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Heterologous expression of the lipid transfer protein CERT increases therapeutic protein productivity of mammalian cells

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ABSTRACT

Recent studies have demonstrated that the introduction of transgenes regulating protein transport or affecting post-translational modifications can further improve industrial processes for the production of therapeutic proteins in mammalian cells. Our study on improving therapeutic protein production in CHO cells by heterologous expression of the ceramide transfer protein (CERT) was initiated by the recent discovery that CERT is involved in protein kinase D (PKD)-dependent protein transport from the Golgi to the plasma membrane.

We generated a set of CHO DG44 cell lines by stable integration of constructs expressing either CERT wild-type or CERT S132A, a mutant conferring increased lipid transfer activity, or a mock plasmid. CHO cells expressing heterologous CERT demonstrated significantly higher specific productivities of the therapeutic protein HSA when grown in inoculum suspension cultures. This effect translated into significantly increased overall HSA titers in a fed-batch format where cells are grown in chemically defined serum-free media. Furthermore, we could show that CERT also enhanced monoclonal antibody secretion in two IgG production cell lines with different basal productivities.

The data demonstrate the potential of CERT engineering to improve mammalian cell culture production processes to yield high amounts of a therapeutic protein product of desired quality. To our knowledge, this is the first study showing a bottle neck in recombinant protein secretion at the Golgi complex in mammalian cells.

1. Introduction

A majority of therapeutic proteins is produced from mammalian cell cultures in controlled bioreactors. Generally, proteins are secreted into the cell culture fluid by the host cell and the product can therefore be easily separated from the cells prior to the purification process leading to the drug substance (Bergemann et al., 2007). All secreted proteins are co-translationally targeted to the endoplasmic reticulum (ER) (Palade, 1975; Blobel, 1983) and then translocated across the membrane of the ER to the Golgi apparatus via the ER–Golgi intermediate compartment (ERGIC) (Hauri and Schweizer, 1992). After crossing the Golgi complex from cis to trans, the proteins are further processed within the tubular structure called the trans-Golgi network (TGN) before they are packed into secretory vesicles that dock to and fuse with the plasma membrane and expel their contents to the outside (Burgess and Kelly, 1987; Jahn and Sudhof, 1999).

Only recently a complex regulatory network has been described that controls key steps during protein transport at the Golgi (Ghanekar and Lowe, 2005). Central to this pathway is the recruitment of members of the protein kinase D (PKD) family to the trans-Golgi network by binding to diacylglycerol (DAG) (Baron and Malhotra, 2002). Once translocated to the TGN membrane, PKD is activated by PKC-eta – mediated phosphorylation (Diaz Anel and Malhotra, 2005). PKD in turn phosphorylates and activates the lipid kinase phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) which appears to be crucial for the TGN-to-plasma membrane delivery of secretory proteins (Hausser et al., 2005). Another key player in secretory transport is the ceramide transfer protein CERT also known as COL4A3BP (collagen, type IV, alpha 3 (Goodpasture antigen) binding protein), GeneID no. 10087). CERT is a member of the family of steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) proteins (Soccio and Breslow, 2003) and mediates ATP-dependent ceramide transport from the ER to the Golgi complex (Hanada et al., 2003). A recent study identified CERT as an *in vivo* PKD substrate and showed that phosphorylation on serine 132 reduces both, the affinity of CERT to Golgi membranes and its ceramide transfer activity (Fugmann et al., 2007). The two proteins appear to be the essential part of a regulatory feedback loop as

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in turn CERT is critical for PKD activation driven by the availability of ceramide as a source for DAG. Using a secreted HRP fusion protein as reporter for constitutive protein secretion it was furthermore shown that the PKD – CERT interaction regulates the secretory activity of the Golgi complex in human cells (Fugmann et al., 2007).

In this study, we investigate the role of CERT in increasing heterologous protein secretion from mammalian cells grown in suspension in chemically defined serum-free media. We created a set of genetically engineered Chinese hamster ovary (CHO) protein producer cell lines expressing two variants of CERT: wild-type CERT and the phosphorylation site mutant S132A that confers increased ceramide transfer activity. We assessed the possibility to improve fed-batch processes for manufacturing of therapeutic proteins by stable expression of these CERT variants. We can show that this approach leads to elevated specific productivities of the plasma protein HSA as well as of two different monoclonal antibodies in inoculum suspension cultures. We furthermore demonstrate that CERT engineered cells yield increased product titers in chemically defined fed-batch processes. These data demonstrate that the level and activity of key molecules regulating Golgi membrane integrity and vesicel transport from the ER via the TGN to the plasma membrane can significantly influence yields in mammalian cell culture production processes.

2. Materials and methods

2.1. Expression constructs

cDNA constructs encoding the human CERT wild-type protein as well as mutant variant bearing a point mutation at position 132 (CERT S132A, described in Fugmann et al., 2007) were subcloned into BI (Boehringer Ingelheim)-proprietary expression vectors. In these constructs, both cDNAs were fused to a N-terminal Flag™ epitope-tag and the expression was driven by a CMV promoter/enhancer combination. All plasmids carry a puromycin N-acetyl transferase gene for selection with the antibiotic puromycin (Sigma, Germany).

2.2. Cell culture and transfection

CHO DG44-derived cells (Urlaub and Chasin, 1980) secreting human serum albumin (HSA) or different human IgG subtype monoclonal antibodies were grown in suspension cultures in BI-proprietary chemically defined serum-free media. The cultures were maintained in shake flasks in Multitron HT incubators (Infors) at 5% CO_2, 37 °C and 120 rpm.

Seed stock cultures were sub-cultivated every 2–3 days and re-seeded with 3×10^5 cells/ml for a 2-day passage and 2×10^5 cells/ml for a 3-day passage. At the end of each culture passage, the concentration of secreted protein product was measured by ELISA, cell numbers were counted and used to calculate the specific productivity.

Cell transfections were carried out using Lipofectamine™ and PLUS™ Reagents (both Invitrogen, Germany) according to the guidelines provided by the manufacturer.

For fed-batch cultivation, cells were seeded at 3×10^5 cells/ml into 125 ml shake flasks in 30 ml of BI-proprietary production medium without antibiotics or MTX. Cultures were agitated at 120 rpm in 37 °C and 5% CO_2 which was later reduced to 2%. Culture parameters including pH, glucose and lactate concentrations were determined daily and the pH was adjusted to 7.0 using bicarbonate solution as needed. Starting on day 3, cells were fed every 24 h using a constant volume of BI-proprietary feed solution. Cell density and viability were determined by trypan-blue exclusion using an automated CEDEX cell quantification system (Innovatis). For product titer determination, samples were collected from the cell culture fluid on days 3, 5 and 7 and product concentrations were measured by ELISA.

2.3. ELISA

Quantitative determination of HSA secretion was performed using the Human Albumin ELISA Quantitation Kit (Bethyl Labs, TX, USA) according to the manufacturer's instructions.

Antibody titers in samples of cell culture fluid were measured by a sandwich ELISA using goat anti-human IgG Fc-fragment antibody (Dianova, Germany) for capture and alkaline-phosphatase conjugated goat anti-human kappa light chain antibody (Sigma–Aldrich, Germany) for detection. Colorimetric titer determination was performed by incubation with 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich, Germany) as a substrate and subsequent absorbance measurement at 405/492 nm using a Rainbow absorbance reader (Tecan, Germany).

The specific productivity was calculated by dividing product concentration by average cell number over the 2- or 3-day passage for serial cultures or by the integral of viable cells (IVC) for fed-batch processes.

2.4. Detection of CERT expression by intracellular FACS staining and Western blotting

For intracellular staining, 1×10^6 cells were fixed in 1% paraformaldehyde in PBS for 20 min and permeabilized in PBS containing 0.05% Tween-20. Subsequently, cells were resuspended in 1% BSA/PBS and stained using an anti-Flag M2 monoclonal mouse antibody (Sigma) and an Alexa488-labeled goat anti-mouse antibody (Invitrogen). The fluorescent signal was quantitatively analysed by flow cytometry (FACS calibour, Coulter).

Whole cell extracts for Western blotting were prepared by lysis of 5×10^6 cells in NP40-buffer [1% (v/v) NP40, 50 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 25 mM NaF and 40 μl/ml Complete® protease inhibitor solution (Roche)] for 15 min on ice. Lysates were cleared by centrifugation at 16,000 × *g* for 10 min and the protein concentration in the samples was determined using a BCA1 assay kit (Sigma). For Western blot analysis, equal protein amounts were separated with MOPS buffer on NuPAGE 10% Bis–Tris gels (Invitrogen) according to the manufacturer's protocol. Protein was transferred onto PVDF membranes (Millipore) using transfer buffer in XCell II blot module (Invitrogen). After blocking for 1 h at room temperature with blocking agent (Invitrogen), membranes were probed with anti-Flag M2 antibody (Sigma). Proteins were visualised with peroxidase-coupled secondary antibody using the ECL Plus chemoluminescence detection system (Amersham Pharmacia).

3. Results

3.1. Heterologous expression of CERT in CHO cells increases secretion of the single-chain protein HSA

Transient expression of the ceramide transfer protein CERT in adherently growing HEK293T cells was recently shown to lead to enhanced secretion of a co-transfected reporter protein into the culture medium (Fugmann et al., 2007). Based on these results, we set out to analyse the potential of CERT as a transgene to improve recombinant protein secretion from CHO cells. For this purpose, we made use of a monoclonal CHO DG44 cell line stably producing human serum albumin. HSA is an abundant, 66.5 kDa unglycosylated single-chain protein of the blood serum, which is responsible for maintaining the colloid osmotic pressure as well as for transport