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CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion

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ABSTRACT

The ability to efficiently produce recombinant proteins in a secreted form is highly desirable and cultured mammalian cells such as CHO cells have become the preferred host as they secrete proteins with human-like post-translational modifications. However, attempts to express high levels of particular proteins in CHO cells may consistently result in low yields, even for non-engineered proteins such as immunoglobulins. In this study, we identified the responsible faulty step at the stage of translational arrest, translocation and early processing for such a "difficult-to-express" immunoglobulin, resulting in improper cleavage of the light chain and its precipitation in an insoluble cellular fraction unable to contribute to immunoglobulin assembly. We further show that proper processing and secretion were restored by over-expressing human signal receptor protein SRP14 and other components of the secretion pathway. This allowed the expression of the difficult-to-express protein to high yields, and it also increased the production of an easy-to-express protein. Our results demonstrate that components of the secretory and processing pathways can be limiting, and that engineering of the secretory pathway may be used to improve the secretion efficiency of therapeutic proteins from CHO cells.

1. Introduction

Mammalian cells are currently widely used for the production of recombinant therapeutic proteins in the biopharmaceutical industry, because they can perform the complex post-translational modifications that are often required for drug efficacy and stability. Great improvement of transcription and translation have been achieved through DNA vector engineering, yielding efficient production of many recombinant proteins from cultured mammalian cells. More recently, considerable efforts have been directed to obtaining higher number of transgene copies stably integrated in the genome, to targeting transgenes at genomic locations favorable to high-level transcription, and/or to include chromatin regulatory elements such as MAR (Mariati et al., 2012; Nehlsen et al., 2009).

Although major successes have been achieved, particularly with monoclonal antibodies and Fc-fusion proteins, increased transcriptional activity does not always enhance the amount of secreted recombinant proteins. Amplification procedures to increase the copy number of the gene of interest eventually reach a plateau beyond which no increases in protein output can be achieved. Thus, very high transgene copy number and mRNA levels may not correlate with a similarly elevated protein yield. In addition, such approach can fail for some specific proteins, for which even moderate levels of secretion cannot be achieved. In such cases, the cells may be unable to cope with the synthesis or processing of particular heterologous proteins, which may elicit cellular stress responses and cell toxicity effects.

Additional cellular bottlenecks may involve downstream cellular machineries such as those mediating polypeptide processing, modification and/or secretion. For instance, limitations on the expression of recombinant proteins may reflect the cell's inability to handle the necessary post-transcriptional events sufficiently rapidly or precisely to keep up with the high supply of the mRNA and polypeptide. In such cases, particular post-translational steps may become limiting, yielding proteins bearing inhomogeneous or variable modifications. Thus, reaching consistent and high-yield production of recombinant proteins requires the identification of these limiting bottlenecks, as well as specific engineering strategies to modify and improve the post-translational protein processing and secretion machineries.

The secretion of proteins by mammalian cells is a complex pathway involving polypeptide translocation from the cytosol into the lumen of the endoplasmic reticulum (ER), where they fold and assemble before being targeted to their final destination.

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The first step of protein secretion depends on the signal peptide, which is a specific sequence at the polypeptide amino-terminus that mediates association of the translating ribosome to the signal recognition particle (SRP) (Keenan et al., 2001). Association of SRP causes translation arrest by the ribosome and its docking to the SRP receptor (SR) on the ER membrane (Walter and Blobel, 1981). Translation then resumes, as the nascent polypeptide is funneled into the ER lumen through the translocon channel (Gilmore et al., 1982; Walter et al., 1982).

Distinct signal peptide sequences show variable efficiency for the secretion of heterologous proteins, but the signal peptides of e.g., interleukins or immunoglobulins, are commonly used to direct the secretion of heterologous recombinant proteins. Despite similarities, these sequences do not promote efficient secretion of all proteins, possibly because the native signal peptide may not function correctly out of the native context or because of differences linked to the host cell or to the secretion process. The choice of an appropriate signal sequence for the efficient secretion of a heterologous protein may be further complicated by the interaction of the signal peptide sequence with the following parts of the polypeptide during translation, such as the variable portions of immunoglobulins (Johansson et al., 1993).

Recent studies reported that the bioengineering of host cell lines may improve the modification or the secretion of heterologous proteins and of other therapeutics (Baik et al., 2012; Goh et al., 2010; Lim et al., 2010; Peng and Fussenegger, 2009; Zhang et al., 2010). Functional proteins involved in the various post-translational steps of the secretory pathway and of exocytosis have been studied, and some of these pathways could be successfully engineered to solve bottlenecks and cellular limitations caused by recombinant protein overflow (Mariati et al., 2010; Peng et al., 2010). It was shown that chaperones, unfolded protein response, and stress-mediated apoptosis pathway ER components could be co-expressed to create novel host cell lines. For instance, CHO cells were engineered to express various protein disulfide isomerases or the X-box-binding protein 1 (Xbp1), a transcription factor that regulates secretory cell differentiation, ER maintenance and expansion, to decrease ER stress and increase protein processing and secretion (Borth et al., 2005; Davis et al., 2000; Mohan and Lee, 2010; Tigges and Fussenegger, 2006). Other attempts included the expression of the chaperones ERp57, calnexin, calreticulin and BiP1 in CHO cells (Chung et al., 2004; Hwang et al., 2003; Morris et al., 1997). Finally, expression of a cold shock-induced protein, the cold-inducible RNA-binding protein (CIRP), was shown to increase the yield of recombinant γ-interferon (Tan et al., 2008). However, the molecular mechanisms by which these ER proteins may improve protein secretion and their ability to act on various recombinant proteins have not been studied systematically.

In this study, we studied a difficult-to-express immunoglobulin that consistently yields low secretion levels from CHO cell lines. We characterized the faulty step as an improper functioning of the SRP complex, leading to the lack of signal peptide removal, to the accumulation of the polypeptide within precipitated inclusions bodies in the ER, and to low secretion levels. We show that proper protein secretion can be triggered by the overexpression of specific secretory proteins of the SRP pathway. CHO cells engineered to express SRP proteins such as SRP14 gained the ability to properly process and secrete the difficult-to-express immunoglobulin, and they also displayed improved secretion of an easy-to-express antibody. This indicates that the CHO cell secretion pathway may generally limit the expression of heterologous proteins, and that high-level secretion can be achieved even for hard-to-express proteins from CHO cells, when they are metabolically engineered to overexpress SRP proteins.

2. Materials and methods

2.1. Plasmids and relative quantitative PCR analysis

Cloning vectors used in this study are the Selexis mammalian expression vectors SLXplasmid_082 and SLXplasmid_104. The luciferase sequence of pGL3-Control Vector (Promega) was replaced by a eukaryotic expression cassette composed of a human CMV enhancer and human GAPDH promoter upstream of the EGFP coding sequence followed by a SV40 polyadenylation signal, the human gastrin terminator and a SV40 enhancer. Two human MAR-derived genetic elements are flanking the expression cassette and a puromycin resistance gene expressed from the SV40 promoter, whereas the SLXplasmid_082 and SLXplasmid_104 vectors differ by the type of the MAR element located upstream of the expression cassette (hMAR 1-68 and hMAR X-29; Girod et al., 2007).

The trastuzumab and infliximab heavy and light chains cDNAs were cloned in expression vector SLXplasmid_104 to replace EGFP. A vector carrying both the heavy and light chain expression cassette of each IgG was made by combining heavy and light chain expression cassettes together on one plasmid vector. The signal peptide sequence of all heavy and light chains are identical, as are the constant portions of the light chains. The constant portions of the heavy chains differ at several amino acid positions (DEL vs. EEM variants).

PCR amplification primers and GenBank accession numbers of the SRP9, SRP14, SRP54, SRPRalpha, SRPRbeta, SEC61A1 and SEC61B cDNAs are listed in Suppl. Table S1. The PCR products encoding secretion proteins were cloned into SLXplasmid_104 to replace the EGFP sequence. When multiple secretion proteins were co-expressed, the inverted terminal sequences of the *piggyBac* transposon were integrated into SLX plasmid_104 plasmids to bracket the expression cassette, and the puromycin resistance gene was removed. The resulting vectors were co-transfected with a *piggyBac* transposase expression vector, to improve transgene integration and to obviate the need for the inclusion of antibiotic selection genes on the plasmids (Ley et al, manuscript in preparation).

For relative quantitative PCR analysis, total RNA was extracted from 1×10^5 cells and reverse transcribed into cDNA using the FastLane Cell cDNA Kit (Qiagen) according to the manufacturer's instructions. The expressions of SRP14 and GAPDH were quantified by qPCR using the Rotor Gene Q (Qiagen) and the LightCycler®480 SYBR Green I Master (Roche) using primers listed in Suppl. Table S1. Messenger RNA levels of SRP14 were normalized to that of GAPDH using the Rotor-Gene Q Series Software (Qiagen).

2.2. Cell culture, stable transfection and subcloning of CHO cell lines

Suspension chinese hamster ovary cells (CHO-K1) were maintained in SFM4CHO Hyclone serum-free medium (ThermoScientific) supplemented with L-glutamine (PAA, Austria) and HT supplement (Gibco, Invitrogen life sciences) at 37 °C, 5% CO_2 in humidified air. CHO-K1 cells were transfected with trastuzumab or infliximab heavy and light chains expression vectors bearing puromycin resistance gene by electroporation according to the manufacturer's recommendations (Neon devices, Invitrogen). Two days later, the cells were transferred in T75 plates in medium containing 10 μg/ml of puromycin and the cells were further cultivated under selection for two weeks. Stable individual cell clones expressing Trastuzumab and Infliximab IgG were then generated by limiting dilution, expanded and analysed for growth performance and IgG production levels. Trastuzumab and Infliximab IgG-producing cell clones expressing the highest IgG levels were selected for further biochemical experiments. Some of these

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