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SCIENCE DIRECT
Molecular Immunology 43 (2006) 1573–1578
Molecular Immunology
www.elsevier.com/locate/molimm

Identification of peptide ligands to the chemokine receptor CCR5 and their maturation by gene shuffling

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Received 16 August 2005; accepted 28 September 2005
Available online 8 November 2005

Abstract

The determination of protein–protein interactions and their role in diverse pathophysiological processes is a promising approach to the identification of molecules of therapeutic potential. This paper describes the identification of peptidic CCR5 receptor ligands as potential drug leads against HIV-1 infection using in vitro evolution based on phage display. A phage-displayed peptide library was used to select for anti-CCR5 peptide. Further in vitro evolution of the peptide by exon shuffling was performed to identify peptides with optimized characteristics for CCR5 receptor. This peptide inhibited HIV coreceptor activity in a cell fusion assay with an IC_{50} of 5 μM. It did not exhibit either agonistic or antagonistic activity on CCR5 in the concentration range used. To our knowledge, this is a first report that describes the identification of peptide ligands specific to the CCR5 receptor from a phage-displayed library and the maturation of the selected peptide sequence by gene shuffling.

Keywords: CCR5 receptor; Peptide; Phage display; Gene shuffling; In vitro maturation; HIV

1. Introduction

The CCR5 receptor is a G protein-coupled receptor (GPCR) belonging to the family of chemokine receptors which are responsible for modulating immune and inflammatory responses by regulating the activation and directed migration of leukocytes (Signoret et al., 2000; Stantchev and Broder, 2001). CCR5 is thought to be involved in the recruitment of leukocytes in a growing number of inflammatory diseases, such as rheumatoid arthritis (Pokorny et al., 2005), asthma (Srivastava et al., 2003), Crohn's disease (Oki et al., 2005) and may also be associated with cancer development (Robinson et al., 2003). Furthermore, it plays a major role in the entry of the HIV virus into the host cell (Feng et al., 1996). This key role in the HIV infection has brought CCR5 forward as a major target for drug development aiming at blocking viral infection.

A number of small molecule CCR5 inhibitors have been identified (Rusconi et al., 2004), but a number of these have proven to be toxic during development, owing to interactions with other structures other than CCR5 (Cooley and Lewin, 2003). The screening of chemical libraries remains the most widely used approach to identify ligands as potential drug leads, the use of biological compounds is gaining in interest. Peptides can be evolved in vitro by phage display techniques towards higher efficacies or affinities to their receptor with little prior knowledge of their interaction mechanisms. A pertinent advantage of peptide ligand drug discovery in this context is that it is often possible to identify molecules that bind specifically to functional sites on target proteins (Gron and Hyde-DeRuyscher, 2000).

Combinatorial infection and in vivo recombination was first suggested as a strategy for making large phage-displayed antibody libraries (Waterhouse et al., 1993). A modified gene shuffling strategy involving a self-splicing group I intron was subsequently used for the construction of very large peptide repertoires for the selection of enzyme substrate (Fisch et al., 1996).

The aim of this study was to use a phage display gene shuffling approach to isolate and optimize peptide ligands capable of blocking the ability of HIV to use CCR5 to infect target cells.

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doi:10.1016/j.molimm.2005.09.025

2. Results

2.1. Selection of phage-displayed peptide library

An M13 phage library was constructed to display peptides as a fusion to the N-terminal extremity of the phage capsid protein 3 (pIII). The library displayed the following peptide sequence: $CDX_3KPCALLRYX_{10}$ (where X represents any amino acid). Sequencing of randomly picked colonies indicated that the complexity of the library was $0.5–1.0 \times 10^9$ different peptide sequences with 70% of phage displaying the full-size peptide. The library was subjected to cycles of selection on CHO cells stably expressing the CCR5 receptor. To decrease the background of phages binding to other cell surface proteins, the phage library was pre-adsorbed on wild-type (CCR5-negative) CHO cells before each of selection cycle.

n-Nonanoyl-RANTES (NNY-RANTES) is a high affinity synthetic analog of the natural chemokine RANTES. NNY-RANTES inhibits HIV entry and replication at low nanomolar concentrations (Mosier et al., 1999) and was therefore chosen as a competitive inhibitor to release phage bound to CCR5 (see Fig. 1, selection 1). Elution with NNY-RANTES at 100 nM yielded an enrichment of 100-fold of the phage population binding to the CCR5-expressing cells, suggesting effective panning and selection. Twenty randomly picked phage clones, isolated after four rounds of selection, contained an identical DNA sequence encoding the 15-mer peptide sequence ALLRYNPFYYLSFSP (peptide CR1-lin). The peptide sequence containing the N-terminal cyclic domain did not seem to provide the correct structural motifs required for binding to the CCR5 receptor and therefore was probably deleterious during the selection. The disulfide bond of the peptide might generate a steric hindrance preventing thus binding to an accessible domain on the receptor.

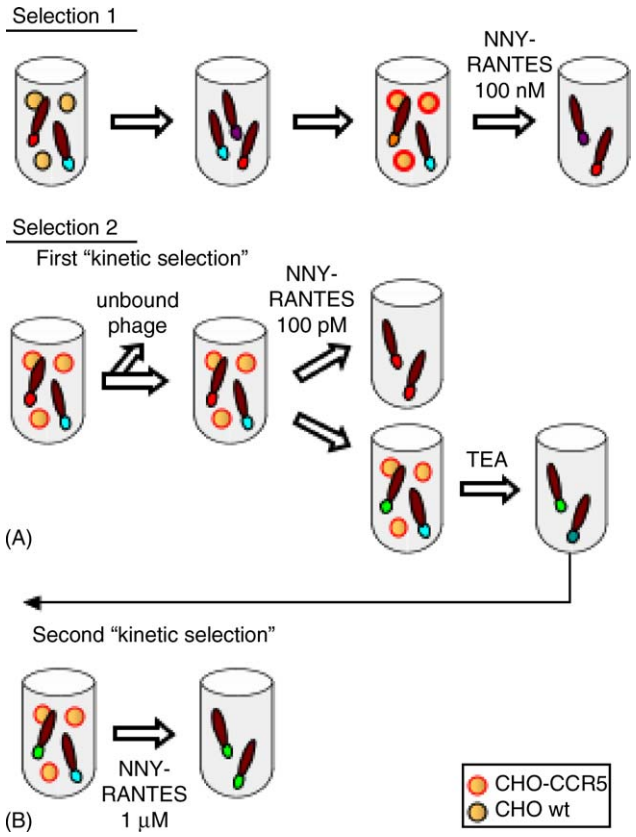

Fig. 1. Selection strategies used for the selection of peptide libraries. *Selection 1*: cell surface selection. After absorption on CHO *wt* cells, the phage library is incubated with CHO-CCR5 cells and phage are eluted with 100 nM NNY-RANTES. *Selection 2A*: first "kinetic selection". The phage library is incubated with CHO-CCR5 cells for 30 s. Bound phage are eluted with 100 pM NNY-RANTES followed by 200 mM TEA. *Selection 2B*: second "kinetic selection". TEA eluted phage (rounds 3 and 4) from first "kinetic selection" are incubated with CHO-CCR5 cells for 5 min. Bound phage are eluted with 1 μM RANTES.

2.2. In vitro evolution and maturation of CCR5 peptide ligands

In vitro evolution can be used to increase the binding affinity and biological activity of a selected peptide sequence (Fisch et al., 1996). Using the exon shuffling method, a new peptide library based on the sequence of peptide CR1-lin was constructed, with an additional 10 amino acids random library appended at its N-terminus: X_{10}-CR1. After Cre-mediated site-directed recombination between the plasmid encoding for the 10 random amino acids and the phage vector harboring the peptide CR1 (NPFYYLSFSP), the size of the recombined library X_{10}-CR1 was determined to be 1×10^9 different clones, with about 44% of recombined phage clones (data not shown).

An initial selection of this novel peptide library using the same conditions as before led to the isolation of non-recombined peptides harboring only the CR1-lin sequence (data not shown). To modify the stringency of selection, a new screening procedure was adopted that favored the selection for slower off-rate phage peptides binding to the receptor. The phage library was incubated with CHO-CCR5 cells without pre-adsorption to non-expressing cells. Phage washed out with NNY-RANTES at 100 pM were discarded and the remaining phage were eluted with a high pH and used for further rounds of selection (see Fig. 1, selection 2A). To further increase the affinity for the CCR5 receptor, the phage population of rounds 3 and 4 were used for additional rounds of selection on CHO-CCR5 (see Fig. 1, selection 2B). At this stage, phage were eluted with 1 μM NNY-RANTES. For both selection schemes, an enrichment of the specific phage population was observed (162-fold for selection A and 15–100-fold for selection B). Sequencing of phage clones from round 4 revealed that 94% and 81% of the sequences corresponded to one single amino acid sequence: LLDSTFFTADALLRYNPFYYLSFSP, peptide CR2.

3. Functional characterization of selected peptides

3.1. Binding affinity

To determine the affinity of the CR2 peptide, a competition assay was performed using radioiodinated RANTES (Fig. 2) as a tracer. Binding of ^{125}I-RANTES to CCR5 cells was almost completely inhibited by CR2 at 100 μM, the highest concentration at which the peptide remains fully soluble. The control peptide