J Biomol NMR (2012) 53:43–51
DOI 10.1007/s10858-012-9619-4

ARTICLE

High-yield *Escherichia coli*-based cell-free expression of human proteins

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Received: 24 January 2012 / Accepted: 1 March 2012 / Published online: 15 March 2012

Abstract Production of sufficient amounts of human proteins is a frequent bottleneck in structural biology. Here we describe an *Escherichia coli*-based cell-free system which yields mg-quantities of human proteins in N-terminal fusion constructs with the GB1 domain, which show significantly increased translation efficiency. A newly generated *E. coli* BL21 (DE3) RIPL-Star strain was used, which contains a variant RNase E with reduced activity and an excess of rare-codon tRNAs, and is devoid of lon and ompT protease activity. In the implementation of the expression system we used freshly in-house prepared cell extract. Batch-mode cell-free expression with this setup was up to twofold more economical than continuous-exchange expression, with yields of 0.2–0.9 mg of purified protein per mL of reaction mixture. Native folding of the proteins thus obtained is documented with 2D [^{15}N,^{1}H]-HSQC NMR.

Keywords Batch-mode cell-free protein expression · *Escherichia coli* S30 cell extract · Stable-isotope labeling · Structural biology of human proteins

Abbreviations

2-ME	β-Mercaptoethanol
BMCF	Batch mode cell-free
bp	Base pairs
CECF	Continuous-exchange cell-free
kDa	Kilo-Dalton
DEPC	Diethylpyrocarbonate
DHFR	Dihydrofolate reductase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FABP4	Adipocyte fatty acid-binding protein 4
FKBP	Human peptidyl-prolyl cis–trans isomerase FKBP1A
GB1	B1 domain of protein G from *Streptococcus sp*
GILT	γ-Interferon-inducible lysosomal thiol reductase
HSQC	Heteronuclear single quantum coherence
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani
MMCE	Mitochondrial methylmalonyl-CoA epimerase
MTHFS	Methenyl-THF synthetase
OAc	Acetate
PBS	Phosphate-buffered saline
PCP2H	Purkinje cell protein 2 homolog
ppiB	Peptidyl-prolyl cis–trans isomerase B
PYG	Phosphate/yeast extract/glucose
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SF20	Stromal cell-derived growth factor
T7 RNAP	RNA polymerase from bacteriophage T7
TBS	Tris-buffered saline

Electronic supplementary material The online version of this article (doi:10.1007/s10858-012-9619-4) contains supplementary material, which is available to authorized users.

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Introduction

In structural and functional studies of human proteins, the production of sufficient amounts of soluble, active protein is a frequent bottleneck. This is due to generally low yields

of eukaryotic expression hosts such as yeast, insect or mammalian cells, whereas the use of prokaryotic hosts such as *Escherichia coli* is often limited by reduced protein solubility due to misfolding or aggregation, or by protein degradation through endogenous bacterial proteases. Here we explore the use of cell-free expression for work with small, soluble human proteins.

Cell-free expression has become popular in a variety of different applications, as it allows expression of toxic proteins (Xu et al. 2005), offers fast microliter-scale screening for optimizing expression conditions, and enables simple purification and easy scale-up (Sitaraman and Chatterjee 2009). For NMR studies, low metabolic activity of the cell extract further allows efficient stable-isotope labeling without significant isotope scrambling between amino acids (Ozawa et al. 2004; Staunton et al. 2006; Vinarov et al. 2006). Except for the use of complex semi-continuous procedures that yielded milligram quantities per mL of reaction mixture of some human proteins (Vinarov et al. 2006), cell extracts from eukaryotic organisms have been reported to provide only microgram amounts of protein per mL reaction mixture (Endo et al. 1992; Kawasaki et al. 2003; Henshaw and Panniers 1983), and major efforts are required to prepare the cell extract (Ezure et al. 2006; Anderson et al. 1983). We therefore decided to optimize an *E. coli*-based cell-free system for production of milligram amounts of soluble human proteins, in an attempt to take advantage of its high intrinsic productivity and relatively simple preparative demands (Kigawa et al. 2008; Michel-Reydellet et al. 2004; Etezady-Esfarjani et al. 2007; Torizawa et al. 2004; Ozawa et al. 2004).

Materials and methods

Our protocol for cell-free protein expression includes that the cell extract was home-made, both to ensure high activity of the freshly prepared extract and for economic reasons. Throughout all procedures used, we exercised extreme care to avoid RNase contamination.

Generation of an *E. coli* BL21 (DE3) RIPL-Star strain for S30 extract preparation

Escherichia coli BL21 (DE3) CodonPlus cells were grown overnight in 5 mL LB medium, harvested by centrifugation, and the pACYC- and pSC101-based plasmids (3.5 and 4.7 kbp, respectively), which harbour genes encoding rare-codon tRNAs, were isolated with a plasmid miniprep (NucleoSpin Plasmid Kit, Macherey–Nagel). All culture media and media plates were supplemented with 20 μg/mL chloramphenicol and 20 μg/mL streptomycin, and they were incubated at 37 °C. Plasmids were separated on a 0.8 % agarose gel, extracted from the gel (QIAEXII, Qiagen), and co-transformed into *E. coli* BL21 (DE3) Star cells (Stratagene). Positive transformants were used to inoculate 5 mL LB cultures, which were then grown with shaking to an OD_{600} of ca. 1.0. The cell suspensions were supplemented to a final concentration of 30 % (v/v) with glycerol and stored at −80 °C in 1 mL aliquots, to be used for inoculation of S30 extract cultures.

S30 extract preparation

The protocols proposed by Chen and Zubay (1983) and by Kainosho and coworkers (Torizawa et al. 2004) were adopted as the starting platform for obtaining cell extract from the *E. coli* BL21 (DE3) RIPL-Star strain. All equipment used for extract preparation was treated sequentially with RNase-AWAY (Molecular BioProducts) and DEPC-treated water and, unless stated otherwise, all solutions other than the culture media were prepared with DEPC-treated water. All preparative steps after cell harvest were performed on ice, and centrifugation and dialysis were carried out at 4 °C.

9 L of PYG medium (5.6 g/L KH_2PO_4, 28.9 g/L K_2HPO_4, 10 g/L yeast extract, and 1 % (w/v) glucose) for the *E. coli* cultures was supplemented with 20 μg/mL streptomycin and 20 μg/mL chloramphenicol, and cells were grown to mid-log phase and harvested as described (Etezady-Esfarjani et al. 2007). The cell pellet was washed twice by resuspending in 500 mL S30 buffer (10 mM Tris-OAc at pH 8.2, 60 mM KOAc, 14 mM $Mg(OAc)_2$, 7.15 mM 2-mercaptoethanol, 1 mM DTT) using a Polytron PT-3100 cell homogenizer at 3,500 rpm followed by centrifugation. The washed cell pellet was resuspended in 2 mL S30 buffer per gram of cells, followed by cell disruption with a single passage through a French Press at 16000 psi (Thermo Electron Corporation). The lysate was centrifuged twice for 30 min at 30,000×*g*, and each time the supernatant was carefully pipetted into a new tube. The cleared lysate was then incubated for 40 min at 30 °C with 0.25 volumes pre-incubation mixture (293.3 mM Tris-OAc at pH 8.2, 84 mM PEP, 13.17 mM ATP, 9.24 mM $Mg(OAc)_2$, 4.4 mM DTT, 40 μM of each of the 20 proteinogenic amino acids, 6.67 U/mL of pyruvate kinase). After 10 min of centrifugation at 4,000×*g*, the pre-incubation supernatant was dialyzed for 1.5 h with a 12–14 kDa MWCO SpectraPor4 dialysis membrane, first against 1 L of S30 buffer and then overnight against 2 L of fresh S30 buffer. The extract was finally concentrated in a 10 kDa MWCO Amicon Ultra-15 ultrafiltration device (Millipore) to an A_{260} of 350–450, and it was cleared by centrifugation for 10 min at 4,000×*g* to remove insoluble particles. 500 μL aliquots of cell extract were frozen by

immersion into liquid nitrogen and stored at −80 °C until needed.

Preparation of the vectors pCFX1 and pCFX3 and cloning of target genes

The B1 domain of the protein G was PCR-amplified from pET30a-GB1 (Zhou et al. 2001), using the 5′- and 3′-oligonucleotide primers 5′-ATA AGA ATG CGG CCG CCA GTA CAA ACT GAT CCT GAA CGG-3′ and 5′-GGA ATT CCA TAT GGC TGC CGC GCG GCA CCA GGC CGC TGC TTT CGG TAA CGG TGA AGG TTT TGG-3′. It was then inserted into pIVEX2.4, using the NotI and NdeI restriction sites to yield the vector pCFX1. The vector pCFX3 was prepared from pCFX1 by site-directed mutagenesis (QuikChange II XL, Stratagene), using 5′-CC GAA AGC AGC GGC GAA AAC CTG TAC TTC CAG AGC GGC AGC CAT ATG ACC-3′ and 5′-GGT CAT ATG GCT GCC GCT CTG GAA GTA CAG GTT TTC GCC GCT GCT TTC GG-3′ as 5′- and 3′-oligonucleotide primers, respectively.

The cDNA encoding the human target proteins was purchased from ImaGenes (Berlin). pET21-DHFR was a gift of Dr. A. Horwich. All target proteins were amplified by PCR (Hotstart) from their respective source DNA, using the oligonucleotide primers (Microsynth) listed in Table S1, and were inserted into pIVEX2.4d (Roche) and pCFX1 using the NdeI and BamHI (NEB) restriction sites. All constructs were sequence-verified at Microsynth (Switzerland). Plasmids for cell-free expression were prepared using the Qiagen Plasmid Maxi Kit (Qiagen).

Cell-free expression

With the exception of the S30 extract, all stock solutions required for cell-free synthesis were stored in appropriate aliquots at −20 °C and were thawed on ice prior to use. The reaction mixtures were prepared with the components listed in Tables S2 and S3. T7 RNA polymerase was expressed and purified as previously described (Etezady-Esfarjani et al. 2007). Analytical-scale batch-mode reactions (50–100 µL) were carried out in 1.5 mL tubes in a Thermomixer Comfort (Eppendorf) at 30 °C for 2.5 h with gentle agitation; preparative-scale reactions (10 mL) were incubated in 15 mL tubes. For continuous-exchange cell-free expression, the reaction solution was transferred to a 50 kDa MWCO SpectraPor Float-A-Lyzer (Spectrum Labs) that was completely immersed in a 14 mL round-bottom culture tube (Greiner Bio-One) containing a ten-fold excess of feeding solution (Table S4). The reaction chamber was sealed tightly with parafilm and was incubated for 20 h at 30 °C with gentle agitation. After the reaction was completed, the reaction and feeding solutions were both pooled and used for subsequent purification.

Monitoring cell-free expression mixtures with SDS-PAGE and Western Blot

After cell-free expression, soluble and insoluble protein was separated by centrifugation (3 min at 14,000×*g*). For protein production we worked only with the soluble fraction. 50 µL of the supernatant was mixed with 500 µL of acetone at −20 °C, the mixture was incubated for 5 min on ice, and centrifuged for 5 min at 14,000×*g*. The pellet was collected and residual acetone removed in an airstream before resuspension in 100 µL SDS-PAGE loading buffer (150 mM Tris–HCl at pH 6.8, 6 % (v/v) 2-ME, 1.2 % (v/v) SDS, 30 % (v/v) glycerol, bromophenol blue) for analysis by SDS-PAGE.

To investigate the impact of fusion with the GB1-domain, we also analyzed the pellet containing the protein which precipitated during the cell-free reaction. The pellet was washed by re-suspension in 100 µL water, collected again by centrifugation for 5 min at 14,000×*g*, and dissolved in 100 µL SDS-PAGE loading buffer.

The solutions in SDS-PAGE loading buffer were incubated at 95 °C for 3 min, which was followed by vortexing for 10 min and clearing by centrifugation for 3 min at 12,000×*g*. The resulting samples were applied either on NuPAGE 4–12 % Bis–Tris gels (Invitrogen) using MES running buffer (50 mM MES, 50 mM Tris, 1 mM EDTA, 0.1 % (w/v) SDS), or on 12 % SDS-Tris-Laemmli gels (Laemmli 1970) using a Tris-Tricine running buffer (100 mM Tris, 100 mM Tricine, 0.1 % (w/v) SDS).

Western blots were prepared following the protocol by Towbin et al. (Towbin et al. 1979) and were incubated sequentially with 1:5,000 diluted murine anti-polyHistidine antibody (Sigma) and anti-mouse IgG (Fc specific) alkaline phosphatase conjugate (Sigma). Protein bands were visualized in alkaline phosphatase reaction buffer (100 mM Tris–HCl at pH 9.5, 100 mM NaCl, 5 mM $Mg(Cl)_2$) supplemented with 100 µM BCIP (Roche) and 100 µM NBT (Sigma).

Purification of the cell-free expression product

All purification steps were performed at 4 °C, using an Aekta-prime FPLC system (GE Healthcare) equipped with A_{280} and conductivity-measurement monitoring devices. The protein solutions obtained after clearing the preparative-scale cell-free reaction mixtures were applied to a 5 mL HisTrap HP column (GE Healthcare) pre-equilibrated with buffer A (50 mM sodium phosphate at pH 7.2, 30 mM imidazole, 500 mM NaCl, 10 µM NaN_3). After washing with 100 mL buffer A, the target protein was

eluted in a 100 mL linear gradient of 30–500 mM imidazole in buffer A. Fractions with absorbance at 280 nm were subjected to SDS-PAGE analysis.

Proteolytic cleavage of the GB1 fusion domain

Initially, the GB1 fusion domain was cleaved with thrombin. For this, ten units of thrombin were added per mg of fusion protein, and the cleavage reaction was dialyzed either for 4 h at room temperature or overnight at 4 °C against 2 L of PBS (11.8 mM sodium phosphate at pH 7.4, 4.4 mM KCl, 136.9 mM NaCl, 10 μM NaN_3) with a 12–14 kDa MWCO SpectraPor4 dialysis membrane. The reaction was quenched with 1 mM PMSF, and the solution was passed through a 5 mL HisTrap HP column pre-equilibrated with buffer A. The flow-through containing the target protein was collected.

Alternatively, we have used TEV protease for fusion protein cleavage. For this, 0.1 mg of $(His)_6$-tagged TEV was added to each mg of purified fusion protein and the cleavage reaction was dialyzed overnight with a 3.5 kDa MWCO SpectraPor3 or a 12–14 kDa MWCO SpectraPor4 dialysis membrane against 2 L of TEV cleavage buffer (50 mM sodium phosphate at pH 7.4, 100 mM NaCl, 2 mM 2-mercaptoethanol, 0.5 mM EDTA, 10 μM NaN_3). After cleavage the solution was passed through a 5 mL HisTrap HP column (GE Healthcare) and the flow-through containing the desired target protein was collected.

For production of TEV protease, the plasmid pET19-TEV, obtained as a gift from Dr. E. Weber-Ban, was transformed into *E. coli* BL21 (DE3) CodonPlus cells. Cells were grown at 37 °C in LB medium containing 100 mg/L carbenicillin to an OD_{600} of ca. 0.6–0.8 and were then induced with 0.125 mM IPTG for 12 h at 20 °C. The cell pellet collected by centrifugation was resuspended in 80 mL buffer A (50 mM sodium phosphate at pH 7.4, 30 mM imidazole, 500 mM NaCl) and passed four times through the cell cracker at 75 psi. After centrifugation at 30,000 × *g* and 4 °C for 30 min, the supernatant was applied to a HisPrep FF 16/10 column (GE Healthcare). After washing with 100 mL buffer A, TEV protease was eluted with a 150 mL linear gradient from buffer A to buffer B (50 mM sodium phosphate at pH 7.4, 500 mM imidazole, 500 mM NaCl). The fractions containing TEV protease were pooled and dialyzed overnight at 4 °C with a 12–14 kDa MWCO SpectraPor4 dialysis membrane against buffer C (25 mM sodium phosphate at pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, 10 μM NaN_3). The buffer was then exchanged with 2 L of new buffer C and dialysis was continued for 8 h. The resulting solution was concentrated in a 10 kDa MWCO Vivaspin-20 ultracentrifugation device to ca. 0.8 mg/mL, diluted with glycerol to a final concentration of 0.5 mg/mL TEV, and stored at −80 °C until needed.

NMR sample preparation

For each protein in Table S1, a 10 mL BMCF reaction with ^{15}N-labeled amino acids (Spectra Stable Isotopes) was set up. The purified target proteins were dialyzed overnight at 4°C against 2 L of NMR buffer (depending on the protein: between 20 and 50 mM sodium phosphate, 20 and 100 mM sodium chloride, 100 μM EDTA, 10 μM NaN_3, 5 % (v/v) D_2O, with the pH of the solution adjusted in accordance with the pI of the protein, and DTT or 2-ME added for proteins containing cysteine residues). Protein constructs with a molecular mass below 15 kDa were dialyzed in a 8 kDa MWCO SpectraPor Biotech RC membrane, while larger constructs were dialyzed in 12–14 kDa MWCO SpectraPor4 membranes (both from Spectrum Labs). After overnight dialysis, the dialysis tubing was transferred into 2 L of fresh NMR buffer for another 4 h. The protein solution was then concentrated using a 3 kDa or 10 kDa MWCO ultracentrifugation device (Millipore) to a final volume of ca. 500 μL, supplemented with 5 % (v/v) D_2O and transferred into a 5TA NMR sample tube (Armar Chemicals).

NMR spectroscopy

NMR measurements were performed either on a Bruker DRX-500 spectrometer equipped with a Cryoprobe, or on Bruker DRX-600 and DRX-750 spectrometers equipped with room-temperature triple resonance probes with shielded z-gradient coils. The measurement temperature was in the range from 20 to 37 °C, depending on the protein. Spectra were processed either with the program PROSA (Güntert et al. 1992) or with TOPSPIN 2.0 (Bruker-Biospin), and spectral analysis was carried out using the program CARA (Keller 2004).

Results and discussion

S30 cell extract preparation

For cell extract preparation we generated the new strain *E. coli* BL21 (DE3) RIPL-Star by introducing two plasmids that encode rare-codon tRNAs from *E. coli* BL21 (DE3) CodonPlus cells into competent *E. coli* BL21 Star cells (Fig. 1). This strain contains basic features that have previously been shown to be favorable for cell-free synthesis. This includes that the strain has reduced RNase E activity (Jermutus et al. 1998; Kigawa et al. 2004) to increase mRNA half-life (Pratt 1984; Torizawa et al. 2004), and is deficient in both OmpT and lon protease activities to reduce proteolytic degradation of the target protein (Kigawa et al. 2004).

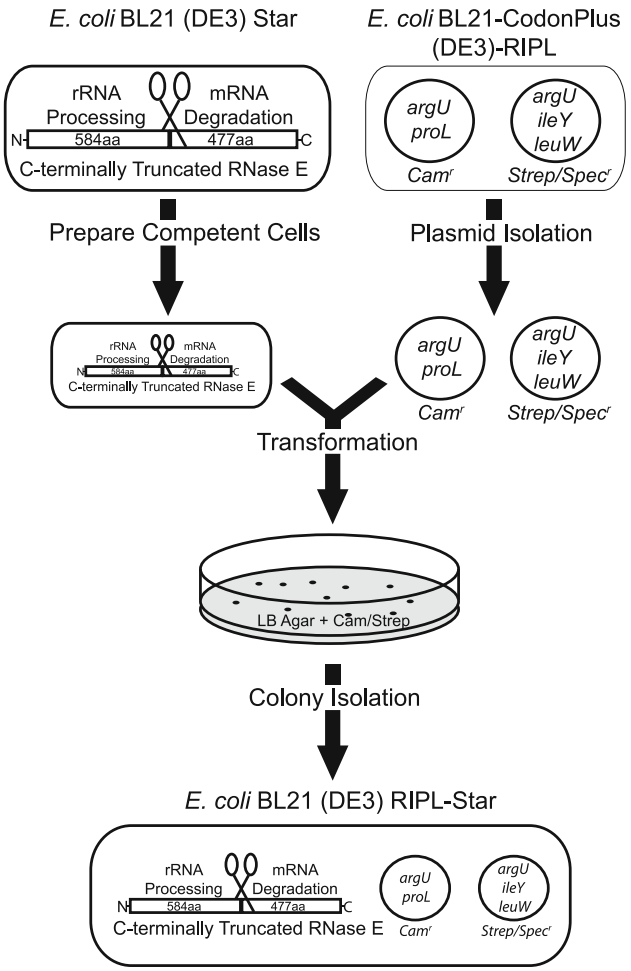

Fig. 1 Flow diagram of the preparation of the *E. coli* BL21 (DE3) RIPL-Star strain

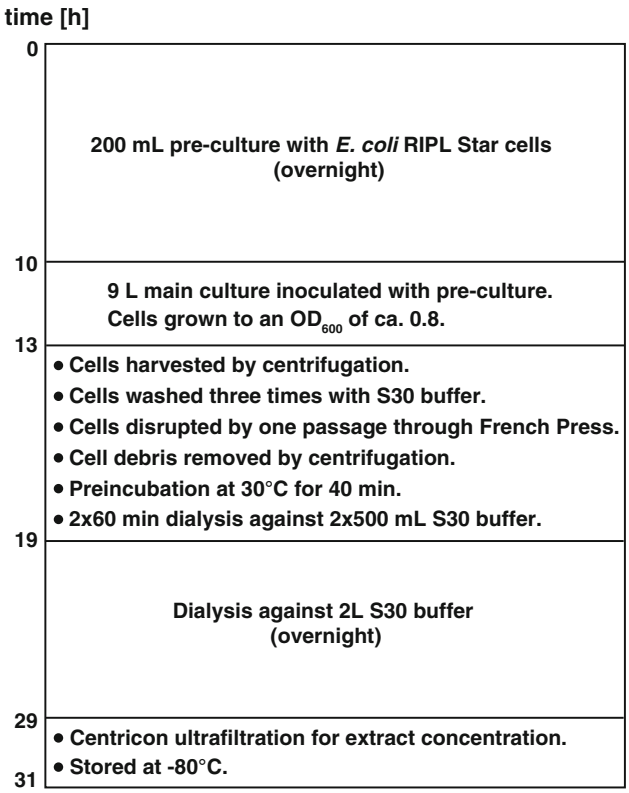

Fig. 2 Flow diagram of the S30 cell extract preparation. On the *left* the approximate times needed for individual steps of the protocol are indicated. *Bullets* indicate the manipulations that require constant involvement of the operator

The S30 extract preparation was based on protocols proposed by Nirenberg and Matthaei (1961) and Chen and Zubay (1983). Adjustments include that the cells are harvested, washed and disrupted within 1 day, followed by pre-incubation of the cell extract and overnight dialysis against S30 buffer (Fig. 2). Thereby, pre-incubation of the cell-extract was shortened from 80 to 40 min and the incubation temperature was reduced from 37 to 30 °C, so as to reduce exposure of the cell extract to conditions that could lead to degradation of the translation machinery. After an overnight pre-culture, highly active cell extract was produced in one working day, with a yield of about 2.5 mL cell extract per liter of cell culture (Fig. 2).

Expression vectors and protein purification

Two new cell-free expression vectors, pCFX1 and pCFX3, containing a 55-amino acid N-terminal GB1-solubility domain with a flexible linker to the target protein were prepared, starting from the commercially available pIVEX2.4 vector (Fig. 3). The vectors provide a multiple-cloning site (MCS) for insertion of target genes with the use of common restriction enzymes, and enable proteolytic removal of either the $(His)_6$-tagged GB1-domain with thrombin (pCFX1) or tobacco etch virus protease (pCFX3), or the $(His)_6$-tag with Factor Xa protease.

Cell-free expression of human proteins

Ten human proteins intended for NMR investigation, with molecular mass below 25 kDa, were used in this study (Table S1), including the four proteins DHFR, complexin-1, ppiB and FABP4 with known structures (Stockman et al. 1992; Chen et al. 2002; Mikol et al. 1994; Marr et al. 2006). The proteins were cloned into both the pIVEX2.4 and pCFX1 vectors. Initially, 100 µL small-scale test expressions were carried out for 2.5 h at 30 °C, and the soluble and insoluble protein products were analyzed with SDS-PAGE and Western blot (Fig. 4). Compared to pIVEX2.4, cell-free protein expression using the pCFX1 vector resulted in up to fivefold increased yields (Fig. 4a, b). With the sole exception of the methenyl-THF synthetase, the presence of the GB1 tag, which is normally considered to enhance solubility, was

found not to influence the amount of insoluble protein (Fig. 4c, d). We conclude that although the GB1-fusion results in increased yields of soluble protein, as expected (Zhou et al. 2001), it also leads to increased total protein synthesis due to enhanced translation efficiency (Butt et al. 1989; Laursen et al. 2005; Goerke and Swartz 2008).

For a quantitative comparison, 10 mL batch-mode reactions of the proteins ppiB, FABP4, SF20, and MTHFS were set up with both pIVEX2.4 and pCFX1 constructs. After purification the protein yields were quantified by measuring the UV absorption at 280 nm. In all cases the protein yield was increased at least twofold when the targets were expressed as GB1 fusion proteins in the pCFX1 vector (Figure S1).

Purification of the cell-free expression product, proteolytic removal of the GB1 domain or the $(His)_6$-tag (Fig. 3), and final purification of the target protein were performed as described in Materials and Methods. For most of the targets the fusion construct with the GB1 domain as well as the free protein were prepared.

NMR characterization of proteins from cell-free expression

The 2D [^{15}N,^{1}H]-HSQC spectra of nine proteins are shown in Fig. 5. No spectrum of GILT could be obtained because the protein aggregated when the solution was concentrated after purification. For some of the proteins the GB1-domain, which usually stabilizes the proteins in solution, was not removed, since it did not interfere with the analysis of the spectrum. For dihydrofolate reductase (Fig. 5a) NMR assignments are available (Stockman et al. 1992). Comparison of the cell-free produced dihydrofolate reductase in complex with methotrexate with the literature data showed that the native fold, capable of binding methotrexate, was obtained after cell-free expression.

The 2D [^{15}N,^{1}H]-HSQC spectra of the proteins shown in Fig. 5b–f, all show chemical shift dispersion typical of globular domains. In the GB1-fusion constructs the subspectrum of this domain can readily be recognized.

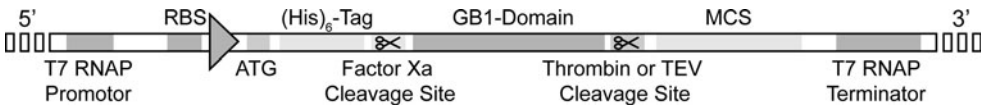

Fig. 3 Cell-free expression vectors pCFX1 and pCFX3. Based on the commercially available vector pIVEX2.4, they both contain a multiple-cloning site (*MCS*) for insertion of the target gene and a GB1 solubility domain which precedes the MCS. The GB1 domain can be cleaved either with thrombin (pCFX1) or with TEV (pCFX3). The N-terminal hexahistidine tag can be proteolytically removed with Factor Xa. T7 RNAP is the RNA polymerase from bacteriophage T7, RBS the ribosomal binding site, and ATG the start codon for translation

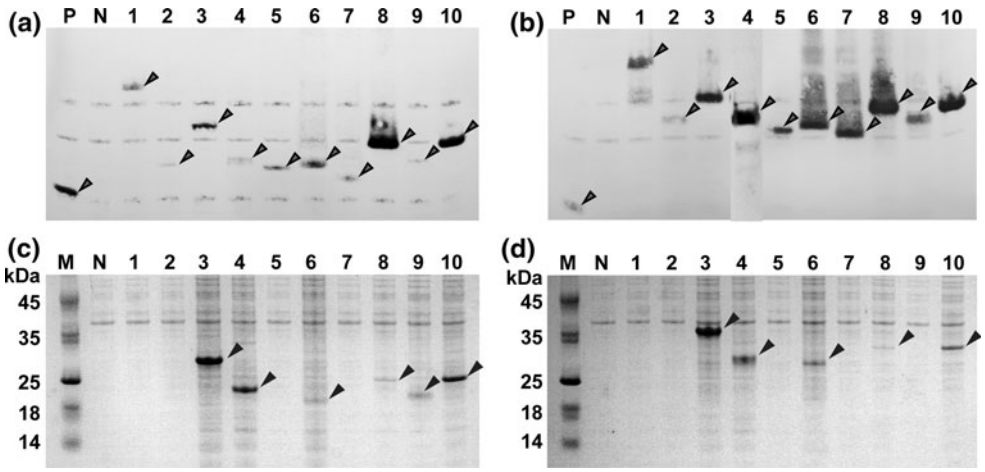

Fig. 4 Comparison of cell-free expression yields for the 10 targets of Table S1 without and with N-terminal fusion with the GB1 domain. Shown are Western blot analyses of the soluble protein obtained from cell-free expression using either the pIVEX2.4 vector (**a**) or the pCFX1 vector (**b**). **c**, **d** SDS-PAGE analyses of the insoluble protein obtained from the same cell-free expression reactions with the pIVEX2.4 (**c**) or pCFX1 (**d**) vectors. The reactions were carried out for 2.5 h at 30 °C with gentle agitation. The synthesis mixture for production of dihydrofolate reductase (*lane 10*) was supplied with 440 μM methotrexate prior to reaction initiation. The applied sample volume on each lane corresponds to 2.5 μL reaction mixture in **a** and **b**, and to 5 μL reaction mixture in **c** and **d**. *Arrows* indicate the bands corresponding to the target proteins. Symbols used: *M* protein molecular weight marker; *N* negative control; *P* positive control protein FKBP; *1* MARCKS-related protein (macrophage myristoylated alanine-rich C kinase substrate); *2* purkinje cell protein 2 homolog; *3* methenyl-THF synthetase (5-formyltetrahydrofolate cyclo-ligase); *4* γ-interferon-inducible lysosomal thiol reductase; *5* mitochondrial methylmalonyl-CoA epimerase; *6* uncharacterized human protein C19orf10 (stromal cell-derived growth factor SF20); *7* adipocyte fatty acid-binding protein 4; *8* peptidyl-prolyl cis–trans isomerase B; *9* complexin-1; *10* dihydrofolate reductase

For the proteins complexin-1, MARCKS-related protein, and PCP2H (Fig. 5g–i), which contain a high content of polar and charged amino acids and a low percentage of large hydrophobic residues, characteristic of intrinsically unstructured proteins (Dyson and Wright 2005), the program GlobPlot (http://globplot.embl.de/) (Linding et al. 2003) predicts that these proteins are mostly disordered. In agreement with this prediction, all three proteins give rise to 2D [^{15}N,^{1}H]-HSQC spectra with small dispersion of the ^{1}H chemical shifts, except that the well-dispersed spectrum of GB1 is seen in the fusion constructs of complexin-1 and the GB1-MARCKS-related protein with this domain.

Economic considerations

The tables S6 and S7 show that with the use of in-house prepared cell extract, the expense for chemicals in the present cell-free system is dominated by the isotope-labeled amino acids. In this context we compared cost effectiveness of the popular continuous-exchange cell-free

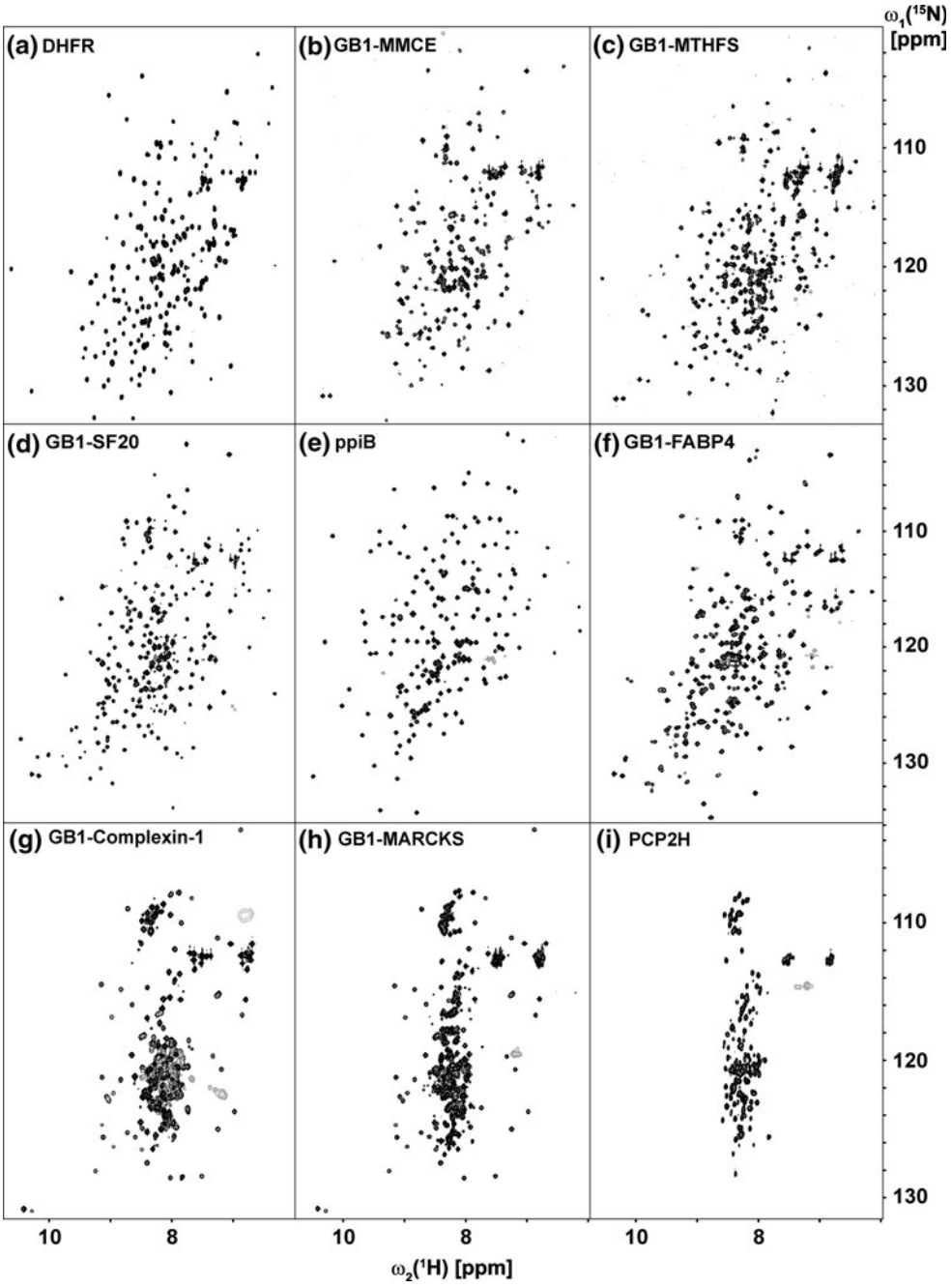

Fig. 5 2D [^{15}N,^{1}H]-HSQC spectra of human proteins prepared by cell-free expression with ^{15}N-labeled amino acids, where for some proteins the fusion construct with GB1 was measured. **a** 325 μM [*u*-^{15}N]-dihydrofolate reductase in complex with methotrexate at 20 °C. **b** 150 μM [*u*-^{15}N]-GB1-methylmalonyl-CoA epimerase at 37 °C. **c** 165 μM [*u*-^{15}N]-GB1-MTHFS at 37 °C. **d** 250 μM [*u*-^{15}N]-GB1-stromal cell-derived growth factor SF20 at 25 °C. **e** 360 μM [*u*-^{15}N]-peptidyl-prolyl cis–trans isomerase B at 20 °C. **f** 400 μM [*u*-^{15}N]-GB1-FABP4 in the presence of 1 mM unlabeled SDS at 20 °C. **g** 500 μM [*u*-^{15}N]-GB1-complexin-1 at 20 °C. **h** 600 μM [*u*-^{15}N]-GB1-MARCKS-related protein at 20 °C. **i** 200 μM [*u*-^{15}N]-purkinje cell protein-2 homolog at 20 °C. The spectra **a**–**f** were recorded at 750 MHz, **g**, **h** at 500 MHz, and **i** at 600 MHz

(CECF) expression mode (Chekulayeva et al. 2001; Schwarz et al. 2007; Torizawa et al. 2004; Vinarov et al. 2006; Ozawa et al. 2004; Kigawa et al. 2004; Spirin 2004) with batch mode synthesis.

To compare productivity and costs, we performed both CECF and BMCF expressions of the soluble protein FKBP (Etezady-Esfarjani et al. 2007). Using CECF, 2 mg of purified protein was obtained per mL reaction mixture, while 0.4 mg of purified protein per mL reaction mixture was obtained using BMCF. However, in spite of the higher productivity, CECF was found to be twofold more expensive than BMCF for the preparation of 1 mg of ^{13}C,^{15}N-labeled FKBP. The higher cost resulted primarily from the increased quantities of labeled amino acids used (Table S7). In addition, based on indirect evidence such as loss of enzyme activity in the expression mixture, we suspect that the prolonged incubation of the protein at 30–37 °C in the highly crowded protein environment of the reaction mixture may lead to protein inactivation or precipitation in the CECF assay. In BMCF, the reaction time is shorter, so that after 2.5 h the target protein can be transferred to a molecular environment that supports the integrity and stability of the target protein.

Acknowledgments We thank Cristina Stocker for help with cell extract preparations, Dr. Arthur Horwich for providing the pET21-DHFR plasmid, Dr. Eilika Weber-Ban for providing the pET19-TEV plasmid, and the Swiss National Science Foundation and the ETH Zürich for financial support through the National Center of Competence in Research (NCCR) Structural Biology.

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