Cell-Free Protein Expression Based on Extracts From CHO Cells

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ABSTRACT: Protein expression systems are widely used in biotechnology and medicine for the efficient and economic production of therapeutic proteins. Today, cultivated Chinese hamster ovary (CHO) cells are the market dominating mammalian cell-line for the production of complex therapeutic proteins. Despite this outstanding potential of CHO cells, no high-yield cell-free system based on translationally active lysates from these cells has been reported so far. To date, CHO cell extracts have only been used as a foundational research tool for understanding mRNA translation (Lodish et al., 1974; McDowell et al., 1972). In the present study, we address this fact by establishing a novel cell-free protein expression system based on extracts from cultured CHO cells. Lysate preparation, adaptation of in vitro reaction conditions and the construction of particular expression vectors are considered for high-yield protein production. A specific in vitro expression vector, which includes an internal ribosome entry site (IRES) from the intergenic region (IGR) of the Cricket paralysis virus (CrPV), has been constructed in order to obtain optimal performance. The IGR IRES is supposed to bind directly to the eukaryotic 40S ribosomal subunit thereby bypassing the process of translation initiation, which is often a major bottleneck in cell-free systems. The combination of expression vector and optimized CHO cell extracts enables the production of approximately 50 μg/mL active firefly luciferase within 4 h. The batch-type cell-free coupled transcription–translation system has the potential to perform post-translational modifications, as shown by the glycosylation of erythropoietin. Accordingly, the system contains translationally active endogenous microsomes, enabling the co-translational incorporation of membrane proteins into biological membranes. Hence, the presented in vitro translation system is a powerful tool for the fast and convenient optimization of expression constructs, the specific labeling of integral membrane proteins and the cell-free production of post-translationally modified proteins.

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KEYWORDS: cell-free protein synthesis; Chinese hamster ovary cells; intergenic region internal ribosome entry site; in vitro protein expression

Introduction

The growing demand for therapeutic proteins promotes the development of technologies for high quality protein production. Mammalian cell-based expression systems are the preferred choice for the synthesis of biologically active proteins. This is mainly due to their capacity to perform correct protein folding, assembly, and post-translational modifications. Among well-established mammalian cell-lines, Chinese hamster ovary (CHO) cells are most widely used for transfection, expression, and large-scale recombinant protein production. Nearly 70% of all recombinant protein therapeutics are produced in CHO cells resulting in current annual sales for biologics of more than US$ 30 billion worldwide (Jayapal et al., 2007). However, protein expression in vivo goes along with a considerable investment of time and resources for process optimization. In contrast, cell-free protein expression provides a more rapid access to the protein of interest. This potential turns a CHO cell-free system into a valuable tool to speed up the entire process and product-development timeline. The cell-free system can be applied for the optimization of DNA constructs prior to in vivo expression, the production of difficult-to-express proteins such as toxic proteins and membrane-spanning proteins as well as the synthesis of proteins with improved properties by incorporating non-canonical amino acids.
In this study, we describe the development of an in vitro protein expression system based on extracts from CHO cells. The batch-based system combines transcription and translation in a single step to save user’s time and effort. Many issues, such as lysate preparation, optimization of reaction conditions, and the design of the expression vector are taken into consideration. Particularly, the latter is a crucial aspect for high-yield in vitro protein production. Several eukaryotic in vitro expression vectors include an internal ribosome entry site (IRES) to enable translation initiation in a cap-independent manner (Kobayashi et al., 2007, 2011, 2013; Mikami et al., 2010). We choose to study particular IRESs from the intergenic region (IGR) of the Dicistroviridae family, as these IRESs recruit ribosomes in the absence of initiation factors, implying that the RNA elements within the IRES harbor all the properties to bind to and manipulate eukaryotic ribosomes (Jan, 2006). Therefore, it can bypass the process of translation initiation, which is often a major bottleneck in cell-free protein expression (Mikami et al., 2006b; Swartz, 2009; Zeenko et al., 2008). IGR IRES elements are divided into two classes according to differences in two key structures (Hertz and Thompson, 2011; Jan, 2006). Firstly, the PKI domain of class II IRES elements contains an additional stem loop that probably plays a crucial role for IRES activity (Hatakeyama et al., 2004; Pfingsten et al., 2007). Secondly, each class of IGR IRES is characterized by a conserved bulge sequence, which is larger in class II (Jang and Jan, 2010; Jang et al., 2009). An IGR IRES element from the Plautia stali intestine virus (PSIV) has been previously used for the cell-free synthesis of proteins lacking an N-terminal methionine in a wheat germ-based cell-free system (Shibuya et al., 2004). Here we investigated the activity of one class I member (Cricket paralysis virus, CrPV) and two class II members (Israeli acute paralysis virus, IAPV, and Taura syndrome virus, TSV).

The performance of the cell-free system is evaluated by synthesizing functional cytosolic proteins, posttranslationally modified proteins and membrane-spanning proteins. Additionally, the application of the system as a powerful tool for the time-saving construction and evaluation of templates suitable for efficient protein expression in vivo is investigated.

Materials and Methods

Materials

Complementary DNAs (cDNA) encoding enhanced yellow fluorescent protein (eYFP), the fusion product of eYFP and heparin-binding EGF-like growth factor (Hb-EGF-eYFP), human erythropoietin (EPO), and firefly luciferase (LUC) were subcloned into EasyXpress pIX 3.0 and pIX 4.0 vectors (Qiagen, Hilden, Germany), respectively. For some constructs the native signal peptide was replaced by the melittin signal sequence (Mel-Hb-EGF-eYFP) or melittin signal peptide (Mel) was added to the N-terminus (Mel-eYFP) to enforce translocation into endogenous microsomes present in the CHO cell lysate. cDNA of CrPV IGR IRES (GenBank accession no. AF218039, nucleotides 6,025–6,216; see Suppl. Fig. 1), IAPV IGR IRES (GenBank accession no. NC009025, nucleotides 6,399–6,617) and TSV IGR IRES (GenBank accession no. AF277675, nucleotides 6,741–6,952) were synthesized and cloned into the pMA vector by Life Technologies (Darmstadt, Germany).

Generation of Expression Vectors

Individual coding sequences were fused to cDNAs of IGR IRESs by a three-step overlap extension (oe) E-PCR. In the first PCR step, the coding sequences of the individual IGR IRES and the gene of interest were amplified separately (IRES-specific primers: CrPV (ATG)-oe-EPO-R, CrPV (ATG)-oe-LUC-R, CrPV (ATG)-oe-Mel-R, CrPV (GCT)-oe-EPO-R, CrPV (GCT)-oe-LUC-R, CrPV (GCT)-oe-Mel-R, IAPV (ATG)-oe-LUC-R, TSV (ATG)-oe-LUC-R, X-CrPV-F, X-IAPV-F, X-TSV-F; gene-specific primers: EPO-F, LUC-F, Mel-F, X-EPO-R, X-eYFP-R, X-LUC-R; Suppl. Table I). In the second PCR step, IRES specific forward primers and gene-specific reverse primers (X-CrPV-F, X-IAPV-F, X-TSV-F, EPO-R, X-eYFP-R, X-LUC-R; see also Suppl. Table I) were used to amplify and fuse the gene of interest to the individual IRES. Additional templates were designed with an ATG-to-GCT mutation, thereby replacing the initial methionine by alanine. The resulting fusion constructs were used as templates in PCR step three. In this PCR step, regulatory sequences containing the cloning sites EcoRI and Xhol were added at the 5' and 3' non-coding regions of the final template (RS 5', RS 3'; Suppl. Table I). Amplified sequences were digested with EcoRI and Xhol restriction nucleases and the resulting fragments were cloned into the EasyXpress pIX 3.0 vector. Wild type as well as mutated LUC templates including or excluding the CrPV IGR IRES were amplified from the EasyXpress pIX 3.0 constructs with specific forward and reverse primers (EcoRI-LUC-F, EcoRI-CrPV-F, Xho-LUC-R). Amplified sequences were cloned into the vector pCDNA3.1 (+) (Life Technologies) after restriction enzyme digestion with EcoRI and Xhol. Nucleotide sequences of cloned constructs were confirmed by DNA sequencing.

CHO Cell Lysate Preparation Procedure

CHO cells were grown exponentially in suspension cultures in well-controlled fermenters at 37°C using the Power CHO-2 chemically defined, serum-free medium (Lonza, Cologne, Germany). CHO cells were harvested at a density of approximately 2 × 10^6 cells/mL. Collected cells were centrifuged at 200g for 10 min and washed once with a buffer consisting of 40 mM HEPES-KOH (pH 7.5), 150 mM NaOAc, and 4 mM DTT. The cell pellet was resuspended to achieve a density of approximately 5 × 10^6 cells/mL. Cell disruption was accomplished by syringeing the harvested cell pellet through a 20-gauge needle, followed by centrifugation at 10,000g for 10 min to remove the nuclei and cell debris. The supernatant was applied to a size-exclusion
chromatography column (Sephadex G-25, GE Healthcare, Freiburg, Germany) and the elution fractions (each 1 ml) with an RNA content above an absorbance of 100 at 260 nm were pooled. Residual mRNA was digested by micrococcal nuclease (S7) treatment. In this respect, 10 U/ml S7 nuclease (Roche, Mannheim, Germany) and 1 mM CaCl₂ (final concentration) were added to the eluate and the reaction mixture was incubated for 2 min at room temperature. The reaction was inactivated by the addition of 6.7 mM EGTA (f.c.). Finally, translationally active lysates were immediately shock-frozen in liquid nitrogen and stored at −80°C in order to preserve maximum activity.

**Cell-Free Protein Synthesis**

Coupled transcription–translation reactions were performed in 25 µL batch volume. Protein production was operated at 33°C in a thermomixer (Thermomixer comfort, Eppendorf, Hamburg, Germany) with gentle shaking at 500 rpm. Reactions were composed of 25% (v/v) CHO cell lysate, canonical amino acids (100 µM), nucleoside triphosphates (1.75 mM ATP, 0.30 mM CTP, 0.30 mM GTP, and 0.30 mM UTP), vector DNA and 1 U/µL T7 RNA-polymerase (Agilent, Waldbronn, Germany). To monitor protein quality and quantity, reaction mixtures were supplemented with 14C-labeled leucine (specific radioactivity 75.0 dpm/pmol). As a comparison of the productivity, commercially available coupled cell-free expression systems based on Spodoptera frugiperda 21 cells (TNT T7 Insect Cell Extract Protein Expression System, Promega, Mannheim, Germany), rabbit reticulocytes (TNT T7 Coupled Reticulocyte Lysate System, Promega), and wheat germ (TNT T7 Coupled Wheat Germ Extract System, Promega) were purchased and in vitro reactions were performed according to the manufacturer’s instructions. All systems were used with the recommended individual LUC-encoding expression template provided by the manufacturer. In the case of the TNT T7 Coupled Reticulocyte Lysate System, the provided Luciferase T7 Control DNA was applied. The TNT T7 Coupled Wheat Germ Extract System and the TNT T7 Insect Cell Extract Protein Expression System were used with the provided T7 Linear Control DNA and Luciferase ICE T7 Control DNA, respectively.

**Determination of Protein Yield**

Aliquots of 5 µL of the CHO cell-free reaction mixtures were assayed for luciferase activity (luminometer LB941, Berthold, Bad Wildbad, Germany) using luciferase assay reagent (Promega). Total protein yields in translation mixtures (TMs) and microsomal fractions (MFs) were determined by hot trichloroacetic acid (TCA) precipitation followed by liquid scintillation counting as described previously (Brödel et al., 2013; Stech et al., 2012).

**SDS–PAGE and Autoradiography**

Aliquots of 7 µL of CHO cell-free reaction mixtures were subjected to cold acetone precipitation. After centrifugation of the samples for 10 min at 16,000 g and 4°C, protein pellets were resuspended in 20 µL of 1× sample buffer (NuPAGE® LDS Sample Buffer, Life Technologies) and loaded on a precast SDS–PAGE gel (Nu PAGE 10% Bis–Tris gel, Life Technologies). Gels were run in a MES SDS buffer for 35 min at 200 V. Subsequently, gels were stained using SimplyBlue Safe Stain (Life Technologies) and then dried for 70 min at 70°C (Unigelder 3545D, Uneiqi, Planegg, Germany). Bands of the SeeBlue Plus2 Pre-Stained Standard were labeled using a radioactive marker in order to identify the molecular masses of the synthesized target proteins. Finally, radioactively labeled proteins were visualized using the phosphor-imager system (Typhoon TRIO+ Imager, GE Healthcare) after a minimum of 2 days of incubation.

**Deglycosylation Assays**

Glycosylation of 14C-leucine-labeled, de novo synthesized EPO was investigated by PNGase F (NEB, Frankfurt, Germany) and Endo H (NEB) treatment of a 7 µL aliquot of the TM and subsequent analysis by autoradiography. Samples were treated according to the manufacture’s instructions. Additionally, EPO was synthesized in a microsome-enriched CHO cell extract. The microsomes tend to form large aggregates, which can be collected by centrifugation (10 min, 16,000g). Microsomes from 30 µL crude CHO cell lysate were pelleted and the resulting MF was resuspended in 10 µL TM in order to prepare a microsome-enriched CHO cell extract. TMs were loaded on SDS–PAGE gels before and after deglycosylation of radiolabeled, de novo synthesized EPO and samples were subsequently analyzed by autoradiography.

**Fluorescence Analysis**

Translocation of Mel-eYFP and Mel-Hb-EGF-eYFP fusion proteins into microsomal membranes was visualized by confocal laser scanning microscopy (LSM 510, Zeiss, Jena, Germany). Samples of TMs were transferred to ibidi slides (μ-slide, 18-well, Ibidi, Planegg, Germany) and eYFP was excited at 488 nm using an argon laser. Emission signals were acquired with a long pass filter in the wavelength range above 505 nm. No template controls were prepared in the same way as the samples with the exception of the DNA template which was replaced by RNase-free water.

**In vivo Assay**

DNA transfections were performed using Lipofectamine LTX transfection reagent (Life Technologies) according to the manufacturer’s protocol with slight adjustments. CHO cells were placed in a 24-well plate in Opti-MEM I reduced serum medium (Life Technologies) at a density of 500,000 cells/mL. Three microliters of Lipofectamine LTX and 750 ng of the respective expression vector were added to 150 µL medium and the mixture was incubated for 30 min at room temperature. DNA/Lipofectamine complexes were added to
the 24-well plate and CHO cells were incubated at 37°C. Forty-eight hours after transfection, CHO cells were harvested, washed once with PBS (w/o Mg²⁺ and Ca²⁺) and lysed by 100 µL cell culture lysis buffer (Promega). Extracts were cleared by centrifugation at 12,000g for 2 min and 10 µL aliquots were analyzed for LUC activity.

**Results**

**Optimization of Initial Reaction Conditions**

Cell-free protein synthesis was performed in coupled transcription–translation reactions with gentle shaking at 500 rpm. Initial reaction conditions were optimized for the expression of active LUC in the batch-based CHO cell-free system (Figs. 1 and 2). Protein expression levels were monitored after 3 h of incubation at 33°C.

The construction of an efficient expression vector was performed by investigating the influence of LUC encoding expression constructs harboring different IGR IRES elements (Fig. 1A). The activity of one class I (CrPV) and two class II (IAPV and TSV) members of the IGR family were taken into consideration. EasyXpress pIX3.0 vector constructs without IRES elements were used as a control in order to demonstrate the impact of the individual IGR IRES on LUC expression. Highest LUC activity was monitored using the expression vector equipped with the CrPV IGR IRES sequence (Fig. 1A). The CrPV IGR IRES-based plasmid enabled a more than 200-fold increased expression of the target protein in comparison to the control vector without the IRES sequence. Replacement of the initial ATG-codon to a GCT-codon resulted in a further improvement of protein expression levels (Fig. 1B). An optimal pIX3.0 CrPV IGR IRES (GCT) vector concentration of 60 ng/µL (20 nM) was determined (Fig. 1C). Increased plasmid concentrations did not result in higher LUC activities.

All reaction buffers were optimized in terms of their composition and compound concentrations in order to obtain highest yields of functional LUC. The influence of various Mg²⁺ concentrations on LUC expression was investigated (Fig. 2A). Highest LUC activity was obtained at a final concentration of 3.9 mM magnesium acetate, having a constant quantity of 1.75 mM ATP, 0.30 mM CTP, 0.30 mM GTP, and 0.30 mM UTP. An optimum KOAc concentration of 150 mM was determined and higher quantities led to a decrease in protein expression (Fig. 2B). Reaction mixtures were incubated at four different temperatures (Fig. 2C). Highest LUC activity was achieved by cell-free expression at 33°C and dropped to <50% of the maximum value with a temperature increase to 37°C. For the regeneration of ATP and GTP, 20 mM creatine phosphate and 100 µM creatine kinase were applied to the reaction mixtures. Optimal reaction conditions in the CHO cell-free expression system are summarized in Table I.

**Productivity of the CHO-Based Cell-Free System**

The initial optimization process was followed by an evaluation of the productivity. Total cell-free synthesis of LUC was investigated by performing a time course analysis within 4.5 h of incubation (Fig. 3A). The optimized cell-free system led to 51.3 µg/mL de novo synthesized LUC after 3.5 h of incubation. Extended reaction times did not further increase the yield of the cell-free expressed protein. Additionally, the percentage of active LUC was investigated (Fig. 3B). 48.7 µg/mL active LUC was monitored after 4.5 h, thus resulting in an active target protein of 95%. To test for protein integrity, ¹⁴C-leucine-labeled, de novo synthesized LUC was visualized by autoradiography and gel electrophoresis (Fig. 3C). Three bands of the SeeBlue Plus 2 Pre-Stained Standard (S) were labeled using a radioactive marker in order to identify the molecular masses of the de novo synthesized LUC.

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**Figure 1** Evaluation of IGR IRES-based in vitro expression vectors. **A:** Comparative analysis of 5’ IRES sequences in LUC encoding expression constructs cloned into the EasyXpress pIX3.0 vector backbone. **B:** Impact of the initiation codon on the translation efficiency of the CrPV IGR IRES vector. **C:** Influence of the pIX3.0 CrPV IGR IRES (GCT) vector concentration on the protein expression level. The data shown in Figure (A) and (B) were obtained using 180 ng/µL of the respective expression vector. Reactions mixtures were incubated for 3h in the coupled CHO cell-free system and the amount of active LUC was determined from three independent experiments using a LUC reporter assay.
target protein. Full-length LUC was observed at 61 kDa without any signs of proteolysis or fragmentation.

The productivity of the CHO-based cell-free system was compared to commercially available in vitro transcription–translation platforms derived from Sf21 cells, rabbit reticulocytes, and wheat germ (Fig. 3D). The lowest yield of de novo synthesized active LUC was 1.1 µg/mL obtained using the TNT Coupled Reticulocyte Lysate System. The use of the TNT Coupled Wheat Germ Extract System and the TNT Insect Cell Extract Protein Expression System resulted in 1.3 and 14.3 µg/mL of active LUC, respectively. The highest productivity of 48.7 µg/mL active LUC was obtained using the CHO-based cell-free protein production platform.

Table I. Optimum conditions in the batch-based CHO cell-free expression system.

| Component        | Optimum concentration |
|------------------|-----------------------|
| Vector           | 20 nM                 |
| T7 RNA polymerase| 1 U/µL                |
| HEPES KOH, pH 7.6| 30 mM                 |
| KOAc             | 150 mM                |
| Mg(OAc)$_2$      | 3.9 mM                |
| ATP              | 1.75 mM               |
| CTP, GTP, UTP    | 0.3 mM                |
| Creatine phosphate| 20 mM                |
| Creatine kinase  | 100 µg/mL             |
| Spermidine       | 0.25 mM               |
| Amino acids      | 100 µM                |
| DTT              | 2.5 mM                |
| Temperature      | 33°C                  |

Parameters were optimized for the cell-free expression of active LUC in a coupled transcription–translation system using the pIX3.0 CrPV IGR IRES (GCT) vector as a template. Reactions were incubated for 3 h in a thermomixer with gentle shaking at 500 rpm.

Translocation of Target Proteins Into Endogenous Microsomes

Protein translocation into endogenous microsomes was investigated by studying the production of eYFP in presence and in absence of the melittin signal sequence, respectively (Fig. 4). In this context, the influence of the initial ATG-to-GCT mutation on translocation of the target protein was evaluated. 14C-leucine-labeled, de novo synthesized eYFP and Mel-eYFP harboring the initiation codon ATG and GCT, respectively, were visualized by autoradiography after gel electrophoresis (Fig. 4A and B). Full-length eYFP and Mel-eYFP were observed at 27 and 29 kDa, respectively, without any signs of proteolysis or fragmentation. Protein expression levels were increased by the ATG-to-GCT mutation of the initiation codon. In addition, protein translation efficiency was affected by the melittin signal sequence, resulting in an increased band intensity of Mel-eYFP compared to eYFP. This observation was verified by quantification of the de novo synthesized proteins leading to 30.7 µg/mL Mel-eYFP (GCT, SD 1.8) and 9.5 µg/mL eYFP (GCT, SD 0.3). In the case of the Mel-eYFP, protein translocation was not affected by the ATG-to-GCT mutation, as can be seen from the band intensities of the MFs. Autoradiographic data were complemented by fluorescence microscopy. Target proteins synthesized from the expression constructs harboring the ATG codon were investigated by confocal laser scanning microscopy. Fluorescent vesicles indicated the translocation and incorporation of de novo synthesized Mel-eYFP into the lumen of the microsomal vesicles present in the CHO cell-free system (Fig. 4D). Unlike Mel-eYFP, cell-free synthesized eYFP was not localized inside these microsomes (Fig. 4C).

Expression of Membrane Proteins

The expression of membrane proteins and their insertion into endogenous microsomes was studied by the synthesis of...
type I transmembrane protein heparin-binding EGF-like growth factor (Hb-EGF). The native signal peptide of Hb-EGF was replaced by the melittin signal sequence and eYFP was fused to the C-terminus of the type I transmembrane protein in order to facilitate microscopic investigations. Quantitative analysis of cell-free expressed and 14C-leucine-labeled Mel-Hb-EGF-eYFP in the TMs and MFs was performed by liquid scintillation counting after hot TCA precipitation. ATG-to-GCT mutation of the initiation codon led to an improved expression up to 41.0 \mu g/mL de novo synthesized target protein. The analysis of the MFs yielded 10.0 \mu g/mL (ATG-codon) and 17.1 \mu g/mL (GCT-codon) of the cell-free expressed membrane protein, respectively. In addition, TMs and MFs of the cell-free expressed Mel-Hb-EGF-eYFP fusion proteins were visualized by autoradiography after separation by SDS–PAGE (Fig. 5B). A single band that migrated at 51 kDa corresponded to the calculated molecular mass of the target protein. Full-length Mel-Hb-EGF-eYFP was observed without any signs of proteolysis or fragmentation. Autoradiographic data were complemented by fluorescence microscopy (Fig. 5C). The MF of the cell-free expressed Mel-Hb-EGF-eYFP harboring methionine as the first amino acid was analyzed. Microsomes appeared with a fluorescent membrane by confocal laser scanning microscopy (CLSM), thereby indicating successful cell-free expression, translocation, and membrane integration of the eYFP-tagged target protein.

Expression of Glycoproteins
The expression of glycoproteins was investigated by the synthesis of human erythropoietin (EPO). EPO harbors three...
N-linked glycosylation sites and migrates on SDS–PAGE gels at 21 kDa in its unprocessed form. 14C-leucine-labeled, cell-free synthesized eYFP (27 kDa; A) and Mel-eYFP (29 kDa; B) harboring either ATG or GCT as initiation codon. Target proteins were visualized by autoradiography after gel electrophoresis. In the case of Mel-eYFP, TMs and MFs were investigated. C and D: Fluorescence analysis of de novo synthesized eYFP and Mel-eYFP. Proteins translated from the expression constructs harboring the initial ATG codon were investigated. CLSM images illustrate the expression of functional and soluble eYFP (C). CLSM analysis of expressed Mel-eYFP (D). Fluorescent vesicles indicate the expression and translocation of Mel-eYFP into the lumen of endogenous microsomal vesicles present in the CHO cell-free system.

Figure 4. Translocation of de novo synthesized Mel-eYFP into endogenous microsomes present in the coupled CHO cell-free system. Qualitative analysis of 14C-leucine-labeled, cell-free synthesized eYFP (27 kDa; A) and Mel-eYFP (29 kDa; B) harboring either ATG or GCT as initiation codon. Target proteins were visualized by autoradiography after gel electrophoresis. In the case of Mel-eYFP, TMs and MFs were investigated. C and D: Fluorescence analysis of de novo synthesized eYFP and Mel-eYFP. Proteins translated from the expression constructs harboring the initial ATG codon were investigated. CLSM images illustrate the expression of functional and soluble eYFP (C). CLSM analysis of expressed Mel-eYFP (D). Fluorescent vesicles indicate the expression and translocation of Mel-eYFP into the lumen of endogenous microsomal vesicles present in the CHO cell-free system.
Figure 5. Synthesis of the membrane protein Mel-Hb-EGF-eYFP in the coupled CHO cell-free system. A: Quantitative investigation of cell-free expressed Mel-Hb-EGF-eYFP harboring either ATG or GCT as initiation codon. Analysis of $^{14}$C-leucine-labeled proteins was performed by liquid scintillation counting after hot TCA precipitation. B: Qualitative analysis of $^{14}$C-leucine-labeled, de novo synthesized Mel-Hb-EGF-eYFP (51 kDa) harboring either ATG or GCT as initiation codon by autoradiography. TMs and MFs were investigated. C: CLSM analysis of de novo synthesized Mel-Hb-EGF-eYFP (ATG). Microsomes appear with a fluorescent membrane, thereby indicating the expression and translocation of the target protein into the vesicles present in the CHO cell-free system.

Figure 6. Cell-free expression of the human glycoprotein EPO. $^{14}$C-leucine-labeled, de novo synthesized EPO (21 kDa, non-glycosylated) harboring either ATG (A) or GCT (B and C) as initiation codon was visualized by autoradiography after gel electrophoresis. C: Cell-free expression of human EPO in a microsome-enriched extract. EPO is presented before (−) and after (+) deglycosylation with PNGase F. D: De novo synthesized EPO is illustrated before (−) and after (+) deglycosylation by Endo H treatment.
upper band was converted into a band at a lower apparent molecular mass, confirming the removal of sugar moieties.

**Evaluation of Expression Constructs for the Synthesis of Proteins In Vivo and In Vitro**

A comparative analysis of different LUC-encoding expression constructs using the CHO cell-free platform (Fig. 7A) and a CHO in vivo expression system (Fig. 7B) was performed. In this context, the impact of the EasyXpress pIX3.0 and the pcDNA3.1 vector backbone including or excluding the CrPV IGR IRES sequence on protein expression was investigated. The final constructs harbored either an initial ATG or GCT in the LUC encoding sequence.

Highest LUC activity was obtained using the pIX 3.0 CrPV IGR IRES (GCT) construct in the cell-free system (Fig. 7A). pcDNA3.1 and EasyXpress pIX3.0-based constructs without CrPV IGR IRES resulted in <0.3 μg/mL de novo synthesized and active LUC, demonstrating the necessity of the CrPV IGR IRES for high-yield protein synthesis in the CHO cell-free system. pcDNA3.1 constructs equipped with the CrPV IGR IRES enabled the synthesis of approximately 70% active LUC compared to the pIX3.0 CrPV IGR IRES construct when expressing the proteins in vitro. CrPV IGR IRES-mediated translation was improved by the ATG-to-GCT mutation of the initiation codon in both vector backbones (Fig. 7A).

In vitro data were complemented by protein expression in vivo (Fig. 7B). The pcDNA3.1 construct led to highest yields of de novo synthesized active LUC in vivo. As expected, EasyXpress pIX3.0 vectors could not be applied for protein synthesis in vivo since they do not contain a suitable promoter such as the CMV promoter. CrPV IGR IRES-mediated translation harboring an initial GCT-codon in the pcDNA3.1 backbone did not function in vivo. In contrast, the GCT-to-ATG exchange of the initial codon led to successful LUC production. The overall expression level of LUC was approximately one-third compared to the positive control vector pcDNA3.1.

In conclusion, the pcDNA3.1 CrPV IGR IRES (ATG) construct was identified as a suitable vector for the synthesis of LUC in vivo and in vitro. The design of the vector enables the expression of a target protein in CHO cell-based as well as CHO cell-free systems without recloning.

**Discussion**

We have developed a novel cell-free protein expression system based on translationally active extracts derived from cultured CHO cells. The system combines transcription and translation in a single step (coupled system), as has been already demonstrated for other cell-free protein production platforms (Mikami et al., 2008; Sawasaki et al., 2000; Stech et al., 2012), in order to save user’s time and effort.

For optimal performance, expression vectors equipped with the CrPV IGR IRES (Wilson et al., 2000b) have been constructed. IRES-mediated translation neglects the capping of mRNA which is a time-consuming and expensive production step in protein synthesis (Carlson et al., 2012; Gebauer and Hentze, 2004; Hershey and Merrick, 2000). Additionally, the inhibitory effect of free caps on the initiation factor eIF4E can be avoided. IGR IRES-mediated translation in particular bypasses all steps of conventional eukaryotic
translation initiation (Pestova and Hellen, 2003; Pisarev et al., 2005; Wilson et al., 2000a) which is often a major bottleneck in cell-free protein expression (Mikami et al., 2006b; Swartz, 2009; Zeenko et al., 2008). The majority of IGR IRESs display maximum efficiency in the context of GCU (alanine) as the first translated codon (Hertz and Thompson, 2011). Replacement of the initiation codon ATG to GCT results in a further improvement of protein expression levels in the CHO cell-free system. Protein translocation into endogenous microsomes seems not to be affected by the ATG-to-GCT mutation of the initiation codon. Since T7 RNA polymerase is used for transcription, DNA constructs must contain a T7 promoter. The T7 promoter-driven vector EasyXpress pIX3.0 but also the pcDNA3.1 plasmid, commonly used for protein expression in vivo, facilitate cell-free protein synthesis when combined with the CrPV IGR IRES. Protein expression levels of pcDNA3.1 and pIX3.0-based templates vary probably due to differences in the composition of the transcribed mRNA.

The pcDNA3.1 vector contains a cytomegalovirus (CMV) and a T7 promoter whereas the pIX3.0 is limited to the T7 promoter (Suppl. Fig. 2), thus resulting in different transcription products. The pcDNA3.1 CrPV IGR IRES (ATG) construct can even be applied for the synthesis of proteins in vitro and in vivo without recloning. This enables convenient in vitro screening of efficient templates prior to their use in cultured CHO cells. Some conceivable applications of this method are the investigation of gene-specific mutations, the analysis of purification tags including their terminal position, the optimization of the encoded gene’s codon composition and the investigation of signal peptides in the context of protein translocation and signal peptide cleavage. It is currently not clear why the pcDNA3.1 CrPV IGR IRES (GCT) vector did not promote CHO-based LUC expression in vivo.

The optimization of reaction conditions is essential for obtaining an effective in vitro translation system. In this study, parameters such as ion concentrations, incubation temperature, reaction time, amino acid concentration, and energy supply (ATP and GTP) have been investigated extensively. As protein expression in cell-free systems is sensitive to ions (Clemens, 1983; Jackson, 1991; Kubick et al., 2003) and CrPV IGR IRES-driven translation functions best at comparatively higher quantities of potassium compared to cap-dependent translation (Cevallos and Sarnow, 2005), the concentrations of potassium and magnesium were adjusted to 150 and 3.9 mM, respectively. Highest LUC activity was obtained by cell-free expression at 33°C and decreased significantly with increasing incubation temperatures. This result may be accounted to the decreased thermostability of LUC at temperatures above 30°C (Koksharov and Ugarova, 2011). Optimal expression temperatures for other target proteins may therefore vary slightly. Translation initiation and elongation are strongly affected by changes in the ATP/ADP and GTP/GDP ratios (Rupniak and Quincey, 1975). ATP and GTP concentrations are maintained by an energy regeneration system to ensure increased reaction times. In prokaryotic cell-free systems, the combination of phosphoenolpyruvate (PEP) and pyruvate kinase is usually used for ATP and GTP regeneration, whereas creatine phosphate and creatine kinase are frequently applied in eukaryotic in vitro expression systems (Jermutus et al., 1998; Spirin, 2004).

Based on primary data obtained from an insect cell-free platform (Kubick et al., 2003), creatine phosphate and creatine kinase were supplemented to CHO in vitro reaction mixtures.

The combination of the CrPV IGR IRES-based expression vector and optimized CHO cell extracts enables the synthesis of approximately 50 μg/mL active LUC within four hours. To our knowledge, these are currently the highest yields of active LUC reported for a coupled batch-type mammalian-based cell-free system. As a comparison, the productivity of various cell-free expression systems derived from eukaryotic organisms is summarized in Table II. Cell-free expression of cytosolic eYFP, secreted Mel-eYFP, membrane-spanning Mel-Hb-EGF-eYFP and the glycoprotein EPO was investigated qualitatively by autoradiography. Cell-free synthesized, 14C-leucine-labeled full-length proteins were observed without any indication of proteolysis or fragmentation in all cases, demonstrating the feasibility of the CHO cell-free system to produce a broad range of target proteins including membrane proteins and glycoproteins.

Translocation of translated proteins into the lumen of the endoplasmic reticulum (ER) or, in the case of polytopic membrane proteins, into the ER membrane is an essential

| Cell extract | Mode of operation | LUC (μg/mL) | Refs. |
|--------------|------------------|------------|------|
| Chinese hamster ovary (CHO) cells | Coupled | 49 | This study |
| Mouse embryonic fibroblasts | Linked | 20 | Zeenko et al. (2008) |
| Rabbit reticulocytes | Linked | 5 | Ezure et al. (2006) |
| Saccharomyces cerevisiae | Coupled | 1* | This study |
| Spodoptera frugiperda 21 (Sf21) cells | Linked | 8 | Hodgman and Jewett (2013) |
| Trichoplusia ni (HighFive) cells | Linked | 45 | Ezure et al. (2006) |
| Wheat germ | Coupled | 14 | This study |
| Trichoplusia ni (HighFive) cells | Linked | 71 | Ezure et al. (2006) |
| Wheat germ | Coupled | 1* | This study |

Productivity was determined based on de novo synthesized active firefly LUC.

*Expected yields of active LUC are in the range of 1–10 μg/mL according to the Promega TNT technical manual.
process in eukaryotic cells to obtain posttranslationally modified proteins (Zhang et al., 2006). Translocated proteins undergo co- and post-translational modifications such as glycosylation, which serve highly diverse functions (Apweiler et al., 1999; Helenius and Aebi, 2004). Cell-free protein expression systems require membrane vesicles, so-called microsomes, in order to enable various post-translational modifications, for example, glycosylation (Kubick et al., 2003; Mikami et al., 2006a; Zeenke et al., 2008). These microsomes must either be supplemented to in vitro reactions (Blobel and Dobberstein, 1975; Walter and Blobel, 1983) or are already present in the cell extracts due to a mild preparation procedure as reported for cell-free systems based on lysates derived from insect cells (Kubicki et al., 2009; Sachse et al., 2012). The CHO cell extract analyzed in this study contains translocationally active, endogenous microsomes that are capable to perform post-translational modifications, as shown by the N-glycosylation of human EPO. These microsomes can be fractionated by centrifugation to obtain microsome-enriched cell extracts, thereby increasing the concentration of translocated and glycosylated target proteins. The CHO-based cell-free system enables either a high mannose or a hybrid type of glycosylation which can be concluded from the sensitivity of de novo synthesized EPO to Endo H digestion.

In addition, these microsomes can be used to incorporate cell-free synthesized membrane proteins, especially those that are difficult to express in living cells, into a native-like lipid environment. Protein translocation and incorporation into microsomes requires an efficient signal peptide such as the melittin signal sequence, as shown by the comparative study of de novo synthesized eYFP and Mel-eYFP. Membrane incorporation of cell-free expressed proteins was tested by studying the production of the type I transmembrane protein heparin binding EGF-like growth factor using the Mel-HB-EGF-eYFP construct. Microsomes appear with a fluorescent membrane by CLSM, thereby indicating the cell-free expression, translocation, and incorporation of the target protein into the microsomal vesicles present in the CHO cell-free system. Cell-free expression of transmembrane proteins represents the basis for further downstream characterization of their functionality.

In conclusion, this wide range of applications turns the novel CHO cell-free system into a powerful alternative to already established in vitro translation systems. It offers a tool for the fast and convenient synthesis of recombinant proteins, not only for the time-saving evaluation of efficient in vivo expression constructs, but also for functional and structural studies.

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