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Chemokine receptor-5 (CCR5) is a receptor for the HIV entry inhibitor peptide T (DAPTA)

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Abstract

The chemokine receptor CCR5 plays a crucial role in transmission of HIV isolates, which predominate in the early and middle stages of infection, as well as those, which populate the brain and cause neuro-AIDS. CCR5 is therefore an attractive therapeutic target for design of entry inhibitors. Specific rapid filtration binding assays have been useful for almost 30 years both for drug discovery and understanding molecular mechanisms of drug action. Reported in 1986, prior to discovery of chemokine co-receptors and so thought to act at CD4, peptide T (DAPTA) appears to greatly reduce cellular viral reservoirs in both HAART experienced and treatment naïve patients, without toxicities. We here report that DAPTA potently inhibits specific CD4-dependent binding of gp120 Bal (IC_{50} = 0.06 nM) and CM235 (IC_{50} = 0.32 nM) to CCR5. In co-immunoprecipitation studies, DAPTA (1 nM) blocks formation of the gp120/sCD4 complex with CCR5. Confocal microscopic studies of direct FITC–DAPTA binding to CCR5+, but not CCR5−, cells show that CCR5 is a DAPTA receptor. The capability of DAPTA to potently block gp120–CD4 binding to the major co-receptor CCR5 explains its molecular and therapeutic mechanism of action as a selective antiviral entry inhibitor for R5 tropic HIV-1 isolates.

Keywords: Envelope; gp120; Binding; CCR5; Chemokine; Receptor; HIV-1; Entry; Treatments

1. Introduction

High affinity binding of gp120 to CD4 receptor molecules induces conformational changes in both molecules (Myszka et al., 2000; Rizzuto and Sodroski, 2000; Lin et al., 2001), which results in enhanced binding of gp120 to one of several co-chemokine receptors (Trkola et al., 1996; Olson et al., 1999; Berger et al., 1999; Bieniasz et al., 1997; Littman, 1998).

The β-chemokine receptor CCR5 is the major co-receptor for macrophage-tropic (R5) strains, which are predominant during the asymptomatic stage of infection, and play a crucial role in the transmission of HIV-1 (Olson et al., 1999; Berger et al., 1999; Moore et al., 1997; Littman, 1998; Meyer et al., 1996; He et al., 1997). T-cell tropic viruses utilizing CXCR4 co-receptor (X4-tropic viral strains) usually emerge concomitant with the decline of CD4+ T-cells in the symptomatic stages of HIV-1 infection (Connor et al., 1997).

Molecules that specifically block HIV envelope protein (gp120) binding to CCR5 comprise a new class of receptor-based therapeutic agents for HIV-1 infection (Moore et al., 1997) and gp120-mediated pathogenesis (Brenneman et al., 1988b; Mulroney et al., 1998; Mankowski et al., 2002; Lipton et al., 1995). Several diverse inhibitors of CCR5 or CXCR4 co-receptors have been identified and include small molecules (Strizki et al., 1997; Baba et al., 1999), peptides (Kilby et al., 1998) chemokines and their modified analogs (Simmons et al., 1997; Aquaro et al., 2001) and antibodies (Wu et al., 1997a; Olson et al., 1999). Drugs which block entry can reduce viral burden in treatment experienced patients (Lalezari et al., 2003).

DAPTA, a non-toxic experimental antiviral entry inhibitor (Pert et al., 1986), which is derived from the V2 region, near

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the stem of HIV-1_{SF-2} env protein (amino acids 185–192) (Sanchez-Pescador et al., 1985) blocks infection of R5 and dual tropic (R5/X4) HIV-1 strains in monocyte-derived macrophages, microglia and primary CD4+ T-cells (Ruff et al., 2001) and is an antagonist of CCR5-mediated chemotaxis (Redwine et al., 1999). A pre-HAART era placebo-controlled trial showed that DAPTA caused cognitive improvements (Heseltine et al., 1998), and a later analysis of stored samples suggests a clinically significant reduction in plasma viral load (K. Goodkin, University of Miami, personal communication). A more recent uncontrolled clinical trial reports that DAPTA substantially suppressed virus in persistently infected cellular reservoirs (Pert et al., 1986) in both HAART experienced and naïve to treatment patients.

In order to determine possible clinical mechanisms we studied the receptor target of DAPTA action. We here describe CD4-dependent binding of two M-tropic gp120 molecules to CCR5 expressing cells that is potently inhibited by DAPTA. In an immunoprecipitation assay we show that DAPTA, at low concentrations, previously shown to block infection, prevents binding of solubilized gp120/sCD4 complex to both detergent solubilized and membrane bound CCR5 receptor. These data therefore indicate that the mechanism of DAPTA inhibition of R5-tropic HIV-1 infection is to prevent binding of the gp120/sCD4 complex to CCR5. The results implicate a functional role for the DAPTA epitope in gp120/sCD4 complex and CCR5 co-receptor interactions, and suggest clinical usefulness of DAPTA as an HIV entry inhibitor and gp120 antagonist by blocking envelope binding to the co-receptor CCR5.

2. Materials and methods

2.1. Compounds

D-Ala_1-peptide T-amide (D-A_1STTTNYT-NH_2) or "DAPTA" (MW 846) was synthesized under GMP conditions by Bachem (Torrence, CA), purified to >95% homogeneity and structure was verified by HPLC isolation, amino acid analysis and sequencing using ABI 470A gas-phase sequencer with on-line HPLC. 0.1 mM stock solutions in sterile water are stored at −20 °C.

Chemokine MIP-1β, recombinant soluble gp120 env protein from clade B HIV-1 Bal isolate, human HIVIg and an anti-CD4 polyclonal antibody T4–4 (R. Sweet SmithKline Beechman) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH (Bethesda, MD). A clade E HIV-1 CM235 was a gift from Christopher C. Broder (USUHS, Bethesda, MD). Recombinant soluble CD4 (sCD4) was from Protein Sciences Corporation (Meriden, CT). Purified monoclonal antibodies against CCR5, were purchased from: mAb 2D7 MAb, PharMinogen (San Diego, CA); 1801 and 1802, R&D Systems (Minneapolis, MN); anti-CCR5 MAb (NT), Peninsula Lab. Inc. (San Carlos, CA). The 1D4 MAb against the bovine rhodopsin C9 peptide (TETSQVAPA) tag was obtained from the Biovest International Inc./National Cell Culture Center (Minneapolis, MN). A goat polyclonal anti-CCR5 antibody CKR5 (C20), rabbit polyclonal anti-hCD4 antibody (H-370) and protein A/G agarose were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Cells

HOS cell lines expressing CD4 (HOS CD4) and CD4 and CCR5 (HOS CD4·CCR5) receptors (contributor Dr. Nathaniel Landau), GHOST CD4 and GHOST CD4·CCR5 lines (contributors Dr. V. Kewal Ramani and Dr. D. Littman) and the canine thymocyte cell line Cf2Th/synCCR5 (contributors Dr. T. Mirzabekov and Dr. J. Sodroski), and its parental Cf2Th (CD4−, CCR5−) cell line were used in binding assays. Cell lines were obtained from the NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH (Bethesda, MD). Cells were cultured in DMEM (Gibco) supplemented with 10% FBS in presence of selective antibiotics according to the published protocols.

2.3. Binding assay

The binding method was modified from Pert et al. (1986) and Doranz et al. (1999). We prepared a novel FITC-labeled tracer from soluble gp120 proteins (25 μg/ml) using a Fluorescent protein labeling kit (Roche Diagnostics GmBH, Indianapolis, IN), according to the manufacture's instructions. Uncoupled FLUOS was removed by Sephadex G-10 column filtration. The molar ratio between FLUOS-labeling molecules and protein was from 3.5 to 4.5 fluorescence molecules per molecule of gp120. The concentration of fluorescent-labeled proteins was measured by Bradford assay (Bio-Rad) and Western blotting by using calibrating amounts of soluble molecules with known concentration. Binding assays were performed in binding buffer described elsewhere (Doranz et al., 1999), in final volume 100 μl. Binding was carried out for 1 h at 37 °C in 96-well filter plates (Millipore). Unbound-labeled proteins were removed by rapid vacuum filtration and washing using a 96-well plates manifold. Each binding mix was washed five times with 0.2 ml (total volume of 1.0 ml/well) cold washing buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$). Filters were counted with a fluorescent plate reader (Hewlett Packard) at 495/530 nm.

2.4. Immunoprecipitation assay

The immunoprecipitation assay with solubilized CCR5 receptor molecules was performed as described by Moulard et al. (2002). Briefly, the cell monolayers (approximately 10^7 cells/well) of thymocyte cells Cf2Th (CD4−, CCR5−) and transfectants Cf2Th/synR5 were washed twice with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (1% Brij97, 5 mM iodoacetamide, added immediately before use in 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA,

and protease inhibitors) at 4 °C for 40 min. The nuclei were pelleted by centrifugation at 14,000 rpm for 25 min in a refrigerated centrifuge. The 1D4 antibody (Mirzabekov et al., 1999) at concentration 7.25 μg/ml and protein A/G agarose (20 μl), pre-washed with PBS, were added to the cell lysate and incubated at 4 °C for 14 h. The beads, washed three times with ice-cold lysis buffer were incubated with gp120/sCD4 complex formed in advance at room temperature (RT) using equal amounts of gp120 and sCD4 (5 μg/ml) in 0.5 ml lysis buffer, in presence or absence of DAPTA at concentration 10 or 1 nM per reaction mix for 4 h at 4 °C. After a final three-fold wash with cold PBS the beads were resuspended in 25 μl 4× sample buffer (SB) buffer. The bead-associated gp120/CD4/CCR5 complexes eluted by incubation overnight at 37 °C were run on a 10% SDS–PAGE gel (Invitrogen, Carlsbad, CA) and electrophoretically transferred to PVDF membranes. The membranes were blocked with Tris buffer saline containing 0.1% Tween-20 (TBST) and 5% non-fat powdered milk. The membranes were incubated with human HIVIg (50 μg/ml) or anti-CCR5 antibody (against EL2 or N-terminus) at dilution 1:1000.

A related procedure was used for immunoprecipitation of gp120 monomeric proteins derived from HIV-1 Bal or CM235 virus strains bound to GHOST CD4 or HOS CD4·CCR5 expressing cells according Lapham et al. (1996), with a modification. The cell monolayers were treated or not with DAPTA at concentrations of 10 nM and 1 nM for 1 h at 37 °C. Monomeric gp120 protein was added at a concentration 10 μg/ml and the incubation continued for 2 h at 37 °C. After washing with PBS, cells were lysed (as above) and immunoprecipitated with the anti-CD4 OKT4 (ascites fluid), 50 μl per 0.5 ml or MAb 1801 against CCR5 ECL2 (R&D Systems) at 15 μg per 0.5 ml overnight at 4 °C in lysis buffer. Protein A/G agarose was used for precipitation of the specific complexes. After washing five times with PBS, samples ($1–2 \times 10^7$ cells/line) were separated on the gel and transferred to PVDF membranes. The membranes were incubated with the human HIVIg or a rabbit polyclonal antibody against CCR5 (NT). CD4 receptor was visualized by incubation of the membranes with rabbit polyclonal antibody T4–4 or CD4 (H-370), with the same results.

The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed by using the super signal chemiluminescent substrate from Amersham Biosciences (ECL Western Blotting Detection Reagents).

2.5. Double-color immunofluorescence analysis by confocal laser scanning microscope

Cells from both thymocyte cell lines (CCR5-positive and the parental, CCR5-negative) were grown on glass cover slips and pre-incubated with normal dog IgG (Santa Cruz Biotechnology) for 1 h at 37 °C. FITC-labeled DAPTA (DAPTA-FITC), manufactured by United Biochemical Research Inc. (Seattle, WA), was purified to >95% HPLC and shown to have the expected molecular mass of 1226 by mass spectrum analyses. The labeled DAPTA was tested for biological activity in an infection assay in vitro and was found to inhibit infection with potency comparable with unlabeled DAPTA (data not shown). The glass cover slips with cells were incubated with DAPTA-FITC in concentration 10 nM in DMEM medium supplemented with 0.1% BSA (200 μl) for 1 h at 37 °C. After washing with PBS, 0.05% Tween-20 cells were incubated with 1D4 MAb 14.5 μg/ml in PBS and 0.2% BSA overnight at 4 °C in a humidified chamber. After washing the cells were incubated at 4 °C for 2 h with anti-mouse IgG-rhodamine (Sigma Chemical Co.–Aldrich Chemicals) at final dilution 1:200. The coverslips were washed and fixed in 4% paraformaldehyde for 10 min before being mounted in aqueous mounting medium with anti-fading agents (Biomedia Corp., Foster City, CA).

The images were taken on a Carl Zeiss LSM 510 Laser Scanning Confocal Microscope. Detection was done on two channels using a 488/543 nM Main Dichroic Beam Splitter. The first channel used the 488 nM Ar/Kr laser with a 500–550 nM band pass emission filter. The second channel used a 543 nM HeNe laser with a 565–615 nM band pass emission filter. All images were taken with a 40× Plan-NEOFLUAR oil immersion objective with a numerical aperture of 1.3 providing an optical slice of 1 μM. Images were acquired sequentially for each fluorochrome to avoid cross-talk between the two channels. Two sections spaced at 0.6 μm were taken in the horizontal plane and these were reintegrated into a single image using the maximum protection algorithm to give a representation of full membrane staining.

2.6. Statistical analysis

Binding experiments were carried out in triplicate and the data were graphed and analyzed (Scatchard, EC50, *P*-values) using the GraphPad Prism 4.0 software.

3. Results

3.1. Specific association of gp120/sCD4 complexes to cellular CCR5

Fluorescently labeled gp120 (monomeric) proteins from the R5 tropic HIV-1 viruses, Bal (clade B) and CM235 (clade E) were used in binding reactions conducted with CD4-negative canine thymocyte cells (Cf2Th), or those cells expressing high levels of CCR5 (Cf2Th/synR5). GHOST CD4 cells and GHOST CD4·CCR5, which express CD4 and chemokine receptors at level comparable to the levels expressed in PBMC's (Trkola et al., 1999) were also studied.

Fluorescently labeled gp120 (FITC–gp120) efficiently bound to the Cf2Th/synCCR5 cells only in presence of sCD4 (Fig. 1). Binding conditions were optimized by varying the concentration of added sCD4. Binding was nearly undetectable when sCD4 was not present in the assay. Maximal

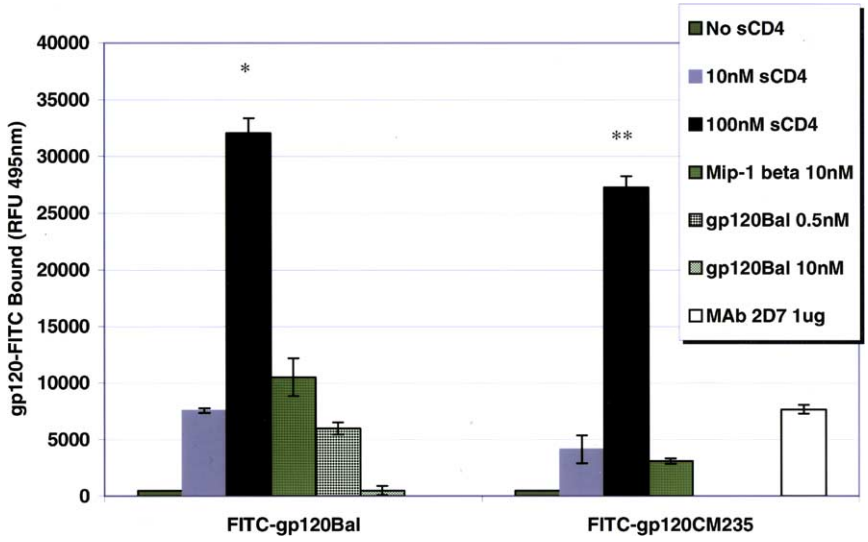

Fig. 1. CD4-dependent binding of gp120 envelope proteins to CCR5 chemokine receptors. Fluorescently labeled gp120 envelope proteins from the M-tropic HIV-1 viral isolates (HIV-$1_{Ba\text{-}L}$ and HIV-1_{CM235}) were tested for binding to cells expressing CCR5 (Cf2Th/synR5) receptors in presence or absence of sCD4 where indicated. R5-tropic envelope proteins from Bal ($^{*}P < .05$) and CM235 ($^{**}P < .03$) bind to CCR5 expressing cells only in the presence of sCD4 and there is no binding to CCR5-negative thymocyte cells Cf2Th (not shown). Specificity of binding inhibition by unlabeled gp120 protein, MIP-1β (10 nM) or MAb 2D7 (1 μg/ml) was performed using added sCD4 (100 nM). The mean total binding of the indicated gp120's, with the S.E.M. from triplicate determinations, is presented.

binding of FITC–gp120 was obtained with 100 nM sCD4, the dose used in subsequent experiments. Specificity of gp120 (Bal and CM235) binding was shown by competition with non-labeled HIV-1Bal, or MIP-1β, a CCR5 selective ligand (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996) (Fig. 1). Binding of labeled FITC–$gp120_{Bal}$ (0.5 nM) was inhibited by over 80% in the presence of an equimolar amount (0.5 nM), or reduced to background levels with 20-fold excess (10 nM) of non-labeled HIV-1Bal. Binding of both gp120 proteins Bal and CM235 were inhibited with the CCR5 specific ligand MIP-1β (10 nM), as previously described (Samson et al., 1996; Combadiere et al., 1996; Raport et al., 1996). Binding of FITC–gp120CM235 was also inhibited by incubation of the Cf2Th/synR5 cells with the 2D7 antibody to CCR5 (Fig. 1), which blocks gp120 binding (Wu et al., 1997b). The FITC–gp120 proteins did not bind appreciably to the parental, CCR5-negative, canine thymocyte Cf2Th cells in the presence of 100 nM sCD4.

These studies show CD4-dependent binding of gp120–FITC to CCR5 receptor and are in agreement with the results presented by others that infection of different cell types, as well as gp120 binding, to CCR5 receptors is strictly dependent on the presence of CD4 receptor or sCD4 (Doranz et al., 1999; Martin et al., 1997; Trkola et al., 1999; Wu et al., 1997b; Moore and Sodroski, 1996). Saturation binding conditions for each of two CCR5 binding gp120 proteins (Bal, CM235) were studied by adding increasing amounts (0.25–2.5 nM) of FITC-labeled gp120 to (Cf2Th/synR5) cell (Fig. 2). Non-specific binding was determined in the absence of sCD4 and was subtracted from binding, in presence of 100 nM sCD4. The same experiments were carried out with the parental cell line Cf2Th (R5−), with no specific binding observed. R5-tropic gp120 proteins showed differences in their affinity for the CCR5 receptor expressed at high density on the surface of the thymocyte cells as well as their B_{max}.

The maximal specific binding of labeled proteins was determined to be approximately 0.5 nM for gp120 Bal and 1 nM for gp120 CM235. Scatchard analysis (Fig. 2, insets) supports a single-site model for gp120/sCD4 binding to CCR5 over the range of concentrations studied. Saturable and high affinity binding of $gp120_{Bal}$ occurred with K_d of 0.46 ± 0.17 nM ($P < .05$), and with K_d of 0.77 ± 0.35 nM ($P < .05$) for $gp120_{CM235}$, results that are in agreement with others (Doms, 2000; Doranz et al., 1999; Mondor et al., 1998).

3.2. DAPTA inhibits gp120 envelope protein binding to CCR5

The lack of CD4 expression on the thymocyte line (Cf2Th/synR5) allowed binding of gp120 directly to CCR5. In order to define the potency of peptide inhibitor of gp120 binding to CCR5 receptors, inhibition studies were carried out using a fixed concentration of sCD4/gp120 complex, in the presence of increasing concentrations of the CCR5 selective chemokine ligand MIP-β, and DAPTA. Total specific binding was defined as the difference in binding of FITIC–gp120 with or without added sCD4 (100 nM). The binding of $gp120_{BaL}$/sCD4 to CCR5 (Cf2Th/synR5) cells was completely inhibited

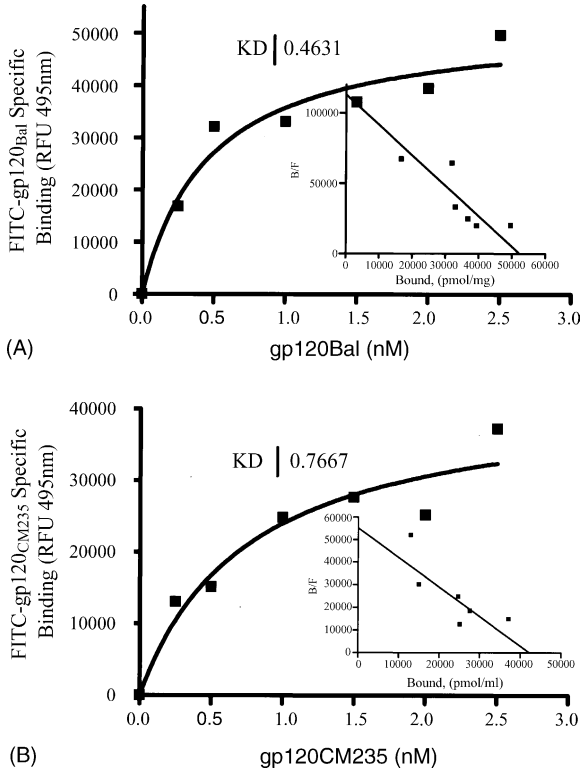

Fig. 2. Specific CD4-dependent binding of gp120–FITC-labeled Env proteins to CCR5 receptors. A competitive binding assay was performed by adding an increasing amount of FITC-labeled gp120 proteins (Bal or CM235) to Cf2Th/synR5 (CCR5+) and Cf2Th (CCR5−) intact cells. Total binding was determined in presence of 100 nM sCD4. Non-specific binding was determined in absence of sCD4 and was subtracted from the total binding to determine the specific binding. Results are expressed as the mean and S.E.M. from two independent experiments (each in triplicate). Scatchard analysis (inset) supports a single-site model for gp120/sCD4 binding to CCR5 over the range of concentrations studied.

by MIP-1β ($IC_{50} = 1.5 \pm 0.002$ nM, $P < .05$) and DAPTA ($IC_{50} = 55 \pm 0.08$ pM, $P < .05$) (Fig. 3A). The Hill slope for DAPTA was −1.07, consistent with a one-site competitive binding model. Similarly, we studied binding inhibition of $gp120CM_{CM235}$/sCD4 to the same cells and again showed substantial (>80%) inhibition of specific binding by MIP-1β ($IC_{50} = 1.8 \pm 0.006$ nM, $P < .05$) and DAPTA ($IC_{50} = 0.32 \pm 0.03$ nM, $P < .05$) (Fig. 3B). We also show that binding of $gp120_{CM235}$/sCD4 to a different CD4 expressing cell line (GHOST CD4·CCR5) was also inhibited by MIP-1β ($IC_{50} = 0.43 \pm 0.07$ nM, $P < .05$) and DAPTA ($IC_{50} = 51 \pm 0.09$ pM, $P < .05$) (Fig. 3C).

These results showed DAPTA to be a potent antagonist of M-tropic gp120 binding (Bal and CM235), on two CCR5 expressing lines, one of which (GHOST) also expresses CD4. Both DAPTA and MIP-1β were more potent inhibitors of $gp120_{Bal}$ compared to $gp120_{CM235}$. Differences in the potency and efficacy of MIP-1β inhibition of gp120 binding to CCR5 in an envelope variant and target cell dependent manner has been noted by others (Wu et al., 1997a; Van Riper et al., 1993; Wu et al., 1997b; Trkola et al., 1998).

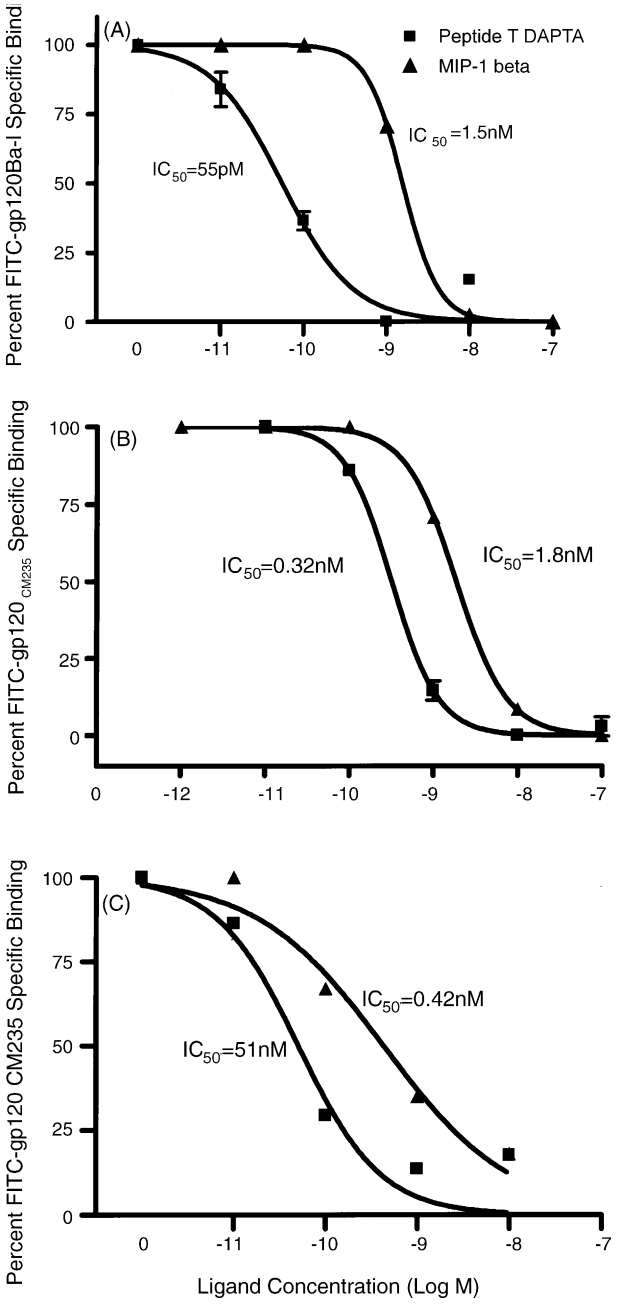

Fig. 3. DAPTA and MIP-1β potently inhibit binding of R5-tropic gp120/sCD4 complexes to CCR5 Receptors. A competitive binding assay of gp120/sCD4 complexes (Bal and CM) to CCR5 cells (Cf2Th/synR5 and GHOST CD4·CCR5) was performed in presence of DAPTA or MIP-1β. Specific binding to Cf2ThR5 cells of: (A) FITC–$gp120_{Bal}$/sCD4, or (B) FITC–$gp120_{CM235}$/sCD4. (C) Specific binding of FITC–$gp120_{CM235}$ to GHOST CD4·CCR5 cells. Data are the mean and S.E.M. from two independent experiments, each with triplicate determinations.

3.3. DAPTA prevents co-immunoprecipitation of gp120–sCD4 with CCR5

To confirm that DAPTA prevents binding of gp120/sCD4 complex specifically to CCR5, we used a soluble CCR5 binding assay, in which binding of gp120/sCD4 complex to

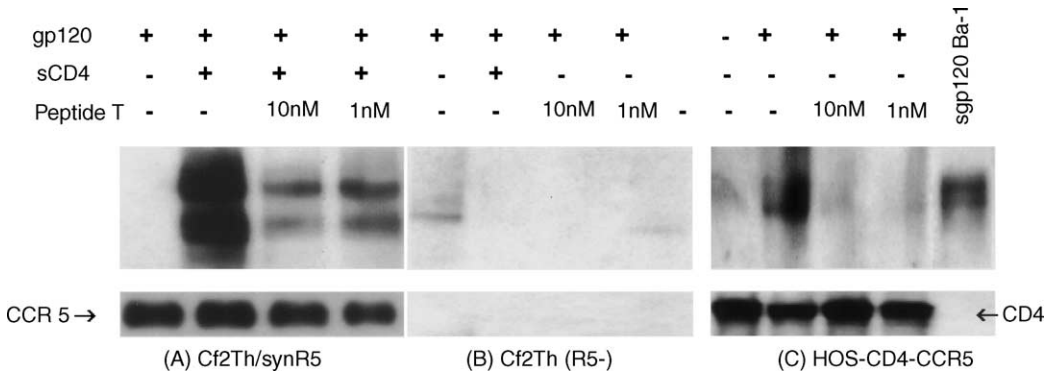

Fig. 4. DAPTA prevents co-immunoprecipitation of gp120/CD4 complex with CCR5 receptor. (A) Solubilized CCR5 receptors from Cf2Th/synR5 cells were captured onto protein A/G agarose and incubated with $gp120_{Bal}$/sCD4 complex in presence or absence of DAPTA (1–10 nM). Receptor (bead) bound gp120 was then detected by Western blotting with a human anti-HIVIg. The same membranes were stripped and hybridized with a rabbit polyclonal antiserum against CCR5 (A, bottom panel) to show equivalent CCR5 loading. (B) Cell lysates prepared from the Cf2Th (R5−) cell line failed to co-precipitate gp120. (C) Intact HOS CD4·CCR5 cells were treated with DAPTA and incubated with $gp120_{CM235}$ before solubilization, as above. Stripped blots (lower panel) were incubated with an anti-CD4 mAb to show equivalent loading. A gp120 standard is presented in the far right lane.

CCR5 was determined after immunoprecipitation by anti-CCR5 mAb and Western blot, using anti-gp120 antisera. DAPTA at 1 nM and 10 nM was added simultaneously with gp120/sCD4 complex to solubilized CCR5, which had been captured onto agarose beads using a specific mAb against a small C-terminal peptide tag.

The results showed that DAPTA greatly reduced binding of recombinant $gp120_{Bal}$ protein to solubiliized CCR5 receptor in presence of sCD4 (Fig. 4A). The same analyses were performed using membrane bound CD4 and CCR5 receptors on HOS CD4·CCR5 cells. In these experiments DAPTA also substantially inhibits binding of gp120 to membrane bound CD4/CCR5 complex (Fig. 4C).

To test whether DAPTA blocked the interaction between gp120 and CD4 receptors we used HOS CD4 (R5−) cells and carried out the same analyses. HOS CD4 cells ($1–2 \times 10^7$ per reaction) were pre-incubated with dilutions of DAPTA (range from 1 μM to 0.1 nM) and after 1 h at 37 °C, gp120 was added (10 μg/ml). The Western blots for gp120 showed that DAPTA does not prevent binding of gp120 to CD4 receptors (Fig. 5).

To further investigate the mechanism by which DAPTA blocks gp120/CD4 complex to CCR5 receptor, we studied direct binding of fluorescent (FITC-labeled) DAPTA. The cells from both thymocyte cell lines Cf2Th/synR5 and Cf2Th cells were incubated with a rhodamine-labeled antibody against CCR5 mAb (Fig. 6A) and FITC–DAPTA (Fig. 6B) and then visualized by confocal microscopy. Colocalization of the peptide with the CCR5 receptor was observed as demonstrated by the yellow (red–green colocalization) staining (Fig. 6C). No binding was detected on the CCR5-negative cells (Fig. 6D). These results indicate that CCR5 is a receptor for DAPTA.

4. Discussion

DAPTA was initially reported to inhibit infection of an uncharacterized early passage patient viral isolate when tested in primary PBMCs (Pert et al., 1986). Although the antiviral effect was not observed for lab adapted HIV isolates grown in T cell lines (Sodroski et al., 1987), these studies were conducted prior to the identification of the chemokine entry co-receptors CXCR4 (Feng et al., 1996) and CCR5 (Choe et al., 1996; Deng et al., 1996; Olson et al., 1999; Berger, 1997; Bieniasz et al., 1997; Moore et al., 1997; Littman, 1998). Our recent reports clarify this discrepancy and show that DAPTA

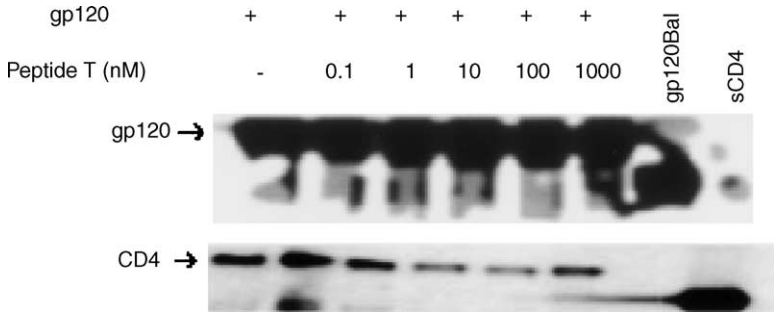

Fig. 5. DAPTA does not prevent binding of gp120 to CD4 receptor. HOS CD4 cells incubated with $gp120_{Bal}$ in presence or absence of increasing concentrations of DAPTA were lysed and immunoprecipitated with an anti-CD4 mAb (OKT4, ortho) and then analyzed by Western blot. Envelope (gp120) was visualized after hybridization with a human anti-HIVIg; CD4 receptors were visualized by hybridization with rabbit polyclonal antiserum against CD4 (H-370).

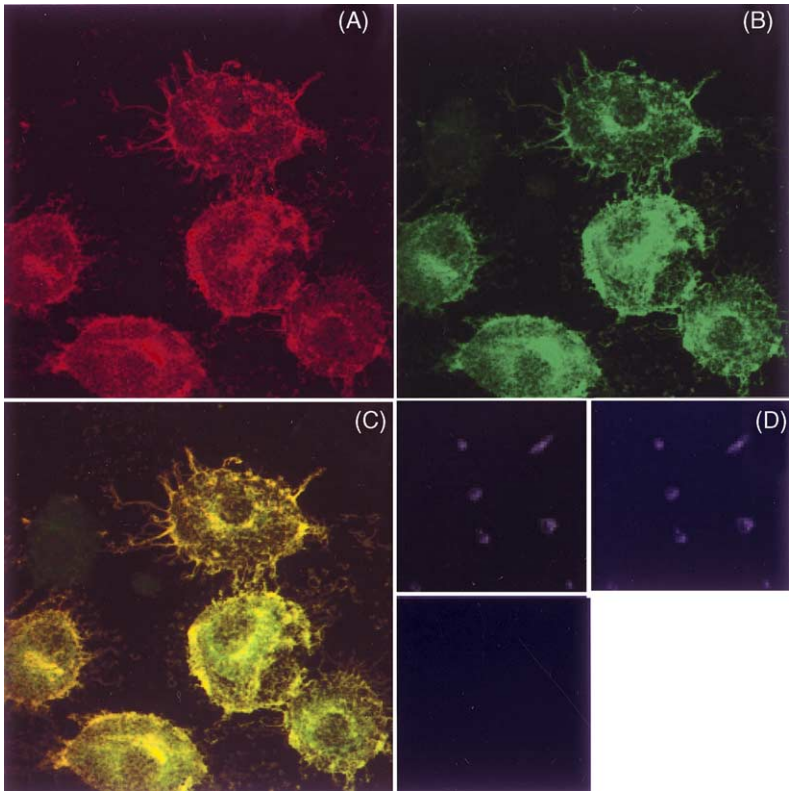

Fig. 6. Co-localization of fluorescently labeled DAPTA and CCR5 receptor. Canine thymocyte cells (CCR5-positive) or (CCR5-negative), incubated with DAPTA–FITC and stained for CCR5 receptor, were analyzed by double color CLSM. Each image represents the superposition of the two scanned sections of the cell membrane. In (A) staining for CCR5 receptor (red); (B) staining for DAPTA–FITC (green); (C) co-localization of FITC–DAPTA with CCR5 receptor on the cell surface is demonstrated by the yellow (red–green co-localization). (D) Cf2Th (R5−) cell stained for CCR5 (top left), FITC–DAPTA (top right), and co-localization (lower left) are shown in low magnification.

is an antagonist of CCR5-mediated chemotaxis (Redwine et al., 1999), and is most effective as an antiviral agent primarily against R5-tropic HIV-1 isolates, with reduced or absent effect for X4-tropic laboratory isolates (Ruff et al., 2001). DAPTA did not inhibit fusion in typical assays, which use high local concentrations of virus and cells, as previously discussed (Ruff et al., 1987; Ruff et al., 2001). Results reported here indicate that the mechanism of antiviral effect for R5 tropic HIV isolates is that DAPTA inhibits binding of viral envelope to CCR5, most likely via competitive binding of DAPTA to CCR5.

We prepared a novel FITC–gp120 tracer which incorporated three to four fluorescent tags and which still retained biological activity. We next verified the specificity of gp120 binding to CCR5 transfected cell lines (Cf2Th/synR5 and GHOST CD4·CCR5) and showed that envelope protein binding in this assay was dependent upon CD4, either endogenous, or exogenous (via added sCD4). Binding also showed the expected specificity in that R5 tropic $gp120_{Bal}$ binding did not occur to CCR5-negative or CXCR4 expressing cells (results not shown), while binding to CCR5 did occur and was inhibited in presence of unlabeled $gp120_{Bal}$ and MIP-1β. We thereby show that high affinity gp120 binding is CD4 dependent, saturable, and specific to CCR5 expressing cells.

The gp120 binding curves differed depending upon envelope protein and the CCR5 receptor density. $GP120_{CM235}$ showed saturable binding over the range 1.0–5 nM, at which point higher amounts of added gp120 revealed a non-saturating lower affinity component, also shown by others (Doranz et al., 1999; Mondor et al., 1998), which we did not study further. The R5-tropic CM235 was less potent in evincing sCD4-dependent R5 binding with a K_d of 0.77 nM in comparison with $gp120_{Bal}$, with K_d of 0.46 nM (Fig. 2).

Having validated the specificity and selectivity of the binding methodology to detect gp120–CCR5 specific binding, we next tested the ability of MIP-1β, a known ligand of CCR5 receptor to inhibit that gp120 binding. Those results confirmed the activity of select β-chemokines to function as inhibitors of CCR5 binding (Cocchi et al., 1995; Wu et al., 1996) and showed that MIP-1β was a potent, albeit partial, inhibitor of $gp120_{CM235}$ binding to GHOST CD4·CCR5 cells. The result is in agreement with earlier studies showing that chemokines do not fully recapitulate the binding interaction of HIV envelope proteins, despite sharing the same receptor (Wu et al., 1997a).

DAPTA also was shown to potently and substantially inhibit binding of two R5-tropic gp120 isolates (Bal, CM235) to CCR5 cells. The binding inhibition (IC_{50}) of DAPTA for $gp120_{CM235}$ was some six-fold weaker (0.32 nM ver-

sus 51 pM) on the Cf2Th/R5 cells compared to GHOST CD4·CCR5 cells. This result is likely related to the greater expression of CCR5 receptors, which typify the Cf2Th/synR5 line compared to GHOST CD4·CCR5 cells (Fig. 3) and is predicted by basic stoichiometric considerations. Furthermore, as the Cf2Th/synR5 cells do not express CD4, there can be no component of gp120 cellular binding which occurs through this moiety. All of the specific gp120 binding occurs via the CCR5 co-receptor. As DAPTA is potent in blocking this reaction we conclude that DAPTA targets the CCR5, not the CD4 interactions of gp120 with cellular targets. Results conducted with HOS CD4 expressing cells (CCR5−) also show that DAPTA does not prevent direct gp120 binding to CD4 receptor (Fig. 5).

An immunoprecipitation assay using solubilized (Cf2Th/synR5 cells) or membrane (native) bound (HOS CD4·CCR5 cells) CCR5 receptors confirmed the results of the rapid filtration binding assay and provides molecular evidence showing DAPTA antagonism of gp120 binding to CCR5. Thus, the CD4-dependent binding of gp120 to CCR5 receptors, as shown by immunoprecipitation, was potently and substantially inhibited in the presence of DAPTA (Fig. 4). These findings show that the block in infection by DAPTA occurs at the early step of viral entry, by inhibiting env binding to CCR5.

DAPTA most likely disrupts the association of gp120/CD4 complex with chemokine receptor CCR5. This could occur by direct binding of DAPTA to CCR5 or an indirect receptor interaction, which shifts the affinity of CCR5 for gp120/CD4 complex. The conclusion that DAPTA prevents R5-tropic HIV infection by direct binding to CCR5 receptors is supported by microscopic visualization of fluorescently labeled DAPTA, which co-localizes with the receptor expressed on the surface of canine thymocyte CCR5-positive cells only (Fig. 6). The result is a confirmation of fluorocytometric analyses that studied direct binding of FITC–DAPTA to the identical cells (Ruff et al., 2003).

While the precise gp120 epitopes involved in CCR5 binding remain to be fully described, peptide T (DAPTA), which has homology to a V2 epitope present in diverse gp120 proteins, is a potent partial antagonist of CCR5 signaling (Redwine et al., 1999; Liapi et al., 1998) which blocks gp120 pathogenic effects (Brenneman et al., 1988a; Zorn et al., 1990; Mulroney et al., 1998; Liapi et al., 1998; Redwine et al., 1999), and is a CCR5 selective HIV entry inhibitor (Pert et al., 1986; Ruff et al., 2001; Ruff et al., 2003). The selective antiviral action of DAPTA for HIV-1 R5-tropic viruses is caused by the disruption of gp120 binding to chemokine receptor CCR5. Resistance development to this therapy was not evident in vitro (Ruff et al., 2003), or in vivo (Polianova et al., 2003). The results additionally show an important role of the DAPTA epitope in co-receptor binding of R5-tropic HIV-1 envelope proteins. Discrete short peptide binding site epitopes (McCarthy et al., 1990) have relevance to the development of novel viral envelope derived peptide entry inhibitors, as well as production of vaccine or therapeutic antibodies which block virus binding to receptors.

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