Engineering the Cellular Protein Secretory Pathway for Enhancement of Recombinant Tissue Plasminogen Activator Expression in Chinese Hamster Ovary Cells: Effects of CERT and XBP1s Genes

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Introduction

Recombinant proteins appear to be the most promising category of pharmaceuticals, because they provide novel preventive and therapeutic opportunities for a large number of human disorders. Today, about 200 recombinant therapeutic proteins have been approved and much more are in clinical trials. Therefore, the development of innovative methods for the rapid production of recombinant proteins is essential [35].

Although a variety of expression systems have been used for the expression of heterologous genes, mammalian cells are considered the most suitable host system for the expression of fully active recombinant proteins with correct posttranslational modifications. Currently, about 60% of recombinant therapeutic proteins are produced in this expression system [4, 34]. Among the available mammalian cell lines, 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. The major disadvantage of CHO cells is their lower protein expression rate in comparison with bacterial and lower eukaryotic expression systems [13, 16].

Different strategies, such as optimization of the cell culture process, adjustment of medium components, generation of more efficient expression vectors, and development of genetically engineered host cells, have been used to improve recombinant protein expression in CHO cells [18, 34]. Cell line engineering approaches have been utilized for the
modulation of the cell proliferation rate [9, 22], the inhibition of apoptosis [6, 31, 36], the enhancement of protein secretion [3, 14], the establishment of new host cells for proper posttranslational protein modifications [25, 33], and decreasing the rate of metabolic waste-products formation [17, 26].

Protein secretion is known as one of the major bottlenecks in the productivity of mammalian cells [2]. Several genes have been used to improve protein secretion from CHO cells, including molecular chaperones and mediators of secretory vesicle formation along the secretory pathway. The influence of genetic modification of the secretory pathway on the productivity of recombinant proteins, however, appears to vary among mammalian cell types, effector genes, and target proteins [7, 15, 27].

Ceramide transfer protein (CERT) has been found to have a critical role in the trafficking of ceramide from the endoplasmic reticulum (ER) to the Golgi, where it is used as a precursor for the synthesis of diacylglycerol (DAG) and sphingomyelin. Accumulation of DAG at the trans-Golgi network (TGN) leads to the recruitment of protein kinase D (PKD). Upon activation at the TGN, PKD plays a central role in protein secretion through activation of the proteins involved in secretory vesicle formation [1, 11]. In addition, CERT is a PKD substrate, and the phosphorylation of CERT at serine 132 reduces its lipid transfer function and its affinity for Golgi [8]. In a report by Florin et al. [7], ectopic expression of CERT and its mutant variant CERT S132A resulted in enhanced secretion of a monoclonal antibody and human serum albumin from recombinant CHO cells.

X-box binding protein 1 (XBP1) is a basic leucine zipper (bZIP) transcription factor that is involved in the mammalian unfolded protein response (UPR). The UPR is a stress response pathway, which is activated by accumulation of misfolded or unfolded proteins in the lumen of the ER. Two major isoforms of XBP1 are known: XBP1u, the unspliced variant that is inactive; and XBP1s, the spliced variant that is a potent transcription factor. Upon UPR activation, XBP1s is generated by removal of a 26 nucleotide intron from the XBP1 transcript. The spliced isoform then stimulates upregulation of the endoplasmic reticulum chaperones and foldases. This protein also induces enlargement in the ER, Golgi, and cell size. Therefore, the result of XBP1s expression would be an improvement in the secretory capacity of the cells [21, 29]. Heterologous expression of XBP1s has been shown to enhance the production of monoclonal antibodies [2], human placental secreted alkaline phosphatase (SEAP), _Bacillus stearothermophilus_ derived _α_-amylase (SAMY), _Bacillus subtilis_ derived _α_-amylase (SAMY), and human vascular endothelial growth factor 121 (VEGF 121) in CHO cells [32]. The human tissue plasminogen activator (t-PA) is a 68 kDa serine protease known as the key component of the fibrinolytic system. t-PA converts the proenzyme plasminogen to plasmin, which in turn mediates the degradation of fibrin. Recombinant human t-PA has been extensively used as a therapeutic agent for the treatment of thrombotic diseases owing to its greater safety and efficiency compared with other plasminogen activators like urokinase and streptokinase. At present, CHO cells are the main source for the commercial production of full-length recombinant human t-PA [28].

In the study presented here, we investigated the effects of CERT and XBP1s, two major proteins involved in mammalian cell protein secretion, on the t-PA expression level in CHO cells. Our results here provide further details about the successful application of the cell line engineering approach for enhancement of the recombinant protein expression.

**Materials and Methods**

**Vector Construction**

The cDNA for human CERT S132A was amplified from the pcDNA3-CERT S132A vector (kindly provided by Dr. Monilola Olayioye, University of Stuttgart, Germany), cloned into the pGEM-T Easy vector (Promega, USA), sequenced, and then cloned into the _Hind_III and _Xba_I sites of the pcDNA3.1/hygro (+) expression vector (Invitrogen, USA). For detection of the CERT S132A protein, a sequence encoding the FLAG epitope was incorporated in the reverse primer. The pcDNA3-FLAG-XBP1s vector, containing the spliced variant of human XBP1s, was a generous gift from Dr. Ling Qi (Cornell University, USA).

**Cell Culture**

CHO 1-5(500) t-PA-producing cells (ATCC CRL-9606) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Gibco, USA) at 37°C in a humidified incubator containing 5% CO₂. Cultures were passaged every 2–3 days at the cell density of 0.2–0.3 × 10⁶ cells/ml. To determine the cell concentration and viability, the trypan blue exclusion method was used.

**Transfection and Development of Stable Cell Lines**

Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer’s protocol. To develop stable CHO 1-5(500) cells overexpressing CERT S132A or XBP1s, cells were transfected with _Bgl_II linearized pcDNA3.1-CERT S132A/hygro or pcDNA3-XBP1s.neo expression vectors, in triplicate. After 48 h, transfectants were diluted in 1:6 ratio and stable cell pools were selected in medium containing 200 µg/ml hygromycin or 400 µg/ml G418 (Invitrogen, USA) for 4 weeks. To confirm the