Protein Expression and Purification 132 (2017) 27–33

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

A highly efficient modified human serum albumin signal peptide to secrete proteins in cells derived from different mammalian species

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ARTICLE INFO

Article history:
Received 29 December 2016
Accepted 6 January 2017
Available online 10 January 2017

Keywords:
Signal peptide
Protein secretory pathway
Mammalian cells
Serum albumin signal peptide

ABSTRACT

Signal peptides (SPs) are key elements in the production of recombinant proteins; however, little information is available concerning different SP in mammalian cells other than CHO. In order to study the efficiency of different SPs to direct the traffic along the secretory pathway of the green fluorescence protein (GFP) and a scFv-Fc fusion protein; CHO-K1, HEK293 and NS0 cell lines were transfected in a transient and stable way. SP of human azurocidin (AZ), modified human albumin (mSA), modified *Cricetulus griseus* Ig kappa chain V III region MOPC 63 like (mIgκ C) and modified human Ig kappa chain V III region VG (mIgκ H) were evaluated. The efficiency of SPs to translocate a propeptide across the ER membrane was evaluated by fluorescence microscopy and flow cytometry for the GFP inside the secretory pathway, and by antigen-specific indirect ELISA for the scFv-Fc outside the cell. The mSA SP was successful in directing the secretion of the active proteins in these different types of mammalian cells, regardless of the transgene copy number. The goal of this work was to demonstrate that a modified version of SA SP might be used in different mammalian cells employing the same expression vector.

1. Introduction

Biopharmaceuticals represent preventive and therapeutic opportunities for a large number of human disorders. Today, about 250 recombinant therapeutic proteins have been approved and many more are in clinical trials [1]. Therefore, the study and development of innovative methods for the rapid production of recombinant proteins are essential [2]. These are mainly produced in mammalian cell lines to guarantee the quality of the post-translational modifications [3].

Chinese Hamster Ovary (CHO) cells are of particular interest for the industrial manufacturing of biopharmaceuticals. The main advantages of CHO cells are the feasibility of gene manipulation, the ability to create human-like glycosylation patterns, and the capacity for growth in high cell densities in serum-free suspension culture. In addition, the retina-derived primary human cells PER.C6, mouse myeloma-derived NS0, baby hamster kidney BHK-21 and human embryonic kidney HEK293 cells received regulatory approval for recombinant protein production [1,4].

Unfortunately, the cultivation of mammalian cells is very expensive and time consuming. In this regard, a variety of vectors and cell engineering strategies have been developed for generating high-producing mammalian cells [3,5–10]. Furthermore, protein secretion is known as one of the major bottlenecks in the productivity of mammalian cells. Several genes have been used to improve secretion from CHO cells, including molecular chaperones and mediators of secretory vesicle formation along the secretory pathway [11–15]. The translocation of secretory protein into the lumen of the endoplasmic reticulum (ER) constitutes the limiting step within the classical secretory pathway [16,17]. The protein secretion efficiency is not ensured by the natural signal peptide (SP) [18–20]. Even many prokaryotic and eukaryotic SPs are functionally interchangeable between different species [21–23]. Kober et al. [24] have selected 16 natural SPs from different species (mammals, fish, scorpions, snails, fungi, plants, viruses and bacteria) and evaluated the efficiency to increase the production of different recombinant proteins, including antibodies, in CHO-K1 cells. They demonstrated that azurocidin and serum albumin preprotein SPs from *Homo sapiens* improved product yield independently of the expressed protein. In this regard, there are reports of SPs evaluation in CHO cells but there is not enough information about these SPs in other mammalian cells [17–19,25]. In order to analyze the secretion efficiency of SPs and the productivity of the different cell lines, the

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http://dx.doi.org/10.1016/j.pep.2017.01.003
1046-5928/

expression of the green fluorescent protein (GFP) and a chimeric protein, based on a murine single-chain variable fragment (scFv) anti recombinant human interferon-α2b (rhIFN-α2b) fused with the fragment crystallizable domain of the human IgG1 (Fcγ1), was assessed.

2. Materials and methods

2.1. Cell lines and culture media

For CHO-K1, cell line growth and maintenance, Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1) supplemented with 2 mM glutamine and 5% (v/v) fetal calf serum (FCS) was used. For HEK293 cell lines, DMEM supplemented with 2 mM glutamine 10% (v/v) FCS was used. For NS0 cell lines, DMEM supplemented with 2 mM glutamine and 20% (v/v) FCS was used. In all cases the pH was adjusted to 7.0. Media, FCS and consumables for cell culture were obtained from Gibco (USA), PAA (Argentina) and Greiner (Germany). Cell lines were cultured at 37 °C in a humidified incubator supplied with 5% CO_2 atmosphere.

2.2. Vector construct

The GFP and the chimeric protein scFv-Fc were used to analyze the traffic efficiency along the secretory pathway of different SPs, listed in Table 1 (AZ and highlighted SPs). Two different types of expression vectors were constructed. The first type of expression vectors were based on a lentiviral (LV) vector containing the GFP coding sequence (Fig. 1A) and the second type of expression vectors were based on a LV vector containing a scFv-Fc coding sequence (Fig. 1B). Both were under the control of Cytomegalovirus (CMV) promoter. The different signal sequences were synthesized by GeneArt™ Gene Synthesis (Thermo Fisher Scientific) and cloned between XbaI and XhoI restriction sites. The secretion efficiency of the modified SPs was checked *in silico* by the SignalP 4.1 bioinformatics prediction software [26]. All constructs were analyzed by DNA sequencing.

2.3. Transient and stable expression

For transient expression CHO-K1, HEK293 and NS0 cells were seeded in a 24-well plate (1×10^5 cells per well) one day before transfection. Experiments were performed with the LipofectAMINE Reagent (Invitrogen) according to the supplier's instructions, using a mix of 1.5 μg of each vector and 1.5 μl of the LipofectAMINE Reagent. Intracellular fluorescence activity was measured on a Guava®EasyCyte™ cytometer (Guava Technology, USA) and analyzed on an Eclipse Ti-U Inverted fluorescence microscope (Nikon) 48 h after transfection. Extracellular scFv-Fc protein was measured by indirect enzyme-linked immunosorbent assay (ELISA).

48 h post-transduction, and in order to get stable expression, media were replaced with fresh growth medium containing 5 or 10 μg/ml puromycin (Sigma Aldrich, USA), depending on the cell line. Selective medium was changed every 3–4 days until control cells were dead. Stably-transfected cells were expanded for scFv-Fc quantitation.

2.4. Fluorescence measurements

Flow cytometry was performed on a Guava® Easy Cyte™ cytometer (Guava Technology, USA). This cytometer has a 488 nm blue laser for access to commonly used fluorescent dyes, and detectors to measure five different parameters (3 fluorescent channels and 2 light scatters). It allows simultaneous measurement of the side scatter (SSC), the forward scatter (FSC) and the green, yellow and red fluorescent emission which are collected by using the optical filters 525/30 nm, 583/26 nm and 690/50 nm, respectively. In addition, this equipment can measure samples from 96-well plates. Data acquisitions and analysis were performed using Guava CytoSoft™ 3.6.1 software. For each sample 5000 events were collected gating on the FSC vs SSC dot plot. Flow calibration and optical alignment was performed with the aid of Flow-Check Fluorospheres (Guava® Check kit, Hayward, CA, USA) before each determination. Cells were evaluated for the GFP signal (means fluorescence intensity multiplied by percentage of GFP positive cells) that is highly proportional to mRNA levels [27]. Data are representative of three independent experiments. The error bar represents the standard error of the mean (SEM).

2.5. scFv-Fc quantitation by indirect specific ELISA

Indirect ELISA was performed in 96-well plates (Greiner). Coating was achieved by incubating the plates with 50 ng per well of an rhIFN-α2b solution in 50 mM carbonate/bicarbonate buffer (pH 9.6) for 1 h at 37 °C and then ON at 4 °C. After blocking 1 h at 37 °C with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 14 mM NaCl, 2.7 mM KCl, pH 7.4), plates were incubated with 2-fold serial dilutions of the chimeric protein standard (mentioned below) or the test samples for 1 h at 37 °C. Then, plates were incubated with an appropriately diluted polyclonal rabbit anti-human immunoglobulin (DAKO, Denmark) for 1 h at 37 °C. Finally, horseradish peroxidase (HRP)-labelled polyclonal goat anti-rabbit immunoglobulins (DAKO) diluted 1:2000 were added to the wells. After 1 h incubation, plates were incubated for 10 min with substrate solution (3 mg/ml *o*-phenylenediamine, 0.12% (v/v) H_2O_2 in 50 mM phosphate-citrate buffer). Absorbance was measured at 492 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). Between every step, plates were washed 6 times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Dilutions of tested samples and antibodies were prepared in PBS-T containing 0.1% (w/v) BSA. Protein A affinity-purified scFv-Fc was used as standard to

Table 1
SPs and SignalP 4.1 scores.

Signal peptide	Protein	GenBank accession number	Organism	Signal peptide sequence	GFP score	scFv-Fc score
AZ	Azurocidin preproprotein	NP_001691	*Homo sapiens*	MTRLTVLALLAGLLASSRA	0.842	0.868
SA	Serum albumin preproprotein	NP_000468	*Homo sapiens*	MKWVTFISLLFLFSSAYS	0.844	0.875
Modified SA				MKWVTFISLLFLFSSSSRA	0.873	0.890
Igκ C	Ig kappa chain V-III region MOPC 63-like precursor	XP_003514704	*Cricetulus griseus*	MGSAALLLWVLLLWVPGSNG	0.937	0.942
ModifiedIgk C				MGSAALLLWVLLLWVPSSRA	0.946	0.950
Igκ H	Ig kappa chain V-III region VG precursor	P04433	*Homo sapiens*	MEAPAQLLFLLLLWLPDTTG	0.832	0.860
ModifiedIgk H				MEAPAQLLFLLLLWLPSSRA	0.889	0.901

Underlined amino acids relate to the introduction of the last four codons from AZ SP 3′ sequence. The GFP and scFv-Fc scores depict the scores obtained after using the online server: SignalP 4.1 [26] to predict the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms. Modified signal peptides scores are highlighted.