Selection of CC Chemokine Receptor 5-Binding Peptide from a Phage Display Peptide Library

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Human CC chemokine receptor (CCR) 5 is a G protein-coupled receptor involved in a broad range of human diseases that mediates HIV-1 viral entry into cells. Certain small molecule receptor antagonists to CCR5 have been useful in therapy for these diseases. In this study, CCR5-expressing CHO cells (CHO/CCR5 cells) were used to select CCR5-binding peptides from a phage-displayed 12-mers peptide library. All of the 30 clones selected from the library showed specific binding to CHO/CCR5 cells by enzyme linked immunosorbent assay (ELISA). Seventeen out of the 30 clones shared the amino acid motif AFDWTFVPSLIL. The motif-containing phages and synthetic peptide AFDWTFVPSLIL blocked the binding of mAb 2D7 to CHO/CCR5 cells and competitively inhibited the ability of chemokine regulated on activation normal T cell expressed and secreted (RANTES) binding to CHO/CCR5 cells. Furthermore, the peptide AFDWTFVPSLIL also inhibited RANTES induced increase in the intracellular Ca2+ level in CHO/CCR5 cells. These results suggest that the peptide AFDWTFVPSLIL was specific for CCR5 and that it might become a CCR5 antagonist.

Key words: phage display; small peptide; screening; human CC chemokine receptor (CCR) 5; CHO cells

Materials and Methods

Generation of the CHO/CCR5 cell line. Chinese

Utilization of combinatorial peptide libraries expressed on filamentous bacteriophages9 is a powerful tool for studies of ligand-receptor interaction and for the discovery of pharmacologically active lead compounds, such as peptide agonists or antagonists.11 In this study, CHO cells stably expressing human CCR5 (CHO/CCR5 cells) were used to select CHO/CCR5-binding peptides from a phage peptide library, and a small peptide specific for CCR5 was identified. Further experiments indicated that the peptide selected blocked the binding of mAb 2D7 and RANTES to CCR5; the peptide also inhibited CCR5-mediated Ca2+-signaling, suggesting that it is a CCR5 antagonist and might be used to develop a new therapeutic agent.

Abbreviations: AOP, aminooxypentane; CCR, CC chemokine receptor; ECL2, the second extracellular loop; ELISA, enzyme-linked immunosorbent assay; gp120, glycoprotein; mAb, monoclonal antibody; MCP, monocyte chemotactic protein; Met, methionine; MIP, macrophage inflammatory protein; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; RANTES, regulated on activation normal T cell expressed and secreted

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hamster ovary (CHO) cells (ATCC, Rockville, MD) were transected with eucaryotic expressing vector pcDNA-ccr5 containing CCR5 cDNA. The coding region of the CCR5 gene was amplified (forward primer, 5'-CTCGGTATTGGGAAAACAAGATGATTAT-3', and reverse primer, 5'-CTCGTGACCATGTGCACA-ACCTGACTG-3') from genomic DNA of human PBMCs, and subcloned in vector pcDNA3.1 with BamHI and XbaI. Selection of transfected CHO cells was made for 18 d with 400 μg/ml G418 (Invitrogen, Carlsbad, CA). Cell surface expression of CCR5 was measured by flow cytometry using mAbs-recognizing extracellular epitope of the receptor.13,14 The phycoerythrin (PE)-conjugated anti-CCR5 monoclonal antibodies 2D7 (a second extracellular loop mAb) and 3A9 (a NH terminus-specific mAb) were purchased from R & D Systems (Minneapolis, MN). CHO cells stably expressing human CCR5 (CHO/CCR5 cells) were under continuous selection in medium containing G418 (400 μg/ml). CHO cells and CHO/CCR5 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and grown at 37°C in the presence of 5% CO₂.

Phage library and bacterial strain. The phage library and the E. coli ER2738 host strain were obtained from New England Biolabs (Ipswich, MA). The Ph.D.-12 Phage Display Peptide Library is based on a combinatorial library of random peptide 12-mers fused to the N-terminal of the minor coat protein (pIII) of the M13 phage. The titer of the library is 10¹³ pfu/ml, and the complexity is 2.7 × 10⁹ transformants. M13KO7 (helper phage, without any peptide insert) is an M13 derivative, that carries the mutation Met40Ile in gII, with the origin of replication from P15A and the kanamycin resistance gene from Tn903 both inserted within the M13 origin of replication.

In vitro selections According to a modified protocol,13,14 before subtraction, the CHO cells were detached with 0.25% trypsin in PBS, counted, and resuspended at 10⁷ cells/ml in PBS and 1% BSA containing 1.5 × 10¹¹ phage/ml. The mix was incubated for 2 h at 4°C with slow shaking, the cells were pelleted in a microfuge at 1,500 rpm for 1 min, and the subtracted supernatant was recovered and used to resuspend another 10⁷ CHO cells. This subtraction cycle was repeated twice. Selection was done by resuspending 5 × 10¹⁰ CHO/CCR5 cells with the subtracted phage supernatant and incubation with slow shaking for 2 h at 4°C. The CHO/CCR5 cells were then washed five times in cold PBS, 1% BSA, and 0.1% Tween-20, and tubes were changed between washes (microfuge, 1,500 rpm, 1 min). Cell-bound phages were eluted with 1.0 ml 0.2 M Glycine–HCl (pH 2.2) and 1 mg/ml BSA for 10 min on ice. The cells were pelleted as above and the supernatant was recovered and neutralized with 150 μl 1 M Tris–HCl (pH 9.1). The recovered pool of phages was titered, amplified (using the E. coli ER2738 host strain and following the supplier’s protocol), and titered again before the next round of subtraction/selection. Two rounds of subtraction were done before each round of selection, and four rounds of selection were performed as above, except that the concentration of Tween-20 was increased to 0.2% in the third and fourth rounds of selection.

Whole-cell ELISA. The exponentially growing CHO and CHO/CCR5 cells were transferred to 96-well microtitre plates (3 × 10⁶ cells/well). Amplified phage clones were randomly selected after the fourth round of selection and preincubated for 30 min with 1 × blocking buffer (2% nonfat dry milk in 1 × PBS). About 2 × 10¹² transducing units (TU) were added to each well and then incubated with the cells for 2 h at room temperature. The cells were centrifuged at 1,500 rpm for 2 min and then washed extensively with wash buffer (1 × PBS with 0.05% Tween-20) the amount of bound phages was detected with horseradish (HRP)-conjugated anti-M13 phage antibody (Amersham Biosciences). The development was performed by the addition of 2',2'-Azino-Bis (3-Ethylbenzthiazoline-6-Sulphonic Acid) diammonium salt (ABTS) (Sigma, Saint Louis, MO), and read at 405 nm in an ELISA Reader (Bio Rad, Hercules, CA). Helper phage M13KO7 was used as a negative control.

DNA and amino acid sequence analysis. DNA phagemids were prepared from identified phage clones by the standard method15 and sequenced with an ABI Prism kit (Perkin Elmer Applied Biosystems, Boston, MA) automatically. The primer used for sequencing was 5'-CCC TCA TAG TTA GCG TAA CG-3' (helper phage, without any peptide insert) is an M13 derivative, that carries the mutation Met40Ile in gII, with the origin of replication from P15A and the kanamycin resistance gene from Tn903 both inserted within the M13 origin of replication.

Determination of phage binding efficiency by titration. After four rounds of subtraction/selection, single plaques were selected for preparation of purified stocks. Phage binding efficiency was determined by incubating 1.5 × 10¹¹ phages with 5 × 10⁶ target or control cells at 4°C. Incubation, washings, and elution conditions were performed as “In vitro selections.” After titering, the phage binding efficiency was calculated by dividing the number of recovered plaque-forming phages (output) by the input number of plaque-forming phages. Helper phage M13KO7 was used as a negative control.
Receptor binding assay. CHO/CCR5 cells were centrifuged at 500 × g for 5 min and washed three times in an isotonic PBS buffer (supplemented with 0.5% BSA) to remove any residual growth factors that might be present in the culture medium. Cells were then resuspended in the same buffer to a final concentration of 4 × 10^6 cells/ml and 25 μl of cells (1 × 10^5) was transferred to a 5 ml FACS tube for staining. 1:5 × 10^11 phages or synthetic peptide was added to the FACS tube and incubated for 30 min at 4°C, and 10 μl phycoerythrin-conjugated anti-CCR5 reagent was then added to the FACS tube and these were incubated for 40 min at 4°C. Unreacted anti-CCR5 reagent was removed by washing the cells twice in 4 ml of the same PBS buffer. The cells were resuspended in 500 μl of PBS buffer for final flow cytometric analysis using CellQuest software (BD FACS Vantage SE). Helper phage M13KO7 was used as negative control.

Competition binding experiment. CHO/CCR5 cells were cultured in 96-well microtitre plates overnight at 37°C, 5% CO2. The second day, after removing the cell culture, RANTES (10 ng) and 1:5 × 10^11 different phage clones or various concentrations of synthetic peptide were added to each well, these were incubated for 1 h at 37°C and then were centrifuged at 500 × g for 5 min, and the RANTES contents of the supernatants were identified with a Quantikine Human RANTES kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The cells were then washed twice with the assay buffer and resuspended at 1 × 10^7 cells/ml in the same buffer. The cells were exposed to synthetic peptide for 50 s and then 50 nM RANTES was added. The relative increase in the cytoplasmic Ca^{2+} level was monitored by confocal microscopy (Zeiss LSM 510 META). Calibration was performed with 10% Triton X-100 for total Ca^{2+} release and with 5 mM EGTA for Ca^{2+} chelation.

Statistical analysis. All values were expressed as means ± SE. Analysis of variance by one-tailed Student's *t* test was used to identify significant differences in multiple comparisons. A level of *P < 0.05* was considered statistically significant.

Results

CCR5 expressing on CHO cells

We examined the expression of CCR5 on the transfected CHO cells using flow cytometry techniques. As Fig. 1 shows, the transfected CHO cells (CHO/CCR5 cells) specifically bound more mAb 3A9 (Fig. 1D), whereas the untransfected CHO cells did not bind mAb.
3A9 (Fig. 1B). This suggests that trypsinisation of the cells does not deplete the CCR5 of a transfected CHO cell (CHO/CCR5 cell) surface.

Specific enrichment of binding phages

In order to enrich CHO/CCR5 cell-binding phages from the phage peptide library, four rounds of selection with CHO/CCR5 cells were performed. The enrichment was determined by the use of the output/input ratio of phages after each round of selection. After four rounds of such selection, an enrichment of approximately $10^5$ was achieved for specifically eluted phages (Table 1), suggesting that biopanning was effective. Conversely, the number of phage particles recovered by nonspecific elution from the CHO/CCR5 cells decreased progressively over the four rounds of selection (data not shown).

Identification of the binding phages

Thirty clones were selected randomly from the selected phages after the fourth round of selection and the specificity was examined by whole-cell ELISA. All of the 30 clones showed binding ability to CHO/CCR5 cells, except for the helper phages M13K07, and the difference was significant ($P < 0.05$).

Analysis of exogenous sequences of positive phage clones

The exogenous DNA sequences of 30 clones showing high affinity to CHO/CCR5 cells were analyzed, and their amino acid sequences were deduced. As shown in Table 2, of the 30 amino acid sequences, 17 shared the motif AFDWTFVPSLIL. Online analysis of the selected motif using Gen-Bank did not reveal any significant homology with known proteins or chemokines, suggesting that the motif is a mimic sequence of CC chemokines epitope that binds to CCR5 molecules on cell membrane.

Binding efficiency of the positive phages

Figure 2 shows the binding efficiency of the nine positive phages, and their exogenous AA sequences are presented in Table 2. The binding efficiency of AFD phage to the CHO/CCR5 cells was about 1,000-fold higher than the CHO cells, and about 20–60-fold higher than the other eight phages. This result suggests that AFD phage harboring the AFDWTFVPSLIL motif has the highest binding efficiency to CCR5. It was further characterized.

The positive phages and synthetic peptide block the binding of mAb 2D7 to the CCR5 receptor on the cell membrane

Phycoerythrin-conjugated 2D7 (the second extracellular loop specific mAb) and 3A9 (the NH2 terminus-specific mAb) were used to examine whether the AFD phage or synthetic peptide AFDWTFVPSLIL would interact with CCR5. As Fig. 3 shows, the AFDWTFVPSLIL motif-containing phage ($1.5 \times 10^{11}$ phages) inhibited the binding activity of 2D7 mAb (C), but not that of 3A9 mAb (A) to CHO/CCR5 cells. Moreover, the AFDWTFVPSLIL peptide markedly antagonized 2D7 binding to CHO/CCR5 cells (D), and the inhibitory effect was detected at low peptide concentrations (10 µg/ml). But the AFDWTFVPSLIL peptide did not affect the binding of 3A9 to CCR5 transfecants even at higher concentrations (1 mg/ml) (B).

| Table 1. Enrichment of Phages for Each Round of Selection from a Displayed 12-mers Peptide Library |
|----------------|----------------|----------------|----------------|
| Rounds         | Selected phages | Eluted phages  | Ratio          |
| 1              | $1.5 \times 10^3$ | $3.4 \times 10^3$ | $2.3 \times 10^{-4}$ |
| 2              | $1.5 \times 10^3$ | $6.2 \times 10^3$  | $4.1 \times 10^{-6}$ |
| 3              | $1.5 \times 10^3$ | $2.5 \times 10^3$  | $1.7 \times 10^{-3}$ |
| 4              | $1.5 \times 10^3$ | $3.0 \times 10^3$  | $2.0 \times 10^{-3}$ |
| *TU: transducing unit |

| Table 2. Exogenous Amino Acid Sequences of Positive Phage Clones Selected from 12-mers Peptide Library |
|----------------|----------------|----------------|----------------|
| Amino acid sequence | Frequency |
| AFDWTFVPSLIL     | 17            |
| SLDWSHPVPLLYL    | 3             |
| GPFNKSGLGSP      | 2             |
| LDVREPWWYPLLP     | 2             |
| LEPPOLWWPWHE      | 2             |
| WSSNLRLVPWPS      | 1             |
| SLYVAPWDDPP       | 1             |
| QLVIFPWNVTYP      | 1             |
| MLTVEPWTISNT      | 1             |

Fig. 2. Binding Efficiency of the Positive Phages.

Selected purified phages were assayed for cell-binding efficiency. The positive phages were AFD, SLD, GPF, LDV, LEP, WSS, SLY, QLV, and MLT (referring to the first three amino acid letters only). Mean values from three independent experiments are shown. H, helper phage (M13K07) without any peptide inserts. The difference between AFD phage-binding CHO/CCR5 cells and binding CHO cell or between AFD phages binding CHO/CCR5 cells and the other eight phages binding CHO/CCR5 cells were significant ($P < 0.05$).
synthetic peptide with the binding of CCR5 to its ligand RANTES, a binding inhibition experiment was carried out. As Fig. 4A indicates, the AFDWTFVPSLIL motif-containing phages competitively inhibited the binding of RANTES to CHO/CCR5 cells and 1.5 × 10¹¹ phages reduced the binding activity of RANTES to CHO/CCR5 cells to 70%. In contrast, the other phage clones had only small effect on the binding of RANTES to CCR5 transfectants. We also tested the ability of the AFDWTFVPSLIL peptide to inhibit RANTES binding to CCR5. As shown in Fig. 4B, the AFDWTFVPSLIL peptide obviously antagonized RANTES binding to CHO/CCR5 cells: half maximal inhibition (IC₅₀) was at 3.6 μg/ml (2.56 μM).

The synthetic peptide AFDWTFVPSLIL inhibits CCR5-mediated Ca²⁺ signaling

In chemokine-induced Ca²⁺ mobilization experiments, RANTES clearly increased the intracellular Ca²⁺ level in CHO/CCR5 cells at a concentration of 50 nM (Fig. 5). The addition of 10 μg/ml peptide AFDWTFVPSLIL did not affect the Ca²⁺ level, but completely abrogated the RANTES-induced increase in the intracellular Ca²⁺ level in CHO/CCR5 cells. This result indicates that peptide AFDWTFVPSLIL can block CCR5-mediated Ca²⁺-signaling.

Discussion

CCR5 is a functional receptor for the CC-chemokines and has been found to act as a coreceptor of human immunodeficiency virus-1 (HIV-1), which mediates the binding of viral envelope protein gp120 to the cell
surface, allowing HIV-1 subsequent entry into the target cells. Resistance to HIV infection was found in individual cells with deletion in the coding sequence of CCR5, resulting in the absence of functional coreceptors at the cell surface, which makes this receptor an attractive target for inhibition of HIV replication. Although the natural ligands for CCR5, such as RANTES, MIP-1α, MIP-1β, and their modifications (Met-RANTES and AOP-RANTES), and the non-peptide CCR5 antagonist TAK-779, are known to block HIV infection, no small peptide CCR5 antagonists from the designed peptide library have been identified. In this study, we identified a novel 12-mers peptide from a phage display peptide library. The peptide selected did not show any significant homology with known proteins or chemokines, but the peptide-containing phage and synthetic peptide AFDWTFVPSLIL competitively inhibited the binding of RANTES to CHO/CCR5 cells. The AFD phage and synthetic peptide also blocked 2D7 mAb binding to CHO/CCR5 cells. Furthermore, the synthetic peptide also abrogates the RANTES-induced increase in the intracellular Ca²⁺ level in CHO/CCR5 cells.

2D7 mAb is a monoclonal antibody directed to the second extracellular loop of CCR5. The selected peptide inhibits the binding of 2D7 mAb to CHO/CCR5 cells, but no inhibition of the peptide on the binding of another anti-CCR5 mAb 3A9 (directed to the N terminal domain of CCR5) to CHO/CCR5 cells was found, suggesting that the selected peptide AFDWTFVPSLIL interacts/


