Selection of Active ScFv to G-Protein-Coupled Receptor CCR5 Using Surface Antigen-Mimicking Peptides†

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ABSTRACT: This study describes the use of cyclic peptides for use in the selection of single-chain (ScFv) antibodies specific for the HIV-1 coreceptor CCR5, a representative G-protein-coupled receptor (GPCR). A tandem ligation strategy was developed for preparing biotinylated cyclic peptides, first through an orthogonal end-to-end ligation and then a chemoselective ligation with functionalized biotin. Cyclic peptides mimicking the extracellular loops of CCR5 and their unconstrained counterparts were then used for solution-phase selection of ScFv antibodies from a phage display antibody library. Antibodies reactive with CCR5 on cells were detected using a homogeneous high throughput assay. Of 19 isolated ScFv antibodies that bound to CCR5+ cells, three inhibited CCR5-mediated but not CXCR4-mediated HIV infection. Only ScFvs selected by binding to cyclic constrained peptides exhibited inhibitory activity. Our results demonstrate that surface-antigen mimetics of a GPCR are effective tools for selecting active, site-specific ScFv antibodies that hold promise as immunological reagents and therapeutics.

As a family, G-protein-coupled receptors (GPCRs)[1] mediate the majority of cellular responses induced by hormones and neurotransmitters. Targeting GPCRs has been a successful therapeutic intervention strategy in developing agonists and antagonists of GPCRs with known ligands. However, over 75% of GPCRs now identified are orphan receptors with unknown ligands and functions. To facilitate structure−function analysis and drug discovery, we have focused on streamlining the selection and isolation of biologically active single-chain (ScFv) antibodies directed to GPCRs using GPCR-mimicking conformation-constrained peptides.

The chemokine receptor CCR5, a class-A GPCR, was selected as a representative GPCR. CCR5 is the major human immunodeficiency virus type 1 (HIV-1) coreceptor on macrophages (*1*, *2*) with CCR5-tropic isolates accounting for most sexually transmitted infections (*3*). Thus, CCR5 is an attractive therapeutic target. The importance of CCR5 in HIV-1 transmission is prominently evidenced by the observation that individuals homozygous for a defective CCR5 allele remain uninfected despite repeated exposure to HIV-1 (*4*−*8*). Blocking CCR5 activity inhibits progression of simian immunodeficiency virus (SIV) infection in macaques (*9*, *10*). Similarly, individuals possessing autoantibodies specific for CCR5 are resistant to HIV infection (*11*−*14*).

Since validation of CCR5 as a therapeutic target, various modalities have been developed for discovering drug candidates aimed at blocking HIV-1 entry to hosts (*12*, *15*−*21*). They include small molecules, peptides, proteins, and antibodies. Indeed, several groups have reported producing monoclonal and recombinant antibodies that bind to CCR5 to prevent HIV infection. Direct antibody production has generally relied on the use of CCR5+ cells or recombinant CCR5 as antigens (*11*, *12*, *22*) along with painstaking monoclonal antibody isolation procedures. Thus far, even with steady progress in developing anti-CCR5 antibodies, there are still few highly active monoclonal antibodies available against the extracellular domains of CCR5, particularly against the ectodomain loops ECL1 and ECL3 (Figure 1). Such site-specific antibodies could immensely aid research in elucidating the fundamental roles of the receptor's ectodomains.

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[1] Abbreviations: AG, ampicillin and glucose; AI, ampicillin and isopropyl-beta-D-thiogalactopyranoside(IPTG); B-peptides, biotinylated-peptides; CCR5, CC-chemokine receptor 5; CXCR4, CXC chemokine receptor; ECL, ectodomain loop; GPCR, G-protein coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV-1, human immunodeficiency virus Type 1; HoBt/HBTU, 1-hydroxybenzotriazole/*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; L-peptide, linker-peptide; Nin-Cys, ninhydrin-protected Cys; PEO-maleimide, 3-maleimidopropionamidyl-3,6-dioxaoctanediamine; PIPES, piperazine-1,4-bis(2-ethanesulphonic acid); ScFv, single chain recombinant antibody; TM, transmembrane.

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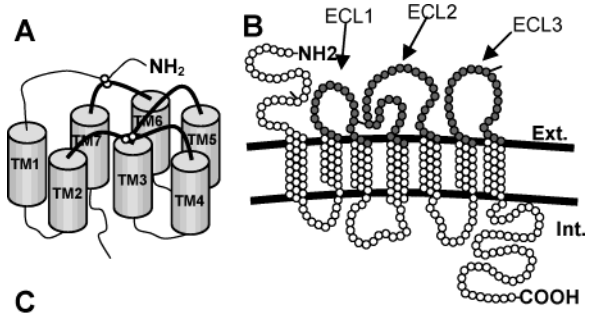

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Domain	Amino acid sequence
ECL1	^{86}*WAHY*AAAQWDFGNTMCQ*LLP*^{105}
ECL2	^{165}*IFT*RSQKEGLHYTCSSHFPYSQYQFWKNFQTLK*IVI*^{200}
ECL3	^{248}*LLL*NTFQEFFGLNNCSSSNRLDQ*AMQVT*^{275}

FIGURE 1: Schematic representations of GPCR CCR5. (A) Extracellular loops (bold) and four Cys residues (○) are highlighted with TM helical segments represented by cylinders. (B) Structure of CCR5 with residues from ECL1, ECL2, and ECL3 selected for peptide synthesis shown in gray. (C) Amino acid sequences of CCR5 extracellular domains. Italicized residues are from TM segments that support the extracellular loops.

A promising approach is to combine peptide synthesis with a phage display library for high throughput ScFv antibody selection. An obstacle to this approach is the design and synthesis of suitable conformational peptide antigens. Previously, our laboratory has reported various strategies for designing stable conformational peptide and protein antigens to induce high-quality polyclonal antibodies (*23*−*25*). Here, we report the design and synthesis, through tandem ligation, of end-to-end cyclic peptide surface-antigen mimetics of CCR5 ectodomain loops for the selection of ScFv antibodies. Our results demonstrate that cyclic peptides, representing domains of cell surface molecules, can be used to select for ScFv antibodies that exhibit biological activity (e.g., inhibit HIV entry into cells (*19*, *26*)).

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Cyclization. All peptides were synthesized using either standard *N*-fluorenylmethoxycarbonyl (Fmoc) or *N*-*t*-butoxycarbonyl (Boc) chemistry. All resins and reagents were from Novabiochem (San Diego, CA). Nin-Cys was synthesized according to Prota and Ponsiglione (*27*) and was coupled to peptide resins using standard HOBt/HBTU activation with coupling time limited to 20 min. Following cleavage from resin and purification, Nin-protected peptides were dissolved in 60% acetonitrile and then added to HEPES buffer (200 mM, pH 7.7) yielding final peptide concentrations of 50−60 μM. 3-Mercaptopropiosulfonic acid (MPS) and tris-carboxymethylphosphine (both from Sigma, St. Louis, MO) were added to the peptide solution yielding final concentrations of 50 mM and 5 mM, respectively. The solution was shaken at room temperature, and time points were assayed by RP-HPLC.

For preparation of biotinylated peptides (B-peptides), peptides with deprotected thiols were dissolved in guanidine hydrochloride (GuHCl, 6 M) to a concentration of 2 mM and added dropwise to a 3 mM solution of PEO-maleimide activated biotin (Pierce, Rockford, IL) in 3 M GuHCl/200 mM PIPES (pH 6.7) to a final peptide concentration of 0.2 mM. The reactions were complete within 1 h.

All peptides were purified by high performance liquid chromatography (HPLC) on a Vydac (Hesperia, CA) C_{18} reversed-phase preparatory column then characterized by analytical HPLC and matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS).

Cell Lines, Virus, Antibodies. Sup-T1, P4R5, and HeLa cells were obtained from Drs. Christopher Aiken and Derya Unumatz at Vanderbilt University. Sup-T1 cells are a nonadherent human leukemia T cell line. P4R5 cells are HeLa cells with a stably integrated LTR-*LacZ* reporter gene cassette expressing CD4, CXCR4, and CCR5. HIV-1 BaL and NL-43 were provided by Dr. Christopher Aiken. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Cf2Th/syn CCR5 cells from Drs. Tajib Mirzabekov and Joseph Sodroski (*28*) and PM1 cells from Dr. Marvin Reitz (*29*). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Monoclonal antibody 2D7 (*20*) was purchased from Pharmingen (San Diego, CA).

Solution-Phase Selection Method. Spleens from naïve newborn and 3−4 week old outbred mice and rats were used to construct a phage-displayed ScFv recombinant antibody library (~2.9×10^9 members) according to modifications of a previously published protocol (*30*). Antibodies comprising the library are encoded within the Amersham pCANTAB5E phagemid vector. The vector contains an ampicillin-resistance gene to select for bacterial clones that contain the ScFv-encoding phagemid. All ScFv are expressed either as a phage gene 3 fusion protein (for phage display purposes) or as an epitope-tagged ScFv. Rodent ScFv display an epitope (E-tag) recognized by the Anti-E monoclonal and HRP-conjugated Anti-E monoclonal antibody (Amersham Biosciences). Anti-E tag monoclonal antibodies were also conjugated either to FITC at a ratio of 50 μg of FITC to 1 mg of antibody or to FMAT Blue Dye using an FMAT Blue Dye Conjugation Kit (Applied Biosystems cat# 4328853).

The phage antibody library (~10^{13} phage particles) was mixed with approximately 100 pmol of each of linker peptide (L-peptide) and B-peptide in the presence of PBS containing 0.05−0.1% Tween 20 and incubated at room temperature for 2−3 h. The mixture was combined with 100 μL of streptavidin magnetic beads (Pierce) that had been previously blocked for 1 h in PBS containing 0.1% Tween 20 (PBS-T). After a 5 min incubation, a magnet was used to separate phage antibodies bound to B-peptides on streptavidin magnetic beads. The beads were washed 7 times with PBS-T and 1−3 times with PBS. The PBS was removed, and 50−100 μL of 100 mM treithylamine was added to elute bound phage antibodies. After a 10 min incubation, a magnet was used to separate magnetic beads from the supernatant that contained the eluted phage antibodies. The supernatant containing the eluted phage antibodies was removed, neutralized with one-half volume of 1 M Tris-HCl (pH 7.4−8.0), and used to infect *Escherichia coli* TG1 cells. Infected cells were incubated for 1 h at 37 °C, plated onto 2xYT agar plates containing 100 μg/mL ampicillin and 2% glucose (2xYTAG), and incubated overnight at 30 °C. After incubation, cells were scraped, grown in 10 mL of 2xYTAG broth at 37 °C, infected with M13KO7 helper phage, and grown overnight at 30 °C in 10 mL of 2xYT medium containing 100 μg of ampicillin

and 50 μg of kanamycin per mL of culture medium to produce phage-displayed antibodies for a second round of selection.

For the second round of phage antibody selection, phage antibodies were cross-absorbed with either free biotin or the L-peptide and streptavidin magnetic beads, to remove, respectively, antibodies reactive with biotin/streptavidin or the WKGCGKI amino acid sequence used to form the cyclic CCR5 peptides.

After cross-absorption, ~1 mL of phage antibodies in culture supernatant was mixed with 100 pmol of B-peptide and Tween 20 (final concentration of 0.05−0.1%) and incubated for 2−3 h at room temperature. Phage antibodies bound to B-peptides were harvested using streptavidin magnetic beads, eluted, used to infect *E. coli* TG1 cells, and plated as per the first round selection. In the two-step selection procedure, phage antibodies were cross-absorbed with streptavidin magnetic beads prior to selections, free biotin, or the L-peptide to remove, respectively, antibodies reactive with biotin/streptavidin or the WKGCGKI amino acid sequence used to form the cyclic CCR5 peptides.

Assay for ScFv Reactive with CCR5 on Cell Surface. E-Tagged ScFv reactive with cellular CCR5 were detected using an Applied Biosystems FMAT 8100 plate reader and the Anti-E monoclonal antibody that had been conjugated to FMAT Blue Dye using an Applied Biosystems FMAT Blue Dye conjugation kit (cat# 431053C). A Genetix Qpix Colony-picker was used to pick bacterial colonies, stemming from second round selections, from 2xYT AG agar plates to 384 well microtiter plates that contained 100 μL/well 2xYT supplemented with ampicillin (100 μg/mL) and 1 mM IPTG (2xYT AI). Microtiter plates were incubated overnight at 30 °C and then centrifuged (~1000 g, 10 min) to pellet cells. Supernatants were removed, and cell pellets were resuspended in 40 μL of TES [0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose] and 60 μL of TES diluted 1:5 with distilled water to yield a 100 μL of an *E. coli* cell suspension. Resuspended cells were placed on ice for 1 h to prepare soluble E-tagged ScFv in periplasmic extract. HeLa and P4R5 cells (2×10^5 cells/mL of PBS) were plated into Applied Biosystems FMAT 384 well microtiter plates at 20 μL/well after which 25 μL of FMAT Blue Dye conjugated Anti-E monoclonal antibody (250 ng/mL PBS-T) and 25 μL of ScFv in periplasmic extract were added. Plates were incubated for 2 h at room temperature in the dark and then read using the FMAT 8100 plate reader. Bacterial clones producing ScFv reactive with CCR5+ P4R5 cells but not with CCR5− HeLa cells were identified and used to produce ScFv on a large scale for further analysis.

ScFv Expression and Purification. Individual bacterial colonies producing ScFv reactive with CCR5+ P4R5 cells but not with CCR5− HeLa cells were used to inoculate 500−1000 mL flasks containing 250 mL of 2xYT AG broth. Cells were grown overnight at 30 °C with shaking at 100−125 rpm and then centrifuged. Pellets were resuspended in 250 mL of 2xYT AI and then grown overnight as stated previously. Cells were pelleted, resuspended in 4 mL of 1xTES and 6 mL of 1/5 x TES, and placed on ice to produce soluble E-tagged ScFv in periplasmic extract. E-Tagged ScFv were affinity-purified using an Anti-E tag monoclonal antibody column and RPAS Purification Module (Amersham Biosciences) according to the manufacturer's instructions.

ELISA. For peptide ELISAs, 96-well polystyrene plates were coated with synthetic linear and cyclic peptides (100 ng/well) or with unrelated peptide as a control antigen in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) and incubated overnight at 4 °C. After washing 4 times with TBS containing 0.1% Tween 20 (TBST), wells were blocked (5% skim milk powder in TBS) for 30 min and then washed 3 times with TBST. Serial doubling dilutions of ScFv in TBS were added to peptide-coated wells at 50 μL per well and incubated for 2 h at room temperature. Plates were washed 3 times with TBST. The HRP-conjugated Anti-E monoclonal antibody (diluted 1:8000 in TBS) was added to wells at 50 μL per well and incubated for 1 h at room temperature to detect E-tagged ScFv antibody bound to peptides. Plates were washed 3 times with TBST after which 50 μL/well TNB and H_2O_2 were added for color development. Plates were read at 450 nm.

Western Blot. Cf2Th/syn CCR5, PM1, and HeLa cells were washed 3 times (0.1 M PBS, pH 7.0), resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40 containing 100 μg/mL protein inhibitor), and incubated overnight at 4 °C. Treated samples were centrifuged (12 000 rpm, 15 min, 4 °C), and 100 μL of supernatant was transferred into fresh tubes. To each tube, 50 μL of native sample buffer was added to the supernatant and mixed. Twenty μL of the mixture was loaded onto a 12% polyacrylamide gel and electrophoresed for 4 h at 65 V. The fractionated extracts were electrotransferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences) that were then blocked for 1 h with 5% skim milk powder in TBS. ScFv antibody preparations were adjusted to 10 μg/mL in blocking buffer, and the membranes were probed for 2 h at room temperature. Membranes were washed with blocking buffer. Anti-E tag HRP conjugate (1:1000; Amersham Biosciences) was added, and membranes were incubated for a further 1 h. Membranes were washed with blocking buffer, and bound ScFv was visualized using a chemiluminescent substrate kit and film (Amersham Biosciences).

Flow Cytometry. PM1, Sup-T1, and HeLa cells were harvested and washed twice with PBS. Cells (1×10^5) and ScFv (1 ug) were mixed, incubated on ice for 2 h, washed with PBS, and centrifuged (2000 rpm, 5 min). After two washes, Anti-E tag antibody conjugated to FITC was added, and samples were incubated for 2 h on ice. Cells were washed as previously described and immediately assayed using a FACSCalibur flow cytometer. Data were then analyzed using the Becton Dickinson CellQuest software package.

Immunofluorescent Staining. P4R5 cells (2×10^3) were seeded into an eight-well chamber slide and cultured overnight. Nonadherent cells were removed by brief rinsing with PBS, and adherent cells were fixed to slides (2% paraformaldehyde in the PBS, 10 min). Following a gentle wash with PBS, cells were exposed to penetrating buffer (0.01% Triton X-100 in PBS, 5 min). The specimens were incubated in blocking buffer for 2 h. The blocking buffer was replaced with 200 μL of PBS containing 10 μg/mL ScFv or a 1:200 dilution of monoclonal antibody 2D7 for control. Following a 2 h incubation at room temperature with the primary reagent, the specimens were stained with a 10 μg/mL solution (100 μL) of anti-E tag FITC. The slides were mounted with 50% glycerol in PBS and

examined immediately under a fluorescence microscope (Nikon, Japan).

HIV-1 Infection Assay. A quantitative method based on that of Kimpton and Emerman (*31*) was used to determine the biological activity of ScFv to inhibit HIV infectivity. P4R5 cells were seeded into 48-well plates at 4×10^4 cells per well in 200 μL of medium containing DEAE-dextran (20 μg/mL) without selective antibiotics. Twenty to 24 h later, the cells were infected with either HIV-1 BaL or HIV-1 NL-43 at approximately 1000 focus-forming units per well, in the presence of ScFv antibodies (100 μg/mL). Three days after infection, the cells were rinsed with PBS and fixed with paraformaldehyde as described previously and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside). The number of HIV-infected cells was determined by enumerating the number of blue nuclei (representing infected cells) using an automated optical imager (NIH image system). Results are expressed as number of blue or infected cells per culture well as compared to control wells in which the virus was incubated either without ScFv or in the presence of the CCR5-specific mAb 2D7.

RESULTS

Tandem Ligation to Prepare End-to-End Cyclic Ppeptides using Thioester Chemistry and a Novel Thiol-Reversible Cysteinyl Protecting Group. GPCRs, including CCR5, contain seven transmembrane (TM) helical segments that support four extracellular domains, namely, the N-terminus and three extracellular loops termed ECL1, ECL2, and ECL3 (Figure 1). Our approach was to prepare conformationally constrained peptides that mimic the surface antigens within the three loops by using end-to-end cyclic peptides and their linear counterparts for comparison. These loop peptides termed E_1, E_2, and E_3 linear (L) or cyclic (C) contained the entire CCR5 loop sequences along with parts of the CCR5 TM helical bundles that stabilize the ectodomain loops (Figure 1C). To aid solubility and design of a reverse turn at the shortened TM helical bundle of cyclic peptides, the residues GK were included at the N- and C-termini of all peptides.

End-to-end cyclic peptides were generated by a Cys-thioester method (orthogonal ligation) using unprotected peptide segments with an N-terminal Cys and a C-terminal thioester (*32*, *33*). This is a critical component of the design strategy as it allows the resultant Cys thiol to participate in a second chemoselective tandem ligation with a functionalized biotin without the need for a protecting group scheme in between.

During the synthesis of unprotected peptides, we found that >30% of the N-terminal Cys was irreversibly modified as a thiazolidine residue, a formylation side reaction generated from the His(Bom) protecting group during the HF cleavage (*34*). To avoid this side reaction, we developed a thiol-reversible protecting group for the N-terminal Cys, the ninhydrin-protected Cys (Nin-Cys; Figure 2). Nin-Cys was readily attached to the N-terminal as the final step of solid-phase synthesis with no indication of steric hindrance during the coupling reaction. The presence of the ninhydrin-derived thiazolidine protects the N-terminal Cys residue from formylation generated during the HF-deprotection step of the His(Bom) residues present in the peptide chains. This

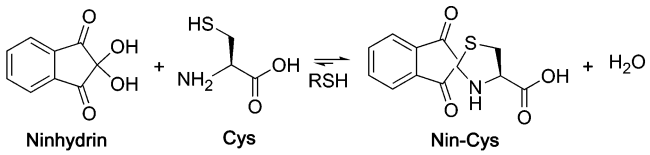

FIGURE 2: General scheme for the synthesis of Nin-Cys.

simplifies the purification by eliminating the need for less effective scavenging reagents. Deprotection of Nin-Cys by a thiol reagent also allows the Cys-thioester peptides to undergo end-to-end cyclization by orthogonal ligation (Figure 3). The orthogonal ligation was performed in aqueous solution and was complete within 10 h. This ligation strategy resulted in straightforward purification of linear components by HPLC (Figure 4) and a high yield of end-to-end cyclic peptides containing a thiol handle.

ScFv Selection Strategy. Figure 5 outlines the entire selection and isolation procedure. Initial attempts to use a direct solid-phase selection method were unsuccessful. When ScFv antibodies were selected with a solid-phase method using peptide antigens coated directly onto beads, a high level of cross-reactivity with nonhomologous peptide antigens containing part of the TM-residues or solid phase-induced denaturation resulted in the selection of many nonspecific ScFv antibodies (data not shown). To obtain antigen-specific phage, a solution-phase selection method was then developed to eliminate solid-phase selection and preserve the conformational integrity of the peptide antigens. To enable the solution-phase selection scheme, we modified peptide antigens by addition of a biotin-tag to produce biotinylated-peptides (B-peptides, Figure 3B). In the case of cyclic peptide antigens, this was conveniently achieved by a thiol addition reaction as the second step in the tandem ligation scheme. For this, the maleimide biotinylated linker was ligated to the thiol moiety resulting from the end-to-end thioester cyclization. We also prepared a bivalent linker peptide (L-peptide, Figure 6) containing nonloop residues (WKGCGKI) as a panning reagent to remove nonspecific binders.

With these reagents, preincubation with L-peptide removed phages specific for the linker residues. Phages subsequently panned with the B-peptides were then captured with streptavidin-coated beads. Phages specific for E_1L, E_1C, E_2L, E_2C, E_3L, or E_3C peptide were eluted with triethylamine.

To increase the probability of identifying biologically active ScFv antibodies, ScFv antibodies were assayed for binding to CCR5+ cells using an Applied Biosystems FMAT 8100 plate reader. CCR5+ and CCR5- cells were diluted to $\sim 2 \times 10^5$ cells/mL in PBS. Twenty μL of diluted cells was added to individual wells of 384-well FMAT microtiter plates. Twenty-five μL of each ScFv in *E. coli* periplasmic extract and 25 μL of FMAT Blue conjugated Anti-E tag monoclonal antibody (diluted to 0.25 μg/mL in PBS) were added to CCR5+ and CCR5− wells and incubated together on cells at room temperature in the dark. After 2 h, plates were read using the FMAT 8100. Nineteen bacterial clones produced ScFv antibodies that stained or interacted with CCR5+ but not CCR5- cells. All 19 bacterial clones were grown on a large scale and induced to express soluble E-tagged ScFv antibodies. Soluble antibodies were purified using the Amersham RPAS Purification Kit and Anti-E tag monoclonal antibody column according to the manufacturer's instructions.

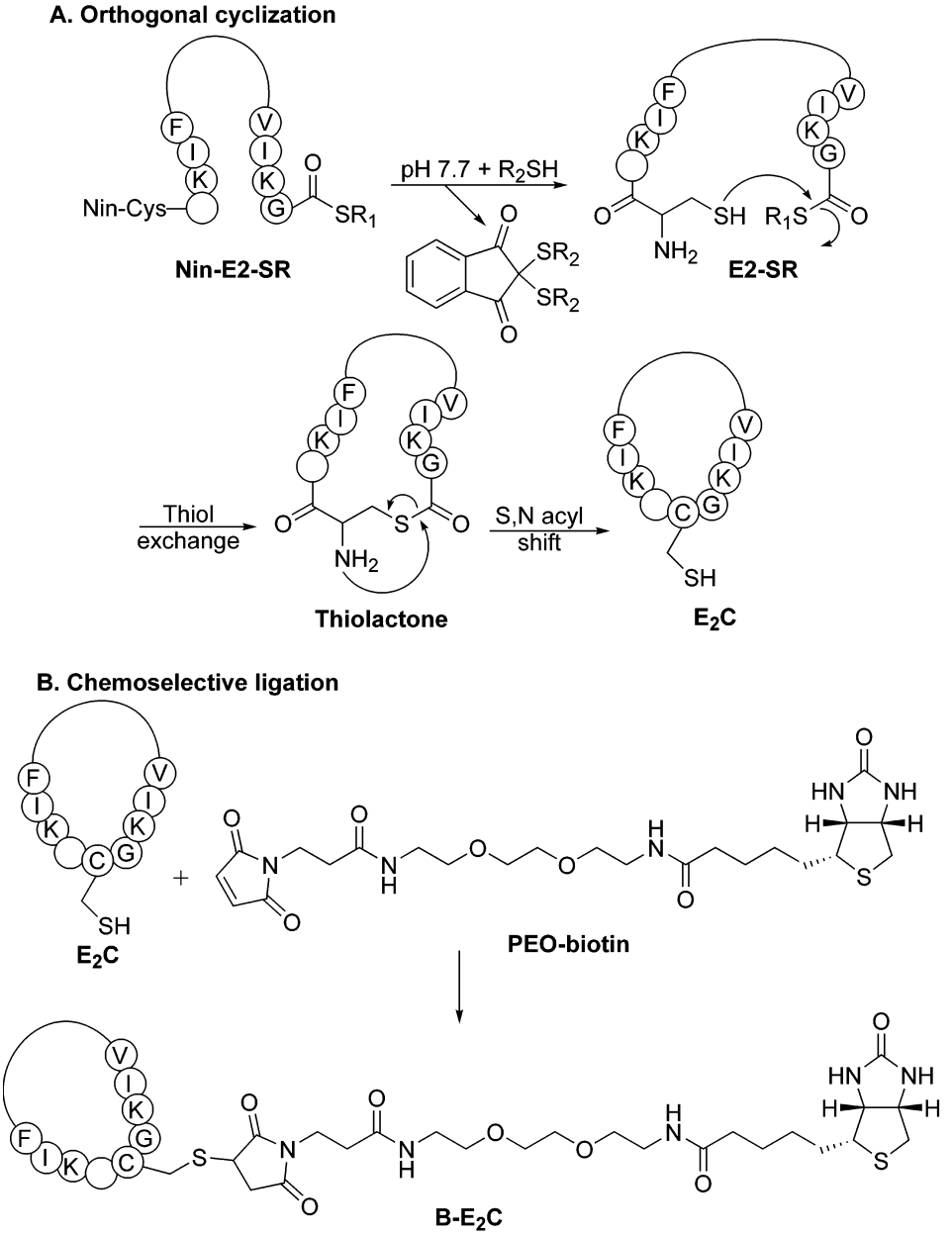

FIGURE 3: Mechanism of the tandem ligation strategy used to produce biotinylated cyclic peptides. (A) Orthogonal ligation. One step deprotection and cyclization of the Nin-E2-SR peptide using a thiol to remove the ninhydrin group as well as regenerate the thioester during the course of the reaction. (B) Chemoselective ligation. Cyclic peptides were biotinylated using PEO-biotin to produce B-peptides.

Cross Reactivity of ScFv Antibodies with Linear and Cyclic Peptides by ELISA. The cross reactivity of 19 purified ScFv antibodies was determined by ELISA by coating linear or cyclic peptides directly onto plastic microtiter wells (Table 1). In some cases, limited quantities of ScFv antibodies precluded use in ELISA. The results fell into four categories with antibodies that bound (a) both linear and cyclic forms, (b) linear but not cyclic, (c) cyclic but not linear, and (d) neither linear nor cyclic. As expected, most ScFv antibodies recognized the linear, conformationally flexible peptides in ELISA (category b). However, there were a few exceptions. For example, the E_1C-selected ScFv antibodies H2 and K1 recognized E_1L but not the expected E_1C. In another example, the E_3C-selected ScFv antibody O17 recognized neither E_3C nor E_3L. These ELISA results support the principle of solution-phase selection by showing that the linear and cyclic peptides possess different physical characteristics when bound as solid-phase antigens to a support such as a plastic surface.

ScFv Antibody Characterization. Western blot characterization of ScFv antibody binding to CCR5 expressed by Cf2Th/syn CCR5 and PM1 cells resulted in five positive ScFv antibodies A1, P21, G11, N18, and O17 (Figure 7A). In control experiments, ScFv antibodies were tested for binding to lysates of CCR5-negative (HeLa) cells, and binding was not observed. Since the selection strategy was designed to favor antibodies specific for structural epitopes, the small number of antibodies capable of binding to isolated CCR5 in Western blot is not unexpected. For Western blotting, the cell line P4R5 was initially used; however, relatively weak CCR5 expression resulted in a change to the cell lines Cf2Th/syn CCR5 and PM1.

Recombinant E-tagged ScFv antibodies characterized for cell binding/staining by FMAT were further characterized

Table 1: Characterization of ScFv Antibodies Directed to CCR5 Extracellular Domains

phage-screening peptide	ScFv	ELISA		Western blot	flow cytometry		immuno-fluorescence	HIV inhibition
		linear	cyclic		increased mean cell fluorescence	increased no. of fluorescent cells		
E1L	G4	+[a]	−[b]	−	−	+	−	−
	M5	−	ND[c]	−	−	+	−	−
	N4	+	+	−	+	−	−	−
E1C	A1	+	+	+	+	−	+	+
	H2	+	−	−	−	+	−	−
	K1	+	−	−	−	+	+	−
E2L	B22	+	ND	−	ND	ND	−	−
	C20	+	ND	−	ND	ND	−	−
	K21	+	ND	−	ND	ND	−	−
	P21	+	+	+	−	+	−	−
E2C	B7	−	−	−	+	+	+	+
	F12	+	+	−	+	+	−	−
	G11	+	−	+	−	+	−	−
	K7	+	−	−	ND	ND	−	−
	K8	+	+	−	+	+	−	−
	L9	+	+	−	−	+	−	+
E3L	M12	−	−	−	ND	ND	−	−
E3C	N18	−	+	+	−	+	−	−
	O17	−	−	+	−	+	−	−

[a] + indicates higher signal as compared to control. [b] − indicates no binding or negative result in test. [c] ND = not determined. Limiting quantity of antibody precluded use.

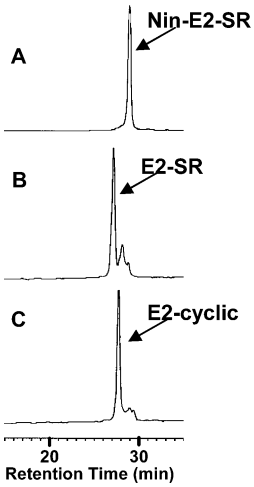

FIGURE 4: RP-HPLC elution profiles of cyclization reaction. (A) Purified Nin-E2-SR peptide. (B) Peptide 10 min after addition of MPS. (C) E_2C formation 2 h after addition of MPS.

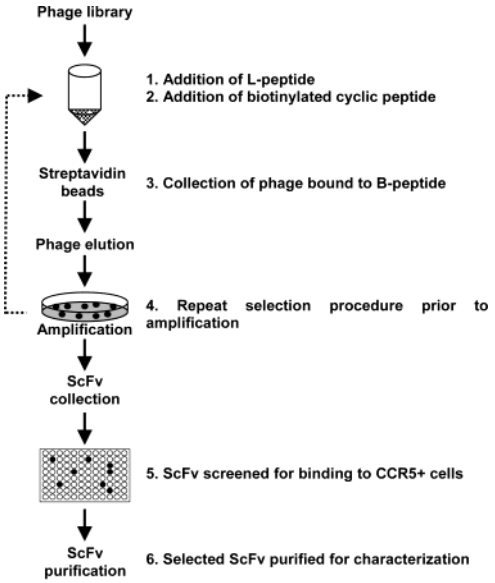

FIGURE 5: Strategy developed for capturing phage expressing ScFv antibodies specific for peptides mimicking CCR5 ectodomains. L-peptide and B-peptides were incubated in solution with phage library before addition to streptavidin magnetic beads. Captured phages were eluted and underwent a second round of selection before amplification. Crude ScFv antibodies were collected and screened for binding to CCR5+ cells. Antibodies that exhibited the strongest binding to CCR5+ cells were purified and used in subsequent assays.

for cell binding using flow cytometry with the FITC-conjugated Anti-E tag monoclonal antibody, nonadherent CCR5+ Sup-T1 cells, and CCR5− HeLa cells. ScFv antibodies stained Sup-T1 CCR5+ but not HeLa CCR5− cells when compared to CCR5+ and CCR5− cells not receiving ScFv. The ScFv antibodies produced three different binding profiles based on the mean cellular fluorescence and/or number of fluorescent cells as compared to samples without antibody (Table 1). Of the 14 antibodies tested, incubation with nine ScFv resulted in an increase in the number of fluorescent cells but not mean cellular fluorescence. This binding profile may be attributed to antibodies binding to epitopes occurring at a very low frequency, leading to a low concentration of antibody binding per cell. However, incubation with two antibodies, N4 and A1, resulted in an increase in mean cellular fluorescence but not an increase in the number of fluorescent cells (Figure 7B). Antibodies N4 and A1 most likely bound to epitopes that exist at a relatively high density on only a limited number of cells. Finally, incubation with antibodies B7, F12, and K8 resulted in both elevated mean cellular fluorescence and number of fluorescent cells. This binding profile suggests that the epitopes recognized by ScFv B7, F12, and K8 occur at high frequency on a large number of cells.

We also characterized ScFv antibodies qualitatively by direct visualization using fluorescence microscopy. While all the ScFv antibodies bound to CCR5+ cells by flow cytometry, only three (A1, B7, and K1 (Figure 7C)) bound

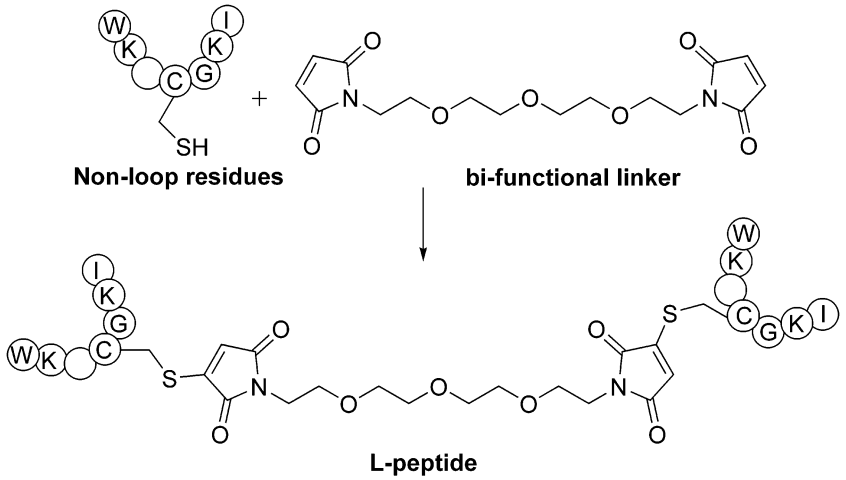

FIGURE 6: Scheme for synthesis of linker-peptide (L-peptide) used during phage selection.

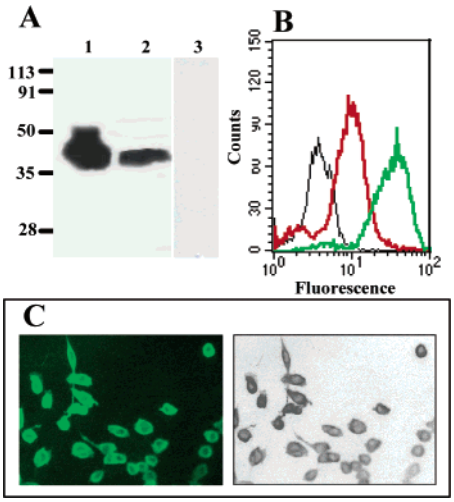

FIGURE 7: Binding of ScFv antibodies to CCR5+ cells. (A) Specific binding of ScFv antibody to CCR5 shown by typical Western blot profile. Cell lysates from Cf2Th/ syn CCR5 cells (lane 1), PM1 cells (lane 2), and CCR5-negative HeLa cells (lane 3) probed with ScFv N18. (B) Nonadherent Sup-T1 cells were either incubated alone (black) or with ScFv A1 (red) or mAb 2D7 (green) and then stained with FITC conjugate and a CCR5+ subpopulation examined by flow cytometry. (C) Indirect immunofluorescent staining of CCR5+ cells with ScFv antibody A1. P4R5 cells were incubated with ScFv antibody, and binding was visualized by addition of anti-E-tag FITC conjugate (magnification ×100). Phase contrast is also shown.

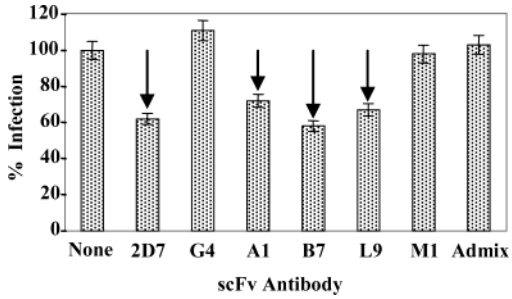

FIGURE 8: HIV inhibitory activity of selected ScFv antibodies. P4R5 cells were treated with ScFv antibody and then infected with CCR5-tropic HIV-1 BaL. The number of infectious centers per well is expressed as a percentage of the number in the control wells that did not receive antibody. Control wells either received monoclonal antibody 2D7 in place of ScFv or no antibody. An admix of the three ScFv antibodies N4, O17, and P21 was also tested: denoted admix. Arrows indicate antibodies that displayed significant inhibitory activity.

to P4R5 cells sufficiently to allow visualization by fluorescence microscopy. All three ScFvs were selected against cyclic peptides, and each displayed different binding profiles in flow cytometric analysis.

ScFv Inhibition of HIV Infection. Three ScFv (designated A1, B7, and L9) of the 19 ScFv antibodies tested specifically inhibited CCR5-tropic HIV-1 BaL but not CXCR4-tropic HIV NL-43 infection (Figure 8). All three of the active ScFv antibodies were obtained by panning against cyclic peptides. The inhibition of infection induced by the ScFv antibodies A1, B7, and L9 was approximately equal to, if not better than, that of mAb 2D7. Additionally, a mixture of three ScFv antibodies, N4, P21, and O17, that tested negative in the infectivity assay and were specific for ECL1, ECL2, and ECL3, respectively, did not prevent viral infection even at high concentrations (100 μg/mL, Figure 8).

DISCUSSION

Peptide antigens are frequently used to induce site-specific antibodies; however, linear peptides, particularly relatively short peptides, are generally unable to mimic structural epitopes. Consequently, whole proteins are preferred antigens for use in the production of polyclonal or monoclonal antibodies or for the selection of recombinant antibodies capable of interacting with native antigens. There are difficulties associated with obtaining purified TM-anchored proteins, such as CCR5, for use as immunogens in the production of biologically active antibodies. These difficulties can be overcome through the use of constrained peptides representing ectodomains on TM-anchored loops. A distinct feature of a looplike cyclic peptide as a surface-antigen mimetic of a TM-anchored protein is that it does not contain any breaks, unlike a linear peptide. Thus, a looped peptide mimetic will contain many more continuous epitopes, and hopefully structural epitopes, than its corresponding linear counterpart and enhance the probability of selecting biologically active antibodies from a phage display antibody library. Our results support the feasibility of using a constrained peptide TM-mimetic approach. We demonstrate that three ScFv (A1, B7, and L9), obtained from a phage antibody library selected on three cyclic or constrained peptide CCR5-mimetics, exhibit biological activity and inhibit HIV cell

infectivity in vitro. These ScFv antibodies are specific for CCR5 and do not inhibit CXCR4-tropic HIV-1 infection.

Our approach requires end-to-end cyclization of ectodomain sequences that range from 25 to 41 residues to produce looplike structures. This end-to-end cyclization results in formation of a peptide bond and is therefore different from conventional peptide cyclization, which is mediated by side chain-to-side chain disulfide constraint. In our study, we used an orthogonal ligation scheme coupled with a Nin-Cys protecting group for thioester chemistries to yield cyclic peptides with a thiol handle. Although end-to-end cyclization of large peptides are not generally favored because of the vast distance between the N-terminal Cys and the C-terminal thioester, the largest of the three ectodomain loops E_2C containing 41 residues is completely cyclized during peptide synthesis within 10 h. Coupled with the absence of end-to-end polymers, this suggests that cyclization was assisted during peptide synthesis using this strategy.

Orthogonal ligation is performed in an aqueous buffer using unprotected peptides under slightly basic conditions. Unlike disulfide formation, orthogonal cyclization has the advantages that the resultant cyclic peptide possesses only peptide bonds and a regenerated Cys residue at the ligation site that can be used for the second chemoselective ligation to yield biotinylated cyclic peptides (Figure 3). The tandem ligation strategy (*32*, *33*) simplifies production of surface-antigen mimetics and provides a useful approach by which peptides can be conjugated to reagents for various purposes by thiol chemistries. For example, conjugation through a disulfide bond or chemoselective ligation by thioalkylation or thiol addition to fluorescent tags, enzymes, substrates, proteins, and reagents such as biotin may find applications where a chemically defined molecule is required. In addition, the disulfide bond can be cleaved using a reducing agent to specifically release peptide-specific ScFv obtained during phage antibody selections on conjugated peptides. Such a selection strategy would not be possible using the conventional side chain-to-side chain disulfide constraint.

Although the inclusion of the TM residues in the surface-antigen mimetics facilitates the loop design and cyclization reaction, these residues are also highly antigenic and complicate the selection process. To eliminate nonspecific binding, we also prepared and preincubated phage with an L-peptide containing the additional residues (Figure 5) to remove phage antibodies that would cross react with the L-peptide WKGCGKI amino acid sequence shared by the biotinylated B-peptides used for phage antibody selections. Surface-antigen-specific phages were eluted, and ELISA results subsequently showed that reliance on solid-phase selection would have resulted in missing active antibodies. These results highlight differences in epitope exposure between solution- and solid-phase assays, justifying our use of a solution-phase selection strategy. Recombinant ScFv antibodies obtained after selection against peptides were characterized for binding activity to cells using an FMAT 8100. Nineteen ScFv reactive with CCR5+ and not CCR5− cells were further characterized for binding activity using Western blots, flow cytometry, and for HIV inhibitory activity (Table 1).

Interestingly, the antibody binding profiles produced by Western blot and flow cytometry do not correlate with HIV inhibitory activity, with Western blot being the least discriminating. ScFv antibodies such as P21, G11, N18, and O17 that bind to CCR5 protein in Western blots and to CCR5+ cells in immunofluorescence assays are inactive in the HIV infectivity assay. The various characterization methods also did not reveal any common similarities among the three inhibitory ScFv antibodies (A1, B7, and L9) that were selected. The A1 ScFv bound to E_1C and bound to CCR5 in Western blot and to CCR5+ cells in flow cytometry and immunofluorescence. The B7 and L9 ScFv antibodies were selected against the E_2C peptide. B7 and L9 did not bind to CCR5 on Western blots. However, B7 bound to CCR5+ cells in flow cytometry and immunofluorescence, whereas L9 bound only in flow cytometry. These results suggest that A1, B7, and L9 were selected by and interacted with different CCR5 epitopes that may or may not be exposed on CCR5 when assayed using different methodologies. These results suggest that epitope exposure varies depending upon the characterization methods used. As a consequence, methods such as Western blots, immunofluorescence, and flow cytometry may not always be optimal methods to screen for biologically active antibodies. Nevertheless, these methods provide support that CCR5 exists in different conformational states on the cell surface (*35*−*39*) and that these conformers can be detected by antibodies specific for various epitopes within CCR5. The fact that two of the three inhibitory antibodies were selected using an ECL2 domain mimetic is in keeping with previous studies that have shown that the CCR5 ECL2 domain is a critical ligand interaction site (*14*, *16*, *40*). Additionally, two HIV-neutralizing murine monoclonal antibodies, designated as PRO140 and 2D7, are both directed to the CCR5 ECL2 domain (*20*, *22*).

It may not be possible to define a suitable secondary screen for a panel of selected ScFv antibodies if the epitope of interest is only accessible during CCR5 activation. This is a realistic scenario in the example presented here, if particular cyclic peptides mimic critical epitopes accessible, or even formed, only after receptor−virus interaction, then there will be difficulties associated with detecting such epitopes on inactive cells even if they do express the requisite CCR5 isomer. Only after the essential epitope mapping studies are completed will it be possible to determine if the epitopes of interest do indeed exist on the surface of CCR5+ cells in the absence of virus−cell interaction.

In conclusion, expression of soluble GPCRs and other TM-anchored receptors is challenging because of the hydrophobic nature of TM helices. However, cyclic peptides as surface-antigen mimetics can be used to select for biologically specific recombinant antibodies to soluble GPCRs such as the CCR5 TM-anchored receptor. This study provides proof of concept by combining chemistry and biology to streamline selection and isolation of ScFv antibodies as HIV infection inhibitors. The design, chemistries, and selection strategy described here for producing surface-antigen mimetics should be applicable to GPCRs and other TM-anchored proteins with known or unknown functions.

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