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A biologically active vMIP-II-IgG3-TfN fusion protein, secreted from methylotrophic yeast *Pichia pastoris*

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ABSTRACT

The viral macrophage inflammatory protein II (vMIP-II) which showed a broad-spectrum interaction with both CC and CXC chemokine receptors including CCR5 and CXCR4, two principal coreceptors for the cell entry of human immunodeficiency virus. To explore the feasibility of using TfN as a carrier moiety for delivery of therapeutic proteins, a genetically engineered vMIP-II-IgG3-TfN fusion gene was loaded into the yeast expression vector pPICZα. The linearized recombinant plasmid pPICZα-vMIP-II-IgG3-TfN was transformed into X33 competent cells. The recombinant protein was expressed in methylotrophic yeast *Pichia pastoris* and was confirmed to have expected molecular mass of 48 kDa by SDS–PAGE. Using methods combining ammonium sulfate precipitation, dialysis, ultrafiltration and affinity chromatography, the vMIP-II-IgG3-TfN fusion protein was successfully purified from the supernatant of the broth. Western-blotting analysis showed that 6× His antibody recognized the purified vMIP-II-IgG3-TfN. CD spectrum revealed a positive peak at 196.5 nm and a negative peak at 209 nm. MALDI-TOF MS analysis showed that the purified vMIP-II-IgG3-TfN was an intact and homogeneous protein. The pepsin digestibility assay showed that the vMIP-II-IgG3-TfN fusion protein could be digested into small fragments by pepsin after 2 min treatment. The vMIP-II-IgG3-TfN fusion protein was found to be stable in human plasma for up to 48 h. Furthermore, in vitro bioactivity assay indicated that the vMIP-II-IgG3-TfN fusion protein can block the chemotaxis of U937 cells induced by SDF1α. In total, this study illustrates the development of an active vMIP-II-IgG3-TfN fusion protein expressed in *P. pastoris*.

Introduction

Chemokines are large family of small chemoattractant proteins (8–10 kDa) encoded by the largest family of cytokine genes, and they play a fundamental role in leukocyte migration, activation and hematopoiesis [1]. The viral macrophage inflammatory protein II (vMIP-II)[1], encoded by K4 sequence in human herpes virus 8 (HHV-8) genome, shows a broad spectrum interaction with both CC and CXC chemokine receptors including CCR5 and CXCR4, two principal coreceptors for the cellular entry of human immunodeficiency virus type 1 (HIV-1) [2,3]. Many studies showed that vMIP-II or peptide derived from vMIP-II can inhibit HIV-1 replication [2,4–7].

Transferrins (Tfs) are a superfamily of single-chain, glycosylated proteins present in all vertebrates that transport iron from plasma to cells or help regulate iron levels in biological fluids [8,9]. Tfs are monomeric proteins of 76–81 kDa, depending on the extent of glycosylation, and consist of two structurally similar lobes (termed the N- and C-lobes) connected by a short peptide linker. Each lobe contains a single iron-binding site. The cDNA sequence coding for the N-terminal domain has been cloned and overexpressed in the methylotrophic yeast, and that the purified protein is capable of reversibly binding iron [10,11]. Tf has been considered as a carrier in drug delivery for either crossing the blood–brain barrier or targeting to tumor cells. Significant progress has been made towards the utilization of Tf or the Tf receptor as a targeting ligand to achieve targeted (site-specific) delivery of a wide range of therapeutic agents [11]. Tf-based recombinant fusion proteins have been further investigated as a novel therapeutic strategy for the treatment of various diseases and a promising approach for future development of orally effective peptide and protein drugs [12–14]. Preclinical studies and clinical results indicate that hTf therapy is safe and effective without obvious toxic side effects [11].

The methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for production of a variety of heterologous functionally active recombinant proteins. This system combines the advantages of high expression levels, easy scale-up, inexpensive growth media, and a capacity to perform most of the post-translational modifications characteristic of higher eukaryotes [15–17].

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[1] *Abbreviations used:* bp, base pair; HRP, Horseradish peroxidase; kb, kilobase pair; Tf, Transferrin; vMIP-II, Viral macrophage inflammatory protein-II.

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Additionally, linearized foreign DNA can be inserted in high efficiency via homologous recombination procedures to generate stable cell lines whilst expression vectors can be readily prepared that allow multiple copies of the target protein, multimeric proteins with different subunit structures, or alternatively the target protein and its cognate binding partners, to be expressed [18].

We describe here expression of vMIP-II-IgG3-TfN fusion protein with IgG3 linker in the methylotrophic yeast *Pichia pastoris*. The vMIP-II-IgG3-TfN fusion gene, under control of the AOX1 promoter, was incorporated into the alcohol oxidase chromosomal locus (AOX1), and protein synthesis was induced with methanol. *P. pastoris* can grow to high cell densities and produce high level of secreted soluble vMIP-II-IgG3-TfN proteins. The pepsin digestibility assay was also conducted to determine the stability of the protein to pepsin protease encountered in the mammalian gastric environment.

Materials and methods

Reagents

XbaI, *Sac I*, CIAP and T_4 DNA ligase were from Takara (Osaka, Japan). KOD-plus are from Toybo (Osaka, Japan). Zeocine was from Invitrogen (Carlsbad, CA, USA). Mouse anti-His monoclonal antibody and rabbit anti-transferrin antibody were from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG was purchased from Proteintech (Chicago, IL, USA). The other reagents used in this study were of analytical grade and were commercially available. The yeast culture media, YPD, BMGY and BMMY were prepared according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA).

Strains and vectors

P. pastoris strain X33 and plasmid pPICZα were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant vectors pMD18T-Tf and pPICZα-vMIP-II were constructed in our laboratory.

Oligonucleotides

Primers were synthesized as Table 1.

Construction of expression vector

A DNA fragment cover the coding sequence of human Tf (1–698 aa) was amplified by PCR (Primer pairs: Tf FP, Tf RP) with cDNA from human fetus liver as a template and cloned into pMD18T by TA cloning. The coding sequence of human Tf (20–357 aa) was amplified by PCR (Primer pairs: P1, P2) with pMD18T-Tf plasmid as a template. Furthermore, the amplified DNA fragment recovery from agarose gel was used as a template for PCR (Primer pairs: P2, P3). The PCR product was digested with *Xba* I and was ligated into the same enzyme digested vector pPICZα-vMIP-II, resulting in the recombinant vector pPICZα-vMIP-II-IgG3-TfN. In the pPICZα-vMIP-II-IgG3-TfN, the vMIP-II-IgG3-TfN was under the control of the AOX1 promoter and in-frame with the α-factor signal sequence at N-terminal and 6× His tag at C-terminal. The recombinant vector was transformed into *Escherichia coli* DH5α for amplification and DNA sequence analysis.

Accession number

The nucleotide sequence of amplified *vMIP-II-IgG3-TfN* gene described in this paper has been submitted to GenBank and assigned an Accession No. JX091745.

Transformation of Pichia pastoris and screening for transformants

Recombinant vector pPICZα-vMIP-II-IgG3-TfN was linearized by *Sac* I and introduced into *P. pastoris* X33 by electroporation according to the manual of Invitrogen. Transformants were initially screened on YPDS plate (Containing 100 μg/ml Zeocine) for their resistance to Zeocine. The positive recombinants were identified by PCR using primer pairs (vMIP-II FP, vMIP-II RP and P1, P2) and PCR products were analyzed on a 0.8% agrose gel.

Expression and purification of vMIP-II-IgG3-TfN

The recombinants were inoculated in 5 ml YPD medium at 28 °C overnight, then 2.5 ml culture were transferred to 250 ml BMGY medium and was incubated at 28 °C overnight. The cells were then collected by centrifugation at 5000 *g* for 10 min and resuspended in 500 ml BMMY medium. The cultures were incubated for 96 h at 28 °C with constant shaking, and 100% methanol was added to a final concentration of 0.5% every 24 h to maintain induction. The supernatant was harvested by centrifugation at 5000 *g* for 10 min and then stored at 4 °C until used. Supernatants were pooled and proteins were precipitated with solid ammonium sulfate, the pellet collected by centrifugation at 15,000 for 20 min, and then dissolved in PBS. After filtration with a 0.22 μm Millipore filter, the dilution was dialyzed overnight against PBS to remove ammonium sulfate. After concentration by ultrafiltration with 30 kDa Vivaflow (Sartorius, Goettingen, Germany), the solution was purified by nickel–nitrilotriacetic (Ni–NTA) (Qiagen, Hilden,

Table 1
List of primers used for PCR.

Primer	Sequence
Tf FP	5' AGTCCGACTGTGCTCGCTGCTCA3'
Tf RP	5' GGTGGCAGCCCTACCTCTGAGAT 3'
P1	5' **GGTGACACAACTCAC**ACATCCGGAGTCCCTGATAAAACTGTGAGATG 3'
P2	5' AGTCTAGACTTTCATCTGTTGGGGCTTCT3'
P3	5' AGTCTAGAGACCCCACTT**GGTGACACAACTCAC** 3'
vMIP-II FP	5'CCGGAATTCGGTGACACCCTGGGTGC3'
vMIP-II RP	5'CAGTCTAGAGAGCGAGCGGTAACCGG3'

Note: Shadow indicates site for *Xba* I sequence, double underline indicates site for *Eco*R I sequence, underline indicates sequence code for IgG3 upper hinge region linker, and bold indicates the overlapped sequence.