Chapter 16

Expression of Multisubunit Proteins in *Leishmania tarentolae*

Marisa Sugino and Tomoaki Niimi

Abstract

Heterologous gene expression in mammalian cells is the first choice for the production of recombinant proteins when posttranslational modifications affect the biological activity of target proteins. However, the expression efficiency of mammalian cells is relatively low compared to other expression systems, such as *Escherichia coli* or yeast. Recently, a novel protein expression system based on *Leishmania tarentolae*, a protozoan parasite of gecko, was developed. This system allows not only easy handling like *E. coli* and yeast, but also full eukaryotic protein folding and the mammalian-type posttranslational modifications of target proteins. Here, we attempt to produce recombinant human laminin (LM)-332, a large heterotrimeric glycoprotein, in the *L. tarentolae* expression system. A recombinant strain harboring three subunits of LM-332 efficiently formed a heterotrimer and secreted it into the medium. The purified rLM-332 showed similar cell adhesion activity to rLM-332 purified from mammalian cells, indicating its proper folding and assembly. In this chapter, we describe a detailed protocol for multiple gene expression in the *L. tarentolae* expression system.

Key words: Trypanosomatidae, Protozoa, Laminin, Basement membrane

1. Introduction

Over the past few decades, various recombinant protein expression systems have been developed using bacteria, yeast, plant, insect, and mammalian cells. Researchers have chosen systems based on their downstream purpose. When posttranslational modifications affect the biological activity of target proteins, heterologous gene expression in mammalian cells is the first choice. However, the expression efficiency of mammalian cells is relatively low compared to other expression systems, such as *E. coli* or yeast. Recently, a novel protein expression system based on *Leishmania tarentolae*,

Argelia Lorence (ed.), *Recombinant Gene Expression: Reviews and Protocols, Third Edition*,
Methods in Molecular Biology, vol. 824, DOI 10.1007/978-1-61779-433-9_16,

a unicellular eukaryotic protozoan parasite, has been established (1–3). Compared to mammalian cell cultures, *L. tarentolae* has the advantage of a higher growth rate with doubling time of 4–5 h in agitated cultures. A unique feature of recombinant proteins produced in *L. tarentolae* is the mammalian-type N-glycosylation pattern. Since the *L. tarentolae* expression system has been used for successful expression of various biologically active proteins, it is an alternative to mammalian cells for production of recombinant proteins (1, 4, 5).

Laminins (LMs) are large glycoproteins that are an integral part of the structural architecture of basement membranes. They consist of three subunits, α, β, and γ chains, which bind to each other via disulfide bonds to form a cross-shaped structure (6–8). To date, 5 α, 3 β, and 3 γ chains have been identified that combine into at least 16 heterotrimeric molecules (9). LM-332, which consists of α3, β3, and γ2 chains, is unique among LM family members in that all three subunits have truncated short arms, making LM-332 the smallest molecule. However, it is difficult to express correctly folded heterotrimeric LMs, even LM-332, in *E. coli* and yeast. Thus, mammalian cells have been used to prepare recombinant LMs for use in biochemical studies. Here, we describe the production of recombinant human LM-332 in the *L. tarentolae* expression system (10).

2. Materials

2.1. Construction of Plasmids

1. pLEXSY-2 expression vectors (Jena Bioscience, Fig. 1): pLEXSY-neo2 encoding the aminoglucoside phosphotransferase, pLEXSY-ble2 encoding the bleomycin resistance gene, and pLEXSY-sat2 encoding the streptothricine acetyltransferase (see Note 1).
2. High-fidelity DNA polymerase.
3. Oligonucleotide primers with appropriate restriction sites.
4. Thermal cycler for polymerase chain reaction.
5. Agarose gel equipment.
6. DNA fragment purification kit (Qiagen).
7. Restriction enzymes.
8. *E. coli* strain DH5α.
9. Luria-Bertani (LB) medium: 10 g/L Bacto-tryptone, 5 g/L bacto yeast extract, and 5 g/L NaCl; autoclave at 121°C for 30 min.
10. LB agar plates containing ampicillin at a final concentration of 100 μg/mL.
11. Miniprep and Midiprep plasmid DNA extraction kits (Qiagen).

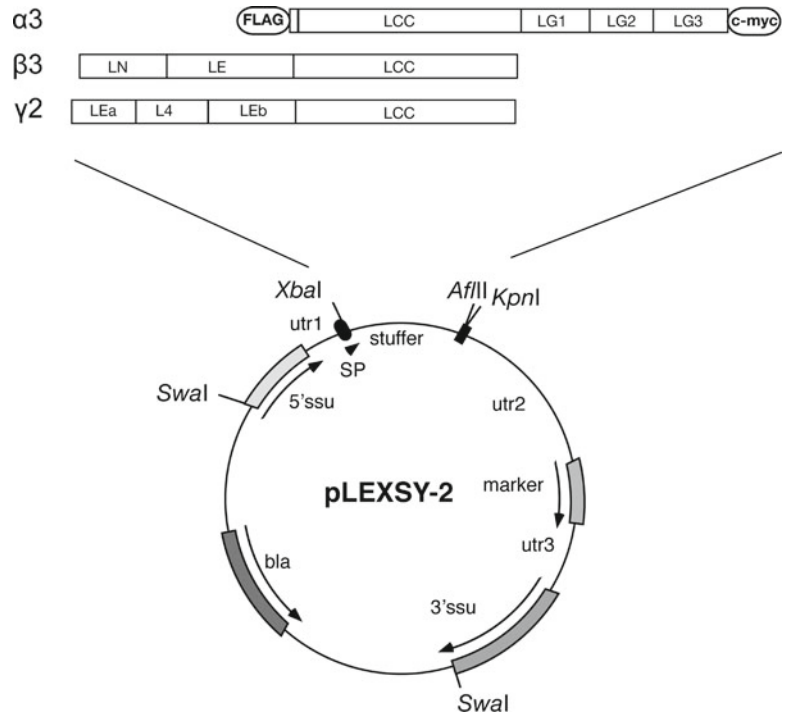

Fig. 1. Construction of expression plasmids for human LM-332 subunits. A map of the pLEXSY-2 vector and the strategy used for plasmid construction are summarized. FLAG and c-*myc* tags were added to the N- and C-terminal ends of the α3 constructs, respectively. The cDNA sequences encoding human LM-332 subunits without signal sequence were inserted in-frame after the signal peptide (SP) of *L. mexicana* secreted acid phosphatase. LM coiled-coil (LCC), LM globular (LG), LM N-terminal (LN), and LM 4 (L4) domains are shown.

2.2. Leishmania Strain, Growth Media, and Additives

1. *L. tarentolae* strain: LEXSY host P10 (Jena Bioscience).
2. LEXSY-BHI liquid medium: Dissolve 37 g LEXSY BHI powder (Jena Bioscience) in 1 L of deionized water and autoclave for 15 min at 121°C. Cool medium to room temperature (RT) before adding of hemin and penicillin/streptomycin.
3. 500× hemin stock solution: 0.25% (v/v) hemin in 50% (v/v) triethanolamine.
4. 200× penicillin/streptomycin stock solution: 10,000 units of penicillin G sodium salt and 10 mg/mL streptomycin sulfate in 0.85% saline.
5. 1,000× G418 stock solution: 50 mg/mL in water.
6. 1,000× bleomycin stock solution: 100 mg/mL in water.
7. 1,000× nourseothricin stock solution: 100 mg/mL in water.
8. LEXSY-BHI agar plates for clonal selection.
   (a) Mix the following components for five plates (50 mL): 35 mL of 2× LEXSY BHI (74 g/L), 10 mL of inactivated

fetal calf serum, 4 mL of 1 M HEPES, pH 7.4, 1 mL of penicillin/streptomycin, 0.2 mL of hemin, and appropriate antibiotics.

(b) Autoclave 50 mL of 2% (w/v) agar and keep at 55°C.

(c) Pour the medium into the warm agar (total 100 mL), mix gently, and distribute 20 mL per 90-mm plastic Petri dish.

(d) Leave the cover off and dry the plate for 10 min after solidifying.

(e) Use the freshly prepared plates immediately.

2.3. Cell Culture, Protein Expression and Detection

1. T25 ventilated tissue culture flask for suspension culture (see Note 2).
2. 125-mL disposable sterile Erlenmeyer flask with vented cap.
3. Incubator at 26°C (see Note 3).
4. Orbital shaker at 26°C.
5. Electroporation device.
6. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) equipment.
7. Western blotting equipment.
8. Nitrocellulose membrane.
9. Anti-FLAG M2 (Sigma), LM β3 (Santa Cruz Biotechnology), and LM γ2 (Santa Cruz Biotechnology) antibodies.
10. Horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare).
11. Chemiluminescence substrate for peroxidase.
12. Chemiluminescence imaging system.
13. TBS buffer: 50 mM Tris–HCl, pH 7.4, and 150 mM NaCl.

3. Methods

3.1. Preparation of the Expression Plasmids for Transfection

The pLEXSY-2 vectors are used for constitutive expression of target proteins following integration of the expression cassette into the chromosomal 18S rRNA locus of *L. tarentolae* (Fig. 1).

3.1.1. Polymerase Chain Reaction Amplification of cDNAs

The primers are designed to ensure in-frame cloning of the cDNA of interest into the expression cassette. The cDNAs are amplified either by reverse transcription-PCR (RT-PCR) from total RNA of human keratinocytes or by PCR using plasmid DNA as a template. The amplification is performed using high-fidelity DNA polymerase to ensure minimal mutation of the sequence during the PCR.

3.1.2. Cloning of cDNAs into the Expression Vectors

We usually add an epitope tag at both the 5′ and 3′ ends of the cDNA, as one of the tags may not be accessible to an antibody if the expressed protein is processed by proteases in *Leishmania*. We use the pSecTag2A-FLAG vector for adding FLAG- and c-*myc*-tags to the 5′ and 3′ ends of cDNA, respectively (10). Once the amplified cDNAs are inserted into the pSecTag2A-FLAG (laminin α3 subunit) or pcDNA3.1(+) vector (laminin β3 and γ2 subunits), further amplification is performed. The resulting PCR products are then ligated into the *Xba*I-*Afl*II- or *Xba*I-*Kpn*I-digested pLEXSY-2 vectors.

3.2. Transfection

The expression plasmids containing cDNAs encoding human laminin β3, γ2, and α3 chains are sequentially transfected into *L. tarentolae* by electroporation as follows.

1. Linearize approximately 5 μg of the expression plasmid with *Swa*I restriction enzyme. This treatment generates a 2.9 kb fragment from *E. coli* and a larger fragment representing the linearized expression cassette with the target gene.
2. Purify the expression cassette with an agarose gel extraction.
3. One day before transfection, inoculate *L. tarentolae* cells at a 1:10 dilution in 10 mL of LEXSY-BHI medium in a T-flask.
4. On the day of transfection, the cell density should be about 6×10^7 cells/mL ($OD_{600} = 1.4$).
5. Centrifuge the cells at $2{,}000 \times g$ for 5 min at RT and remove 50% of the supernatant volume.
6. Resuspend the pellet in the remaining medium and incubate on ice for 10 min.
7. Chill off linearized plasmid DNA (1–2 μg) in another tube on ice.
8. Add 350 μL volume of prechilled cells to the tube with DNA and transfer a 0.2-cm electroporation cuvette on ice.
9. Apply a pulse of 450 V with 450 microF.
10. Place back the cuvette on ice for 10 min.
11. Transfer the cells to 5 mL of LEXSY-BHI medium.
12. Incubate the electroporated cells at 26°C for 24 h.

3.3. Clonal Selection by Plating on Agar Plates

For establishment of the expression strain, we always use the clonal selection method by plating on agar plates with appropriate antibiotics as follows (see Notes 4 and 5).

1. Withdraw 0.5–1.5 mL from the transfected 5 mL of culture.
2. Pellet the cells for 1 min at $2{,}000 \times g$ at RT.
3. Remove the supernatant and resuspend the cells in 50–100 μL of residual medium.

4. Carefully spread the resuspended cells onto freshly prepared LEXSY-BHI agar plates supplemented with the appropriate selective antibiotics.
5. Seal the plates with parafilm and incubate upside down at 26°C for 5–10 days.
6. Transfer five to ten colonies carefully using a micropipette tip to 96-well plate with 200 μL of selective medium.
7. After a 24–48-h incubation, expand the clones to 1 mL of selective medium in a 24-well plate and can be used for evaluation of protein expression by Western blotting.

3.4. Verification of Correct Transformants by Western Blotting

1. Apply the cell lysates and/or conditioned medium of the selected clones after clonal selection to an SDS-PAGE.
2. Transfer proteins to nitrocellulose membranes.
3. Block the membrane with 5% (w/v) nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 for 1 h at RT.
4. Incubate the membrane with primary antibodies against the FLAG epitope (1:1,000 v/v), LM β3 (1:2,000 v/v), or LM γ2 (1:2,000 v/v) for 1 h at RT.
5. Wash the membrane three times with PBS containing 0.1% (v/v) Tween-20.
6. Incubate with HRP-conjugated secondary antibody (1:2,000 v/v) for 1 h at RT.
7. Wash the membrane three times with PBS containing 0.1% (v/v) Tween-20.
8. Develop the membrane using chemiluminescence reagents and detect on a chemiluminescence imaging system.

3.5. Storage of L. tarentolae Host and Recombinants

1. Add 1.2 mL of autoclaved 80% glycerol to a 15-mL tube.
2. Withdraw 3.6 mL of culture grown in LEXSY-BHI medium from the mid-growth phase (see Note 6).
3. Mix with glycerol and dispense 3 × 1.6-mL aliquots into sterile cryovials.
4. Incubate for 10 min at RT.
5. Incubate for 1 h on ice.
6. Incubate for 1 day at –20°C.
7. Transfer to –80°C for long-term storage.

3.6. Protein Expression and Purification

1. Inoculate approximately 3 mL of the static culture from the recombinant strains harboring the three constructs in 30 mL of LEXSY-BHI medium containing half the required concentration of G418, bleomycin, and nourseothricin.

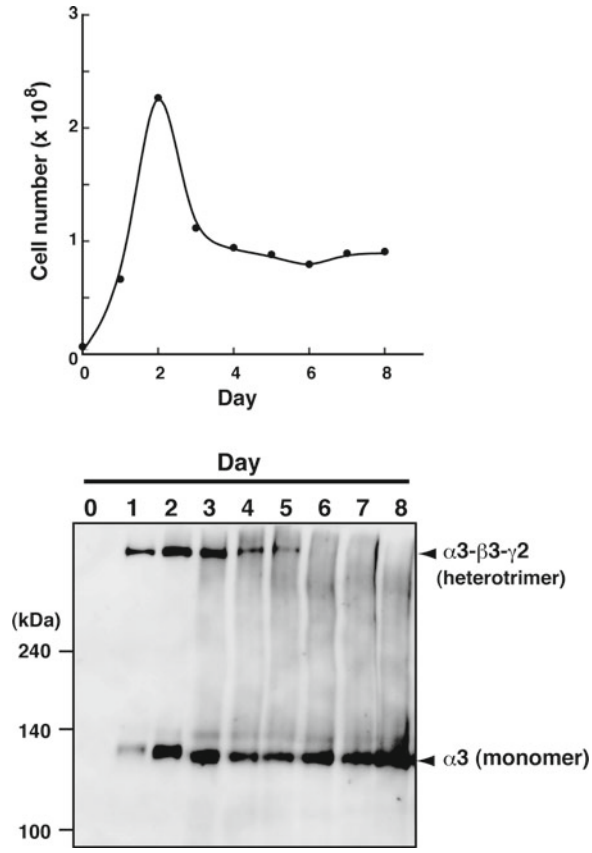

Fig. 2. Production of recombinant human LM-332 in *L. tarentolae* cells. Kinetics of cell growth (*upper panel*) and expression of recombinant LM-332 (*lower panel*) are presented. Plasmids containing cDNA encoding the β3, γ2, and α3 subunits of LM-332 were sequentially transfected into *L. tarentolae* by electroporation. A recombinant strain harboring three constructs was analyzed for the expression of LM-332. Culture medium was analyzed by SDS-PAGE under nonreducing conditions and immunoblotted with anti-FLAG antibody. The bands assumed to be the α3-β3-γ2 heterotrimer and α3 monomer are indicated with arrowheads. Molecular weight markers are indicated to the *left* of the figure.

2. Incubate the cells at 26°C in a 125-mL Erlenmeyer flask with shaking at 140 rpm in an orbital shaker.
3. Check protein expression by Western blotting (Fig. 2) (see Note 7).
4. Harvest the culture medium around 72 h post inoculation by centrifugation at 4,000 × *g* for 15 min.
5. Apply the culture medium to an anti-FLAG M2 affinity column.
6. Wash the column with ten bed volumes of Tris-buffered saline (TBS).
7. Elute the bound proteins with five fractions of FLAG peptide (100 μg/mL) each of half a bed volume.
8. Dialyze the eluate against TBS (see Note 8).

4. Notes

1. A fourth expression vector, pLEXSY-hyg2, encoding hygromycin phosphotransferase is available, which makes it possible to express four genes simultaneously in this system.
2. *L. tarentolae* cells require aerobic conditions. The cells can be maintained continuously in suspension in ventilated tissue culture flasks with regular dilutions at 1:10–1:50.
3. *L. tarentolae* cells must be cultivated in the dark at 26°C because hemin is light sensitive.
4. The frequency of correct integrants is very high in clonal selection by plating on agar plates.
5. The days that colonies begin to appear are different for each clone when using the clonal selection method on agar plates. Some clones can be picked on the fourth day after plating, and some clones need more than 10 days' incubation before they can be picked.
6. The reactivation of *L. tarentolae* strains from glycerol stocks has often failed. Glycerol stock must be prepared from the mid-growth phase culture carefully.
7. It is important to harvest the culture medium in the appropriate growth phase. The LM-332 heterotrimer should be harvested between 2 and 3 days before degradation. If the α3 monomer is required, it may be better to harvest the culture on later days.
8. The final yield of the purified recombinant LM-332 was estimated to be 2.5–5 μg from 30 mL of culture medium. This yield was comparable to that of LM-332-producing mammalian cell lines.

Acknowledgments

This work was supported by a Grant-in-aid (no. 18108003) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Breitling, R., Klingner, S., Callewaert, N., Pietrucha, R., Geyer, A., Ehrlich, G., Hartung, R., Muller, A., Contreras, R., Beverley, S. M., and Alexandrov, K. (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production, *Protein Expr. Purif.* **25**, 209–218.
2. Kushnir, S., Gase, K., Breitling, R., and Alexandrov, K. (2005) Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*, *Protein Expr. Purif.* **42**, 37–46.
3. Fritsche, C., Sitz, M., Weiland, N., Breitling, R., and Pohl, H. D. (2007) Characterization of the growth behavior of *Leishmania tarentolae*: A new expression system for recombinant proteins, *J. Basic Microbiol.* **47**, 384–393.

4. Soleimani, M., Mahboudi, F., Davoudi, N., Amanzadeh, A., Azizi, M., Adeli, A., Rastegar, H., Barkhordari, F., and Mohajer-Maghari, B. (2007) Expression of human tissue plasminogen activator in the trypanosomatid protozoan *Leishmania tarentolae*, *Biotechnol. Appl. Biochem.* **48**, 55–61.
5. Basak, A., Shervani, N. J., Mbikay, M., and Kolajova, M. (2008) Recombinant proprotein convertase 4 (PC4) from *Leishmania tarentolae* expression system: purification, biochemical study and inhibitor design, *Protein Expr. Purif.* **60**, 117–126.
6. Tunggal, P., Smyth, N., Paulsson, M., and Ott, M. C. (2000) Laminins: Structure and genetic regulation, *Microsc. Res. Tech.* **51**, 214–227.
7. Li, S., Edgar, D., Fassler, R., Wadsworth, W., and Yurchenco, P. D. (2003) The role of laminin in embryonic cell polarization and tissue organization, *Dev. Cell* **4**, 613–624.
8. Miner, J. H., and Yurchenco, P. D. (2004) Laminin functions in tissue morphogenesis, *Annu. Rev. Cell Dev. Biol.* **20**, 255–284.
9. Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C., Kleinman, H. K., Marinkovich, M. P., Martin, G. R., Mayer, U., Meneguzzi, G., Miner, J. H., Miyazaki, K., Patarroyo, M., Paulsson, M., Quaranta, V., Sanes, J. R., Sasaki, T., Sekiguchi, K., Sorokin, L. M., Talts, J. F., Tryggvason, K., Uitto, J., Virtanen, I., von der Mark, K., Wewer, U. M., Yamada, Y., and Yurchenco, P. D. (2005) A simplified laminin nomenclature, *Matrix Biol.* **24**, 326–332.
10. Phan, H. P., Sugino, M., and Niimi, T. (2009) The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system, *Protein Expr. Purif.* **68**, 79–84.