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The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system

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ABSTRACT

Laminin (LM)-332 (α3β3γ2), a large heterotrimeric glycoprotein, is an essential component of epithelial basement membranes that promotes cell adhesion and migration. Here, we expressed human LM-332 using a novel protein expression system based on the trypanosomatid protozoan host *Leishmania tarentolae*. Plasmids containing cDNA encoding full-length β3 and γ2 subunits and truncated α3 subunit were sequentially introduced into *L. tarentolae*. A recombinant strain harboring the three subunits of human LM-332 efficiently formed heterotrimer and secreted it into the culture medium. Heterotrimeric recombinant LM-332 (rLM-332) could be purified from culture medium with one-step immuno-affinity chromatography. The eluted fraction contained all three subunits, as confirmed by immunoprecipitation and immunoblotting. The purified rLM-332 showed similar cell adhesion activity to rLM-332 purified from mammalian cells, indicating its proper folding and assembly. The obtained expression level was not high; however, we suggest that this expression system has the potential for mass production of LMs for tissue engineering.

Introduction

Basement membranes (BMs)[2] are thin sheet-like structures that form a highly specialized region of extracellular matrix (ECM) located at the epithelial–mesenchymal interface of most tissues. BMs serve a number of cellular functions including proliferation, survival, and differentiation through interactions with cell surface receptors such as integrins and dystroglycans [1]. The major constituents of BMs are laminin (LM), type IV collagen, heparan sulfate proteoglycan (perlecan), and nidogen. Matrigel™, a solubilized extract derived from the mouse Engelbreth-Holm-Swarm (EHS) tumor, is rich in these proteins, so it has been widely used as an active BM model [2,3]. However, the source of BMs is almost exclusively limited to the EHS tumor, because there are few other sources that produce large amounts of BM proteins. Thus, the development of a large-scale production system for BM proteins is required to provide them as resources for tissue engineering.

LMs are large glycoproteins that are an integral part of the structural architecture of BMs [4]. They consist of three subunits, α, β and γ chains, which bind to each other via disulfide bonds to form a cross-shaped structure with three short arms and one rod-like long arm. The α chain comprises a large globular domain (LG) in its C-terminal region, which consists of five homologous globular subdomains (LG1–5). To date, five α, three β, and three γ chains have been identified to combine into at least 16 heterotrimeric molecules [5]. LM-332 (formerly known as LM-5), 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. Furthermore, proteolytic cleavage, which has been shown to play a key role in cell migration as well as tumor progression and metastasis, occurs in the α3 and γ2 chains [6].

There are various recombinant protein expression systems using *Escherichia coli*, yeast, plant, insect, and mammalian cells. These systems are now being used to provide relatively large proteins, such as collagen, for biomaterials [7]. However, it is difficult to express correctly folded heterotrimeric LMs in *E. coli* and yeast. Thus, mammalian cells have been used to prepare recombinant LMs that used for biochemical studies [8–10]. Recently, a novel protein expression system based on *Leishmania tarentolae*, a protozoan parasite of lizards, was developed [11]. This system allows not only easy handling like *E. coli* and yeast, but also full eukaryotic protein folding and the mammalian-type posttranslational modification of target proteins. Therefore, we attempted to produce recombinant human LM-332 in the *L. tarentolae* expression system. A recombinant strain harboring three subunits of LM-332 efficiently formed heterotrimer and secreted it into the medium.

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[2] *Abbreviations used:* BMs, basement membranes; ECM, extracellular matrix; LM, laminin; EHS, Engelbreth-Holm-Swarm; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate.

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Heterotrimeric rLM-332 could be purified from culture medium and this showed similar cell adhesion activity to rLM-332 prepared from 293-F mammalian cells.

Materials and methods

Plasmid construction

Expression vectors for human LM α3A (GenBank database Accession No. NM_000227) subunit lacking LG4–5 modules and most of the LEc domain with an N-terminal FLAG-tag and a C-terminal c-*myc*-tag were prepared as follows. cDNA encoding truncated α3 chain was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of human keratinocytes using the primers 5′-AAGATATCAGATAGCAGCCCTGCAGAAGAATG-3′ and 5′-AAGCGGCCGCCTGCAACAGCTGGTTGATACG-3′ (amino acids residues 192–1337). The PCR product was digested with EcoRV–NotI and inserted into the corresponding restriction sites of pSecTag2-FLAG to generate pSecTag2-FLAG-hLAMA3. pSecTag2-FLAG was generated by subcloning annealed oligonucleotides encoding the FLAG epitope (DYKDDDDK) into the HindIII–BamHI sites of pSecTag2A mammalian expression vector containing c-*myc* epitope-tag (Invitrogen, Carlsbad, CA). Then, cDNA encoding truncated α3 chain with an N-terminal FLAG-tag and a C-terminal c-*myc*-tag was amplified by PCR using pSecTag2-FLAG-hLAMA3 as a template and the primers 5′-AAATCTAGAGGATTACAAGGATGACGACGATAAG-3′ and 5′-AAACTTAAGTCAATGATGATGATGATGATGGTC-3′. The PCR product was digested with XbaI–AflII and inserted into the corresponding restriction sites of pLEXSY-sat2 expression vector (Jena Bioscience, Jena, Germany).

Expression vectors for human LM β3 (NM_000228) chain were prepared as follows. cDNA encoding full-length β3 chains was amplified by RT-PCR from total RNA of human keratinocytes using the primers 5′-AAAAGATCTCCCCATTGGCTGAAGATGAGACC-3′ and 5′-AAATCTAGACTACTTGCAGGTGGCATAGTAG-3′. The PCR product was digested with BglII–XbaI and inserted into the BamHI–XbaI sites of pcDNA3.1 (+) mammalian expression vector (Invitrogen) to generate pcDNA3.1-hLAMB3. Then, cDNA encoding full-length β3 chain without signal sequence was amplified by PCR using pcDNA3.1-hLAMB3 as a template; the PCR product was digested with XbaI–KpnI and inserted into the corresponding restriction sites of pLEXSY-neo2 expression vector (Jena Bioscience). The primers used were 5′-AAATCTAGACCAACAAGCCTGCTCCCGTGGGG-3′ and 5′-AAAGGTACCTCACTTGCAGGTGGCATAGTAGAGC-3′ (residues 18–1172).

Expression vectors for human LM γ2 (NM_005562) chain were prepared as follows. A partial cDNA encoding the γ2 chain was purchased from Open Biosystems (Huntsville, AL), and the remaining portion of the cDNA was amplified by RT-PCR and ligated in tandem to create a full-length cDNA. The resulting cDNA was inserted into the EcoRI–XhoI sites of pcDNA3.1 (+) (Invitrogen) to generate pcDNA3.1-hLAMC2. Then, cDNA encoding full-length γ2 chain without signal sequence was amplified by PCR using pcDNA3.1-hLAMC2 as a template; the PCR product was digested with SpeI–AflII and inserted into the XbaI–AflII sites of pLEXSY-ble2 expression vector (Jena Bioscience). The primers used were 5′-AAAACTAGTACCTCCAGGAGGGAAGTCTGTG-3′ and 5′-AAACTTAAGCTTCACTGTTGCTCAAGAGCCTGGG-3′ (residues 22–1193).

Protein expression and purification

rLM-332 was produced using LEXSYcon2 Expression Kit (Jena Bioscience). Plasmids containing cDNAs encoding human LM β3, γ2, and α3 chains were sequentially transfected into *L. tarentolae* strain by electroporation, and stable transfectants were selected with G418 (50 μg/ml), bleomycin (100 μg/ml), and nourseothricin (100 μg/ml), respectively. Recombinant strains harboring the three constructs were cultured in brain–heart-infusion based medium, supplemented with hemin (5 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 26 °C in the dark with shaking (140 rpm). rLN-332 was also produced using the Free-Style™ MAX 293 Expression system (Invitrogen) according to the manufacturer's instruction.

For purification, culture medium was applied to an anti-FLAG M2 affinity column (Sigma, St. Louis, MO). The column was washed with TBS (50 mM Tris–HCl, pH7.4, 150 mM NaCl), and bound LMs were eluted with FLAG peptide (100 μg/ml), and dialyzed against TBS. The purified proteins were analyzed by 5% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing or non-reducing conditions, and separated proteins were visualized by silver staining.

Immunoblot analyses

Protein samples were separated on 5% SDS–polyacrylamide gels under reducing or non-reducing conditions, and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20, followed by incubation with first antibodies against FLAG epitope (1:1000) (monoclonal anti-FLAG M2; Sigma), LM β3 (1:2000) (H-300; Santa Cruz Biotechnology), and LM γ2 (1:2000) (B-2; Santa Cruz Biotechnology) for 1 h at room temperature. Membranes were washed with PBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) (GE Healthcare). Membranes were then developed using ECL Western Blotting Detection Reagents (GE Healthcare) and imaged on an LAS-4000mini Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

Immunoprecipitation

Culture medium was immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) at 4 °C overnight, then centrifuged at 3000 rpm at 4 °C for 30 s. The resin was washed three times with TBS, and boiled in SDS sample buffer. The immunoprecipitated samples were separated on 5% SDS–polyacrylamide gels under reducing conditions, and immunoblotted with anti-LM β3 or anti-LM γ2 antibodies.

Cell adhesion assay

Cell adhesion assays were performed using HT1080 human fibrosarcoma cells and A431 human epidermal carcinoma cells. Briefly, 96-well plates were coated with rLM-332 at 4 °C overnight and blocked with 1% BSA for 1 h at room temperature. The cells were harvested with PBS containing 1 mM EDTA, suspended in serum-free DMEM at a density of 3×10^5 cells/ml, and then plated in the wells. After incubation in a CO_2 incubator at 37 °C for 1 h, the attached cells were stained for 30 min with 0.4% crystal violet in 50% methanol. After washing with distilled water, cells were dissolved in 50 μl of 0.1 M sodium citrate in 50% ethanol, and the absorbance was measured at 595 nm. For inhibition assay, the cells were treated with 5 μg/ml of function-blocking anti-integrin antibodies against α3 (P1B5; Millipore, Billerica, MA) and α6 (GoH3; eBioscience, San Diego, CA) subunits for 20 min at room temperature before inoculation.

Results

Expression of human LM-332 in L. tarentolae cells

The full-length cDNAs of LM β3 and γ2 chains without signal sequence were cloned into the *L. tarentolae* expression vector pLEX-