Study on CCR5 analogs and affinity peptides

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The G protein-coupled receptor of human chemokine receptor 5 (CCR5) is a key target in the human immunodeficiency virus (HIV) infection process due to its major involvement in binding to the HIV type 1 (HIV-1) envelope glycoprotein gp120 and facilitating virus entry into the cells. The identification of naturally occurring CCR5 mutations (especially CCR5 delta-32) has allowed us to address the CCR5 molecule as a promising target to prevent or resist HIV infection in vivo. To obtain high-affinity peptides that can be used to block CCR5, CCR5 analogs with high conformational similarity are required. In this study, two recombinant proteins named CCR5 N-Linker-E2 and CCR5 mN-E1-E2 containing the fragments of the CCR5 N-terminal, the first extracellular loop or the second extracellular loop are cloned from a full-length human CCR5 cDNA. The recombinant human CCR5 analogs with self-cleavage activity of the intein Mxe or Ssp in the vector pTwinI were then produced with a high-yield expression and purification system in Escherichia coli. Experiments of extracellular epitope-activity identification (such as immunoprecipitation and indirective/competitive enzyme-linked immunosorbent assay) confirmed the close similarity between the epitope activity of the CCR5 analogs and that of the natural CCR5, suggesting the applicability of the recombinant CCR5 analogs as antagonists of the chemokine ligands. Subsequent screening of high-affinity peptides from the phage random-peptides library acquired nine polypeptides, which could be used as CCR5 peptide antagonists. The CCR5 analogs and affinity peptides elucidated in this paper provide us with a basis for further study of the mechanism of inhibition of HIV-1 infection.

Keywords: CCR5 antagonist/epitope-activity identification/extracellular domains/human chemokine receptor 5 analogs

Introduction

During the entry of human immunodeficiency virus type 1 (HIV-1) into host cells, the virus gp120 glycoprotein molecule binds to two cell-surface receptors, CD4 and a co-receptor; the latter could be either chemokine receptor 5 (CCR5) or CXCR4 (Alkhatib et al., 1996; Deng et al., 1996). CCR5 and CXCR4 are G protein-coupled receptors characterized by seven transmembrane alpha-helices, an extracellular N terminus (Nt), an intracellular C terminus, three intracellular loops and three extracellular loops (ECLs). However, high-resolution structures of CCR5 and CXCR4 are still lacking, although some insight has revealed the crystal structures of other family members such as bovine rhodopsin (Palczewski et al., 2000), two adrenergic receptors (Cherezov et al., 2007) and the adenosine receptor (Rasmussen et al., 2007). The approximate structure of CCR5 has been modeled based on the similarity revealed by the structures of these related proteins (Lodowski and Palczewski, 2009).

Furthermore, studies on CCR5 function have to rely on alanine scanning study (Blanpain et al., 1999), molecular homology modeling technique, site-directed mutagenesis experiment and crystal structure analysis. Multiple CCR5 domains directly or indirectly contribute to their co-receptor activity. And the N-terminal (Nt), the first extracellular loop (ECL1) and the second extracellular loop (ECL2) of CCR5 are known to play a privileged role in ligand binding and viral entry (Liu et al., 1996; Dragic, 2001; Pastori et al., 2008). Engagement of CCR5 at both Nt and ECL2 triggers additional conformational changes leading to HIV-1 entry (Thompson et al., 2002). And the Nt of CCR5, which contains several sulfated tyrosines, interacts with a highly conserved four-stranded bridging sheet in gp120, which assembles upon CD4 binding, whereas the ECL2 region interacts with the tip of the immunodominant V3 loop in gp120 and the natural ligands [regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β] (Lam et al., 2008). ECL2 containing 32 amino acid residues is also thought to play an important role in CCR5 interactions with HIV-1 envelope and natural ligands (RANTES and MIP-1) binding (Navenot et al., 2001). In recent years, many studies have demonstrated that CCR5 antagonist could be used in clinical treatment, such as maraviroc.

On the basis of the arguments presented above, this study constructed two recombinant human CCR5 analogs to select small affinity peptides by phage display technology. These peptides could be used as the peptide antagonist against CCR5. And CCR5 analog itself could also be potentially used to inhibit HIV entry.

Materials and methods

Bacterial strains, plasmid and reagents

The template pcDNA3.0 ccr5 which corresponds to full-length CCR5 gene was donated by Professor Tianyuan Zhang (Key Laboratory of Genetic Engineering of Ministry of Education, Sun yat-sen University) (Wang et al., 2006).
Molecular modeling of CCR5 was determined using Swiss-PDB Viewer 3.7.

The *Escherichia coli* DH5α strain (Novagen, Germany) was used for the preparation of plasmid and cloning, and *E.coli* BL21(DE3) (New England BioLabs, USA) was applied to express fusion protein. The vector pTwin1 (IMPACT CN System, New England BioLabs) was used for constructing recombinant genes. *Escherichia coli* BL21 (DE3) carrying recombinant plasmid were cultivated in high nutritional LB (1.2% tryptone, 0.6% yeast extract and 0.8% NaCl) with 100 μg/ml ampicillin. CCR5 ECL2 monoclonal antibody (mAb) 3A9 and CCR5 Nt mAb 2D7 were purified from mouse IgG2a and purchased from BD Pharmingen in USA. The random phage-displayed peptide library (PhD-12) was purchased from New England BioLabs. Detection module recombinant phage antibody system (GE Company, USA) was used for the identification of positive phages.

**Construction of expression vectors**

Based on the known sequence of ccr5 gene (GeneBank Accession No. NM0000579) and an additionally designed flexible peptide sequence GlyGlyGlySer-GlyGlyGlySer-GlyGlyGlySer, target gene ccr5 N-Linker-E2 was amplified from pcDNA3.0 ccr5 by regular PCR and SOE-PCR (splicing by overlapping extension PCR). The two pairs of primers used for ccr5 N-Linker-E2 were as follows: UP1 (5’T-ATTCGATCATATGATGGATTATCAAGTG-3’), DOWN1 (5’T-TCAGGCGGAAGGTTCTGGCGGTGGGATCGACCAGATCTCTAAAAG-3’), and DOWN2 (5’T-ATAGGAGGAGTATCCTTTATGTCGTT-3’), where the underlined bases are restriction endonuclease cleavage recognition sequences. The final amplified product by SOE-PCR and pTwin1 were digested with restriction endonucleases NdeI and BspQI (New England BioLabs). Both of these digested products were cloned together by T4 ligase, and confirmed by colony PCR and DNA sequencing. The resulting recombinant plasmid pTwin1-CCR5 N-Linker-E2 was then transformed into *E.coli* strain BL21 (DE3) with cultivation containing 100 μg/ml ampicillin.

Based on ccr5 N-Linker-E2, another target gene named ccr5 mN-E1-E2 was constructed by means of deletion, addition, or mutation. In brief, sequences of the first three amino acid residues in Nt and amino acid residues from Gln189 to Ile198 in ECL2 were deleted from ccr5 N-Linker-E2, and then sequences from ECL1 (Ala90-Gln102) and other flexible peptide GlyGlyGlySer-GlyGlyGlySer-GlyGlyGlySer were added for solution expression. Specific primers used for ccr5 mN-E1-E2 were UP1’ (5’T-AAGGCTCTTCCACCAAGTTGTC-3’), DOWN1’ (5’T-TATTACAACCAAATCTCCACCTGGCGCAAGCCGATACCCGCAAG-3’), UP2’ (5’T-AGTGGGATTTTGGTAAATACTGTTGAGGTGGGCTCAG-3’) and DOWN2’ (5’T-CAGAGATCCCTTATATTGATCTGACGTA-3”). The underlined bases are restriction endonuclease cleavage recognition sequences of restriction endonucleases BspQI and BamHI (New England BioLabs). The other procedure was the same as the above one.

**Large-scale expression of the chitin-binding domain–intein fusion protein**

The cells were recovered at 37°C in 30 ml Luria Broth (LB) media containing 100 μg/ml ampicillin overnight. This culture was then amplified in 1.2 l fresh high nutritional LB with 100 μg/ml ampicillin until the A600nm reached 0.6–0.8, and isopropylthio-β-D-galactosidase (IPTG) was added at different concentrations and at appropriate temperature to induce expression. After induction, cells were harvested by centrifugation at 5000 r.p.m. for 10 min and the sample of whole cell lysates was analyzed by 16.5% Tricine–SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blotting (WB) using anti-chitin-binding domain (CBD) antibody (IMPACT CN System, New England BioLabs) for the fusion CBD-tag.

**Purification of recombinant proteins CCR5 N-Linker-E2 and CCR5 mN-E1-E2**

The purification method of the recombinant protein CCR5 N-Linker-E2 was as follows. The pellet of *E.coli* cells was resuspended in Buffer B2 (20 mM Tris-Cl, 250 mM NaCl, 1 mM EDTA (ethylenediamine tetraacetate acid, pH 7.0)) coldly and sonicated at 200 W intermittently for 2 h. Then, the cell debris was removed by centrifugation at 10 000 r.p.m. for 10 min at 4°C. The supernatant was collected and loaded onto a 10 ml chitin bead affinity column (New England BioLabs) preequilibrated with 10 column volumes of Buffer B2 at the flow rate of 0.5 ml/min. Protein flow-through was collected as the supernatant passed through the column and analyzed with 16.5% Tricine–SDS–PAGE. After loading was completed, an additional incubation of 15 min was followed. Afterwards, the chitin column was washed with Buffer B2, at least 10 column volumes, until UV280nm absorbance was close to stable or even zero. The column was further washed with five column volumes of Buffer B3 (20 mM Tris-Cl buffer containing 500 mM NaCl, 1 mM EDTA, 60 mM DTT (1,4-dithiothreitol), pH 8.5) at 2 ml/min. The two outlets were then closed so as to incubate the column at a specific temperature of 8°C for 24 h for self-cleavage. After cleaving, the recombinant protein was then eluted with Buffer B3 (non-additive DTT) at the flow rate of 1.5 ml/min. Finally, the fractions of the recombinant protein CCR5 N-Linker-E2 were loaded on Sephadex G-25 resin (GE Healthcare, USA) for getting rid of DTT with Buffer B3 (non-additive DTT) at 1 ml/min and analyzed by SDS–PAGE with 16.5% Tricine–SDS–PAGE.

The other purification method for the recombinant protein CCR5 mN-E1-E2 was the same as the above one except for some differences, using Buffer B2 (20 mM Tris-Cl buffer containing 250 mM NaCl, pH 8.5) as the solution buffer, loading buffer and washing buffer and using Buffer B2 at a lower pH as the buffer for intein self-splicing (20 mM Tris-Cl buffer containing 250 mM NaCl, pH 7.0) without DTT or MESNA (2-mercapto-ethanesulfonic acid).

**High-performance liquid chromatography and mass spectrometry analysis**

The purified proteins were further analyzed by using a reversed-phase C18 high-performance liquid chromatography (HPLC), which is a simple, specific, rapid and effective method validated for protein purity determination, and further characterized by using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), which is a mature and reliable technique for molecular weight determination.
Immunoprecipitation with CCR5 antibodies

Immunoprecipitation (IP) was performed to identify the binding activity between CCR5 mAbs and the recombinant proteins and to examine the quantity or physical characteristics of the recombinant proteins. Approximately 500 μg of the recombinant protein CCR5 N-Linker-E2 or CCR5 mN-E1-E2 was added into 40 μl of Protein G PLUS-Agarose (Santa Cruz Biotechnology, USA) and 2 μg of primary antibody CCR5 ECL2 mAb 3A9 (purified mouse IgG2a, κ isotype, BD Pharmingen) or CCR5 Nt mAb 2D7 (purified mouse IgG2a, κ isotype, BD Pharmingen). Cap tubes and incubate at 4°C overnight. Collect immunoprecipitates by centrifugation at 3500 r.p.m. (~1000 g) for 5 min at 4°C. Carefully aspirate and discard redundant supernatant. Wash pellet four times with 1.0 ml 1× PBS (phosphate-buffered saline), each time repeating the centrifugation step above. After final washing, aspirate and discard supernatant, and resuspend pellet in 50 μl of 1× PBS buffer. Boil samples with 50 μl 1× loading buffer and analyze by 16% Tricine–SDS–PAGE, followed by silver staining analysis.

Indirective ELISA analysis with mAbs 3A9 and 2D7

Microtiter plates (JET BIOFIL, China) coated with 10 μg/ml of the purified protein CCR5 N-Linker-E2 or CCR5 mN-E1-E2 were blocked with coating buffer (0.1 M sodium bicarbonate, pH 8.6). mAb 3A9 or mAb 2D7 was then added and incubated for 2 h at room temperature. The wells were washed for five times with washing buffer PBST (1× PBS containing 0.1% Tween-20) so that the secondary antibody horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG2a (Proteintech Group, China) could bind to the primary antibody effectively. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Wuhan Boster Biological Technology, China) was added into the wells and incubated at 37°C for 30 min. The reaction was stopped by adding 1 M sulfuric acid and absorbance was measured at 450 nm.

Indirective competitive ELISA analysis of Met-RANTES binding

It was proved that modified Met-RANTES derived from RANTES can also slow down breast tumor growth and reduce the macrophage infiltrate as CCR5 chemokine (Robinson et al., 2003). For indirective competitive enzymelinked immunosorbent assay (ELISA) of Met-RANTES with the recombinant protein CCR5 N-Linker-E2, 1 μg of Met-RANTES (R&D Systems, USA) dissolved in coating buffer (0.1 M sodium bicarbonate, pH 8.6) was coated on microtiter wells of ELISA plates by incubating overnight at 4°C. Blocking was done by adding 5% bovine serum albumin (BSA) in 1× PBS and incubating for 1 h at 4°C; all wells were then washed with the washing buffer PBST (1× PBS containing 0.1% Tween-20) for five times. After adding 0.5 μg of the primary antibody anti-RANTES (Wuhan Boster Biological Technology), antigen CCR5 N-Linker-E2 was competitive binding to Met-RANTES in a concentration gradient of 250, 100, 50, 25, 20 and 0 ng/ml, while 5 μg of the primary antibody anti-RANTES (Wuhan Boster Biological Technology) was added together for 2 h at room temperature. Repeating the washing steps, sequentially 1: 20 000-dilution HRP-conjugated goat anti-rabbit IgG (Wuhan Boster Biological Technology) of the second antibody was also supplied for 2 h at room temperature. Washing was performed as above and 50 μl of TMB substrate was added to each well and incubated at 37°C for 30 min. Color development was stopped by adding 50 μl of 1 M sulfuric acid before absorbance detection at UV450nm.

For the indirective competitive ELISA of Met-RANTES with the recombinant protein CCR5 mN-E1-E2, 0.25 μg of Met-RANTES (R&D Systems) dissolved in coating buffer were coated on microtiter wells of ELISA plates by incubating overnight at 4°C. After adding 0.5 μg of the primary antibody anti-RANTES (Wuhan Boster Biological Technology), the antigen CCR5 mN-E1-E2 was competitive binding to Met-RANTES in a concentration gradient of 10 000, 1000, 500, 250, 100, 50 and 0 ng/ml for 2 h at room temperature. The main procedure was the same as the above one.

All assays were performed in triplicates and their mean values were estimated by calculating mean value ± standard deviation using Microsoft Excel. Statistical analyses were performed using the Student’s t-test. A P value of <0.05 was considered as statistically significant.

Affinity screening of phage library

The random phage-displayed peptide library (PhD-12) expressing peptides at the Nt of the PIIE coat protein of the filamentous coliphage M13 was screened for peptides with high affinity to the CCR5 analogs, by biopanning against the CCR5 analogs for four successive enrichment rounds. Phages were propagated in E.coli strain ER2537 provided with the library kit as a non-competent glycerol stock.

The DNA inserts were sequenced from the library to confirm the existence of the random-peptide sequences. All the phages examined contained inserts, although several had the amber stop codon (TAG) that is expressed as glutamine in these libraries.

Microrider wells were coated with 100 μl of 50 μg/ml recombinant protein CCR5 N-Linker-E2 or CCR5 mN-E1-E2 in coating buffer (Na2CO3, pH 9.6) at 4°C overnight, then blocked with 5% BSA in 1× tris-buffered saline (TBS) for 1 h at 4°C, followed by the addition of 10 μl phage library and incubation at room temperature for 60 min. Unbound phages were eliminated by washing with buffer TBST (1× TBS containing 0.1% Tween-20) for six times, and then washed with elution buffer (0.1 N Gly-HCl, pH 2.2, containing 1 mg/ml BSA) at 37°C for 10 min to collect the high-affinity bound phages, which were neutralized with 15 μl of 2 M Tris per 100 μl phage eluate and were used to infect ER2537 bacterial cells in log phase. The bacterial cells were cultured for 4.5 h and then cells were pelleted and discarded by centrifugation twice at 4000 × g for 15 min. Phages in the supernatant were precipitated by using 25% polyethylene glycol 6000 and 2.5 M NaCl on ice for 1 h and pelleted by centrifugation for 15 min at 10 000 × g. The phage pellet was resuspended in buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The precipitation step was repeated and the final phage pellet was resuspended in PBS/0.02% NaN3 and used for further rounds of biopanning (according to the manufacturer’s instructions). After three or four rounds of biopanning, the phage clones were sequenced and used for affinity-capture phage ELISA of the positive phage clones.
Phages used for ELISA were derived from clones selected after three or four rounds of biopanning. Microtiter wells were coated overnight with 7.5 μg of the diluted recombinant protein CCR5 N-Linker-E2 or CCR5 mN-E1-E2 and then blocked with blocking buffer (5% BSA in 1×TBS). The phages (1 × 10^{12} PFU/150 μl/well) diluted in TBS were added and incubated for 2 h at room temperature. The plates were washed six times with TBST (1×TBS containing 0.1% Tween-20), and sequentially 1:5000-dilution HRP-conjugated anti-M13 mAb was supplied for 2 h at room temperature. Washing was performed as above with 150 μl of TMB substrate and color development was detected at UV₄₅₀nm absorbance.

Homologous analysis and multiple sequence alignment were done using BLAST from Swissprot database and Multalin soft to determine the motifs of related affinity peptides.

Results

Expression and purification of recombinant proteins

Recombinant genes ccr5 N-Linker-E2 and ccr5 mN-E1-E2 were constructed by SOE-PCR and regular PCR amplifications, with the full-length pcDNA3.0 ccr5 as the template, choosing the most possible sequences of antigenic-epitopes in the extracellular domains (Fig. 1). To mimic CCR5 more closely and to obtain recombinant proteins with more stable conformation so as to be used as CCR5 analog for target antigen in phage screening, a distinctive construction strategy was adopted that optimized the flexible peptide and chose a 12-amino acid sequence as flexible peptide (Kikuchi et al., 2005). Special primers for PCR were synthesized based on the sequence of Nt and ECL2 of CCR5.

To produce small molecular weight recombinant proteins, we had constructed plasmids with many vectors, such as pET21b, pET22a, pET22b or pET30a in different host strains such as Rosetta (DE3), Origami (DE3) or BL21 (DE3). Unfortunately, we could not acquire suitable soluble and quantitative expression in these ways (data not shown). Finally, the CBD-intein purification method was adopted, which was proved to provide us with high product yield and purity under a wide range of buffer compositions (Gillies et al., 2008). The pTwinI vector with intein-mediated self-cleavage activity was especially ideal for small molecular weight proteins with the advantage of single-step purification (Fong et al., 2010).

Expression conditions including IPTG concentration, induction time and culture temperature were optimized to obtain a better yield of the recombinant proteins. The result showed that the fusion protein CCR5 N-Linker-E2-Mxe intein-CBD was expressed more soluble with culture temperature at 16°C than at higher temperatures. And 0.05 mM of IPTG was the best concentration at this temperature (Fig. 2). Another fusion protein, CBD-Ssp intein-CCR5 mN-E1-E2, was optimized at 22°C culture temperature with 0.1 mM of IPTG concentration (Fig. 2). However, induction time with IPTG had little effect on solubility but protein yields. The supernatants and pellets of the sonicated product from engineered bacterial cultures were subjected to 16.5% Tricine–SDS–PAGE and WB, which showed a band of about 36.526 kDa for the fusion protein CCR5 N-Linker-E2-Mxe intein-CBD, and a band of ~33.8 kDa for the fusion protein CBD-Ssp intein-CCR5 mN-E1-E2 (Fig. 2).

The recombinant proteins were expressed and purified by the intein-CBD system according to the protocol provided by New England BioLabs with some modifications to adapt different fusion strategies, i.e. with some different purification steps for CCR5 N-Linker-E2 and CCR5 mN-E1-E2.

The affinity chromatography column was pre-equilibrated with a low concentration of NaCl at 250 mM so that the loaded protein containing the CBD-tag would bind to the chitin column easily. The loaded column was extensively washed with a high concentration of NaCl at 500 mM until UV invariant to eliminate any non-related proteins. The target protein could be obtained by adding nucleophilic agents such as DTT, hydroxylamine or cysteine to generate the thioester intermediate required for the initiation of self-catalytic intein-mediated cleavage reaction (Morassutti et al., 2002). The time and temperature for self-cleavage induction were crucial. The target protein CCR5 N-Linker-E2-Mxe intein-CBD with C-terminal fusion strategy was released by adding 60 mM DTT and modulating the pH to 5.5 at 4°C and incubated for 24 h in favor of Mxe intein cleavage (Fig. 3A), while CBD-Ssp intein-CCR5 mN-E1-E2 with Nt fusion strategy was released by just modulating the pH to 7.0 under 4°C and incubated for 40 h (Fig. 3B). Since CCR5 N-Linker-E2 was sensitive to temperature change, 1 mM EDTA was added to the Buffer B3 to prevent the protein from degradation to smaller fractions. And since the solution for CCR5 N-Linker-E2 purification contained a low concentration of DTT, elution peaks were further passed through a Sephadex G-25 column to get rid of DTT for subsequent immunological identification (Fig. 3A).

The purified proteins were then analyzed by MALDI-TOF MS, which demonstrated a molecular weight of 8.193 kDa for CCR5 N-Linker-E2 and 8.6546 kDa for CCR5 mN-E1-E2, approximately in accord with the prediction values within a systematic error of 5%, which could be due to disturbance by the ion exchange of Na⁺/H⁺ in the buffer. The result suggested that the acquired CCR5 analogs were monomers.
Analysis by rapid reversed-phase HPLC (RP-HPLC) showed a single highest peak, demonstrating the high purity of the recombinant proteins, with 90% purity for CCR5 N-Linker-E2 containing NaCl (Fig. 3C) and 95% purity for CCR5 mN-E1-E2 without NaCl (Fig. 3D). The result supported the advantage of using the intein-mediated self-cleavage system to obtain secreted recombinant proteins with high purity with ease.
**Immunological identification of conformational epitopes for the recombinant proteins**

The purified CCR5 analogs were analyzed by IP with the murine mAbs 3A9 and 2D7, which were the two most potent inhibitors of R5 virus cell entry by recognizing a conformational epitope in the Nt and ECL2, respectively, to confirm the similarity in conformational epitope between CCR5 analogs and natural CCR5, which is a practical method for this purpose (Nisius et al., 2008).

The result showed that both 3A9 and 2D7 could specifically bind to CCR5 N-Linker-E2 and CCR5 mN-E1-E2, while the control proteins (degraded fractions of target protein) could not bind (Fig. 4A), suggesting that the CCR5 analogs reserved the conformational epitope of natural CCR5 and implying that the degraded fractions of CCR5 analogs lost the natural conformational epitope.

To examine which domain (Nt or ECL2) was dominant on the surface of CCR5 N-Linker-E2, a modified indirective ELISA analysis was performed, which showed a value of 0.43811 ± 0.05561 (mean value ± standard deviation value) for mAb2D7, and 1.409 ± 0.14665 for 3A9, statistically significant with $P < 0.01$ (Fig. 4B). The result suggested that the Nt structure in the protein CCR5 N-Linker-E2 contained a significantly more effective epitope than the ECL2 domain did. A similar experiment showed no difference between the epitopes of the Nt and ECL2 domains for CCR5 mN-E1-E2. Indirect ELISA with 2D7 and 3A9 showed a value of 1.427 ± 0.069376 and 1.561667 ± 0.181236, respectively, statistically non-significant with $P > 0.05$, implying that the epitope conformation of Nt and ECL2 in CCR5 mN-E1-E2 was exposed to the surface at similar opportunities (Fig. 4B). In addition, it is noteworthy that CCR5 mN-E1-E2 bound with mAbs 2D7 and 3A9 more strongly than CCR5 N-Linker-E2 did, which might imply that CCR5 mN-E1-E2 contained more important epitopes related to the mAbs binding, such as ECL1 and disulfide bridge between ECL1 and ECL2.

The affinity binding between CCR5 N-Linker-E2 and Met-RANTES was determined by direct competitive ELISA in a competitive gradient with anti-Met-RANTES. The data obtained through logistical model curve fitting by using CurveExpert 1.3 showed a logarithmic equation of $y = 50.983655/(1 - 1.2575622exp(-0.94197373x))$, with $R^2 = 0.993$, as the best fitted model with high reliability (Fig. 4C). A similar result was obtained for CCR5 mN-E1-E2, which showed a logarithmic equation of $y = 101.10861 - 19.58106x$, with $R^2 = 0.999$ (Fig. 4D). Competitive ELISA experiments showed that the reaction of the CCR5 analogs with Met-RANTES was concentration-dependent. And CCR5 mN-E1-E2 bound with Met-RANTES better than CCR5 N-Linker-E2 did, which suggested that CCR5 mN-E1-E2 was more suitable for Met-RANTES recognition.

**Fig. 4.** Immunoassay of recombinant extracellular domains of CCR5 analogs. (A) IP of recombinant proteins CCR5 N-Linker-E2 and CCR5 mN-E1-E2 with mAbs 2D7 and 3A9 by silver staining in 16.5% Tris–Tricine–SDS–PAGE. (B) Comparison of exposure epitopes between recombinant proteins CCR5 N-Linker-E2 and CCR5 mN-E1-E2 by indirect ELISA analysis, showing significant differences between the two CCR5 analogs binding to mAb 2D7 and between the two antibodies binding to N-Linker-E2 with $P < 0.05$. (C) Competitive ELISA of recombinant proteins CCR5 N-Linker-E2 with Met-RANTES. (D) Competitive ELISA of recombinant proteins CCR5 mN-E1-E2 with Met-RANTES.
The result of the conformational-identification assay suggested that the two CCR5 analogs could be used in screening for affinity peptides and in blocking the cellular-surface epitopes, probably even in competitively binding with HIV-1 gp120, although further experiments are required.

Screening of high-affinity peptides from phage random-peptide library using CCR5 analogs

After four rigid rounds of biopanning against the recombinant proteins in vitro, 15 and 11 phagotopes against N-Linker-E2 and CCR5 mN-E1-E2, respectively, were isolated from the phage random-peptide library and ELISA was used to determine the affinity of the isolated phage clones. The amino acid sequences of screened phagotopes were deduced according to the genetic code table provided along with the kit (Ph.D.12™ Phage Display Peptide Library Kit) by DNA sequencing. Among the 15 phagotopes targeted with the recombinant protein CCR5 N-Linker-E2, seven unique phage clones or peptide sequences were obtained. Positive phage clones no. 1, 3, 4, 11 and 15 (excluding clones with identical exogenous sequences) were further amplified in E.coli ER2738 for the phage titers. Affinity analysis with ELISA indicated that five of the seven clones could bind effectively to CCR5 N-Linker-E2 upon phages titer of 1 x 10^{12}, except clones no. 2 and 14 (Fig. 5A), compared with positive and negative controls. Among the 11 phagotopes targeted with CCR5 N-Linker-E2, seven unique phage clones or peptide sequences were obtained. Similar panning and affinity testing experiments against CCR5 mN-E1-E2 resulted in four clones (no. 1201, 1206, 1212 and 1219) that bound effectively to the recombinant protein.

<table>
<thead>
<tr>
<th>Phagotope</th>
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<th>Motifs</th>
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<td>11</td>
<td>ASMCEVVTGAE(1)</td>
<td>1.740b</td>
<td>SS,SV</td>
</tr>
<tr>
<td>14</td>
<td>QTSRPMRRL(1)</td>
<td>0.528</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>QSPHSISWPR(2)</td>
<td>1.700b</td>
<td>SS,PL</td>
</tr>
</tbody>
</table>

Values indicate units (415 nm) by capture ELISA measured at 30 min.

Phagotope also positive by direct ELISA.

CCR5 mN-E1-E2 (Fig. 5B). Eventually, we got nine phagotopes highly positive by phage capture ELISA.

Homology analysis of the deduced amino acid sequences

Multiple sequence alignment analysis of the affinity-peptide sequences found similar motifs (Table I). The motifs SL, SS, SV or PL contained in all of the positive affinity peptides were found in the conserved structural elements of HIV gp120 or gp41, with SL motif occurring five times, SS motif occurring three times and SV or PL motif occurring twice (Table I), which could be epitope motifs in the 3D structure.
of HIV-1 gp120, implying the potential value of these affinity peptides as a diagnostic and cell-targeting reagent, although further experiments are required.

Discussion

In the present study, we constructed two kinds of recombinant proteins that mimic CCR5 efficient epitopes, particularly domains of the Nt and ECLs, which are known to be involved in gp120 binding. The produced CCR5 analogs were then used to acquire affinity peptides capable of binding to CCR5 for future development of antagonists.

In addition to CD4 receptor, chemokine receptors CXCR4 and CCR5 are known to be important co-receptors for HIV-1 infection (Alkhatib et al., 1996). CCR5 belongs to the superfamily of G protein-coupled receptors. The Nt and ECL2 of CCR5 play an important role in HIV-1 invasion (Dragic et al., 1998; Farzan et al., 1998). At the same time, previous studies showed that the peptide Ala95-96 of CCR5 induces high titer anti-CCR5 antibodies in chicken and therefore anti-ECL1 antibodies are expected to play a role in the natural resistance to HIV infection (Doranz et al., 1997). The results of the above studies suggested that the Nt, ECL1 and ECL2 of CCR5 are ideal targets in the development of CCR5 antagonists, which was the basis for designing the recombinant protein CCR5 N-Linker-E2 in this study. Another CCR5 analog named CCR5 mN-E1-E2 has been designed based on prediction of advantage epitopes by bioinformatics using forecasting software and online databases, such as DNA Star, PDB, SWISS-MODEL and so on, and constructed by linking with flexible peptides for accommodating the space required for its conformational change. The acquired CCR5 analogs were proved functional and could be potential candidates for developing CCR5 peptide antagonist or HIV inhibitors.

Different expression systems have been proved beneficial for GPCR production (Sarramegna et al., 2003), such as baculovirus, the insect cell and the yeast cell. Although E. coli cannot make many posttranslational modifications, expression of recombinant proteins in E. coli is more economical and with high-level yield (Ren et al., 2009). And it did have the ability to secrete target protein with proper folding and disulfide bond formation (Yoon et al., 2010). Even though post-translational sulfation of tyrosines in the Nt regions of chemokine receptors has been shown to be important in the binding affinity for chemokine ligands (Huang et al., 2007; Jen et al., 2009), the function of many CCR5 antagonists such as PSC-RANTES (Kawamura et al., 2004), Met-RANTES (Proudfoot et al., 1996), AOP-RANTES (Mack et al., 1998) and NNY-RANTES (Sabbe et al., 2001) did not completely rely on the sulfated tyrosine residues of Nt, but involved the induction of long-term intracellular sequestration of CCR5. These antagonists induced significant levels of CCR5 internalization without detectable G protein-linked signaling activity for inhibiting HIV entry and infection by competing with the virus for CCR5 binding (Gaertner et al., 2008). The result of this study showed that using E. coli as the expression system did provide us with high yield, easy purification and functional CCR5 analogs. In addition, considering the codon usage bias of E. coli (Bulmer, 1991), the amino acid residues Asp95 and Gly97 in the ECL1 were re-encoded from gac to gat and from gga to ggt, respectively, for high-level expression in E. coli strain BL21 (DE3).

Assays using CCR5 mAbs 2D7/3A9 confirmed the close similarity between antigen–epitope conformations of CCR5 analogs and natural CCR5. mAb 2D7 completely blocked the binding and chemotaxis of the three natural chemokine ligands of CCR5, RANTES, macrophage inflammatory protein MIP-1a and MIP-1b, to CCR5 transfectedants. And mAb 3A9 prevented monocyte infection but only slowed HIV replication in microglia and produced a marked inhibition of viral DNA (Ghospade et al., 1998). When the binding of mAb 2D7 with the second extracellular domain was lost in CCR5 mutants lacking the disulfide bridge between ECL-1 and ECL-2 (Khurana et al., 2005), the epitope on the main Nt binding site and the ECL1 were crucial for binding with mAb 3A9 (Königs et al., 2000). The extracellular CCR5 domains, i.e. CCR5 Nt and the ECL2, were confirmed to interact with the RANTES core where the N-loop/B1-strand region is the major determinant (Vangelista et al., 2008). This study showed that CCR5 mN-E1-E2 bound more strongly with mAbs 2D7 and 3A9 than CCR5 N-Linker-E2 did. It is probable that CCR5 mN-E1-E2 contained more important epitopes related to the mAbs binding, such as the ECL1 and the disulfide bridge between ECL-1 and ECL-2 (Fig. 3B), consistent with previous studies. In fact, the competitive recognition of met-RANTES also showed better results with CCR5 mN-E1-E2 than with N-Linker-E2, supporting the importance of ECL1 and the disulfide bridge between ECL-1 and ECL-2 for CCR5 antigen–epitope conformations.

On the basis of the above conformational-identification results, high-affinity peptides targeted on CCR5 analogs were obtained in this study by phage display technology, which had been applied for screening affinity peptides with anti-CCR5 potency. Patent cooperation treaty manual of WO/2005/06489, WO/2006/138745 and WO/2008/074895 had opened technology separately in multiple peptide molecules from extracellular domains of CCR5, which generated CCR5 neutralizing antibodies for the treatment of inflammation and disease. Currently, there are still no CCR5 peptide antagonists proved by food and drug administration (FDA), although one kind of small molecule/non-peptide antagonist named maraviroc (molecular formula: C29H41F2N5O) has been developed by the Pfizer company, which is the only CCR5 antagonists approved by FDA. In comparison with the small molecule antagonists which might produce toxic side effects, peptide antagonists could bind specifically to the CCR5-specific extracellular domains and exhibit inhibitory activity, without toxic side effects.

A previous study on screening high-affinity peptides from phage peptide libraries had mostly focused on the cell surface directly. Wang (Wang et al., 2006) acquired a CCR5 high-affinity and specific-binding peptide encoding AFDWTVFPSIL on CHO/CCR5 cells from a phage random 12-mer peptide library, and further proved that the 12-mer peptide could bind to ECL2 domain of CCR5 specifically. Comparing with the screening strategy targeting on CCR5 analogs in vitro in this study, screening high-affinity peptides from phage peptide libraries targeting directly on the cell surface could not guarantee the single kind of target molecular CCR5 and thus increased the blind chance of bio-panning. Although the recombinant proteins including the
extracellular domains were not equivalent to full-length CCR5, we evidenced the conformational epitopes of them by trails so that the CCR5 recombinant proteins could be reasonable as antigens. It is worth mentioning that an effective method was used in this study to determine the correct conformational epitope of the recombinant CCR5 analogs in vitro, which ensured that affinity peptides with correct conformational epitopes could be obtained in the subsequent screening against CCR5 analogs. Hopefully the acquired peptides with high affinity and specificity could be used as candidate CCR5 peptide antagonists, although activity experiments such as cellular activity assay of these affinity peptides are still required.

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References