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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-

SY-2 behind the signal sequence of *L. mexicana* secreted acid phosphatase (Fig. 1) [12]. It has been reported that the α3 chain undergoes extracellular proteolytic processing of two sites. One is in the LG3 domain near the junction with the LG4 domain, and the other is in LEc domain near the junction with the long arm region [6,13,14]. Because there is no antibody that detects the α3 chain in the case of cleavage of these two sites in *L. tarentolae* cells, a FLAG-tag and a c-*myc*-tag were added to the N- and C-terminal ends of these cleavage sites, respectively (Fig. 1). Then, the epitope-tagged cDNA of the α3 chain was cloned into pLEXSY-2 vector in the same way as the β3 and γ2 chains. These plasmids were sequentially transfected into *L. tarentolae* cells by electroporation, and stable transfectants were selected by culturing cells on solid media with antibiotics.

The recombinant strain harboring three constructs was analyzed for expression of LM-332 subunits. When the cell lysates and the culture medium were separated by SDS–PAGE under reducing conditions and subsequently immunoblotted with anti-FLAG, -LM β3, and -LM γ2 antibodies, major bands of the expected sizes corresponding to the 145 kDa α3 chain, the 135 kDa β3 chain, and the 150 kDa γ2 chain, were detected (Fig. 2A). When using anti-c-*myc* antibody, the band corresponding to the α3 chain was detected in the cell lysate but not in the medium. This result indicates that the c-*myc* tag at the C-terminal end of the α3 chain was cleaved by a protease after its secretion into the medium (see below).

Next, heterotrimer formation of the α3, β3, and γ2 chains in the medium was analyzed by SDS–PAGE under non-reducing conditions, in which the disulfide bonds are preserved. When immunoblotting with anti-FLAG, -LM β3, and -LM γ2 antibodies major bands corresponding to the α3–β3–γ2 heterotrimer were clearly detected, although some of the α3 chain was secreted into the medium as monomer (Fig. 2B). Immunoprecipitation analysis showed that the α3 chain interacted with the β3 and γ2 chains in the medium (Fig. 2C). These results demonstrated for the first time that the three chains of LMs can form heterotrimer with disulfide bonds in unicellular eukaryote.

When analyzing the recombinant strain harboring only β3 or β3/γ2 subunits, the β3 monomer and the β3–γ2 heterodimer were produced in the cells but not secreted into the medium (data not shown). This suggested that the α3 subunit has an important role for secretion of trimeric LMs.

Purification and characterization of recombinant human LM-332 produced in L. tarentolae

To purify the rLM-332, the culture medium was applied to an anti-FLAG affinity column; then, bound LMs were eluted with FLAG peptide. For comparison, rLM-332 was also purified from 293-F mammalian cells transfected with the same set of LM chains in the mammalian expression vector. The eluted fractions were separated by SDS–PAGE and analyzed by silver staining. Under non-reducing conditions, rLM-332 from 293-F cells separated as two bands corresponding to the α3–β3–γ2 heterotrimer containing the native or truncated γ2 chains (Fig. 3A, left panel) [10]. On the other hand, rLM-332 from *L. tarentolae* separated as a single band. Under reducing conditions, rLM-332 from 293-F cells separated as four bands, the 150 kDa γ2 chain, the 145 kDa α3 chain, the 135 kDa β3 chain, and the 105 kDa truncated γ2 chain, while rLM-332 from *L. tarentolae* separated as two bands (Fig. 3A, right panel). To determine which bands correspond to the three chains of LM-332, the rLM-332 bands were separated by SDS–PAGE under

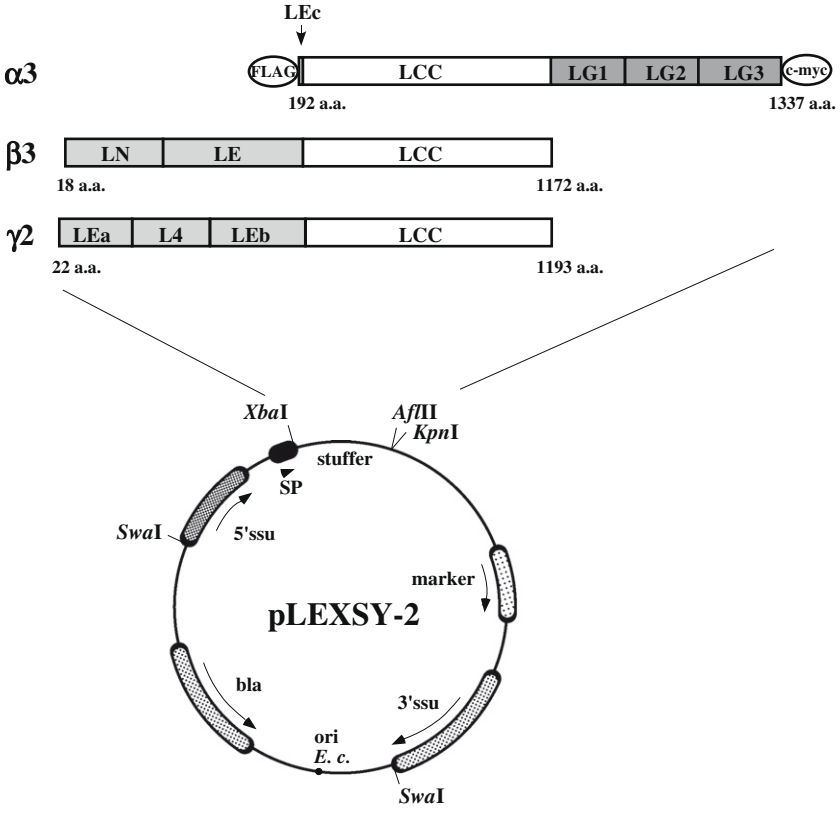

Fig. 1. Construction of expression plasmids for human LM-332 subunits. Map of pLEXSY-2 vector and the strategy used for plasmid construction are summarized. The constituent α3, β3, and γ2 chains with their N- and C-terminal amino acids are shown schematically. FLAG and c-*myc* tags were added to the N- and C-terminal end 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 epidermal growth factor-like (LE), LM coiled-coil (LCC), LM globular (LG), LM N-terminal (LN), and LM 4 (L4) domains are shown.

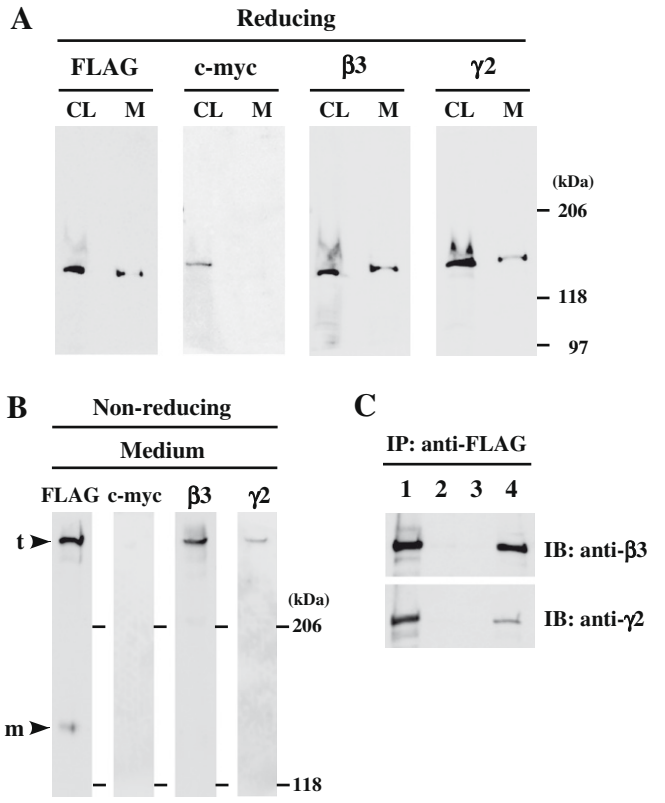

Fig. 2. Expression of human LM-332 subunits in *L. tarentolae*. Plasmids containing cDNA encoding the β3, γ2, and α3 chains were sequentially transfected into *L. tarentolae* by electroporation. A recombinant strain harboring three constructs was analyzed for the expression of LM-332. Cell lysate (CL) and medium (M) were separated by SDS–PAGE under reducing (A) or non-reducing (B) conditions. Membranes were sequentially immunoblotted with anti-FLAG, anti-c-*myc*, anti-LM β3, and anti-LM γ2 antibodies. Molecular weight markers are indicated to the *right*. The bands assumed to be monomer (m) and trimer (t) are indicated with *arrowheads*. (C) The medium was subjected to anti-FLAG immunoprecipitation followed by immunoblotting with anti-LM β3 or anti-LM γ2 antibodies. The samples used were culture medium from 293-F cells transfected with the α3, β3, and γ2 subunits (lane 1), wild-type *L. tarentolae* strain (lane 2), *L. tarentolae* strain transfected with the β3 and γ2 subunits (lane 3), and *L. tarentolae* strain transfected with the α3, β3, and γ2 subunits (lane 4).

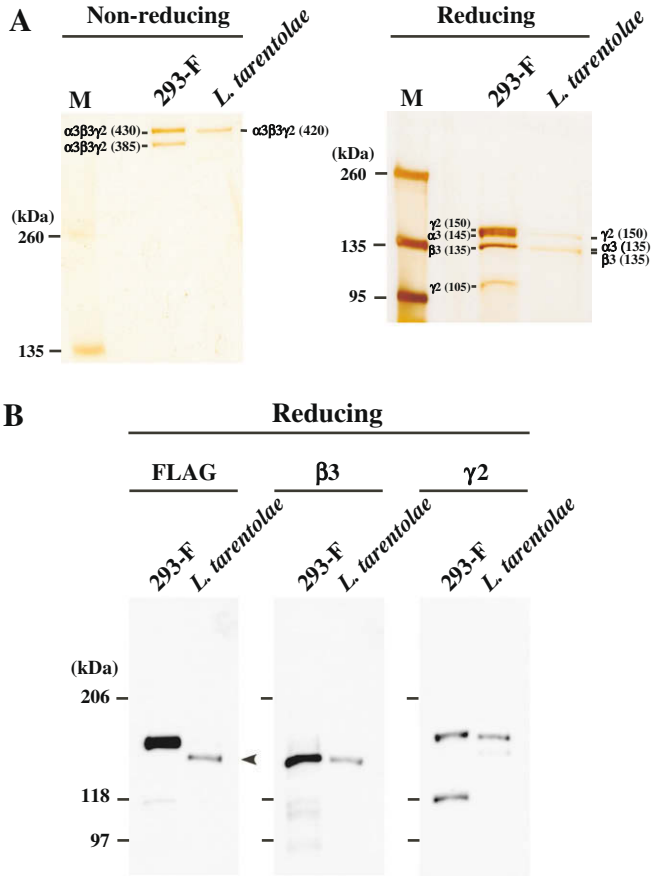

Fig. 3. Analysis of the purified rLM-332 by SDS–PAGE and immunoblotting. (A) Purified rLM-332 from 293-F cells (*left lane*) and *L. tarentolae* (*right lane*) were separated by SDS–PAGE under non-reducing or reducing conditions and analyzed by silver staining. The numbers in parenthesis are the assumed molecular masses in kDa. (B) Purified rLM-332 from 293-F cells (*left lane*) and *L. tarentolae* (*right lane*) was separated by SDS–PAGE under reducing conditions, and subsequently immunoblotted with anti-FLAG (*left*), anti-LM β3 (*middle*), and anti-LM γ2 (*right*) antibodies. Molecular weight markers are indicated to the *left*. Note that the band (*arrowhead*) corresponding to the α3 chain from *L. tarentolae* was smaller than that from 293-F cells.

reducing conditions and subsequently immunoblotted with anti-FLAG, -LM β3, and -LM γ2 antibodies. The band corresponding to the α3 chain from *L. tarentolae* was found to be smaller than that from 293-F cells, suggesting that the C-terminal end of the α3 chain was cleaved to generate the 135 kDa band, the same size as the β3 chain (Fig. 3B).

The final yield of the purified rLM-332 was estimated about 33 μg from 60 ml of culture medium (Table 1). This yield was comparable to that of LM-332-producing mammalian cell lines [10].

Biological activity of recombinant human LM-332

rLM-332 was evaluated for cell adhesion activity using the human fibrosarcoma cell line HT1080 and the human epidermal carcinoma cell line A431. HT1080 cells express α3β1 integrin, while A431 cells express α3β1 and α6β4 integrins, which are two major cell surface receptors for LM-332 [15,16]. Compared with rLM-332 purified from 293-F cells, the cell adhesion activity of rLM-332 purified from *L. tarentolae* was similar or slightly weaker, indicating that it was properly folded (Fig. 4A and B). When A431 cells were pretreated with function-blocking antibodies against integrin α3 or integrin α6 subunit, the cell adhesion to the rLM-332 was partially inhibited (Fig. 4C). It was strongly blocked by a combination of anti-integrin α3 and anti-integrin α6 antibodies. This result showed that the cell adhesion to the rLM-332 purified from *L. tarentolae* is mediated by both α3β1 and α6β4 integrins as in the case of the rLM-332 purified from 293-F cells. Taken together, these observations indicated that the C-terminal truncation of the α3 chain of rLM-332 from *L. tarentolae* did not affect integrin binding.

Table 1
Purification summary.

	Volume (ml)	Total protein (mg)	Estimated target protein (mg)	Yield (%)
Culture supernatant	60	15.3	0.06	100
Affinity chromatography pool	1.5	0.033	0.033	55

Discussion

LM has many cysteine residues that must fold properly via intra-chain disulfide bonds in the short arm region of all three chains, the LG domain of the α chain, and the Lβ domain of the β chain. Furthermore, all three chains are disulfide linked to each other via their long arm regions before secretion. Although synthetic

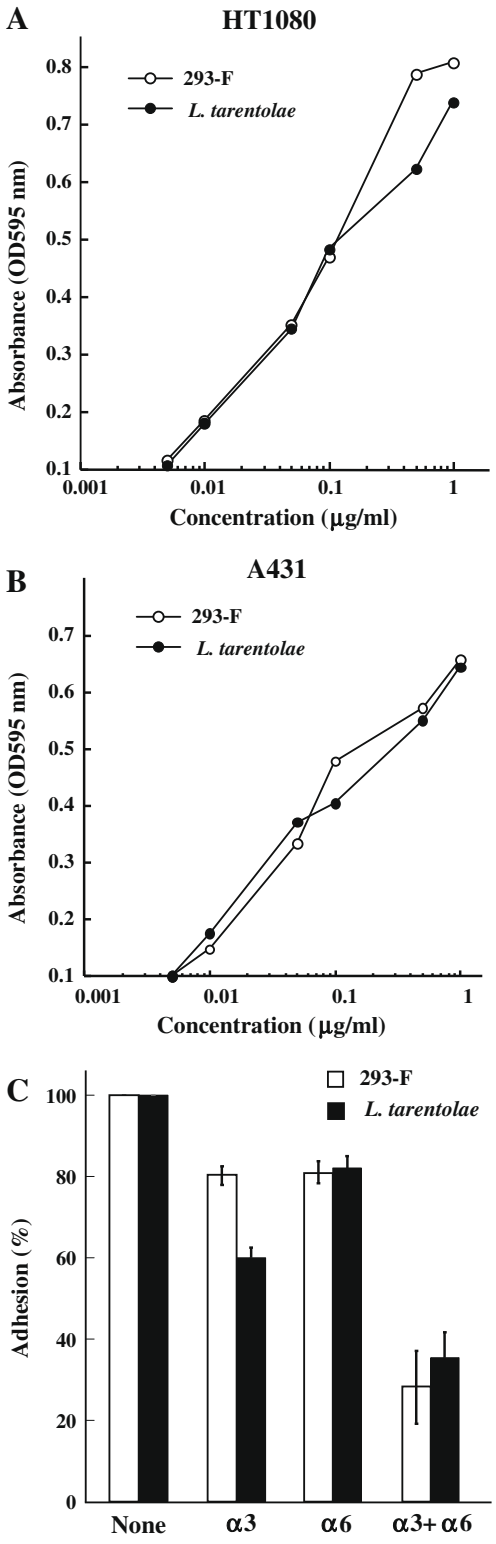

Fig. 4. Cell adhesion activity of rLM-332 purified from *L. tarentolae* and 293-F cells. 96-well plates were coated with increasing concentrations of purified rLM-332 from *L. tarentolae* (closed circles) and 293-F cells (open circles). HT1080 (A) and A431 (B) cells were added and incubated for 1 h. After washing with PBS, the relative numbers of attached cells were assessed by crystal violet staining. Data are expressed as the means of triplicate results. (C) Inhibition of cell adhesion to the rLM-332 using anti-integrin antibodies. A431 cells were incubated with the indicated function-blocking anti-integrin antibodies (5 μg/ml) at room temperature for 20 min. After treatment, the cells were plated on the rLM-332 purified from 293-F cells (white bars) or *L. tarentolae* (black bars), and incubated for 1 h. The relative numbers of attached cells without antibody was taken 100%. Each point represents the means ± standard deviation for triplicate results.

peptides or small fragments of the long arm region of three subunits can assemble themselves *in vitro* through hydrophobic interaction, assembly of the whole chain is difficult to achieve, probably because the individual proteins need to fold correctly first [17–20]. To date, heterotrimeric rLM has not been generated in *E. coli* or yeast, possibly because of these difficulties. In the present study, we employed a *L. tarentolae* expression system to produce human LM-332. A recombinant strain harboring three subunits of LM-332 efficiently formed heterotrimer and secreted it into the culture medium. This suggests that the appropriate molecular chaperones to aid protein folding and a transport system for large proteins are both present in *L. tarentolae* cells.

LM-332 has been reported to undergo extracellular proteolytic processing of the α3 and γ2 chains [21,22]. The α3 chain undergoes processing in the LG domain and the short arm region, while the γ2 chain undergoes processing in the short arm region. In this study, we expressed a fully processed form of the α3 chain with intact β3 and γ2 chains; these chains can be assembled into one of the smallest heterotrimeric LMs (~420 kDa). The successful production of rLM-332 led us to investigate whether the other isoforms of the LM family can be produced in the *L. tarentolae* expression system. When the β1 and γ1 chains were swapped with the β3 and γ2 chains in the recombinant *L. tarentolae* strain, we found that rLM-311 (~525 kDa) was secreted into the culture medium (data not shown). Further studies are required to investigate whether larger isoforms, such as LM-111 (α1β1γ1; ~800 kDa) or LM-511 (α5β1γ1; ~900 kDa), could be produced in this system.

N-glycosylation in *L. tarentolae* is exceptionally homogenous with a higher eukaryote-like biantennary oligosaccharide and the $Man_3GlcNAc_2$ core structure [11]. However, higher branched *N*-glycans were lacking. Recently, Kariya et al. showed that *N*-glycosylation of LM-332 regulates its biological functions [23]. They clearly demonstrated that introduction of a bisecting GlcNAc to LM-332 decreased its cell adhesion, scattering, and migration activities. In this study, we showed that rLM-332 purified from *L. tarentolae* had similar cell adhesion activity as that purified from 293-F mammalian cells. This indicates that the *N*-glycosylation profile of rLM-332 was similar between *L. tarentolae* and 293-F cells; however, further investigation is needed to examine the various biological functions of rLM-332.

LM-332 exerts its functions through binding to several cell surface receptors, including α3β1 and α6β4 integrins [6]. In α3β1 integrin-dependent cell adhesion via LM-332, the combination of the LG2–3 domain of the α3 chain and the C-terminal end of the γ2 chain is important [24,25]. The results of cell adhesion assay indicated that rLM-332 from *L. tarentolae* was properly folded and assembled. However, immunoblot analysis revealed that the α3 chain was cleaved at the C-terminal end, including the c-*myc* tag, after its secretion. Although the exact cleavage site is unclear, the α3β1 and α6β4 integrin binding sites seem to be unaffected. Appropriate protease inhibitors should be added to the culture medium to prevent *L. tarentolae*-specific proteolytic processing of LMs.

It has been demonstrated that *L. tarentolae* can be cultured on a cheap medium and grown to high cell densities (5×10^8 cells/ml) with 4–5 h doubling time [11]. The cells can also be grown on fully defined media so that recombinant proteins are less likely to be contaminated with prions or pathogenic viruses, which may accompany mammalian cell cultures. Thus, the establishment of a mass production system for rLMs using non-pathogenic *L. tarentolae* will be useful for cell biology and tissue engineering applications. In the current study, the obtained expression level was insufficient to mass produce rLMs, but could possibly be increased by improving the vector sequence and culture conditions.

Acknowledgments

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