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Expression of biologically active human colony stimulating factor-1 in *Pichia pastoris*

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ABSTRACT

Colony Stimulating Factor-1 (CSF-1) is involved in proliferation, differentiation, and survival of the mononuclear lineage, in development of the female reproductive system and mammary glands during pregnancy and lactation. It is also implicated in the biology of breast cancer and promotion of its metastasis to bones. Therefore, CSF-1 is required for many applications in cellular and molecular biology studies. Commercial products, usually expressed in prokaryotic systems, are costly, with the likelihood of endotoxin contamination and also lack posttranslational modifications. These considerations provide the rationale to express growth factors in eukaryotic systems. In this study, the biologically active and soluble fragment (residues 33–182) of human (CSF-1) was cloned from K562 cell line and expressed in *Pichia pastoris*. The expression level of the active CSF-1 was about 100 μg/ml of the *P. pastoris* culture medium. Protein analysis revealed that the expressed CSF-1 appears in three bands with apparent molecular weight of 30, 26 and 20 kDa constituting 44%, 25% and 13% of all proteins in the culture medium, respectively. The expressed protein was partially purified and concentrated (10x) by ultrafiltration, then filter sterilized. The product was confirmed to be biologically active by stimulation of its receptor (FMS) autophosphorylation in THP-1 cells and also growth promotion of factor dependent FDC-P1 cells expressing human wild-type FMS (FD-FMS-WT). Therefore, *P. pastoris* is a highly efficient and cost-effective expression system for production of endotoxin-free CSF-1 for research and potentially for therapeutic applications.

Introduction

Colony stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF), is the ligands for FMS, which is a member of the type III receptor tyrosine kinase family. Human CSF-1 is synthesized as a cell surface transmembrane protein of 554 residues (UniProt ID: P09603). The first 32 residues function as a signal sequence and the region 497–517 is the transmembrane domain. Alternative splicing of its mRNA results in two shorter forms of the ligand by removing the region encoding amino acids 182–479 (isoform 3) or 365–480 (isoform 2). All of these isoforms can stimulate the human CSF-1 receptor (FMS) and are considered as biologically active isoforms. The active transmembrane protein can be cleaved from the cell surface, in the juxtamembrane region (in the PQLQE motif) by a membrane-associated protease [1], to make the soluble active form of the ligand (Fig. 1). Therefore it can be concluded that the residues 33–182 constitute the region required for CSF-1 biological activity. This region contains the cysteine residues that make the essential disulfide bonds to maintain the active conformation of the protein. These disulfide bonds, between cysteines 39–122, 80–171 and 134–178 as well as cysteine 63–63 (between two monomers), are required for ligand dimerization [2].

CSF-1 is a hematopoietic growth factor that is involved in the proliferation, differentiation, and survival of monocytes, macrophages [3], bone marrow progenitor cells and also development of the female reproductive system [4]. It has also been demonstrated that CSF-1 and CSF-1 receptor have additional roles in mammary gland development during pregnancy and lactation [5]. They are also involved in the biology of breast cancer in which abnormal expression of CSF-1 and its receptor correlates with tumor cell invasiveness and adverse clinical prognosis [6,7]. Recent findings also implicate tumor-produced CSF-1 in promotion of bone metastasis in breast cancer [8]. The membrane-associated form of CSF-1, however, appears to induce immunity against tumors [9]. Additionally, the CSF-1 signaling pathway can be used to modulate immune responses associated with bacterial lipopolysaccharide-induced septic shock and also immunostimulatory CpG DNA [10,11].

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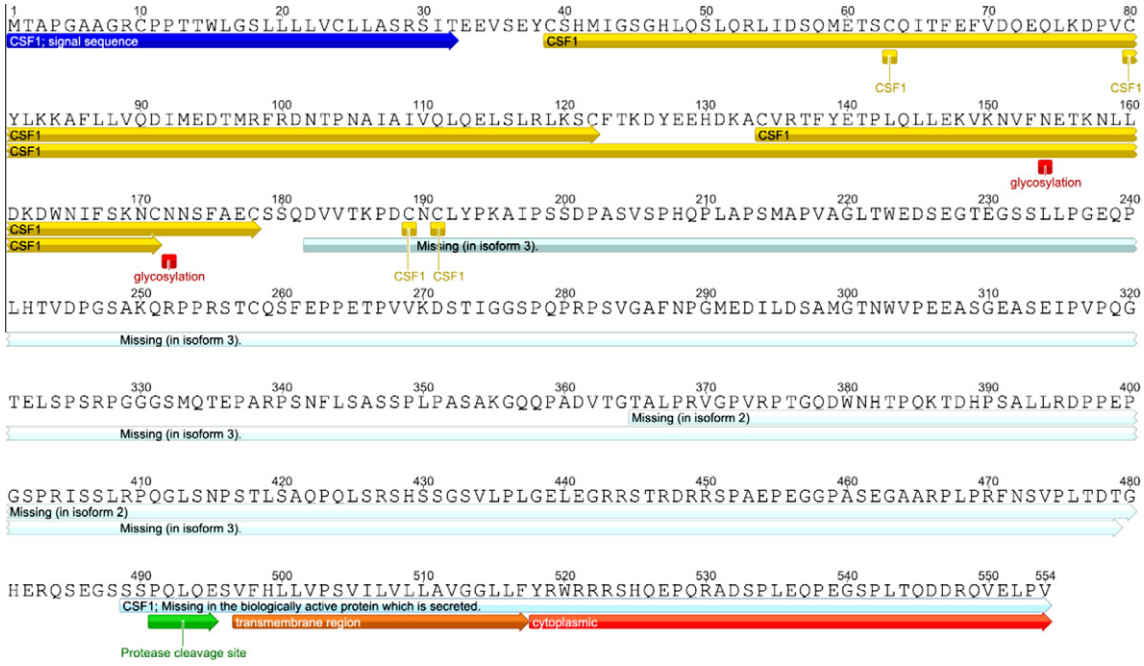

Fig. 1. Different isoforms of human CSF-1 are produced by alternative splicing of mRNA. Parts of molecule indicated with light blue arrows are missing in different splice variants, and in the soluble form of the ligand. Yellow arrows indicate cysteines involved in the formation of crucial disulfide bonds. Potential glycosylation sites (Asparagine 154 and 172) in human CSF-1 are specified in red. The soluble form is produced by protease action on a cleavage site (green) immediately before the transmembrane domain (brown). (For interpretation of the references to color in Figs. 1, 2 and 5 legends, the reader is referred to the web version of this article.)

These findings indicate that CSF-1 has many research applications. Since most commercially available products have been expressed in prokaryotic systems, they lack posttranslational glycosylation which reduces their stability and half-life in the experimental system [12]. Additionally these products may have traces of bacterial lipopolysaccharide (LPS) contamination that severely compromises the accuracy of the experimental results [13–16]. Therefore, we embarked on cloning and expression of biologically active and soluble fragments of human CSF-1 in *Pichia pastoris* to access an unlimited source of glycosylated and LPS-free growth factor.

Materials and methods

Production of CSF-1

Extraction of RNA and cDNA synthesis

The *RNA* used for cloning *CSF-1* cDNA was extracted as described by Chomczynski and Sacchi [17] from cells of the K562 line (**ATCC ID: CCL-243**), a suspension cell line which expresses CSF-1[18]. The quality of the extracted *RNA* was tested by agarose gel electrophoresis and *cDNA* was prepared using Superscript III Reverse Transcriptase system (Invitrogen, Carlsbad, CA) according to the supplier's instruction.

Amplification and cloning of CSF-1

A fragment of CSF-1 (codons 256–741 of GenBank ID: NM_172210) is required for biological activity of the ligand. Two sets of primers (Table 1) were designed and obtained from Sigma-Genosys (Sigma, St. Louis, MO). The first set (called nested-primers) was used to amplify the selected part of the ligand from *cDNA* for cloning the product into the *pGEM-T Easy* vector (Promega, Madison, WI). The second set was used for amplification of the ligand construct from *pGEM-T Easy* and includes the restriction sites for XhoI in the forward and XbaI in the reverse primer.

The insertion of the *CSF-1* fragment into the *pGEM-T Easy* vector (Promega) was confirmed by sequencing construct at Australian Genome Research Facility Ltd (AGRF, Brisbane, Australia) using T7 and SP6 primers (Promega). Then the second sets of primers called recombinant primers (Table 1) were used to amplify the coding fragments of CSF-1 (codons 286-732) for subcloning the products into *pPICZαA* expression vector. Insertion of *CSF-1* fragment in the correct frame in *pPICZαA* was confirmed by sequencing using AOX and α-Factor primers (Invitrogen).

Expression of CSF-1

The constructs of *pPICZαA*-CSF-1 was propagated in *E. coli* DH5α and linearized by PmeI restriction enzyme, then electroporated into the *P. pastoris* electro-competent cells using the Gene Pulser electroporation system (Bio-Rad) with the following parameters: voltage: 1.5 KV, capacitor: 25 μF, time: 8 ms and resistor of 400 Ω. The cells were recovered by adding 1 M sorbitol and incubating at 30 °C without shaking for 2 h before being selected on YPDS plates containing 100 μg/ml of zeocin. Single colonies from YPDS-zeocin plates were used to inoculate BMGY media. The cultures were grown at 30 °C for 36 h in a shaking incubator (to reach OD_{600} of 1.5). Then the BMGY medium was replaced by 2 ml of BMMY containing 0.5% methanol instead of 1% glycerol to stimulate expression of CSF-1. Each day for 5 consecutive days, 0.4 ml of culture was taken and 0.5 ml of fresh BMMY containing 2% methanol was added to the tubes. The samples were centrifuged at 2000 g for 10 minutes and the supernatants were stored at −80 °C for subsequent protein analysis by SDS-PAGE and western blotting. The protein bands intensity was analyzed using Multi Gauge V3.0 (Fujifilm, Tokyo, Japan).

Confirmation of CSF-1 expression

Since the product was expected to be expressed as a fusion protein of CSF-1_c-Myc_His tag, the protein expression was confirmed by western blotting for c-Myc epitope. The supernatant of hybridoma cells clone 9E10, kindly provided by Dr. Rick Thorne, Cancer