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Exploiting *Leishmania tarentolae* cell-free extracts for the synthesis of human solute carriers

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Abstract

Cell-free protein production offers a versatile alternative to complement *in vivo* expression systems. However, usage of prokaryotic cell-free systems often leads to non-functional proteins. We modified a previously designed cell-free system based on the protozoan *Leishmania tarentolae*, a parasite of the Moorish gecko *Tarentola mauritanica,* together with a species-independent translational sequences-based plasmid to produce human membrane proteins in 2 hours reaction time. We successfully established all four commonly used expression modes for cell-free synthesis of membrane proteins with a human organic anion transporter, SLC17A3, as a model membrane protein: (i) As precipitates without the addition of any hydrophobic environment, (ii) in the presence of detergents, (iii) with the addition of liposomes, and (iv) supplemented with nanodiscs. We utilized this adapted system to synthesize 22 human solute carriers from 20 different families. Our results demonstrate the capability of the *Leishmania tarentolae* cell-free system for the production of a huge variety of human solute carriers in the precipitate mode. Furthermore, purified SLC17A3 shows the formation of an oligomeric state.

Keywords: *Membrane proteins, solute carriers, cell-free synthesis*

Introduction

Solute carriers (SLCs) form the second largest group of human membrane proteins, controlling the uptake and efflux of a huge variety of solutes like inorganic ions, nucleotides, amino acids, sugars and drugs (Hediger et al. 2004). Solute carriers are grouped into 51 families by amino acid sequence similarity and substrate specificities, comprising mitochondrial transporters, coupled transporters, exchangers, passive transporters and vesicular transporters. Numerous solute carriers are linked to human diseases like the biotin-responsive basal ganglia disease (BBGD), caused by defects in the thiamine transporter 2, SLC19A3 (Zeng et al. 2005). Furthermore, several transporters can serve as drug targets or as drug delivery systems (Hediger et al. 2004). For example SLC17A3 (NPT4), an organic anion efflux transporter in kidneys and liver was found to transport drugs like *para*-aminohippurate and diuretics as well as endogenous urate in a voltage-driven manner (Jutabha et al. 2010, 2011a, 2011c).

Cell-free systems are completely open systems and thus offer the opportunity for the synthesis of proteins, allowing the addition of additives like protease inhibitors or substrates which can stabilize the produced membrane proteins (Klammt et al. 2004). Providing a hydrophobic environment using detergents, liposomes or nanodiscs, further increases the application range for cell-free synthesis of membrane proteins (Lyukmanova et al. 2012). The recently established supplementation of cell-free reactions with nanodiscs (Cappuccio et al. 2008, Katzen et al. 2008a, 2008b, Yang et al. 2011), lipid bilayer patches, surrounded by a belt of membrane scaffold proteins (Denisov et al. 2004), is a promising tool for the cell-free production of functional membrane proteins.

The most commonly used cell-free system for the synthesis of membrane proteins is based on the prokaryote *Escherichia coli* (Schwarz et al. 2007a, 2007b, 2008, Klammt et al. 2004, 2007). The *E. coli* cell-free system is afflicted with the lack of post-translational modifications and eukaryotic multi-domain proteins tend to missfold in this system due to the absence of chaperones, necessary to fold proteins correctly. Cell-free systems on the basis of eukaryotes like wheat germ extracts (WGE) (Nozawa et al. 2009, 2010, Takai and Endo 2010, Takai et al. 2010,

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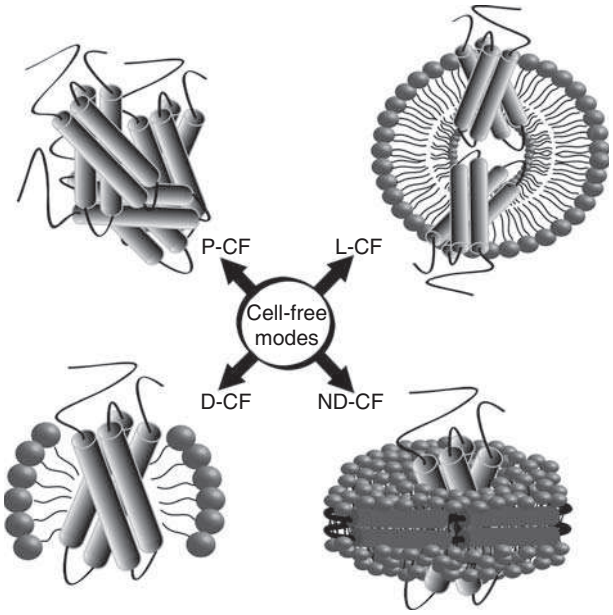

Figure 1. Schematic overview of cell-free production modes for membrane proteins. In the precipitate cell-free (P-CF) mode, membrane proteins aggregate due to the lack of hydrophobic environment. Membrane proteins can be co-translationally produced in a solubilized form by adding detergents (D-CF). Lipids can be provided either as liposomes (L-CF), or as nanodiscs (ND-CF).

Guild et al. 2011, Noirot et al. 2011), insect cell extracts (ICE) (Ezure et al. 2006, Katzen and Kudlicki 2006, Suzuki et al. 2006a, 2006b) or rabbit reticulocyte lysates (RRL) (Hoerz and McCarty 1971, Jackson et al. 1983, Beckler et al. 1995, Olliver and Boyd 1998) provide the right environment, but are often elaborate to prepare or are adversely affected by low yields.

Therefore, Mureev et al. (2009) established a cell-free system based on *Leishmania tarentolae*. The lysates used in this system can be prepared with robust protocols and yield relatively high amounts of protein.

Until now most eukaryotic cell-free systems suffered from the necessity of cap-analogs to enable efficient translation. The bottlenecks of those analogs are high costs and the fact that free cap-analogs inhibit cell-free protein synthesis. Furthermore, Mureev et al. (2009) used the species-independent translational sequences (SITS), which enable cell-free protein synthesis not only in multiple cell-free systems, but also abolish the requirement for cap-analogs in eukaryotic systems.

We successfully adapted this method to the synthesis of human integral membrane proteins. Here we describe the establishment of all four commonly used modes for membrane proteins in cell-free systems: As precipitates (P-CF), in the presence of detergents (D-CF), with the addition of liposomes (L-CF) and nanodiscs (ND-CF) (Figure 1). We use SLC17A3, a human organic anion transporter (Melis et al. 2004, Reimer and Edwards 2004, Jutabha et al. 2010, 2011a, 2011b, 2011c) as a model protein. Furthermore, we report the production of 22 human solute carriers from 20 different families with *L. tarentolae* extracts.

Material and methods

Preparation of DNA constructs

Constructs for cell-free synthesis of human solute carriers were cloned into the vector pLEXSY_invitro-1 (Jena Bioscience, Jena, Germany), which also codes for the enhanced green fluorescent protein (eGFP).

Targets were amplified via standard polymerase chain reactions and restriction sites were inserted with the corresponding forward and reverse primers at the 5′ and 3′ end of the gene, respectively. Genes of interest were cloned into pLEXSY_invitro-1 using the restriction enzymes *Hind*III and *Not*I, resulting in a N-terminal eGFP-fusion and *Nco*I and *Xho*I, achieving a C-terminal eGFP-fusion. Oligonucleotides used for cloning are listed in Table I.

Polymerase chain reaction products were cut with the appropriate restriction nucleases, purified by agarose-gel electrophoresis and ligated into the vector pLEXSY_invitro-1.

Preparation of T7 RNA polymerase

T7 RNA polymerase was prepared from 2 l LB-Medium, inoculated with *E. coli* C43 (DE3) (Miroux and Walker 1996) (Lucigen, Middleton, WI, USA), carrying the plasmid pAR1219 (Sigma, Taufkirchen, Germany). Cells were grown aerobically at 37°C and 180 rpm until an OD_{600} of 0.6 was reached, which was determined using an Ultrospec*2100* pro spectrophotometer (Amersham Biosciences, Freiburg, Germany). Subsequently, gene expression was induced with isopropyl-β-D-thiogalactopyranosid and cells were further incubated for 3 h.

Cells were harvested by centrifugation at 4°C, 5000*g* for 10 min and the pellet was washed by resuspending in TEN buffer (50 mM Tris-HCl pH 8.1, 2 mM Na_2EDTA, 20 mM NaCl) and another centrifugation step as above. Cells were resuspended in 30 ml TEN buffer with additional 0.375 mg/ml lysozyme. Cells were incubated on ice for 20 min, 0.067% sodium-deoxycholate was added and the suspension was again incubated on ice for 20 min. The lysate was sonicated three times for 30 sec. After addition of 5 ml 2 M

Table I. Primers used for cloning.

Primer	Sequence 5′–3′
1A4fwdHind	TCTGCAAGCTTCAGAGAAGAGCAACG
1A4revNot	AGGAGGGCGGCCGCTTACAGAACCGACTC
pInv-4A8-Hind-fwd	TCTGCAAGCTTCACCGGCCGCCGGGAG
pInv-4A8-Not-rev	GGAGGGCGGCCGCTTACTTAGAAGTCCCTCC
5A11fwdHind	TCTGCAAGCTTCAGTGTGGTGGCCAG
5A11revNot	GGAGGGCGGCCGCTTAAGCAAAATAGCC
10A4fwdHind	CTGCAAGCTTCAGACGGCAACGAC
10A4revNot	GAGGGCGGCCGCTTAGAGAGAAGTCTGAGCG
12A1_Hind_fwd	GGTGGAAGCTTCTCACTGAACAACTC
12A1_Not_rev	CTCCAGGCGGCCGCAGAGTAAAATGTCAAG
12A3_Hind_fwd	GGTGGAAGCTTCGCAGAACTGCCCAC
12A3_Not_rev	TCCAGGCGGCCGCCTGGCAGTAAAAGG
Inv_17A3C_fwd	GGAGATCTCGAGCGCCACCAAGACAGAGTTG
Inv_17A3C_rev	CCCTTGCTCGAGTGTAAACGAGTGAGTTTTCTC
Inv_17A5N_fwd	ATCTGCAAGCTTGGTCTCCGGTTCGAGAC
Inv_17A5N_rev	AGGAGGGCGGCCGCTTAGTGTCTGTGTCCATGGTG
pInv-19A3-Hind-fwd	CTGCAAGCTTCAGATTGTTACAGAACTTCAC
pInv-19A3-Not-rev	GGAGGGCGGCCGCTTAGAGTTTTGTTGACATG
pInv-20A1-Hind-fwd	TCTGCAAGCTTCAGCAACGCTGATTACC
pInv-20A1-Not-rev	GGAGGGCGGCCGCTTACATTCTGAGGATGAC
26A6fwdNco	GAAACCATGGCAGGGCTGGCGGATG
26A6revXho	CTTGCTCGAGGTGAGTCTGGTGACC
27A6fwdHind	TCTGCAAGCTTCACTTCTGTCATGG
27A6revNot	GGAGGGCGGCCGCTTAAAGTTTTATTTCC
29A4fwdHind	TCTGCAAGCTTCAGGCTCCGTGGGG
29A4revNot	GGAGGGCGGCCGCTTAGAGGCCTGCGAG
30A7fwdHind	CTGCAAGCTTCATTGCCCCTGTCC
30A7revNot	GAGGGCGGCCGCTTACATGGCTGCAAAG
31A1fwdHind	TCTGCAAGCTTCAGATCATTCCCAC
31A1revNot	GGAGGGCGGCCGCTTAATGGCAATGCTC
pInv-35F5-Hind-fwd	TCTGCAAGCTTCAGTGCCGCCACGACGCC
pInv-35F5-Not-rev	GGAGGGCGGCCGCCTAACTAGCTCCATCCTC
pInv-36A1N-fwd	TCTGCAAGCTTCATCCACGCAGAGACTTCG
pInv-36A1N-rev	GGAGGGCGGCCGCCTATATGAAGGCACAGG
38A2fwdSal	AGCTTGTCGACCAAAGAAGGCCGAAATGG
38A2revNot	GGAGGGCGGCCGCTTAATGGCCACCTCCAG
pInv-39A1-Hind-fwd	TCTGCAAGCTTCAGGGCCCTGGGGAG
pInv-39A1-Not-rev	GGAGGGCGGCCGCTTAGATTTGGATGAAGAG
43A2fwdHind	AGGTGAAGCTTCTGCGCCCACCCTG
43A2revNot	TCCAGGCGGCCGCCTACACGAAGGCC
46A2fwdHind	AGGTGAAGCTTCTAGCCCCGAGGTC
46A2revNot	CTCCAGGCGGCCGCTCATTTCTCTATG
pInv-47A1-Hind-fwd	TCTGCAAGCTTCAGAAGCTCCTGAGGAGCC
pInv-47A1-Not-rev	GGAGGGCGGCCGCTCACTGAATTCTGACATAG

ammonium sulfate, TEN buffer was added to the lysate until a volume of 50 ml was reached. DNA was removed by the addition of 5 ml polymin P (Sigma, Taufkirchen, Germany) and stirring for 20 min on ice.

The solution was centrifuged at 39,000 *g* for 15 min and 0.82 volumes saturated ammonium sulfate solution were added to the supernatant, precipitating the T7 RNA polymerase by stirring on ice for 20 min. After centrifugation at 39,000 *g* for 15 min, the pellet was resuspended in 15 ml C-50 buffer (20 mM sodium phosphate pH 7.7, 1 mM Na_2EDTA, 1 mM DTT, 5% (w/w) glycerol, 50 mM NaCl) and dialysed against C-50 buffer for 4 h. After buffer exchange, dialysis was performed over night. After removal of aggregates, the supernatant was applied to a SP Sepharose FF column (*ca*. 53 ml bed volume, GE Healthcare, Freiburg, Germany). T7 RNA polymerase was eluted via a gradient from 50–500 mM NaCl in C buffer. Fractions containing the T7 RNA polymerase were pooled and dialysed against C-0 buffer (20 mM sodium phosphate pH 7.7, 1 mM Na_2EDTA, 1 mM DTT, 5% (w/w) glycerol) for 4 h. After buffer exchange, dialysis was performed over night. Afterwards, the dialyzed T7 RNA polymerase was pelleted by centrifugation at 12,000 *g* for

15 min. The pellet was resuspended in C-100 buffer (20 mM sodium phosphate pH 7.7, 1 mM Na_2EDTA, 1 mM DTT, 5% (w/w) glycerol, 100 mM NaCl), supplemented with glycerol to a final concentration of 50% (v/v), aliquoted, snap frozen in liquid nitrogen and stored at −80°C.

Preparation of Leishmania tarentolae extracts

Leishmania tarentolae Parrot laboratory strain P10 was obtained from Jena Bioscience GmbH (Jena, Germany). *L. tarentolae* extracts (LTE) were prepared according to previous reports (Mureev et al. 2009, Kovtun et al. 2010, 2011) with slight modifications.

The strain was cultured with agitation in 1 l brain heart infusion-medium, supplemented with hemin and penicillin/streptomycin, at 26°C and 125 rpm to an final OD_{600} of 1.6, which was determined using an Ultrospec*2100* pro spectrophotometer (Amersham Biosciences, Freiburg, Germany).

After harvesting the cells at 2500 *g* and 4°C for 20 min, the pellet was washed twice by resuspending in sucrose extraction buffer (250 mM sucrose, 45 mM Hepes-KOH 7.6, 100 mM potassium acetate, 3 mM magnesium acetate) and harvesting by centrifugation (2500 *g*, 20 min, 4°C). The pellet was resuspended in sucrose extraction buffer to achieve a cell density of approximately 1×10^{10} cells/ml (Scepter™ 2.0 Cell Counter, Merck, Darmstadt, Germany). Cells were incubated at 4°C for 45 min under nitrogen pressure (7×10^6 Pa) and disrupted via a nitrogen cavitation chamber (Parr Instruments, Frankfurt, Germany). The cell lysate was centrifuged at 10,000 *g* for 20 min and the supernatant was again centrifuged (30,000 *g*, 4°C, 20 min). Finally, the lysate was supplemented with anti-splice leader DNA oligonucleotide to a final concentration of 20 µM (Sigma, Taufkirchen, Germany), divided into aliquots, snap frozen in liquid nitrogen and stored at −80°C.

Cell-free synthesis of human solute carriers

Cell-free protein synthesis was performed either in batch mode or in the continuous exchange mode (CECF) (Kim and Choi 1996) using a D-tube dialyzer (Merck, Darmstadt, Germany), containing a semi-permeable membrane with a molecular weight cut-off of 12–14 kDa to separate the reaction mix (RM), which contains all high-molecular weight components from the feeding mix (FM), comprising low-molecular weight components.

Batch reactions were set up for screening, detergent cell-free and nanodisc cell-free reactions, while CECF reactions were performed for L-CF reactions.

Previously reported concentrations of components for batch reactions were used (Kovtun et al. 2010, 2011).

Screening batch reactions contained 1.2 mM ATP, 0.134 mM GTP, 0.25 mM spermidine, 2 mM DTT, 40 mM creatine phosphate, 3.5 mM magnesium acetate, 30.25 mM Hepes-KOH pH 7.6, 0.01% (v/v) PEG3000, 1× complete protease inhibitor, 0.136 mM amino acid mixture, 0.5 mM rNTPs, 50 mM potassium acetate, 0.01 mM anti-splice leader DNA oligonucleotide, 2.5 µl T7 RNA polymerase, 0.04 U/µl creatine kinase, 0.02 µM plasmid, 50% (v/v) LTE and 25 U RNase inhibitor in a final volume of 20 µl. Reaction batches were incubated at 26°C with gentle shaking at 300 rpm in a thermomixer (Eppendorf, Hamburg, Germany) for 2–3 h.

The RM:FM ratio was 1:10. CECF RM contained 1.2 mM ATP, 0.134 mM GTP, 0.25 mM spermidine, 2 mM DTT, 40 mM creatine phosphate, 3.5 mM magnesium acetate, 30.25 mM Hepes-KOH pH 7.6, 0.01% (v/v) PEG3000, 1× complete protease inhibitor, 0.136 mM amino acid mixture, 0.5 mM rNTPs, 50 mM potassium acetate, 0.01 mM anti-splice leader DNA oligonucleotide, 2.5 µl T7 RNA polymerase, 0.04 U/µl creatine kinase, 0.02 µM plasmid, 50% (v/v) LTE and 25 U RNase inhibitor in 100 µl reactions, while the corresponding 1 ml FM contained 1.2 mM ATP, 0.134 mM GTP, 0.25 mM spermidine, 2 mM DTT, 40 mM creatine phosphate, 3.5 mM magnesium acetate, 30.25 mM Hepes-KOH pH 7.6, 0.01% (v/v) PEG3000, 1× complete protease inhibitor, 0.136 mM amino acid mixture, 0.5 mM rNTPs, 50 mM potassium acetate. CECF reaction batches were incubated in a thermomixer at 26°C with gentle shaking at 750 rpm over night for 12–16 h.

Precipitate cell-free (P-CF) mode and subsequent solubilization

P-CF reactions were performed without the addition of detergents or liposomes.

Precipitated membrane proteins were spun down in a tabletop centrifuge at 20,000 *g* at 4°C for 30 min. Pellets were solubilized in different detergents with a final concentration of 2% (w/v) in solubilization buffer (50 mM Hepes-KOH pH 7.6, 150 mM NaCl) in equal volumes to the RM. After incubation at room temperature for 2 h, non-solubilized protein was removed by centrifugation (20,000 *g*, 4°C, 30 min) and an equal amount of sample buffer was added to the pellet. Solubilization efficiencies were analyzed by SDS-PAGE, followed by Western blotting, immunodetection with an anti-GFP primary antibody from mouse (Roche Diagnostics, Mannheim, Germany)

and an alkaline phosphatase coupled anti-mouse IgG secondary antibody from goat (Sigma, Taufkirchen, Germany) and comparison of intensities of corresponding protein bands in pellet and supernatant fractions.

Detergent cell-free (D-CF) mode

For the production of directly detergent-soluble membrane proteins, detergents were added to cell-free reactions. Afterwards reaction batches were centrifuged at 100,000 *g* for 1 h. Pellets were resuspended in an equal volume to the reaction volume of sample loading buffer. Soluble and insoluble fractions were analyzed by SDS-PAGE, followed by Western blotting and detection with an anti-GFP primary antibody (Roche Diagnostics, Mannheim, Germany) and an alkaline phosphatase coupled anti-mouse IgG secondary antibody (Sigma, Taufkirchen, Germany).

Liposome preparation and liposome cell-free (L-CF) mode

Liver polar lipid extract (bovine), brain polar lipid extract (porcine) and heart polar lipid extract (bovine) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), solubilized in chloroform. The lipids were dried under vacuum. The lipid film was hydrated by shaking in 50 mM Hepes-KOH pH 7.6 and adjusted to a final concentration of 20 mg/ml.

For L-CF synthesis, unilamellar liposomes were prepared from the corresponding polar lipid extracts by extruding 21 times through a 400 nm Whatman polycarbonate filter (Whatman, Freiburg, Germany) in an Avanti Polar Lipids mini extruder (Alabaster, AL, USA). Liposomes were added to the cell-free reaction to a final concentration of 2.8 mg/ml and incubated in a thermomixer (Eppendorf, Hamburg, Germany) with gentle shaking at 750 rpm over night at 26°C. For further analysis, L-CF reaction mixtures were separated via PD-10 columns (GE Healthcare, Freiburg, Germany) and elution fractions were analyzed by light scattering and SDS-PAGE, followed by Western blotting, immunodetection with an anti-GFP primary antibody from mouse (Roche Diagnostics, Mannheim, Germany) and an alkaline phosphatase coupled anti-mouse IgG secondary antibody from goat (Sigma, Taufkirchen, Germany).

Purification of membrane scaffold protein 1D1 (MSP1D1) and nanodisc assembly

E. coli BL21-Gold (DE3) (Agilent Technologies, Waldbronn, Germany), carrying the plasmid pMSP1D1 (Addgene, Cambridge, MA, USA), was grown in baffled flasks at 37°C and 175 rpm to an OD_{600} of 0.6, which was determined using an Ultrospec*2100* pro spectrophotometer (Amersham Biosciences, Freiburg, Germany). Gene expression was induced with isopropyl-β-D-thiogalactopyranosid for 4 h and cells were harvested by centrifugation. The pellet was resuspended in buffer A (20 mM Tris-HCl pH 7.5, 150 mM KCl, 20 mM imidazole) and cells were disrupted in two cycles of $4.1 \times 10^6 - 6.9 \times 10^6$ Pa pressure in a french press. The lysate was cleared by centrifugation at 100,000 *g* at 4°C for 30 min. His-tagged MSP1D1 was affinity purified with Ni-NTA material, washed each with 7 column volumes buffer A, buffer B (20 mM Tris-HCl pH 7.5, 150 mM KCl, 30 mM imidazole), buffer C (20 mM Tris-HCl pH 7.5, 150 mM KCl, 50 mM imidazole) and eluted with 8 column volumes buffer D (20 mM Tris-HCl pH 7.5, 150 mM KCl, 500 mM imidazole). Elution fractions were dialysed against buffer A without imidazole, aliquoted, snap frozen in liquid nitrogen and stored at −80°C.

Lipids for nanodisc assembly were treated as for liposome preparation and finally resuspended in a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM KCl and 200 mM sodium deoxycholate to a concentration of 100 mM. MSP1D1 and the corresponding lipids were mixed in different molar ratios and incubated on ice for 2 h. Methanol-activated Bio-Beads SM-2 (Bio-Rad Laboratories, Munich, Germany) were added and the suspension was incubated at 4°C with agitation over night. After removal of the Bio-Beads, nanodisc formation was checked via size exclusion chromatography, Coomassie-stained SDS-PAGE and thin layer chromatography. Prepared nanodiscs were stored at 4°C for up to two weeks.

Nanodisc cell-free (ND-CF) mode

For ND-CF production, 250 µl batch reactions were supplemented with 30 µl of prepared nanodiscs. The reactions were incubated in a thermomixer (Eppendorf, Hamburg, Germany) at 300 rpm at 26°C for 3 h. Nanodisc-transporter complexes were affinity purified with Ni-NTA material, washed with 10 column volumes buffer A and 5 column volumes buffer B and eluted with 10 column volumes buffer D.

SDS-PAGE and Western blotting

Cell-free samples were mixed with 5× loading buffer (25% glycerol, 12.5% β-mercaptoethanol, 7.5% SDS, 0.25 mM Tris-HCl pH 8.0, 0.5 mg/ml

Table II. Overview of produced human solute carriers.

No.	Target	Alternative name(s)	Length	kDa	TMHs*
1	1A4	Neutral amino acid transporter A	532	56	9
2	4A8	Electroneutral sodium bicarbonate exchanger 1	1093	123	11
3	5A11	Sodium/myo-inositol cotransporter 2	611	74	14
4	10A4	Na(+)/bile acid cotransporter 4	437	47	8
5	12A1	SLC12A1	1099	121	12
6	12A3	SLC12A3	1030	113	12
7	17A3	Sodium-dependent phosphate transport protein 4, Na(+)/PI cotransporter 4, Sodium/phosphate cotransporter 4	420	46	8
8	17A5	Sialin	495	55	12
9	19A3	Thiamine transporter 2	496	56	12
10	20A1	Sodium-dependent phosphate transporter 1	679	74	10
11	26A6	Solute carrier family 26 member 6	759	83	9
12	27A6	Long-chain fatty acid transport protein 6	619	70	2
13	29A4	Equilibrative nucleoside transporter 4	516	58	10
14	30A7	Zinc transporter 7	376	42	6
15	31A1	High affinity copper uptake protein 1	190	21	3
16	35F5	Solute carrier family 35 member F5	523	59	10
17	36A1	Proton-coupled amino acid transporter 1	476	53	11
18	38A2	Sodium-coupled neutral amino acid transporter 2	506	56	11
19	39A1	Zinc transporter ZIP1	324	34	8
20	43A2	Large neutral amino acids transporter small subunit 4	569	62	12
21	46A2	Thymic stromal cotransporter homolog	475	51	12
22	47A1	Multidrug and toxin extrusion protein 1	570	62	13

*TMHs, transmembrane helices.

Bromophenol blue) and loaded onto 4–12% NuPAGE® Bis-Tris gels (Life Technologies, Darmstadt, Germany). After separation proteins were transfered onto Immobilon™ PVDF transfer membranes (Merck Millipore, Darmstadt, Germany) when necessary. Protein gels were stained with Coomassie blue R250 (Carl Roth, Karlsruhe, Germany).

Purification of SLC17A3-eGFP via anion exchange chromatography

SLC17A3 was purified via anion exchange chromatography. Therefore 250–500 µl D-CF reaction aliquots were dialyzed against 500 ml ion exchange buffer (50 mM Hepes KOH pH 7.6, 0.01% Brij-78, 5% glycerol) at 4°C for 2 h. Buffer was exchanged and dialysis was repeated over night.

Aggregates were removed by centrifugation at 20,000 *g* for 10 min and the supernatant was applied to a HiTrap Q Sepharose FF column (GE Healthcare, Freiburg, Germany). The column was pre-equilibrated with 5 column volumes ion exchange buffer. Proteins were eluted with a step-gradient ranging from 0 mM KCl in ion exchange buffer to 800 mM KCl in ion exchange buffer. Each elution fraction (5 column volumes) was concentrated via an Amicon Ultra Centrifugal Filter Unit (Millipore, Darmstadt, Germany) with a 100 kDa cut-off and analyzed via SDS-PAGE and subsequent Western blotting.

Results

Optimization of reaction conditions and cell-free synthesis of 22 human solute carriers

For the evaluation of production levels, all target sequences were cloned into the plasmid pLEXSY_invitro-1, containing the species-independent translational sequences, as either N-terminal or C-terminal eGFP-fusions.

In the first step, critical parameters for cell-free synthesis like incubation temperature, reaction duration, Mg^{2+} and K^{+} concentration and the amount of added LTE were optimized with eGFP as a reporter protein. Under these conditions, the yield of cell-free produced eGFP was approximately 40 µg/ml, which is in good agreement with the amount reported by Kovtun et al. (2010).

In a second step, human solute carriers were screened for synthesis in the *L. tarentolae* cell-free system in a final volume of 20 µl in batch reactions. Overall, 22 human solute carriers from 20 different families were investigated for successful synthesis to test the universal usefulness of the *L. tarentolae* cell-free system. We chose human SLCs ranging from 31–121 kDa molecular masses, comprising 2–13 predicted transmembrane helices (TMHs), transporting a multiplicity of solutes. The calculated molecular masses of all targets as well as their alternative names and predicted THMs are listed in Table II.

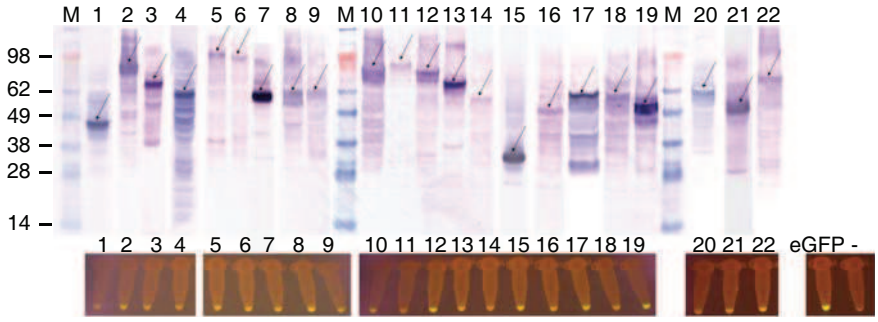

Figure 2. Production of human solute carriers in LTE as shown by immunoblot and eGFP-fluorescence. Human transporters were either produced as N-terminal or C-terminal eGFP-fusion proteins in *L. tarentolae* cell-free system in 20 µl batch reactions, loaded onto a gel, blotted and detected via an anti-GFP antibody. Arrows indicate the position of the produced membrane proteins. Below the Western blot, eGFP-fluorescence of each sample is shown. Table II lists the synthesized membrane proteins. Molecular masses of marker proteins are indicated (M). Negative control: no plasmid (-).

The successful synthesis of these transporters was confirmed via SDS-PAGE, Western blotting and immunodetection with an anti-GFP antibody and an alkaline phosphatase-coupled anti-mouse IgG antibody. For all tested membrane proteins, a significant band was visible in the Western blots (Figure 2, top). The amounts of synthesized membrane protein varied widely, independent of their size or numbers of TMHs. Besides that no correlation of yield and transported substrate was found. For some SLCs, shorter fragments were observed, possibly due to premature translation termination.

Fluorescence of eGFP was visible in all reactions (Figure 2, bottom), providing first evidence for correct folding of at least the eGFP-fusion part. Amounts of synthesized transporters were estimated by fluorescence quantification and comparison with a GFP-standard curve, revealing synthesis yields of up to 50 µg/ml of membrane protein.

Moreover, the successful synthesis of selected transporters was confirmed by peptide mass fingerprinting analysis (data not shown).

As SLC17A3 showed the highest production levels among all tested transporters, all further studies were carried out with this SLC member as a model membrane protein. Furthermore, activity assays from *in vivo* produced SLC17A3 are already available (Jutabha et al. 2010, 2011a, 2011c) which might simplify the establishment of transport and binding assays from *in vitro* produced protein.

Evaluation of detergents for post- and co-translational solubilization of SLC17A3-eGFP produced in P-CF and D-CF mode

For P-CF mode, SLC17A3 with a C-terminal eGFP-fusion was produced in LTE. After solubilization of the pellets, supernatants and pellet fractions were analyzed via SDS-PAGE, Western blotting and immunodetection (Figure 3). Screening revealed that harsh detergents like LMPG (1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]) and SDS (sodium dodecylsulfate) have optimal solubilization efficiencies whereas Fos-12 (N-Dodecylphoscholine) could only partially solubilize SLC17A3 precipitates. Consistent with solubilization screens from *E. coli* cell-free produced membrane proteins (Schwarz et al. 2007b, Kai et al. 2010), other detergents like OG (n- Octyl-ß-D-glucoside) or DM (n-Decyl-ß-D-maltoside) were not capable of solubilization. The solubilization efficiencies of different

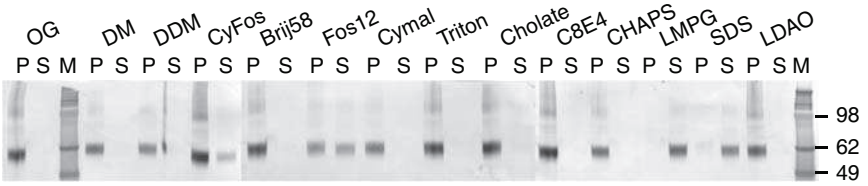

Figure 3. Solubilization of SLC17A3-eGFP produced in the P-CF mode by different detergents as shown by immunoblotting. Non-soluble membrane proteins were spun down and solubilized in different detergents with a final concentration of 2% for 2 h. Non-solubilized protein was removed by centrifugation and an equal amount of sample buffer was added. Same volumes of supernatant (S) and pellet (P) were loaded onto a 4–12% NuPAGE® gel, blotted and detected via an anti-GFP antibody. Used detergents are indicated above the corresponding lanes and listed in Table III.

Table III. Used detergents for solubilization of P-CF produced solute carriers.

Detergent	Short name	Charge*	CMC [%]	% used for solubilization	x CMC	Efficiency
n-Octyl-β-D-glucoside	OG	N	0.7	2	2.86	-
n-Decyl-β-D-maltoside	DM	N	0.087	2	22.99	-
n-Dodecyl-β-D-maltoside	DDM	N	0.006	2	326.16	+
7-Cyclohexyl-1-pentylphosphocholine	CyFos5	Z	0.15	2	13.33	+
Polyethylene glycol hexadecyl ether	Brij-58	N	0.009	2	222.22	-
N-Dodecylphoscholine	Fos-12	Z	0.047	2	42.55	++
Cyclohexyl-pentyl-β-D-maltoside	Cymal5	N	0.12	2	16.67	-
α-[4-(1,1,3,3-tetramethylbutyl)-phenyl]-ω-hydroxy-poly(oxy-1,2-ethandiyl)	Triton X-100	N	0.02	2	100	-
Deoxycholic Acid, Na salt	Deoxycholat	A	0.24	2	8.33	-
Octyltetraoxyethylene	C_8E_4	N	0.22	2	9.09	-
3-[N-(cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS	Z	0.49	2	4.08	-
1-myristoyl-2-hydroxy-sn-glycero-3-[phosphor-rac-(1-glycerol)]	LMPG	A	0.075	2	26.74	+++
Sodium dodecyl sulfate	SDS	A	0.035	2	57.14	+++
N, N-Dimethyl-dodecylamine-N-oxide	LDAO	Z	0.026	2	76.92	-

*N: non-ionic; Z: zwitter-ionic; A: anionic.

detergents, their charges as well as their critical micellar concentrations (CMC) are listed in Table III.

Alternatively, SLC17A3-eGFP was produced in the presence of detergents (D-CF mode). In total, 13 different detergents were tested for their ability to solubilize the synthesized membrane protein during cell-free synthesis by providing a hydrophobic environment in the *L. tarentolae* cell-free system (Figure 4). Astonishingly, the produced membrane protein stayed partly in the supernatant without the addition of any detergent after centrifugation at 100,000 *g*.

In general, the most frequently used non-ionic Brij-derivates were suitable for D-CF reactions in LTE. These findings are in good agreement with previous reports concerning the *E. coli* system (Kai et al. 2010). Furthermore, Pluronic (polyoxyethylene-polyoxypropylene block copolymer) was acceptable for D-CF mode, while the steroid derivative digitonin apparently reduced protein synthesis. No capability for obtaining the SLCs in a soluble form was shown for CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and $C_{12}E_9$ (nonaethylene glycol monododecyl ether). Some detergents like DDM (n-dodecyl-ß-D-maltoside) which is widely used in membrane protein research, Fos-14 (N-Tetradecylphoscholine), sodium deoxycholate, S-M (sucrose-monolaurate), CyFos5 (7-Cyclohexyl-1-pentylphosphocholine) or Cymal5 (Cyclohexyl-pentyl-β-D-maltoside) inhibited cell-free protein synthesis completely. The solubilization efficiencies of different detergents, their charges as well as their critical micellar concentrations and their effect on cell-free protein synthesis are listed in Table IV.

Purification of SLC17A3-eGFP and formation of an oligomeric state

As the expression plasmid did not code for a purification tag, the eGFP-fusion part was used for purification. Several protocols exist for the purification of GFP via strong anion exchange columns (Cabanne et al. 2004).

Figure 4. Cell-free production of SLC17A3-eGFP in the D-CF mode investigated by immunoblotting. Detergents were added to cell-free reaction mixes and the reaction mix was incubated at 26°C and 300 rpm over night. Reaction mixes were then centrifuged at 100,000*g* for 1 h. Pellets were resuspended in an equal volume of sample loading buffer. Same volumes of pellet (P) and supernatant (S) were loaded onto a 4–12% NuPAGE® gel. SLC17A3-eGFP was detected with an anti-GFP antibody. Molecular masses (M) of the marker proteins are indicated on the right side. Detergents and their final concentrations in the reaction are listed above the corresponding lanes and in Table IV.

Table IV. Used detergents for soluble production of solute carriers in D-CF.

Detergent	Short name	Charge*	CMC [%]	% for D-CF	x CMC	Solubilization efficiency	Inhibition of protein synthesis
Nonaethylene glycol monododecyl ether	$C_{12}E_9$	N	0.003	0.1	33.3	-	No
n-Dodecyl-β-D-maltoside	DDM	N	0.006	0.1	16.7	-	Yes
Polyoxyethylene-polyoxypropylene block copolymer	Pluronic	N	n.a.	0.1	n.a.	+	No
N-Tetradecylphoscholine	Fos-14	Z	0.0046	0.1	21.7	-	Yes
N-Tetradecylphoscholine + 3-[N-(cholamidopropyl)dimethylammonio]-1-propanesulfonate	Fos-14 + CHAPS	Z + Z	0.0046 + 0.49	0.1 + 0.1	21.7 + 0.2	-	Yes
3-[N-(cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS	Z	0.49	0.1	0.2	-	No
Polyoxyethylene-(20)-cetyl-ether	Brij-58	N	0.009	1.5	166.7	+++	No
Polyoxyethylene-(20)-stearyl-ether	Brij-78	N	n.a.	1	n.a.	+++	No
Digitonin	Digitonin	N	n.a.	0.5	n.a.	-	Slightly
No detergent	(w/o)	-	-	-	-	++	No
Sodium deoxycholate	Cholate	A	0.24	1	4.2	-	Yes
Sucrose-monolaurate	S-M	N	n.a.	0.5	n.a.	-	Yes
n-Octyl-β-D-glucoside	OG	N	0.7	0.5	0.7	-	Slightly
7-Cyclohexyl-1-pentylphosphocholine	CyFos5	Z	0.15	0.5	3.33	-	Yes
Cyclohexyl-pentyl-β-D-maltoside	Cymal5	N	0.12	0.5	4.2	-	Yes

*N: non-ionic; Z: zwitter-ionic; A: anionic. n.a.: not available.

SLC17A3-eGFP was produced in D-CF mode and applied to a HiTrap Q Sepharose FF column. The fusion protein was capable of binding to the column material and was eluted by increasing salt concentrations in ion exchange buffer. The main part of the solute carrier-fusion protein eluted from 150–350 mM KCl, which was confirmed by SDS-PAGE, subsequent Western blotting and immunodetection via an anti-GFP antibody (Figure 5).

To get information on the oligomeric state, the 150 mM elution fraction was loaded onto a 4–16% NativePAGE™ gel and clear native gel electrophoresis was performed. After Western blotting, a single band with an apparent molecular mass of approximately 600 kDa was detected with an anti-GFP antibody, indicating the formation of an SLC17A3-eGFP oligomer.

Effects of lipidic environments on cell-free reactions

The influence of liposomes on LTE reactions was investigated, as reconstituted transporters are a prerequisite for the functional characterization by transport measurements. For liposome preparations, complex liver, brain and heart lipid polar extracts were used instead of single lipid species to mimic the native lipidic environment of the transporters. Cell-free reactions containing preformed liposomes were applied to PD-10 columns to separate solute carrier-containing liposomes from non-incorporated transporters and analyzed by SDS-PAGE and Western blotting. We found that the application of unilamellar liver, brain or heart vesicles did not inhibit cell-free protein synthesis in LTE (Figure 6). Moreover, liposomes and transporter co-eluted in the same fractions, indicating an attachment of the membrane protein to the liposomes, at the very least. Furthermore, the insertion into the liposomes of one eukaryotic solute carrier was confirmed by freeze fracture electron microscopy (data not shown).

To cover all four commonly used cell-free modes for membrane proteins, the influence of nanodiscs on cell-free protein synthesis in *L. tarentolae* extracts was evaluated.

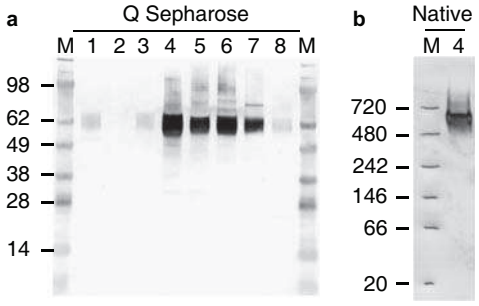

Figure 5. Purification of SLC17A3-eGFP via anion exchange chromatography, produced in the D-CF mode as shown by immunoblotting and NativePAGE. SLC17A3 was produced in the D-CF mode, subsequently purified via HiTrap Q Sepharose FF column and detected via an anti-GFP antibody. M, molecular weight marker. 1–8: Elution fractions; (1) 0 mM KCl; (2) 20 mM KCl; (3) 50 mM KCl; (4) 150 mM KCl; (5) 250 mM KCl; (6) 350 mM KCl; (7) 500 mM KCl; (8) 800 mM KCl. (a) SDS-PAGE of elution fractions. (b) NativePAGE of elution fraction number 4.

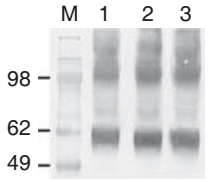

Figure 6. Cell-free production of SLC17A3-eGFP in the L-CF mode as shown by immunoblotting. Liposomes were added to CECF reaction batches and incubated at 26°C and 750 rpm over night. L-CF reaction mixtures were separated via PD-10 columns and aliquots of the elution fractions were loaded onto a gel. The synthesis of protein was detected via an anti-GFP antibody. The addition of liposomes did not inhibit the synthesis of SLC17A3-eGFP. M, molecular weight marker. (1) heart lipid liposomes; (2) liver lipid liposomes; (3) brain lipid liposomes.

For this purpose, different MSP1D1:lipid ratios were tested for nanodisc formation. To identify the influence of nanodisc lipid composition, lipids with a different charge and degree of saturation (POPC, DMPC, liver polar extract, brain polar extract) were used. All tested MSP1D1:DMPC ratios resulted in the assembly of nanodiscs, which was confirmed via size exclusion chromatography (Figure 7a), Coomassie-stained SDS-PAGE and detection of lipids in the peak fractions via thin layer chromatography. Similar results were obtained for a MSP1D1:POPC ratio of 1:40 (Figure 7b). All three evaluated MSP1D1:liver lipid, respectively MSP1D1:brain lipid ratios did not show a monodisperse nanodisc peak by size exclusion chromatography (Figure 7c, 7d) due to aggregation.

Nevertheless, all four assembled nanodiscs (POPC, DMPC, liver polar extract, brain polar extract) were added to the cell-free reactions. Subsequently, the His-tagged MSP1D1 was affinity-purified via Ni-NTA material and flow through, washing and elution fractions were checked for eGFP-fluorescence and analyzed by Western blotting. If nanodiscs were added to the cell-free reaction mix, the flow through and the elution fractions mainly showed eGFP-fluorescence (Figure 8a–d, above), while – without the addition of nanodiscs – the major fraction of

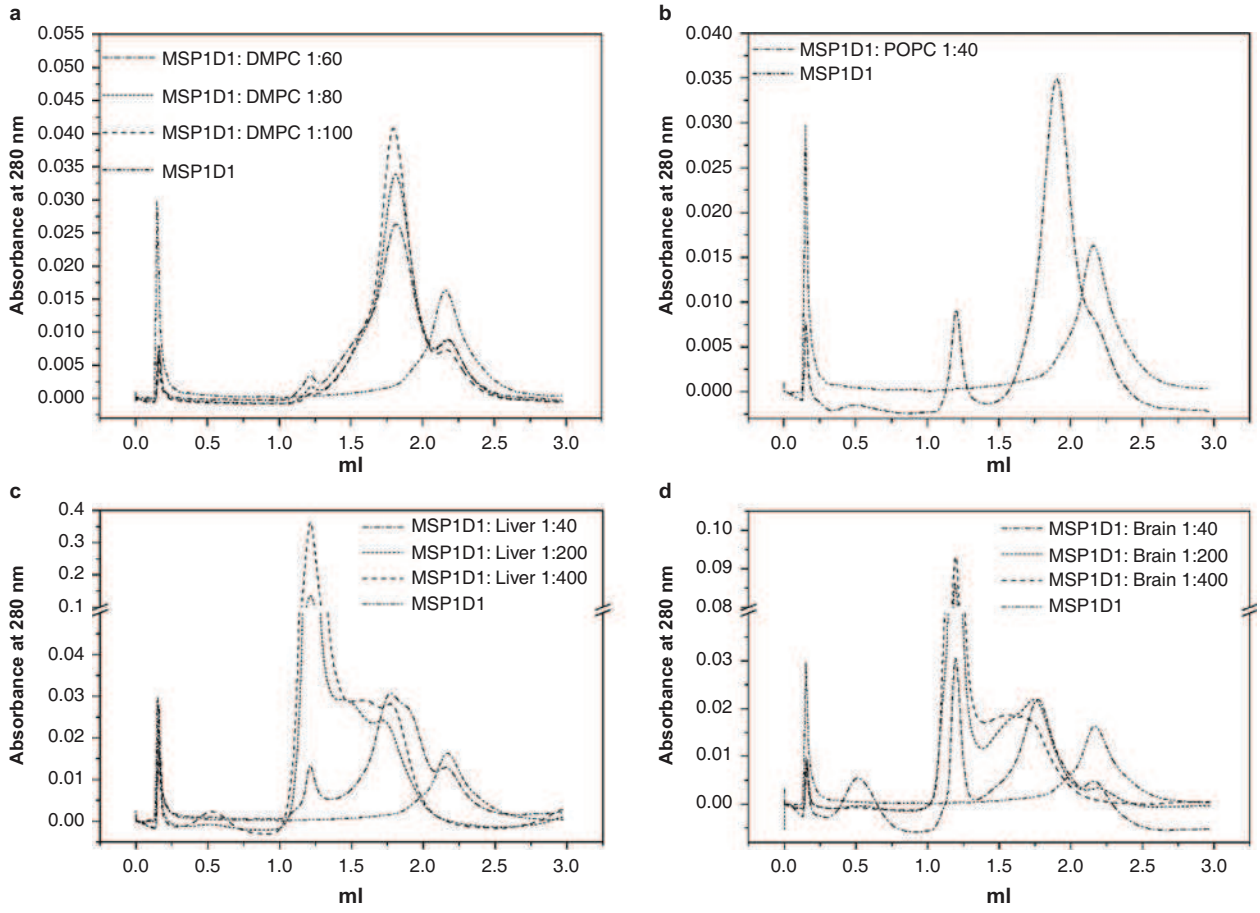

Figure 7. Nanodisc assembly investigated by gel filtration. Different MSP1D1:lipid ratios were tested for nanodisc assembly and analyzed via size exclusion chromatography (Superdex 200 column, GE Healthcare, Freiburg, Germany). MSPs were detected via absorbance at 280 nm. (a) MSP1D1:DMPC; (b) MSP1D1:POPC; (c) MSP1D1:liver; (d) MSP1D1:brain.

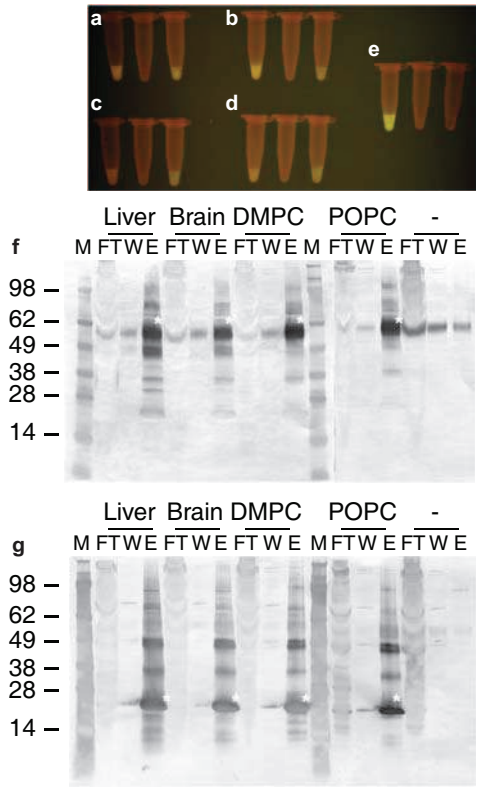

Figure 8. Purification of SLC17A3-eGFP, produced in the ND-CF mode as shown by eGFP-fluorescence and immunoblotting. SLC17A3 was produced in the ND-CF mode and subsequently purified via His-tagged MSP1D1 using Ni-NTA. Liver (a), Brain (b), DMPC (c), POPC (d) indicate the lipid sources, used for nanodisc formation. Negative control: No nanodiscs supplemented (-). FT, flow-through fraction; W, wash fraction; E, elution fraction; M, molecular weight marker. Asterisks indicate SLC17A3-eGFP and His-tagged MSP1D1, respectively. (f) Detection of SLC17A3-eGFP via anti-GFP antibody. (g) Detection of His-tagged MSP1D1 via anti-His antibody.

synthesized SLC17A3-eGFP was found in the flow through (Figure 8e, above). Depending on nanodisc addition, His-tagged MSP1D1 and transporter were detected via Western blotting in the same fractions (Figure 8f, 8g), providing evidence for an insertion of SLC17A3-eGFP into all four kinds of nanodiscs. These results suggest that the lipid composition of the nanodiscs is negligible for all lipids used in this work. If no nanodiscs were added to the cell-free reaction, only a minor part of SLC17A3 could be detected in the elution fraction by Western blotting (Figure 8g, no nanodiscs supplied), which is probably caused by unspecific binding of the transporter to the column material.

Discussion

Solute carriers are present in eukaryotes in a huge variety of tissues and are involved in a multiplicity of uptake and efflux processes (Schlessinger et al. 2010). Overall, approximately 400 human solute carriers are classified into 51 families by sequence and substrate similarities. In agreement with this crucial role in transporting solutes, defects in many solute carriers cause diseases. For example Batter's syndrome type I is caused by a defect in SLC12A1 (Daniluk et al. 2004, Watanabe and Uchida 2006), and the sialic acid storage disease (Salla disease) by a defect in SLC17A5 (Yano and Ohno 1995).

To date, little is known about many solute carriers. In fact, numerous transporters are still orphan. *In vivo* expression often suffers from low yield due to toxicity, aggregation and incorrect trafficking (Gubellini et al. 2011) and is not an easy task, in particular in human cell lines.

In contrast, cell-free systems circumvent cytotoxic effects which often accompany membrane protein production and offer the opportunity to synthesize co-translationally solubilized membrane proteins by the direct addition of detergents to the cell-free reaction. Critical parameters like salt concentrations, incubation temperature or any desired additive can be analyzed in high-throughput-screens, accelerating membrane protein production tremendously. Thereby the successful crystallization of the human voltage-dependent anion channel-1 (hVDAC-1) was achieved (Deniaud et al. 2010). Nevertheless synthesis in the *E. coli* cell-free system is often adversely affected by the prokaryotic source of the extract. Therefore establishing an easy cell-free system for membrane proteins based on eukaryotic extracts is desirable.

This work demonstrates the possibility of producing human solute carriers in *L. tarentolae*-based cell-free systems. We were able to produce 22 human solute carriers from 20 different families reaching concentrations up to 50 µg/ml in the P-CF mode in the *L. tarentolae* cell-free system. Furthermore, we implemented the four most commonly used cell-free production modes for membrane proteins in the *L. tarentolae* cell-free system with one member of the SLC family. Compared to the high-yield *E. coli* cell-free system, of up to several mg/ml of synthesized protein these yields are relatively low. Taking into account the eukaryotic source of the extract, a comparison with the wheat germ cell-free system seems to be more appropriate. In the wheat germ system Nozawa et al. (2011) obtained different SLCs in L-CF mode in amounts comparable to the *L. tarentolae* cell-free system.

We have chosen transporters from different families with diverse molecular weights and numbers of transmembrane segments (Table II) in order to examine the limitations of the *L. tarentolae*-based cell-free

system. As had to be expected, the target proteins were produced in varying amounts (Figure 2). We tested whether this variation is caused by the different molecular weights of the proteins. Actually, SLCs with high molecular weights like SLC12A1 (121 kDa) or SLC12A3 (113 kDa) were produced in lower amounts than SLC31A1 (21 kDa) (Figure 2, lanes 5, 6 and 15). However, this variation could not be correlated solely with the molecular weight as the overall amount of produced protein differed significantly for SLC17A3 and SLC10A4, both membrane proteins with calculated masses of 46 kDa and 47 kDa, respectively (Figure 2, lanes 7 and 4). Independence of *in vivo* production of membrane proteins and the number of transmembrane helices was shown by Tate (2001), whereas a negative correlation of the molecular weight of the produced proteins and the yield was shown in the cell-free *E. coli* system (Keller et al. 2008). Such a relationship could not be detected in the cell-free *L. tarentolae* system. SLC17A3 and SLC10A4 both comprise eight predicted transmembrane helices and were produced with varying amounts (Figure 2, lanes 7 and 4), for example. Furthermore, SLC31A1, possessing three transmembrane helices was synthesized in bigger amounts than SLC27A6 with only two transmembrane helices. In contrast to Tate (2001), White et al. (2007) showed a correlation between the number of transmembrane helices and the *in vivo* levels. In addition, they confirmed the existence of a relationship between the molecular weight, the overall hydrophobicity, the number of transmembrane helices and the amino acid composition of the transmembrane helices and the protein levels in *S. cerevisiae*. Such a correlation could not be seen in the *L. tarentolae* cell-free system. Besides, it was checked if the production level correlates with the endogenous tissues of the transporters. SLC17A3, which was produced in high amounts in the cell-free *L. tarentolae* system, is synthesized in humans mainly in the liver and kidneys. In contrast, SLC26A6, which is endogenously produced in high amounts in the kidneys and pancreas, was produced in the cell-free *L. tarentolae* system in low amounts. Although both transporters are synthesized in high amounts endogenously in the kidneys, their production levels diverged *in vitro*. Therefore no precise correlation between the endogenous tissue and the cell-free amount could be detected. Probably RNA secondary structures or half lives of the RNAs influence the *in vitro* production, which has to be demonstrated in the future. In addition, the effect of the diverging codon usage of *Homo sapiens* and *L. tarentolae* should be taken into account. Analysis of the target sequences and the codon usage of *L. tarentolae* provided no significant evidence for such a correlation. Probably, several hundreds of targets have to be analyzed in the future to render a conclusive statement.

In general, we showed that the *L. tarentolae* cell-free system is capable of expressing solute carriers with a wide spectrum of calculated molecular masses of up to 121 kDa (SLC12A1) and as little as 21 kDa (SLC31A1) (Figure 2, lanes 5 and 15). As functional data are still missing, a comparison with the *E. coli* cell-free system is difficult. Nevertheless, we suppose that the eukaryotic source of the extract might advantageously affect the native folding of human membrane proteins which could also be influenced by the presence of eukaryotic chaperones. Drew et al. (2001) reported that the GFP-moiety can serve as a folding reporter for the fusion protein. Jaehme and Michel (2013) showed that there was no fluorescence detectable of a GltSM-GFP fusion protein which was produced using an *E. coli* cell-free system. In contrast to this, all of our tested transporter-GFP fusion proteins synthesized in the *L. tarentolae* cell-free system showed GFP-fluorescence. These promising findings support the hypothesis that the *L. tarentolae* cell-free system might be superior to the *E. coli* cell-free system with regard to folding. Furthermore, post-translational modifications may be achieved in eukaryotic rather than in prokaryotic cell-free systems.

With the P-CF mode, solute carriers were produced as precipitates and subsequently one SLC member was solubilized with detergents exemplarily. Only harsh detergents like SDS or Fos-12 were suitable for the post-translational solubilization of SLC17A3, while other detergents like OG, DM or DDM, which would be more suitable for structural and functional experiments, failed (Figure 3, Table III).

In the D-CF mode, it was possible to obtain soluble membrane protein (Figure 4, Table IV) directly. For D-CF production of membrane proteins in particular Brij-derivates turned out to be well suited, which is in good agreement with previous findings for the *E. coli* cell-free system (Berrier et al. 2004, Klammt et al. 2005, Gourdon et al. 2008, Kaiser et al. 2008, Junge et al. 2010). It is well known, that several detergents can inhibit protein synthesis in cell-free systems. Such an inhibition was also found for the *L. tarentolae* extracts. Despite its similarity to natural lipids, Fos-14 inhibited the protein production completely. Genji et al. (2010) reported, that the inhibitory effects of Fos-choline and CHAPS can counteract each other, thereby allowing efficient synthesis of bacteriorhodopsin. Hence we tested the combination of these two detergents in the *L. tarentolae* cell-free system. The detrimental effects of Fos-14 and CHAPS were still observed in this system.

We supplemented cell-free reactions with the alkylglycoside detergent DDM, the most frequently used detergent for investigations of membrane proteins. However, addition of DDM did not lead to a soluble form of SLC17A3. Moreover protein production was abolished completely (Figure 4, lanes DDM). These findings differ from those of a previous report for the *E. coli* system (Elbaz et al. 2004), where addition of DDM yielded soluble transporters. This observation may indicate that specific detergents are required for each membrane protein.

Without the addition of any detergent, a minor part of SLC17A3 stayed in the supernatant (Figure 4, lanes w/o). This behavior possibly is due to remaining small membrane patches in the extract (data not shown), which is also known for *E. coli* extracts (Schneider et al. 2010). The *L. tarentolae* cell-free system has not been characterized in detail to date; therefore, other explanations might be possible.

Analysis of anion exchange purified SLC17A3-eGFP revealed the formation of an oligomeric state (Figure 5b). Further studies have to address whether this oligomeric state is the functional form or an artefact due to the method of protein production. Nevertheless, functional assays to verify an active state still have to be performed.

Insertion of membrane proteins directly into liposomes is a promising tool for activity assays. Cell-free synthesis in the presence of liposomes of a variety of membrane proteins like bacteriorhodopsin from *Halobacterium salinarium* (Kalmbach et al. 2007) in the *E. coli* cell-free system turned out to be successful. Therefore, the L-CF mode was investigated in the *L. tarentolae* cell-free system. The synthesis of one solute carrier and the incorporation into liposomes with different lipid compositions was validated, showing no dependence on the lipid composition of the liposomes (Figure 6).

Nanodiscs were found to be an eligible tool for the incorporation of membrane proteins like bacteriorhodopsin from *Exiguobacterium sibiricum* or the homodimeric transmembrane domain of the human receptor tyrosine kinase Erb3B (Lyukmanova et al. 2012). In fact, natively folded membrane proteins (Marin et al. 2007, Bayburt and Sligar 2010), capable of the formation of native oligomeric states (Bayburt et al. 2006) could be obtained. Moreover, nanodiscs were used for the structural investigation of beta-barrel membrane proteins (Raschle et al. 2009). The successful combination of nanodiscs and the *E. coli* cell-free system was already shown (Cappuccio et al. 2008, Katzen et al. 2008a, Yang et al. 2011, Lyukmanova et al. 2012), but has been missing for the *L. tarentolae* cell-free system. Our findings concerning the L-CF and ND-CF mode for the *L. tarentolae* system are consistent with recent data for the *E. coli* cell-free system (Lyukmanova et al. 2012), showing that the yield of soluble membrane protein does not strongly depend on the charge and saturation of the lipids used. Saturated lipids (DMPC) were as applicable as unsaturated lipids (POPC). Even complex lipid mixtures like liver polar lipid extract and brain polar lipid extract were suitable for the production of solubilized SLC17A3 (Figure 8). In contrast to the inhibition of some detergents in D-CF mode, the lipids used did not influence the yield of the produced membrane protein.

With the establishment of the four most commonly used modes for membrane proteins (Figure 1) in the *L. tarentolae* cell-free system, valuable novel tools for the analysis of membrane protein function were created. In particular, the L-CF mode might prove to be useful for setting up transport assays, allowing us to unravel the function of many transporters, while the ND-CF mode could be a worthwhile tool for binding assays.

Conclusions

All four commonly used modes for the cell-free synthesis of membrane proteins, P-CF, D-CF, L-CF and ND-CF, were established for the *L. tarentolae* cell-free system with the transporter SLC17A3 as a model membrane protein. Moreover, first purification-trials for SLC17A3 were successful and revealed the formation of an oligomeric state. The convenience of extract preparation argues in favor of the *L. tarentolae* cell-free system compared to other eukaryotic cell-free systems like the WGE or RRL. Furthermore, the eukaryotic source of the *L. tarentolae*-based cell-free system might be advantageously in terms of eukaryotic membrane protein production compared to the prokaryotic *E. coli* cell-free system with regard to folding. This hypothesis is supported by the fact that fluorescence was only detected for transporter-eGFP-fusions produced in the *L. tarentolae* cell-free system, but not in the *E. coli* cell-free system (Jaehme and Michel 2013).

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