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When less becomes more: Optimization of protein expression in HEK293–EBNA1 cells using plasmid titration – A case study for NLRs

Els F. Halff [1], Marian Versteeg, T. Harma C. Brondijk, Eric G. Huizinga *

Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, 3584 CH Utrecht, The Netherlands

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ABSTRACT

Transient transfection of the human HEK293–EBNA1 cell line using polyethyleneimine is widely adopted for recombinant protein production. Whereas high expression of many targets is achieved, purification yields of some highly expressed proteins remain low due to aggregation. We hypothesized that for these proteins the expression rates achieved at standard transfection conditions are too high, causing an overload of the protein folding machinery. Here we present plasmid titration as an efficient method to vary expression rates for the optimization of soluble protein expression. In plasmid titration a dilution series of expression vector mixed with dummy plasmid is transfected in small scale cultures. Application to GFP shows that plasmid titration achieves a wide range of expression levels while maintaining high transfection efficiencies even at 500-fold plasmid dilution.

Application of plasmid titration to selected Nod-like receptors (NLRs), which at standard conditions are highly expressed but poorly soluble, delays the onset of NLR aggregation and improves cell viability and the buildup of biomass. The amount of soluble protein depends on the combination of dilution factor and harvest day in a protein specific manner. For NOD1 50-fold plasmid dilution increases the amount of soluble protein approximately 5-fold. Due to its association with chaperones at all dilution factors tested we were unable to purify NOD1 to homogeneity. For NLRC4, which did not associate with chaperones, 10-fold plasmid dilution increased the purification yield 2-fold. This improvement, obtained with minimal effort due to the simplicity of the method, shows that reducing total expression may increase soluble protein yield.

Introduction

Structural and functional characterization of proteins using biochemical and biophysical techniques often requires their purification in large quantities and high purity. Expression levels and purification efficiencies of endogenous proteins are frequently too low to obtain sufficient amounts of native protein. Recombinant protein expression provides the advantage of enhanced expression, and the possibility to genetically alter proteins such as the addition of purification tags, or the introduction of mutations that alter protein behavior. The Human Embryonic Kidney 293 (HEK293)[2] cell line expressing Epstein–Barr virus nuclear antigen 1 (EBNA1) is well established for recombinant expression of secreted, membrane, and intracellular proteins [1–3]. Binding of EBNA1 to an expression vector containing OriP ensures episomal replication of the vector during mitosis. High expression in HEK293E cells is furthermore obtained by using optimized OriP-based expression vectors containing the strong CMV promoter. Efficient transient transfection of milliliter to liter volumes of HEK293E cells can be achieved using polyethyleneimine (PEI), which circumvents the tedious selection procedure required for stable transfection. Using this system protein production up to 50 mg per liter of cell culture has been reported for intracellular proteins [1,4].

We used the HEK293E system to express Nod-like receptors (NLRs), which are cytosolic receptors of the innate immune system that are activated by pathogen and danger associated molecular patterns (reviewed in [5]). NLRs are 100–160 kDa proteins consisting of a C-terminal leucine-rich repeat domain presumably responsible for ligand binding, a central AAA+-type ATPase domain and one or more N-terminal effector binding domains, the identity of which varies between NLRs [5,6]. Activation of NLRs results in either increased production of pro-inflammatory molecules via

* Corresponding author. Tel.: +31 302532866.
E-mail address: e.g.huizinga@uu.nl (E.G. Huizinga).
[1] Current address: Department of Neuroscience, Physiology and Pharmacology, Division of Biosciences, University College London, London WC1E 6BT, United Kingdom.

[2] *Abbreviations used:* NLRs, Nod-like receptors; HEK293, Human Embryonic Kidney 293; EBNA1, Epstein–Barr virus nuclear antigen 1; PEI, polyethyleneimine; ptf, post transfection; SEAP, secreted alkaline phosphatase.

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NF-κB or the induction of inflammatory cell death via caspase-1 activation. Mutations that compromise NLR-function have been associated with a broad range of chronic inflammatory disorders [7–9]. Among the most intensively studied NLRs are NOD1, NOD2, NLRP3, and NLRC4. When we transfected these NLRs in HEK293E cells we found them to be highly expressed, however, nearly all of the protein was aggregated and the viability of NLR-expressing cells decreased rapidly.

We suspected that aggregation of NLRs and other abundantly expressed proteins may result from an overload of the protein folding machinery due to fact that at the standard transfection conditions, which have been optimized for efficiently folding proteins like SEAP, very high transcription and translation rates are achieved [1,10]. The effect of different expression rates on protein aggregation has not been investigated in the context of transient expression.

Here we present a straightforward method to systematically vary expression levels in transient transfections by stepwise dilution of the expression plasmid with non-coding dummy plasmid. We show that this procedure, coined plasmid titration, achieves a wide range of protein expression levels while maintaining high transfection efficiencies. In the case of NLRs, plasmid dilution delays the onset of protein aggregation, improves cell viability, and increases the amount of soluble protein. At optimized expression plasmid concentrations a 2-fold improvement in yield of NLRC4 is obtained. This illustrates that more soluble protein can be obtained by expressing less protein.

Materials and methods

Cell culture and transfection

Suspension growing HEK293E cells were cultured at 120 rpm in Freestyle medium (Invitrogen), containing 0.2% FCS and 50 μg/ml G418 disulfate in a humidified 5% CO_2 atmosphere at 37 °C. Adherent HEK293E cells were cultured in Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose (DMEM + Glutamax-II, Gibco), 10% FCS, 100 units/ml penicillin G sodium (Gibco), and 100 μg/ml Streptomycin sulfate (Gibco).

Small scale cultures (4 and 20 ml) were performed in 6-well culture plates and 150 ml polycarbonate Erlenmeyer culture flasks (Corning, New York, USA), respectively; large scale (1 L) cultures were performed in 3 L polycarbonate Fernbach culture flasks (Corning). Transfections of suspension growing HEK293E cells were performed according to Durocher et al. [1], using 0.5 μg DNA/ml cell culture and 3 μg PEI/μg DNA. Series of transfections in which the amount of vector was systematically varied (referred to as plasmid titration) were performed by replacing expression vector DNA by empty pCR4-TOPO vector (Invitrogen), while keeping the total amount of DNA constant. Cell viabilities and cell counts were measured using a Casy Model TTC cell counter (Schärfe Systems, Germany).

Plasmids

cDNA of human *Nlrc4* and human *Nlrp3* was purchased from Invivogen (Toulouse, France). cDNA of human *Nod1* and human *Nod2* was a kind gift from M.G. Netea (Dept. of Medicine, Radboud University Nijmegen Medical Center, The Netherlands). The coding sequence for bovine cGMP-dependent protein kinase I alpha (PKG) was a kind gift from U-Protein Express BV (Utrecht, The Netherlands).

Coding sequences were amplified by PCR using forward primers that introduce a *BamHI* restriction site while omitting the start codon, and reverse primers that introduce a *NotI* restriction site and omit the stop-codon. At the protein level this procedure results in the introduction of a Gly-Ser sequence at the N-terminus and three Ala residues at the C-terminus. PCR products were subcloned into pCR4-TOPO vector (Invitrogen). The *BamHI*/*NotI* DNA fragments were subcloned into pUPE vectors (U-Protein Express BV, The Netherlands) that contain an expression cassette under control of the CMV promoter. Expression vectors encoded an N-terminal His_6-$StrepII_3$-TEV tag for *Nod1* and *Nod2*, and a C-terminal $StrepII_3$-His_6 tag for *Nlrp3* and *Nlrc4*, unless indicated otherwise. For fluorescence microscopy, each NLR and luciferase were expressed with an N-terminal His_6-TEV-eGFP tag. The empty pCR4-TOPO vector used as dummy DNA was created by restriction of the two *EcoRI* sites that flank the TA cloning site, followed by self-ligation.

Cell harvest and lysis

For analysis of total and soluble expression in small scale (4 or 20 ml) cultures, cells were harvested by centrifugation at 600×*g* for 2 min. Sample size was 400 μl and 2 ml for 4 and 20 ml cultures, respectively. Cell pellets were stored for 30 min up to 1 week at −20 °C. Prior to cell lysis, pellets were thawed at room temperature. Cytoplasmic extracts were obtained using a method described by Tsai et al. [11], with omission of the PBS washing step. This lysis method leaves the nuclear membranes intact. In short, cell pellets were resuspended in 1/10th of the culture volume in cold small scale lysis buffer containing 10 mM HEPES pH 7.6, 5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, 1 μg/ml DNase, 0.3% NP40, and 1 tablet Complete mini, EDTA-free tablets (Roche, Basel, Switzerland) per 20 ml buffer. Cleared lysates were obtained by centrifugation at 10,000×*g* for 10 min.

Protein purification and quantification

$StrepII_3$-tagged proteins were purified from the cleared lysates using StrepTactin Sepharose beads (GE Healthcare). After incubation for 1–2 h at 4 °C, beads were washed in StrepTactin wash buffer (100 mM NaCl, 25 mM HEPES pH 7.5, 5 mM benzamidine, 5% glycerol, 2 mM DTT), and subsequently protein was eluted in wash buffer supplemented with 5 mM *d*-desthiobiotin (Sigma).

For the purification of NLRC4-$StrepII_3$-His_6 from 200 ml HEK293E cultures, cells were harvested by centrifugation at 600×*g* for 15 min. Cell pellets were stored for 1 h up to 2 days at −80 °C. Cell lysis was performed as described above. The cleared lysates was obtained by centrifugation at 22,000×*g* for 15 min. The protein was purified as described above. The protein concentration in samples of purified NLRC4 was determined from the absorbance at 280 nm as measured on a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies; Wilmington, Delaware) using a value for A_{280} (1 mg/ml) of 0.9.

Gel electrophoresis and protein detection

Prior to gel electrophoresis, samples of total cell lysate were diluted 10-fold with lysis buffer to obtain a volume equal to the original harvest volume. The soluble fraction was obtained from the cleared lysates or, for the gel depicted in Fig. 1B, diluted 10-fold. Reduced protein samples were separated on standard Laemmli 9% SDS–PAGE gels. Gels were silver stained, Coomassie stained, or transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). Proteins were detected on Western blot using a mixture of mouse anti-polyHistidine (Sigma, catalogue number H1029) and mouse anti-Penta-His (Qiagen, Germany, catalogue number 34660) as primary antibodies and rabbit anti-mouse-HRP (Dako, Denmark) as the secondary antibody. The signal was detected using ECL (GE Healthcare). Quantification of stained