD4. The conformation de-

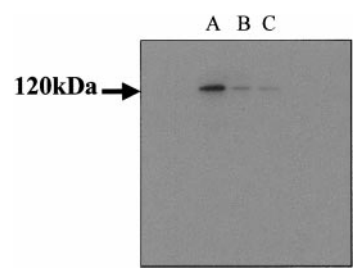

FIG. 3. Coprecipitation of solubilized CXCR4 and HIV-1 gp120. 150 μg of solubilized CEM membrane protein were incubated with 100 nM sCD4 and 6.4 nM ^{125}I-gp120$_{LAI}$ in the absence (A) or presence of 100 nM unlabeled gp120 (B) and 500 nM SDF-1α (C). CXCR4 was precipitated with 3 μg of goat polyclonal anti-CXCR4 antibody and 20 μl of Protein G–Sepharose beads. Lanes were exposed to film for 72 h.

pendent antibody 12G5 could not be used since it blocks both functions of CXCR4. Also, SDF-1α, the only known endogenous ligand for CXCR4, blocked coprecipitation of gp120.

It will be of high interest to characterize further the binding properties of gp120 for solubilized CXCR4. It has been suggested that the interaction of the HIV-1 envelope with its co-receptor induces a conformational change in the envelope protein, mediating fusion of cell and virus membranes. Fusion-competent vaccines led to broad neutralization of primary isolates of HIV-1 in transgenic mice (16). Characterization of the interaction of viral envelope and purified co-receptor may impact on vaccine development and may facilitate the search for HIV-1 entry blockers.

In conclusion, we have characterized the equilibrium-binding parameters of SDF-1α to membrane preparations and developed a protocol for successful solubilization of conformationally relevant CXCR4. This protocol, producing stable, solubilized CXCR4 and development of a binding assay will be valuable tools for the purification and detailed molecular characterization of this receptor.

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