Supplemental Data

Small-Molecule CD4 Mimics Interact with a Highly Conserved Pocket on HIV-1 gp120

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids Expressing HIV-1 Envelope Glycoproteins

The wild-type and mutant HIV-1 envelope glycoproteins were expressed from the pSVIIIenv vector. The glycoprotein mutants were created by site-directed mutagenesis, as previously described (Kunkel et al., 1987; Olshevsky et al., 1990; Xiang et al., 2002). The residue numbering is based upon that of the prototypic HIV-1_{HXBc2} envelope glycoproteins, according to current convention (Korber, 1998). The *env* genes of the mutated plasmids were sequenced to verify the presence of the desired mutation and the absence of unwanted changes. The mutants are designated by the following nomenclature: wild-type amino acid in single-letter code, residue number, and amino acid to which the residue has been changed.

Recombinant Luciferase Viruses

Using the Effectene transfection reagent (Qiagen, Valencia, CA), 293T human embryonic kidney cells were cotransfected with plasmids expressing the pCMVΔP1ΔenvpA HIV-1 Gag-Pol packaging construct, the wild-type or mutant HIV-1_{YU2} envelope glycoproteins or the envelope glycoproteins of the control amphotropic murine leukemia virus (A-MLV), and the firefly luciferase-expressing vector at a DNA ratio of 1:1:3 μg. For the production of viruses pseudotyped with the A-MLV glycoprotein, a *rev* expressing plasmid was added. The single-round, replication-defective viruses in the supernatants were harvested 24-30 hours after transfection, filtered (0.45 μm), aliquoted, and frozen at -80°C until further use. The reverse transcriptase (RT) activities of all viruses were measured as described previously (Rho et al., 1981).

Radiolabeling of the gp120 Glycoprotein

Approximately 3.5 x 10^6 293T cells were seeded in a T75 tissue culture flask one day before transfection. Cells were cotransfected with 9 μg of pSVIIIEnv expressing the full-length HIV-1_{YU2} envelope glycoproteins and 1 μg of pLTR-Tat using the Polyfect transfection reagent (Qiagen). One day after transfection, the cells were labeled for 48 hours with [^{35}S]-Express protein labeling mix (30 μCi/mL) (Perkin-Elmer, Waltham, MA). The supernatant was harvested 48 hours later, cleared by centrifugation at 2,000 rpm for 5 minutes, and stored at 4°C. The amount of labeled gp120 was quantitated by immunoprecipitation with a

mixture of sera from HIV-1-infected individuals and protein A-Sepharose beads (Amersham Bio-Sciences, Piscataway, NJ), followed by SDS-PAGE and autoradiography.

gp120-CCR5 Binding Assay

Cf2Th cells expressing high levels of CCR5 were lifted using 5 mM EDTA, pH 7.5. The cells were washed with serum-free DMEM, added to microcentrifuge tubes (2-3 x 10^6 cells/tube), and incubated with 500 μL of radiolabeled HIV-1_{YU2} gp120 in the absence or presence of varying concentrations of sCD4 or NBD-556 at 37°C for 1.5 hours with gentle agitation. The supernatants were removed following incubation and the cells were washed twice with cold DMEM before lysis in 0.5 mL of IP buffer containing 0.5 M NaCl, 10 mM Tris, pH 7.5, 0.5% [vol/vol] NP40 and a cocktail of protease inhibitors. The cells were incubated in IP buffer for 30 minutes at 4°C with gentle agitation. The lysates were cleared by centrifugation at 14,000 x g for 30 minutes at 4°C. Bound gp120 was precipitated by a mixture of sera from HIV-1-infected individuals and protein A-Sepharose beads and visualized by autoradiography of a 3-8% SDS-polyacrylamide gel.

Production and Purification of the HIV-1 gp120 Glycoprotein

The wild-type gp120 and gp120 variants with C-terminal $(His)_6$ epitope tags were expressed from plasmids containing a codon-optimized (GeneScript Corp., Piscataway, NJ) *env* gene. Plasmids expressing codon-optimized gp120 glycoproteins were transfected into 293F cells using 293fectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Seven days later the supernatant expressing the envelope glycoproteins was harvested and filtered using 0.45 μm filters. The supernatant was concentrated 2-3-fold using Centricon plus-80 (Amicon, Billerica, MA) filters. Ni-NTA beads (Qiagen) were added to the concentrated supernatant and incubated overnight at 4°C with gentle shaking. The supernatant/bead mixture was poured into a small column, washed with 20 mM imidazole in buffer A (150 mM NaCl, 20 mM Tris-HCl, pH 7.4), and eluted by gravity flow with 200 mM imidazole in buffer A. The eluant was concentrated with Centriprep-30 (Amicon) and dialyzed with a 10K-cutoff dialysis cassette (Pierce, Rockford, IL) in 20 mM Tris-HCl, pH 7.4 and 150 mM NaCl.

Production and Purification of the HIV-1 gp120 Core Protein

The gp120 core protein from HIV-1_{YU2} (Kwong et al., 2000) was expressed in Drosophila Schnieder 2 cells under the control of an inducible metallothionein promoter. The secreted gp120 core protein was purified by affinity chromatography with the F105 antibody covalently coupled to Sepharose. Following extensive washing with phosphate-buffered saline containing 0.5 M NaCl, the gp120 core protein was eluted with 0.1 M glycine, pH 2.8, followed by immediate neutralization with Tris buffer. After spin concentration with Centriprep 30 filters (Amicon), the gp120 core protein was dialyzed into phosphate-buffered saline and stored at -20ºC in aliquots.

Compound Binding Assay

BioSpin columns (Bio-Rad) capable of separating low-molecular-weight compounds from large macromolecules were used to measure the binding of [^{3}H]-NBD-556 or [^{3}H]-BMS-806 (ViTrax Radiochemicals, Placentia, CA) to the

HIV-1 gp120 envelope glycoprotein. Mixtures (70-90 μL) containing purified HIV-1 gp120 in phosphate-buffered saline (PBS) and [^{3}H]-NBD-556 or [^{3}H]-BMS-806 were incubated for 15-30 minutes at 37°C, applied to a BioSpin column, and centrifuged for 2 minutes at 1200 rpm. The eluate was collected and counted in an LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter).

Optical Biosensor Binding Assay

Optical biosensor (Biacore, Inc., Piscataway, NJ) analysis of the binding of NBD-556 to the HIV-1_{YU2} gp120 core bound to the 17b antibody was carried out as described previously (Zhang et al., 1999). Standard amine coupling was used to immobilize the 17b antibody to CM5 sensor chips at a surface density of approximately 500 response units. The gp120 core protein (200 nM) in the presence of variable concentrations of NBD-556 was passed over the modified sensor chip at 30 μL/min for 5 minutes, followed by a 5-min dissociation phase. Sensorgrams were analyzed using global fitting with BIAevaluation 4.1 software.

Isothermal Titration Calorimetry

Isothermal titration calorimetric experiments were performed using a high-precision VP-ITC titration calorimetric system from MicroCal Inc. (Northampton, MA). The calorimetric cell (~1.4 mL), containing wild-type or mutant gp120 dissolved in PBS (Roche Diagnostics GmbH), pH 7.4 with 2% DMSO, was titrated with the different inhibitors dissolved in the same buffer. The concentration of gp120 was ~3 μM and the concentration of inhibitor in the injection syringe was between 100 and 150 μM. Binding of Mab 17b to core gp120 was studied by stepwise injections of 20 μM 17b to the calorimetric cell containing 3 μM core gp120 by itself or equilibrated with 600 μM NBD-556. Binding of NBD-556 to core gp120 in complex with 17b was studied by titration of 3 μM core gp120 with 20 μM 17b and 300 μM NBD-556, in sequence. The heat evolved upon injection of the inhibitors was obtained from the integral of the calorimetric signal. The heat associated with the binding reaction was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted against the molar ratio, and the values for the enthalpy change (ΔH) and association constant ($K_a = 1/K_d$) were obtained by nonlinear regression of the data. All experiments were carried out at 25 °C.

Modeling the Binding of NBD-556 and Its Analogues to HIV-1 gp120

Small molecule preparation. Molecules were constructed in MOE (MOE Molecular Operating Environment Chemical Computing Group, version 2005.06 (Montreal, Canada) (http//www.chemcomp.com), ionized using MOE's WashMDB function, and hydrogens were added (MOE, 2005). The small molecule conformation was minimized to a gradient of 0.01 in the MMFF94x (Halgren, 1999a; Halgren, 1999b) force field using a distance-dependent dielectric constant of 1 (Halgren, 1999a; Halgren, 1999b).

Protein preparation. Using the x-ray crystal structure of the CD4-bound HIV-1 gp120 core (PDB code 1G9M), hydrogen atoms were added and tautomeric states and orientations of Asn, Gln and His residues were determined with Molprobity (Lovell, 2003; Word, 1999) (http://molprobity.biochem.duke.edu/). Hydrogens were added to crystallographic waters using MOE (MOE, 2005). The OPSLAA (Jorgensen, 1996) force field in MOE was used and all hydrogens were

minimized to an rms gradient of 0.01, holding the remaining heavy atoms fixed. A stepwise minimization followed for all atoms, using a quadratic force constant (100) to tether the atoms to their starting geometries; for each subsequent minimization, the force constant was reduced by a half until 0.25. This was followed by a final cycle of unrestrained minimization.

Docking calculations. Glide(4.018) Water, isopropanol, fucose and n-acetyl D-glucosamine molecules were removed from the coordinates of the minimized protein (1G9M) as described above. The protein was then passed through the protein preparation utility in Glide (Friesner, 2004; Halgren and L. L.; Pollard, 2004) using the OPSLAA (Jorgensen, 1996) force field and using a water solvation model with extended cutoffs. All heavy atoms were constrained with a parabolic potential of 100 kJ/ Å. One-hundred iterations of Polak-Ribiere conjugate gradient (PRCG) minimization were applied. The binding site was defined based on the positions of CD4 Phe 43 and the isopropanol molecule from the 1G9M crystal structure. The Glide grids were computed with a box center at 28.10, -12.35, 81.57 and an inner and outer box range of 14 Å and 36 Å, respectively. Docking calculations were performed in standard sampling mode with maxkeep 5,000 and maxref 1,000.

Gold (version 3.2). The binding site was defined by using the docked conformation of NBD-556 produced with Glide. Docking calculations were performed with crystallographic water molecules in the cavity. Analysis of initial docking calculations with all six water molecules (HOH 6, HOH 77, HOH 134, HOH 313, HOH 327 and HOH 343) toggled on and off during docking and the estimated free energy of binding in Gold indicated that three crystallographic waters behaved as integral parts of the protein (HOH 6**,** HOH 327 and HOH 343) and three were likely to be displaced (HOH 77, HOH 134 and HOH 313). Water HOH 6 forms a bridging hydrogen bond between the backbone carbonyls of Gly 473 and Trp 427, HOH 343 forms a bridging hydrogen bond between Val 425 and Asn 377, and HOH 327 hydrogen bonds with Ser 375 in the base of the cavity. Subsequent docking calculations were performed with HOH 343 left on while waters HOH 6 and HOH 327 were turned on with hydrogen atoms spun. One hundred genetic algorithm (GA) docking runs were performed with the following parameters**:**
initial_virtual_pt_match_max=3.5, diverse_solutions=1, divsol_cluster_size=1, and divsol_rmsd=1.5. All other parameters were set as defaults.

Affinity. Independently of the above modeling, NBD-556 was modeled and docked to the CD4-bound structure of the HIV-1 gp120 core (PDB code 1G9M) using Insight II/ Discover Software (Accelrys Software, Inc., San Diego, CA). The NBD-556 structure model was constructed with Molecular Builder and Sketcher and the gp120 core structure was cleaned with the Biopolymer program. Simulated Annealing docking (SA-docking) was performed with the Affinity program (Halgren, 1999a). The binding site for NBD-556 docking was limited to a 15-Å radius from the center of the Phe 43 cavity, and the final energy minimization was performed in 1,000 steps. Discover/CVFF force field was applied for the docking calculations and the docked poses were analyzed in the Analysis module/Insight II.

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