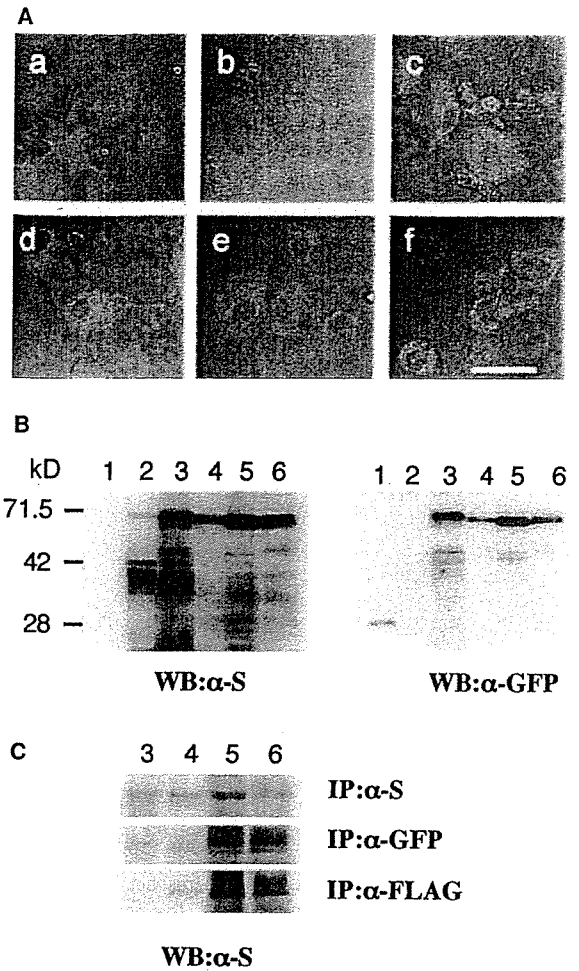

Fig. 2. Expression of L fusion proteins in Cos7 cells. (A) Cells were transfected with plasmids to express (a) EGFP, (b) L protein, (c) L-FLAG–EGFP, (d) L(Δ32)-FLAG–EGFP, (e) L(Δ45)-FLAG–EGFP, and (f) L(Δ54)-FLAG–EGFP, by electroporation. Two days after electroporation, the green fluorescence was observed under a confocal microscope at a 63-fold magnification. The bar scale shows 50 μm. After 3 days, the Cos7 cells were harvested and disrupted by sonication. (B) Cell lysates were probed in western blots with anti-S or anti-GFP IgG. (C) Conditioned media were collected, immunoprecipitated with anti-S, anti-GFP or anti-FLAG IgG, respectively, and subjected to western blotting with anti-S IgG. Lane 1, EGFP; lane 2, L protein; lane 3, L-FLAG–EGFP; lane 4, L(Δ32)-FLAG–EGFP; lane 5, L(Δ45)-FLAG–EGFP; lane 6, L(Δ54)-FLAG–EGFP.

L(Δ45)-FLAG–EGFP particles were immunoprecipitated with two different antibodies and treated with proteinase K. Western blots with anti-GFP IgG showed two digested EGFP bands of I and II (Fig. 5A) when immunoprecipitated with anti-S IgG, but only one band of II when immunoprecipitated with anti-GFP IgG. The only difference between these two bands of I and II should be due to the size of

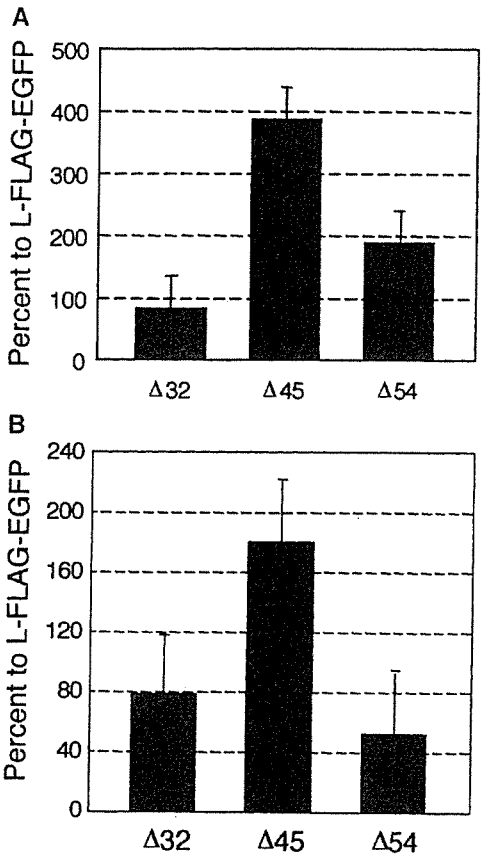

Fig. 3. Secretion of L fusion particles evaluated by enzyme immunoassay and fluorescence. The HBsAg immunoreactivity (A) and the fluorescence (B) in the conditioned medium of the transfected Cos7 cells were measured and the percentages were calculated with the level of L-FLAG –EGFP expression assumed to be 100%. Δ32, Δ45 and Δ54 denote L(Δ32)-FLAG–EGFP, L(Δ45)-FLAG–EGFP and L(Δ54)-FLAG–EGFP, respectively. Each SD was calculated from three independent evaluations.

N-terminal sequence of EGFP protected from proteinase K. This result indicates that the C-terminus of the L-EGFP fusion protein may exhibit dual topology. Because an anti-S IgG recognizes the immunodominant a-epitope, which is common to all six genotypes of HBV as the major surface region of the HBsAg envelope protein [21,22], this antibody will immunoprecipitate all of the L(Δ45)-FLAG–EGFP particles. By contrast, anti-GFP IgG selectively immunoprecipitate particles that display the EGFP moiety at the C-terminus on their surface. With the immunoprecipitates, the EGFP moiety was processed to band II, the N-terminus of which was not protected from proteinase K (Fig. 5A). Anti-S IgG immunoprecipitates included another topology, which protects the N-terminus of the EGFP moiety from proteinase K. There is no

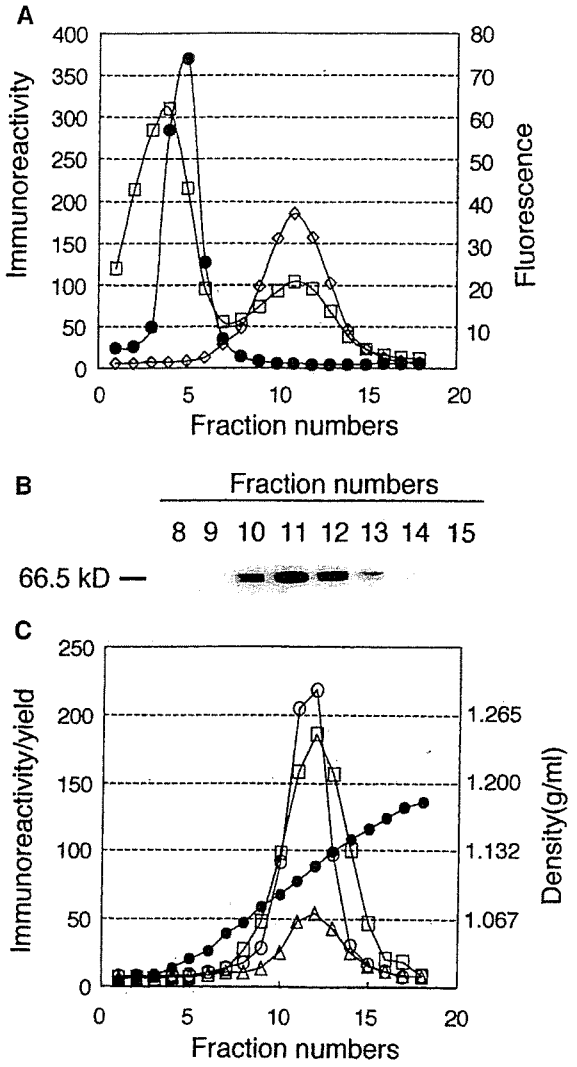

Fig. 4. Particle formation of L(Δ45)-FLAG–EGFP evaluated by sucrose gradient ultracentrifugation. (A) Cell extracts of the Cos7 cells transfected with L(Δ45)-FLAG–EGFP were subjected to sucrose gradient ultracentrifugation and each fraction was evaluated for immunoreactivity (◇) and fluorescence (□). Native EGFP was subjected to centrifugation and the fluorescence from EGFP was simultaneously monitored (●). (B) Fractions that showed both immunoreactivity and fluorescence in (A) were analyzed by western blotting with anti-S IgG. (C) L(Δ45)-FLAG–EGFP particles prepared from conditioned medium (open triangle) and cell extracts [fraction 11 in (B); □] were compared with the BNC prepared from recombinant yeast [6] (○) for immunoreactivity. The density of each fraction was plotted by ●.

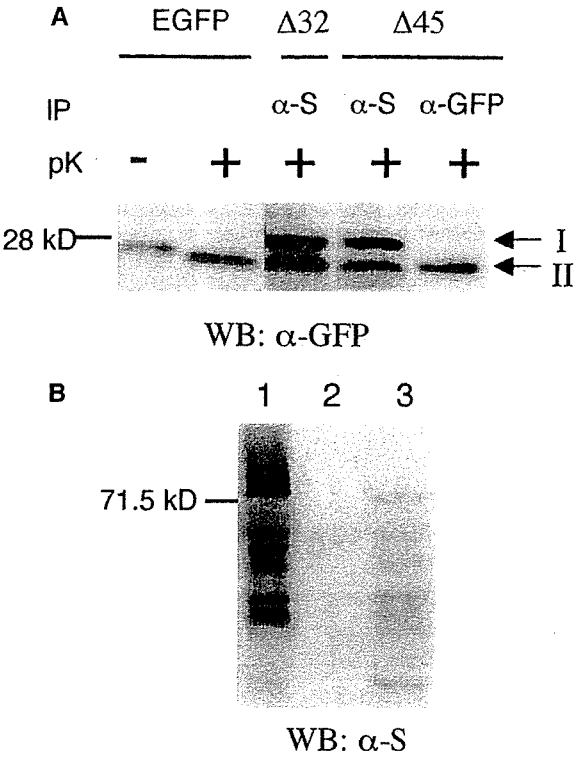

Fig. 5. Evaluation of the C-terminal orientation of the EGFP moiety in L fusion particle. (A) Two different L fusion particles were immunoprecipitated by either anti-S or anti-GFP IgG, and the immunoprecipitates were treated by proteinase K (pK). Δ32 and Δ45 denote L(Δ32)-FLAG–EGFP and L(Δ45)-FLAG–EGFP, respectively. Digested products were detected with anti-GFP IgG. The produced bands were indicated by arrows with I (28 kDa) and II (25 kDa). The native EGFP was treated with or without proteinase K and shown as the reference. In (B) the conditioned medium containing L(Δ45)-FLAG–EGFP particles was immunoprecipitated by anti-GFP IgG (lane 1), and the process was repeated until no L fusion protein could be detected by anti-S IgG (lane 2). The residual supernatant was immunoprecipitated by anti-S IgG (lane 3).

possibility that the dual C-terminal topologies coexist in a particle. However, there should be two types of L(Δ45)-FLAG–EGFP particles, because EGFP moiety-displaying particles immunoprecipitated with anti-GFP IgG showed only single digested product band II. If the two types of C-terminal topology coexist in a particle there should be two digested bands following immunoprecipitation by anti-GFP IgG, as revealed by the anti-S IgG. Thus, we concluded that the EGFP moiety was protected from proteinase K by the membrane when it was located inside the particle, and that it was slightly digested when located on the external side of a particle without membrane protection.

To confirm this finding, we subtracted the L(Δ45)-FLAG–EGFP particles from the conditioned medium with anti-GFP IgG and protein G conjugated to agarose by immunoprecipitation. The residual supernatant was subjected to repeated immunoprecipitation using the same procedure until the L fusion protein could not be detected by western blotting. When the

final subtracted fraction of the supernatant was further immunoprecipitated with the anti-S IgG conjugated to a microparticle, the L fusion protein band was still detected in the fraction, thereby indicating the presence of L fusion particles, which contained EGFP moieties inside (Fig. 5B).

Human hepatocyte specific delivery of EGFP by infection

The preS1 peptide displayed on the surface of L particles recognizes the specific receptor present on human hepatocytes and is essential for HBV infectivity [7,8]. This specific infectivity of the L particle should be independent of the tolerable C-terminus truncation. Cell type-specific infection of L(Δ45)-FLAG–EGFP particles was assessed on various human cancer cells (Fig. 6). After 9 h of incubation with L(Δ45)-FLAG–EGFP particles, EGFP fluorescence was specifically observed in human hepatocellular carcinoma HepG2 cells and NuE cells, whereas no EGFP fluorescence was observed from either human colon adenocarcinoma WiDr cells or human epidermoid carcinoma A431 cells (Fig. 6A). The EGFP fluorescence was not observed in HepG2 cells when incubated with BNC, EGFP or a mixture of BNC and EGFP (Fig. 6B).

Discussion

In this study we attempted to establish a nanocapsule that efficiently delivers protein for tissue- or cell-type-specific targeting. Based on our technology of BNC as a delivery vector, we used a fusion strategy that ensures that the protein of interest is produced as a component of the particle. The fusion proteins between L protein and EGFP (L-FLAG–EGFP) were expressed with or without C-terminal truncation of L protein. It was necessary to truncate the C-terminus of the L protein by 45 amino acids for it to be efficiently secreted from the cells. A EGFP moiety fused to the C-terminus of the L protein appears to prevent secretion, although the mechanism is not currently clear. However, incorrect folding of each half moiety of the fusion protein does not explain the low secretion, because we were able to prepare the particle from both conditioned media and cell extracts. Once prepared,

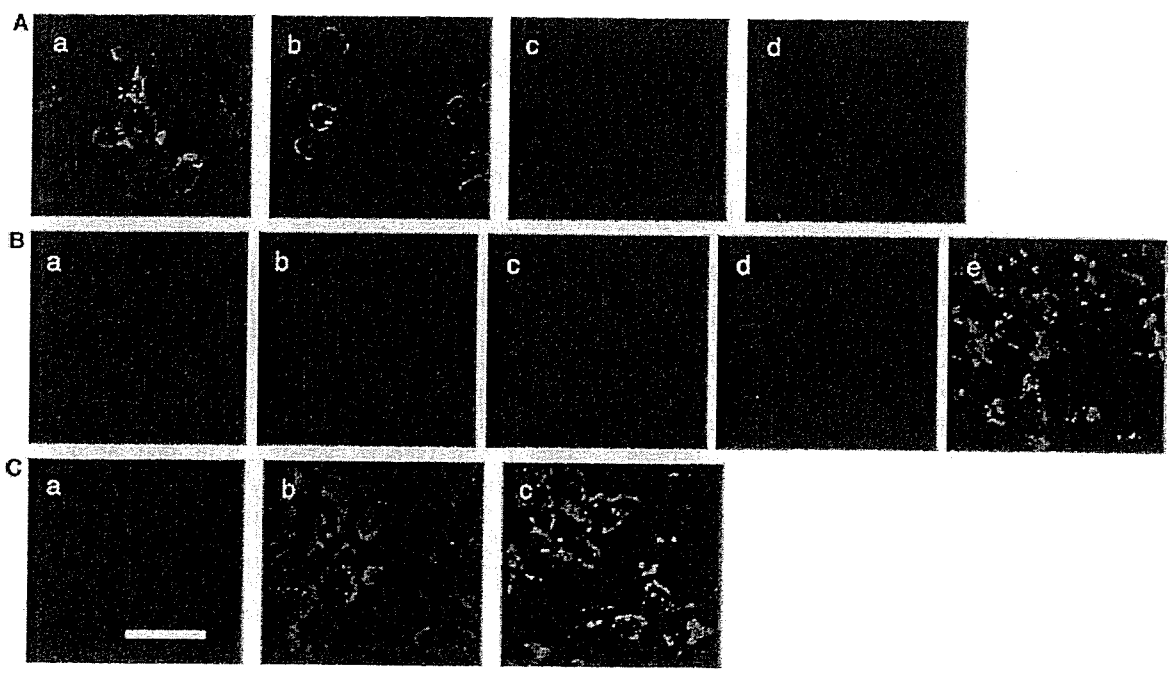

Fig. 6. Infection of L fusion particles *in vitro*. (A) Five nanograms of L(Δ45)-FLAG-EGFP particles obtained from the conditioned medium were added to the culture media of 5×10^4 cells of HepG2 (a), NuE (b), WiDr (c) and A431 (d), respectively. (B) Conditioned medium of nontransfected Cos7 cell (a), 5 ng of BNC from the conditioned medium (b), 100 ng of EGFP (c) and a mixture of 5 ng of BNC with 100 ng of EGFP (d), and 5 ng of L(Δ45)-FLAG-EGFP particles from the conditioned medium (e) were added to 5×10^4 cells of HepG2, respectively. (C) Extracts of nontransfected Cos7 cells (a), 5 ng of L-FLAG-EGFP particles from the extracts of transfected Cos7 cells (b) and 5 ng L(Δ45)-FLAG-EGFP particles from the extracts of transfected Cos7 cells (c) were added to 5×10^4 cells of HepG2. The fluorescence was observed under a confocal microscope at a 63-fold magnification after 9 h infection. Scale bar = 50 μm.

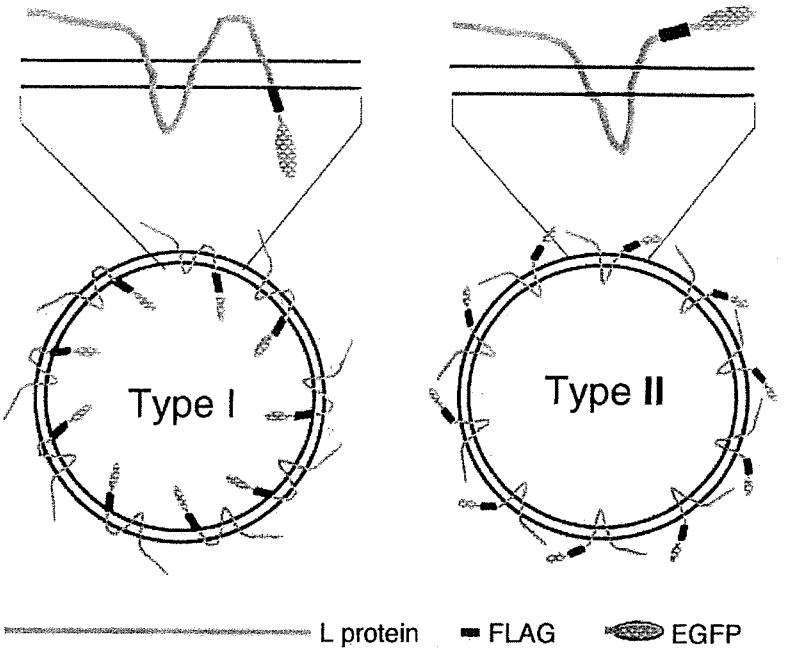

Fig. 7. Proposed models of L fusion particles. Type I, EGFP incorporating particle. Type II, EGFP displaying particle.

particles displayed immunoreactivity in an enzyme immunoassay consistent with the fluorescence intensity, when kept for 3 weeks at 4 °C in the presence of phenylmethanesulfonyl fluoride (PMSF). Furthermore, the cell extract was able to directly infect HepG2 cells (Fig. 6C). These results indicate that the L-FLAG–EGFP particle is stable, if properly prepared, and has the potential to infect cells.

Following optimization of the C-terminal truncation, we attempted to determine the topology of the fused protein in the particle because the purpose of this study was to design a nanoparticle that incorporated a foreign protein using a fusion strategy. One of the purposes of inserting a FLAG-tag between the C-terminus of the L protein and EGFP was to study the topology of the fused protein because we expected enterokinase to specifically recognize and cleave FLAG peptide. Unexpectedly, this protease cleaved other basic residues in the L protein moiety, displaying many degraded products, which confused us. By contrast, the strong resistance of EGFP to proteinase K was extremely useful in studying the C-terminal topology of L fusion protein. Proteinase K treatment of the L(Δ32)-FLAG–EGFP particles showed results similar to those obtained with the L(Δ45)-FLAG–EGFP particle. The hydrophobic sequence of approximately 20–30 amino acids in the C-terminus of the L(Δ32) or L(Δ45) protein may traverse the membrane of lipid bilayer. However, our results show that this terminus is not sufficiently hydrophobic to anchor the C-terminus in the membrane, although it is sufficient to exhibit dual topology. Based on the results of the proteinase K protection assay, we proposed a model of the nanoparticle of L fusion protein (Fig. 7). We designated the particle, whose N-terminal EGFP moiety was incorporated within the membrane, as type I, whereas type II denotes the EGFP moiety displayed on the surface of the particle. Because the particle membrane protected the N-terminal part of the EGFP moiety, proteinase K digestion of type I showed the EGFP moiety to have a slightly higher molecular mass than that produced by the treatment of type II. We scanned the western blots in Fig. 5A and analyzed the densities of the two bands. We found the ratio of band I to band II was 39 : 61. To confirm this ratio, we also compared the immunoreactivity of the conditioned medium containing both type I and type II particles with that of the type II-subtracted medium by the anti-GFP IgG, as shown in Fig. 5B. The ratio of the result is 100 − 36, which means that the ratio of type I to type II is 36 : 64. These different procedures used to estimate the ratio of the particles in two topologies lead to almost equal results. Therefore, we concluded that nearly 40% of the particles are type I. The secretion enhanced by C-terminal truncation might be explained by the fixed topology because we could not detect a particle with a mixed type I and type II topology in one particle. However, it is difficult to find a determinant of the topology of the C-terminal moiety that causes it to be incorporated inside or displayed outside. This unfixed

pattern of topology might clarify the results of previous studies of the topology of the HBsAg protein. It is suggested that the C-terminus of the envelope protein protrudes from the particle in 1987 [12]. Localization of HBV epitope by monoclonal antibodies revealed that the residues 178–186 of the S peptide are exposed on the surface of the virion particle [23]. Kuroda *et al.* described that Asn146 was not glycosylated when the recombinant L particle was prepared from yeast, whereas it was glycosylated when expressed in mammalian cells [3]. This suggests that this aspargine residue is located at the border of the external region and the membrane-bound region. The C-terminal sequence of 56 amino acids from 170 to 226 may be long enough to traverse the membrane twice, although the hydrophobicity is not sufficient to explain the topology precisely. It may be possible to design the C-terminal region as the clear transmembrane region by replacing it with one of the transmembrane-type receptors to limit the topology of the particle to type I.

Our BNC has the same tissue-specific infectivity as HBV because of the N-terminal region of L protein, preS1, which determines its narrow host range and distinct organ tropism. The region from 3 to 77 amino acid residues of preS1 is essential for this specificity [24]. To avoid impairing this selectivity, we fused EGFP to the C-terminal of L protein, which was successfully truncated in order to be secreted from mammalian cells and assembled to an L fusion particle.

We previously reported that BNC containing DNA of interest yielded a very high transfection efficiency with a high specificity of gene transfer to human liver-derived cells [6]. The L fusion particle described here should have equivalent specific transfection efficiency due to the character of the preS1 region of the L protein. The advantage of the fusion particle is that there is no need to incorporate proteins using specialized methods, such as electroporation, for which it is difficult to establish the efficiency needed to transfer genes and drugs into cells. Depending on the cell type, conditions vary and optimization of the conditions may sometimes lead to a 10-fold increase in efficiency. This was also the case with our BNC, and we had to optimize the electroporation conditions, depending on the substances to be incorporated. An efficient procedure is required to eliminate empty particles after electroporation in order to attain the highest efficiency. As for the L fusion particle, all of the particles are destined to convey the protein of interest with a transfection efficiency of nearly 100% directed to human-derived liver cells.

The L fusion particles designed in this study were found to have dual C-terminal topologies, which could easily be separated using antibodies. There was no difference in specific infectivity among them when monitored using EGFP fluorescence (data not shown). This means that it is possible to choose various proteins for the C-terminal moiety of the L fusion proteins, depending on the character of the proteins to be fused. This possibility will include cytoplasmic proteins, as well as cytokines or ligands, for the cell surface. In this context, one of the candidates of the moiety might be interferon (IFN) which is used with ribavirin to treat hepatitis C virus-induced liver disease. This therapy has many does-dependent side effects, such as depression and insomnia. The L–IFN fusion particle would be extremely useful because it targets only the liver so that the dose administered could be low so that the side effects would not be a cause for concern.

Retargeting of BNC by replacing the preS1 region with other targeting moieties or biorecognition molecules, such as ligands, receptors and antibodies as previously proposed [6], should also be applicable to the L fusion particles. The greatest problem in using the particle is that people who have antibodies to HBV are increasing in number due to the widespread hepatitis B vaccination program. Stealth mutations at Gln129 and Gly145 to Arg would not only address this problem, but also lead to a design of the delivery vector with extremely low immunogenicity [25,26]. Thus, we are developing our BNC for its potential to become a practical vector of protein delivery.

Experimental procedures

Cell cultures

Human hepatoma HepG2 cells, human squamous cell carcinoma A431 cells and human colon adenocarcinoma WiDr cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria). Human hepatoma NuE cells were cultured in RPMI-1640 with 10% (v/v) FBS. African green monkey kidney-derived Cos7 cells for particle production were maintained in DMEM supplemented with 5% (v/v) FBS. These cells were maintained at 37 °C/5%CO_2.

Construct of plasmids

The HBV L gene was excised from the plasmid pGLD LIIP39-RcT [7] and inserted into the *Xho*I site of pEGFP-N1 vector (Clontech, Mountain View, CA). Then the synthetic oligo-nucleotide coding FLAG-tag sequence (5′-ATATATTGATTACAAGGATGACGACGATAAGA TA-3′) was inserted between the *Acc*I site close to the C-terminus of the L protein and the *Age*I site at the N-ter-

minus of EGFP in pEGFP-N1. The *Not*I site after the termination codon of EGFP was changed to the *Xho*I site. The resultant ORF of the L-FLAG–EGFP fusion protein was excised with *Xho*I and inserted at the *Xho*I site downstream of the SRα promoter in the plasmid of pBO477, which is a derivative of pTB1455 [27], to construct the plasmid pBO572. We constructed three other expression vectors pBO638, pBO637 and pBO822 for L proteins with truncation by 32, 45 and 54 amino acid residues at the C-terminus, respectively. The resulting three types of L fusion particles were designated L(Δ32)-FLAG–EGFP, L(Δ45)-FLAG–EGFP and L(Δ54)-FLAG–EGFP (Fig. 1).

Preparation of L fusion particles

Five micrograms of expression plasmid DNA were transfected into 5×10^6 of Cos7 cells by electroporation at 300 V/950 μF. Transfected cells were first cultured for 14–16 h in 8 mL of DMEM containing 5% (v/v) FBS in a 100 mm dish. The medium was replaced with 8 mL of CHO-S-SFM II (Invitrogen, Carlsbad, CA) and the cells were cultured for a further 72 h. The conditioned medium was collected and condensed in a Vivaspin concentrator tube (molecular mass cut-off at 100 kDa; Vivaspin, Sartorius, Hannover, Germany) according to the manufacturer's instructions. Cells were harvested by a cell scraper and suspended in 100 μL of NaCl/P_i in DMEM per dish and then sonicated for 30 s. The supernatant from the cell extracts was collected by centrifugation. The concentration of L fusion particles in the conditioned medium and in the cell extracts was independently determined by IMx HBsAg (Abbott Laboratories, Sligo, Ireland). The fluorescence of EGFP from the particles was simultaneously measured by an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Sucrose gradient ultracentrifugation

L fusion particles were analyzed by sucrose gradient ultracentrifugation with himac CP70MX (Hitachi) as described previously [3]. Briefly, transfected cells were harvested by a cell scraper in the NaCl/P_i containing EDTA. The wet cells were suspended in buffer A [0.1 M sodium phosphate, pH 6.8, 15 mM EDTA, 2 mM PMSF, 0.85% (w/v) NaCl and 1% (v/v) Triton X-100], and then sonicated for 30 s on ice. Cell extracts in the supernatant were subjected to sucrose gradient ultracentrifugation at 103 600 *g* for 14 h at 4 °C in 27 mL of sucrose gradient of 10–50% (w/v) in buffer A without Triton X-100. Fractions containing L fusion particle were collected and dialyzed against buffer A without Triton X-100. The dialyzed solution was again subjected to sucrose gradient ultracentrifugation under the same conditions. The conditioned medium was also subjected to sucrose gradient ultracentrifugation after condensation with Vivaspin. Each 1 mL, fractionated from the top of the centrifugation tube, was analyzed for density, immunoreactivity (IMx) and fluorescence. L fusion particle fractions were collected and dialyzed against NaCl/P_i for 16 h at 4 °C. The dialyzed solution was filtered through a membrane filter (0.22 μm, MILLEX-HV, Millipore, Cork, Ireland) and stored at 4 °C.

Protease protection assay

The L fusion particles were immunoprecipitated with monoclonal anti-(S mouse epitope) IgG conjugated to microbeads contained in the IMx kit or with monoclonal anti-(GFP mouse epitope) IgG (Sigma, St Louis, MO) and protein G agarose (Invitrogen). The immunoprecipitates were washed five times with NaCl/P_i, and resuspended in 10 μL of NaCl/P_i. Proteinase K (New England Biolabs, Beverly, MA) was then added to achieve a concentration of 100 $\mu g \cdot mL^{-1}$. The suspension was incubated at 37 °C for 1 h. PMSF was added to 5 mM to stop the digestion.

Transfection of L fusion particle

About 5×10^4 cells of HepG2, NuE, WiDr and A431 were seeded in each well of a Laboratory-Tek chamber slide (Nunc, Naperville, IL) and cultured at 37 °C in 5% (v/v) CO_2. After 12 h, the culture media were replaced with 300 μL of CHO-S-SFM II containing 5 ng of L(Δ45)-FLAG–EGFP particles, and the cells were cultured for a further 9 h. The chambers were subsequently detached and the glass slide was washed with NaCl/P_i. The cells were covered with glass in the presence of NaCl/P_i containing 10% (v/v) glycerol, and the specific EGFP fluorescence was observed under a confocal microscope LSM 510 META (Zeiss, Jena, Germany).

Acknowledgements

The authors thank Mrs Kumiko Soga for her excellent technical assistance, and Mr Masayuki Kita for his continuous encouragement. This project was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Science and Technology Corporation (Research Fund for Patenting).

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IUBMB *Life*, 58(1): 1–6, January 2006

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Critical Review

Engineered Bio-nanocapsules, the Selective Vector for Drug Delivery System

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Summary

The bio-nanocapsule (BNC) is our concept of artificial hollow nanoparticles that have been designed and produced through biotechnological procedures. We proposed an empty virus-like particle, which consists of a recombinant L envelope protein of hepatitis B virus (HBV) and a lipid derived from the host cell, as an engineered BNC. Although this BNC was first developed as an immunogen of hepatitis B vaccine, the pre-S1 region in N-terminus of L envelope protein confers hepatocyte specific infectivity of HBV on the BNC. This recombinant BNC is now being developed as a novel platform of drug delivery system (DDS) vector for selective delivery.

IUBMB *Life*, 58: 1–6, 2006

Keywords Vector; specific infection; delivery; topology.

INTRODUCTION

The drug delivery system (DDS) is one of the areas of intense focus recently in pharmacology. However, the development of DDS technology has concentrated on the controlled release of pharmaceutical drugs encapsulated in a nano-sphere composed of such substances as micelles and liposomes. The targeting potential of the pinpoint delivery in DDS has appeared to be ignored since antibody-based 'missile therapy' has been extensively studied by immunologists. Although live viruses are sometimes toxic or oncogenic to the infected cells, they have been found to be extremely useful for the transfer of genes to the cells. Consequently, the development of artificial viruses as vectors for gene transduction or gene therapy has caused vigorous research worldwide as a part of nanobiotechnology. This new area of science deals with molecules as nanostructures and molecules in nanostructures such as nanoparticles not only for the conventional analyses of molecules but for the extensive development of new tools in biotechnology and medical engineering (*1*). In presenting an overview of the viruses, it may be noted that many of them are known to have host ranges, but to rarely exhibit cell type or tissue type selectivity. Only a few of them exhibit some narrow range of cell type selectivity like HBV or HIV, for example. The HBV is among the best known viral pathogens that affect humans. It has been studied for more than 60 years in the hope of learning how to prevent its infection. HBV is a small enveloped DNA virus of the hepadnaviridae family (*2, 3*). The surface proteins of the virion envelope are found to consist of three types of glycoproteins, which are called small- (S-), middle- (M-) and large- (L-) protein (*4*). These three different proteins, which are cotranslationally integrated into the rough endoplasmic reticulum as transmembrane proteins, are encoded in a single open reading frame of HBV genome with three independent, but in-frame, AUG translational start codons (*5, 6*). The HBV infected cells secrete, not only the complete 42-nm infectious virions, but also a large excess of 22-nm noninfectious empty envelope particles. The latter are composed of S-protein (*7–9*). Valenzuela et al. successfully prepared HBV surface antigens (HBsAg) as recombinant S-protein, which organized itself as a particle having a diameter of 22 nm (*10*). When produced in

Received 21 October 2005; accepted 20 November 2005
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ISSN 1521-6543 print/ISSN 1521-6551 online © 2006 IUBMB
DOI: 10.1080/15216540500484368

yeast, recombinant M- and L-proteins were also found to associate as hollow virus-like particles, although they had been only developed as immunogens for hepatitis B vaccines (*11, 12*). The virus-like particle composed of the recombinant L-proteins had a diameter of approximately 200 nm (*12, 13*). Due to the specific affinity to human hepatocytes localized in the amino-terminus of L-protein, only the nanometer size hollow particle, composed of L-proteins, showed extremely selective targeting potential as a novel type of DDS vector to be directed to the human liver (*14*). Because of its favorable potential, we have proposed a bio-nanocapsule (BNC) concept as an efficient nanomachine to achieve tissue-/cell-type specific delivery of genes, drugs and proteins.

PROPERTIES OF ENGINEERED BIO-NANOCAPSULES

Our BNC is described as a hollow protein nano-scale particle. Some viral core particles, which consist of sole self-organizing proteins, such as HBV core antigen (cAg) and others that also consist of self-organizing proteins with lipids as essential components, like the envelope of hemaglutinating virus of Japan (HVJ), are conceivable. Since the BNC should function as a capsule that contains some encapsulated foreign substances in its interior, the lipid component appears to be essential to confer flexibility in order to maintain a suitable shape as a DDS vector even after the physical implementation of encapsulation such as electroporation. In this context, HBV cAg does not appear to be suitable as a capsule due to the tight association of each component of cAg. The HBV envelope proteins form a BNC, since almost 80% of the components are L-, M- or S-proteins. This BNC of HBV can be formed by a single species of viral surface proteins. Therefore, newly engineered BNC can be designed and produced by modifying the protein in a variety of ways by a simple procedure. The L-protein is composed of three regions, the 108 or 119 amino acids (subtype ayw or adw, respectively) pre-S1 region (*8, 15*) involved in the direct recognition of human hepatocytes, the 55 amino acids pre-S2 region associated with the polymerized albumin-mediated interaction (*16*), and the major 226 amino acids S protein region.

When L-protein was expressed in yeast cells, it failed to assemble into a subviral particle, unlike S protein, although the protein is efficiently expressed (*17, 18*). The synthetic gene for chicken-lysozyme signal (C-SIG) peptide was fused at the 5′-terminal of L gene. The C-SIG peptide was processed correctly by the yeast secretory apparatus and led the pre-S1 region in traversing the ER membrane. Then, the L-proteins assembled themselves into a virus-like particle as a L-BNC. A small region of $Ser^{44}-Thr^{48}$ containing a site sensitive to the trypsin-like protease ($Arg^{48}-Thr^{49}$) has been deleted from pre-S2. The addition of C-SIG peptide and deletion of $Ser^{44}-Thr^{48}$ did not affect the stability of L-protein and polymerized human serum albumin binding activity (*19*).

This engineered L-protein, produced as a L-BNC in recombinant yeast cells, has been purified and characterized. The average molecular mass of a L-BNC was confirmed by equilibrium sedimentation to be 6.4×10^6, which indicates that about 110 molecules of L-protein associate to form an L-BNC. Atomic force microscopy in a moist atmosphere showed the L-BNCs to be spherical particles with diameters ranging from 50–500 nm and an average of 200 nm. The L-BNCs were stable at a high temperature of around 80°C and, for a week, at 4°C, but were found to become unstable as a result of freezing and thawing or treatment with dithiothreitol (*19*).

ENGINEERED BIO-NANOCAPSULE HAS THE SAME SPECIFIC INFECTIVITY AS HBV

HBV, a member of hepadnaviridae, known as a family of hepatotropic, has a very narrow host-range and is limited to infecting naturally humans and some higher primates, such as the chimpanzee (*11, 20, 21*). It has been demonstrated that the L-protein is important in forming an infectious virus particle and essential for receptor interaction (*22–24*). In particular, the 108 or 119 amino acids of pre-S1 domain of glycosylated L-protein have long been considered to be responsible for receptor binding and the host range (*22, 25, 26*), while the 55 amino acids of pre-S2 is dispensable for viral infectivity (*27*). The involvement of L-protein in the infection stage has been confirmed. Furthermore, the segment from 21 to 47 amino acid of pre-S1 was found to be indispensable for HBV infection (*28–31*). However, the latest research demonstrated that more amino acid residues, extending from 3 to 77, are involved in this process (*22*). The pre-S1 region of the L-protein, displayed on the surface as the specific ligand for receptor of human hepatocytes, certainly confers on the L-BNC the high infectivity to hepatocytes limited to humans and primates. Very recently, we have shown that the L-BNC efficiently delivers the gene for green fluorescence protein (GFP) and GFP protein to human liver cells in a cell type specific manner *in vitro* and *in vivo* (*14, 32*). Thus, our engineered L-BNC was demonstrated to be an efficient delivery mimicking HBV.

ENGINEERED BIO-NANOCAPSULE CAN DELIVER SUBSTANCES TO TARGET TISSUE OR CELL

HBV is the only DNA virus that targets the liver where it efficiently infects hepatocytes. This ability permits the development of an HBV-based vector for liver-directed gene transfer with foreign DNA inserted into the virus genome (*33, 34*). However, this viral vector is not suitable for the delivery of substances other than DNA. Since the L-BNC is an empty particle that recognizes human hepatocytes just like HBV, electroporation was used to encapsulate the DNA and small chemical compounds that were inside the L-BNC (*14*). The GFP gene or fluorescent chromophore calcein was successfully encapsulated by electroporation and transferred

to human hepatocytes in culture, or in a xenograft model with the specific infection of the L-BNC. This specificity was shown by the green fluorescence observed only in human hepatocellular carcinomas. Any other cells of human carcinomas or of host tissues did not show positive fluorescence. When the gene for human clotting factor IX was transferred into the xenograft model by the L-BNC, factor IX was produced at a significant level in the sera relevant to the treatment of moderate hemophilia B.

Although electroporation is proposed as one of the procedures suitable for enclosing substances in the particle (*14*), optimal conditions for electroporation appear to be lacking. The empty particles that failed in encapsulation must be removed just prior to infection in order to attain the highest delivery efficiency. If not, they will bind to receptors on the surface of hepatocytes competing with the particles carrying the substances, resulting in low transfer efficiency. A more efficient procedure might be required in place of electroporation.

In the case of delivery of proteins by the BNC, it appears to be difficult sometimes to introduce the proteins into hollow particles with canonical electroporation due to the high-voltage pulse and disordered redox conditions that denature the proteins. The fusion strategy was proposed as a suitable procedure to prepare the BNC that delivers proteins to human hepatocytes. This strategy will ensure the generation of protein for transfer accompanied by the formation of recombinant L-BNCs. The gene of EGFP, an analogue mutant of GFP, fused to the 3' end of L gene, was expressed in mammalian COS-7 cells and the BNCs of L-EGFP fusion were produced and prepared. When infected, these L fusion particles showed green fluorescence only on the human hepatocytes (*32*), indicating that the L fusion particles have equivalent specific transfection efficiency due to the hepatocyte recognition site present in the pre-S1 region. No encapsulation procedure, such as electroporation, is required if the fusion strategy is available for the BNC. All of the particles are destined to convey the protein of interest with a transfection efficiency of nearly 100% to human liver cells.

Thus, it has been demonstrated that the BNC is an ideal vector for the cell- or tissue-type specific delivery of genes, drugs and proteins *in vitro* and *in vivo*. Together with the advantage that it is mass produced in yeast cells and free from viral genomes, the BNC should be an extremely useful novel vector system, which could be introduced to clinical trials in the near future (Fig. 1).

UNIQUE C-TERMINAL TOPOLOGY OF L-PROTEIN IN ENGINEERED BIO-NANOCAPSULE

The topological information of the HBsAg envelope protein came primarily from the research of S-protein (equivalent to the S domain of L-protein). L-protein was found to have a dual N-terminal topology. The N-terminal pre-S domain of L-protein functions as an adapter for a virus receptor when it is located on the surface of a secreted virion particle. While the pre-S domain is located inside a viral envelope, it is believed to function as a matrix protein that mediates contact between viral envelope and nucleocapsid during virion assembly (*35–37*). Two hydrophobic regions in the S domain enable the L-protein to traverse the membrane at least twice. The hydrophilic region flanked by the two hydrophobic regions is considered to be located on the interior side of the BNC. The major HBsAg epitopes in the S domain following the second hydrophobic region are located on the

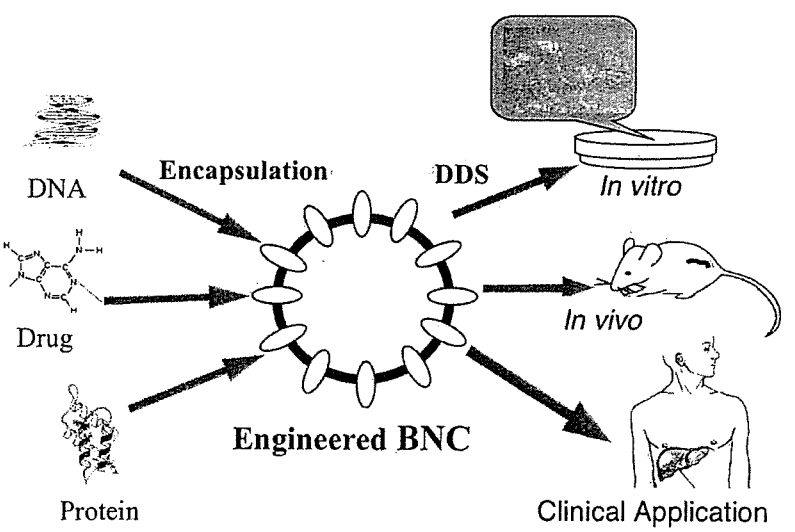

Figure 1. The concept of the application of engineered BNC for DDS. The BNC has the potential to incorporate DNA, drugs and proteins in its interior and to transfer them in a cell-/tissue-type specific manner *in vitro* or *in vivo*.

surface of the BNC. However, there has been no detailed critical description of the hydrophobic C-terminus region of the S domain to date. Eble et al. provided experimental evidence that the C-terminus protrudes from the particle (*38*). Localization of HBV epitope by monoclonal antibodies against HBV surface antigen suggested that epitope from 178 to 186 amino acid of S domain is exposed on the surface of the virion particle (*39*).

During the design of the fusion of the BNC and EGFP, FLAG-tag and EGFP were fused to the C-terminus of L protein with some length truncated. The fusion particles of L-FLAG-EGFP could be immunoprecipitated with the anti-FLAG antibody and anti-GFP antibody (*32*). These results also indicate that the C-termini of L located at the external side of the particle is suitable for a single spanning in the membrane even after the truncation of the C-terminus. However, the protease protection assay revealed the presence of another independent C-terminal topology of L-protein. The results showed that L-EGFP fusion protein takes a dual C-terminal topology, displaying EGFP moiety outside of, or enclosing it inside, a BNC. However, the experiments showed that only single orientation characterizes a particle (Fig. 2).

The dual C-terminal topologies of the fusion BNCs could be applied to various proteins for the C-terminal moiety of the fusion, depending on the character of the proteins. They might include cytoplasmic proteins, as well as cytokines or ligands to cell surface receptors. This fusion design is considered to be the most efficient way to prepare a BNC that delivers proteins to specific cells or tissues.

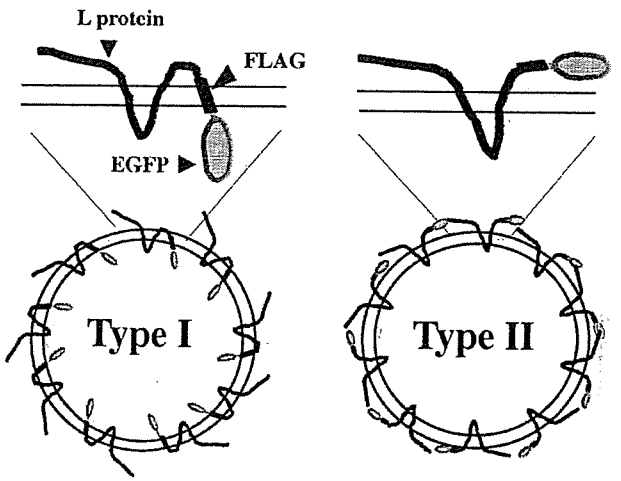

Figure 2. The C-terminal of the EGFP moiety in L-protein fusion has dual topologies. The flexible C-terminus topologies result in the formation of two types of the L-BNC-EGFP. Each of these topology types occupies a particle independently. Type I, EGFP moiety is encapsulated inside; type II, EGFP moiety is displayed outside.

THE TARGET OF ENGINEERED BIO-NANOCAPSULE CAN BE ALTERED

The pre-S1 domain in L-protein contains a specific host cell human hepatocyte recognition site present at the sequence of 3 to 77 amino acid of HBV subtype ayw (*22*, *23*). When the nucleotide sequence in L-protein, which corresponds to this site, was substituted with a synthetic gene encoding 53 amino acids of mature human epidermal growth factor (EGF), the EGF-BNC was expressed and found to display the EGF moiety on the surface of particles. The EGF-BNCs incorporating calcein were mixed with A431 cells, which overexpressed EGF receptors, or hepatic NuE cells. A high level of fluorescence was observed only in A431 cells, whereas no fluorescence was observed in NuE cells, indicating that the EGF moiety functions to target cells in a specific manner (*14*). This result demonstrates that the pre-S1 region in L-protein plays a critical role in cell recognition and that foreign biorecognition molecules can be substituted to alter the specificity of BNCs. Now, we are developing a convenient BNC that has the specific binding site for hepatocytes substituted with a peptide derived from protein A with a specific affinity to the Fc region of IgG molecules (Muraoka et al. manuscript in preparation). Due to the specific affinity to the antibody, the specific tropism of the engineered BNC could be easily altered, depending on the antibody displayed on the surface of the BNC. Recently, we successfully designed a DNA microarray for the screening of cell surface markers (*40*). In the screening of the cell surface marker of SV40 transformed mouse fibroblasts, we found that the expression of CD62L and IL-6 receptor genes were upregulated, in comparison to normal fibroblasts. The antibodies for these markers might be available to target these tumor cells, for example. The search for the cell type-specific surface markers would become a very important component of any efforts to improve the availability of the BNC.

FUTURE PERSPECTIVES

There are 14 cysteine residues in the L-protein, and they are localized only in the S domain. Since Cys48, Cys65 and Cys69 were found to be essential for the secretion of particles (*41*), the other cysteine residues were considered to be the cause of the oligomeric form of L-proteins induced by false intra- or inter-molecular disulfide bonds. We are now attempting to remove the unessential cysteine residues and 8 have been successfully removed (Fukuda et al. submitting to J. B. Chem). The multiple mutations in L-protein enhanced the stability of the BNC and raised the efficiency of encapsulation ameliorating the specific infectivity to target cells.

Because of the worldwide Hepatitis B vaccination program, the application of the BNC will be limited in clinical application. In L-protein, the segment extending from 99 to 169 amino acid of S domain contains a highly conformational

epitope cluster (*42*). The 'a' determinant region extending from 121–149 amino acid especially induces typical protective neutralizing anti-HBs antibodies. It will be important to arrange the sequence in the S domain for engineered BNC, which has less immunogenicity, as a safe BNC. With these sophisticated improvements, the BNC should be extremely useful vectors for academic research and clinical applications.

ACKNOWLEDGEMENTS

We thank Mrs K. Soga, Mr N. Nagatomo, Mr T. Yabuki, and Miss C. Amano for their excellent technical assistance. We also acknowledge Dr S. Muraoka and Prof. H. Yamada for their helpful discussions and Mr M. Kita for his continuous encouragement.

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Appl Microbiol Biotechnol
DOI 10.1007/s00253-006-0486-3

Secretory production system of bionanocapsules using a stably transfected insect cell line

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Received: 25 March 2006 / Revised: 2 May 2006 / Accepted: 4 May 2006

Abstract Bionanocapsules (BNCs) are hollow nanoscale particles composed of L protein of the hepatitis B virus surface antigen that represent specific affinity for human hepatocytes. BNCs can transfer genes and drugs into human hepatocytes efficiently and specifically. BNC can be expressed in yeast cells. In this study, we developed a new L particle production system using a stably transfected insect cell line. For this purpose, we established a host–vector system using the *Trichoplusia ni* insect cell line. L particles were efficiently secreted by the overexpression of the L protein, which was fused to the secretion signal peptide. The concentration of L particles was reached approximately 1.7 μg/ml in 5 days during cultivation in a serum-free medium without antibiotic selective pressure. The production of L particles was maintained for at least 75 days. The secretory production of L particles facilitated their easy purification by chromatography. Furthermore, it was demonstrated that purified L particles can transfect only human hepatocytes. Therefore, an insect cell expression system is an attractive tool for the production of BNC.

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Introduction

Viral and nonviral gene transfer vectors were developed for gene therapy (El-Aneed 2004; Helge 2000; Schmidt-Wolf and Schmidt-Wolf 2003). Viral vectors, such as adenoviruses, adenoassociated viruses, and retroviruses, have some advantages such as high transduction efficiency and stability. However, there are safety concerns for the clinical applications of viral vectors (Schroder et al. 2002; Woods et al. 2003; Li et al. 2002). On the other hand, nonviral vectors, such as naked DNA and liposomes, are generally safe and do not have a limitation in the size of the transgene. However, the efficiency of gene transfer is low (Herweijer and Wolff 2003; Hirko et al. 2003). Furthermore, these vectors transfer genes to not only the target cells but also other cells. Thus, no system has so far fulfilled the required criteria of high cell and tissue specificity, high transgene efficiency, and high safety level.

We previously reported an efficient drug and gene delivery system with L particles derived from the hepatitis B virus (HBV) (Yamada et al. 2003; Yu et al. 2005). HBV is an enveloped DNA virus of Hepadnaviridae. The human hepatocytes infected with HBV synthesize and release not only 42 nm of HBV virions but also noninfectious 22 nm empty subviral particles, which are spherical and filamentous. The HBV genome encodes three envelope proteins: the S protein, which is a major constituent (226 amino acid residues) of the HBV envelope protein and empty HBV surface antigen (HBsAg) particles; the M protein, which

contains 55 additional amino acid residues (pre-S2 peptide) at the N terminus of the S protein; and the L protein, which contains the 108 (subtype *y*) or 119 (subtype *d*) additional amino acid residues (pre-S1 peptide) at the N terminus of the M protein (Heermann et al. 1984). L particles are hollow nanoparticles consisting of only the L protein and endoplasmic reticulum membrane-derived phospholipids (Yamada et al. 2001). L particles have several advantages compared with viral and nonviral vectors. Firstly, L particles show a high infectivity. Secondly, L particles are very safe because they are free from the viral genome. Thirdly, there is no practical limitation in the size of the transgene. Because of these reasons, the applicability of L particles can be extended to the delivery of proteins and chemical compounds. Furthermore, they have specificity for human hepatocytes. The results of in vitro and in vivo transfection experiments demonstrated that genes and drugs are specifically transferred into human hepatocytes using L particles (Yamada et al. 2003). It was suggested that L particles are strongly expected as potential candidates for the specific gene and drug delivery system.

To date, the preparation of HBsAg particles was studied in various expression systems, namely, yeast cells (Valenzuela et al. 1982), mammalian cells (Holzer et al. 2003), plant cells (Richter et al. 2000), and insect cells (Lanford et al. 1989; Hofmann et al. 1995; Deml et al. 1999a,b). We previously demonstrated the efficient production of L particles in recombinant yeast cells and 4 μg of the purified L particles was obtained from 1 ml of the culture medium after 7 days of cultivation (Yamada et al. 2001; Kuroda et al. 1992). However, L particles accumulate in the cytoplasm, therefore, yeast cell disruption and a subsequent purification step are required. On the other hand, the productivity of L particles is very low in mammalian cells (>50 ng/ml). Therefore, insect cells are desired as a new expression system for the production of L particles. A transient expression system using recombinant baculoviruses was reported previously (Lanford et al. 1989; Hofmann et al. 1995). However, insect cells are killed during infection cycle in this system and expressed recombinant proteins tend to be degraded by proteases. A stable expression system in stably transfected cell lines was also reported (Deml et al. 1999a,b). Although S particles or L particles coexpressed with the S protein are produced efficiently, an expression system with only L particles has not yet been established.

In this study, we constructed a new L particle expression system. The L protein was fused to the honeybee melittin secretion signal peptide and we examined the expression of the fused protein in insect cells. The stably transfected insect cell line can efficiently produce L particles and secrete into the culture medium. This production was stable for more than 2 months. Furthermore, it was indicated that the expressed L particles can be used for gene and drug delivery.

Materials and methods

Materials and strains

The *Escherichia coli* strain NovaBlue (*endA1 hsdR17* (rk12 −/mk12+) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proA+B* + *lacIqZ.M15* :: Tn*10* (TcR)]) (Novagen, Darmstadt, Germany) was used as a host for recombinant DNA manipulation. *E. coli* was cultivated in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl; w/v) with 100 μg ampicillin/ml.

Cell culture

The *Trichoplusia ni* BTI-TN-5B1-4 insect cell line (High Five, Invitrogen, Carlsbad, CA, USA) was maintained in a serum-free medium (Express Five SFM, Invitrogen) supplemented with 0.26 g/l L-glutamine and 0.1% gentamicin (Invitrogen) at 27 °C. The human hepatocellular carcinoma cell line NuE was maintained in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2 (Murayama et al. 1999). The human hepatocellular carcinoma cell line HepG2 and human epidermal carcinoma cell line A431 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS.

Plasmids construction

The expression plasmid for the L protein was constructed by inserting a fragment of a large hepatitis B surface antigen (L protein, subtype *adr*) into pXINSECT-DEST38 (Invitrogen) and pIB/V5-His (Invitrogen). This insert was amplified from the plasmid pGLDLIIP39-RcT by polymerase chain reaction (Kuroda et al. 1992) using the forward primer 5′-GGGGGATCCACCATGAAATTCTTAGT CAACGTTGCCCTTGTTTTTATGGTCGTATA CATTTCTTACATCTATGCCATGGGGAC GAATCTTTCTGTTCCC-3′, including the honeybee melittin secretion signal peptide sequence or 5′-GGGGGATC CATGAAGTTATGCATATTACTGGC CGTCGTGGCCTTTGTTGGCCTCTCGCTC GGGATGGGGACGAATCTTTCTGTTCCCAAT-3′, including the *Drosophila* binding protein (Bip) secretion signal peptide sequence, and the reverse primer 5′-CCCGCGGCCGCGTCGACCAGCTTTAACGAACG CAG-3′. The amplified fragment was digested at the *Bam*HI and *Not*I restriction sites and ligated into plasmids pXINSECT-DEST38 and pIB/V5-His. The resulting plas-

mids were designated pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip. To obtain a stable transformant, plasmid pBmA.neo (Invitrogen), which contains the neomycin resistant gene, was used simultaneously with the plasmid pX-Mel and pX-Bip.

Transfection and selection of insect cell line

For the transient expression of L particles, High Five cells were seeded on a 35-mm dish at a density of 2×10^5 cells/ml 24 h before transfection, and the cells were transfected with 1 μg of pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip using FuGENE 6 (Roche, Basel, Switzerland). Three days posttransfection, culture supernatant was recovered and L particles were assayed using an IMx enzyme immunoassay (EIA) kit (Abbott Laboratories, Abbott Park, IL, USA), which can specifically detect the particulate forms of HBsAg but not the free HBsAg protein.

For the generation of stably transfected cell lines, High Five cells were cotransfected with pX-Mel and pBmA.neo or pX-Bip and pBmA.neo with weight ratios of 10:1, 50:1, and 100:1 (pX:pBmA.neo). At 48 h posttransfection, transfected cells were resuspended in 12 ml of a fresh culture medium and distributed on a 12-well plate. After 1 day, the medium was replaced with fresh Express Five SFM containing 1.0 mg/ml G418 (Invitrogen) for selection. The selective medium was replaced every 4 days, and stably transfected cell lines were isolated after 3 weeks of selection with a G418-containing medium. The concentration of L particles was assayed after 4 days of cultivation of cloned cells using an IMx EIA kit.

L particle production with stably transfected High Five cells

For the analysis of the stability of L particle expression, stably transfected High Five cells were seeded on a 12-well plate at a density of 1×10^5 cells/ml in 1 ml of a fresh medium and allowed to grow for 8 days. The concentration of L particles from the culture supernatant was assayed everyday using an IMx EIA kit. The production stability of stably transfected High Five cells was examined for 75 days. The stably transfected cells were subcultured and L particles produced were quantified at 5-day intervals.

Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of L particles in culture supernatants was performed using 10% gel. Western blot analysis was performed using an anti-S protein antibody prepared from an immunized mouse as the primary antibody and an alkaline phosphatase (AP)-conjugated anti-mouse IgG antibody (Promega Co., Madison, WI, USA) as the secondary antibody. The colorimetric detection of AP activity was performed using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega Co., Madison, WI, USA).

Purification of L particles from stably transfected cell culture supernatants

The culture supernatant (150 ml) of stably transfected insect cells was collected and concentrated fourfold by ultrafiltration using a Vivaspin20 device (Sartorius AG, Goettingen, Germany) at 4 °C. After concentration, the supernatant was loaded on sulfate Cellulofine resin (Chisso, Tokyo, Japan) equilibrated in 0.01 M sodium phosphate (pH 7.2) containing 0.15 M NaCl in a disposable gravity column (Clontech Laboratories, CA, USA), and washed. L particles were recovered by elution with 0.01 M sodium phosphate (pH 7.2) containing 0.5 M NaCl.

In vitro transfection of calcein with L particles

Calcein (0.1 mM, bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein); Dojindo, Kumamoto, Japan) was mixed with purified L particles (240 ng) in 200 μl of phosphate-buffered saline, and electroporated into the particles using a Gene pulser II electroporation system (BioRad Laboratories, Hercules, CA, USA) in a 2-mm-gap cuvette, typically at 200 V and 1,000 μF for about 20 ms. The electroporated L particles were centrifuged at 8,000 rpm for 1.5 min and 60 ng of L particles recovered from supernatant were added to 2×10^4 cells of HepG2, NuE, and A431. Fluorescence was observed under an 5-Pa laser-scanning microscope (LSM) (Carl Zeiss, Oberkochen, Germany) at 6 h posttransfection at 37 °C.

Results

Establishment of High Five expression system of L particles

To investigate the dependence of L-particle productivity on the expression cassette, the transient expression of L particles in High Five cells was performed using pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip. The expression plasmid pX contains the silkmoth (*Bombyx mori*) cytoplasmic actin gene promoter and pIB contains the promoter derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) (Fig. 1). In addition, for the efficient secretion of L particles, two types of secretion signal peptide, namely, the honeybee melittin signal peptide sequence (Mel signal) and the *Drosophila* Bip signal sequence (Bip signal), were investigated. The concentration of secreted L particles from transfected cells was deter-

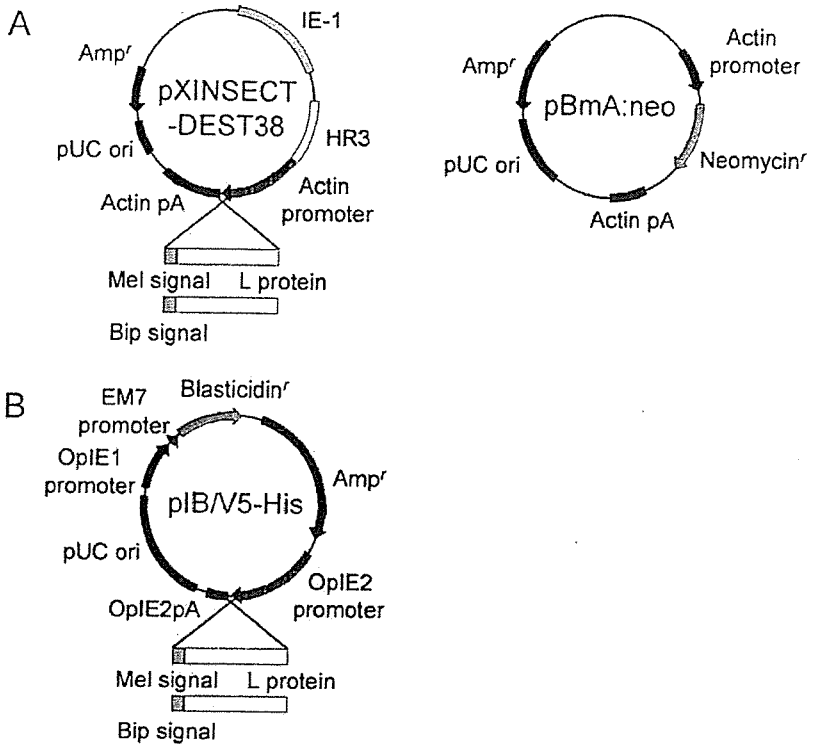

Fig. 1 Schematic representation of expression vector for L protein. One system based on pXINSECT-DEST38 including silkmoth (*Bombyx mori*) cytoplasmic actin gene promoter. In this system, pBmA.neo is used for antibiotic selection (**a**). Another system based on pIB/V5-His including promoter derived from baculovirus *Op*MNPV (**b**). The L protein was fused to the honeybee melittin secretion signal peptide and *Drosophila* Bip secretion signal peptide at its N terminus. Constructed L protein expression plasmids were named pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip

mined 3 days after transfection using an IMx EIA kit (Fig. 2a). Results indicated that pX-Mel is more efficient than other expression plasmids. Furthermore, the results of Western blotting demonstrated that pX-Mel is the optimal expression plasmid for the secretory expression of L particles in High Five cells (Fig. 2b). Therefore, pX-Mel was used in the subsequent studies.

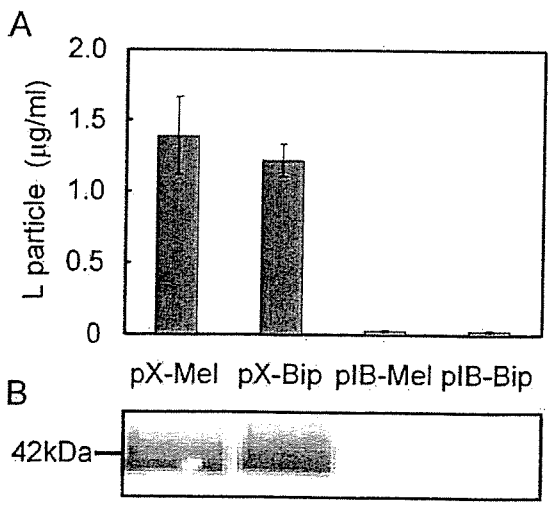

Fig. 2 Transient expression of L particles. pX-Mel, pX-Bip, pIB-Mel, and pIB-Bip were transfected into High Five cells using FuGENE 6. At 3 days after posttransfection, secreted L particles were quantified using an IMx EIA kit (**a**). The data points represent the average of three independent experiments. Western blot analysis was performed using the anti-S protein antibody (**b**)

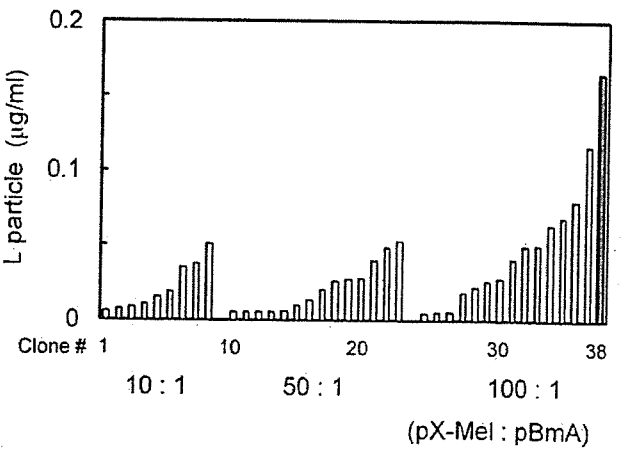

Fig. 3 Distribution of expression levels of L particles in stably transfected cell lines. High Five cells were cotransfected with pX-Mel or pX-Bip and pBmA.neo at the weight ratio of 10:1, 50:1, and 100:1 (expression plasmid pX:antibiotic resistance plasmid pBmA.neo). The amount of L particles was assayed after 3 days of subculture using an IMx EIA kit. The high-expression cell line #38 is represented by a *filled bar*

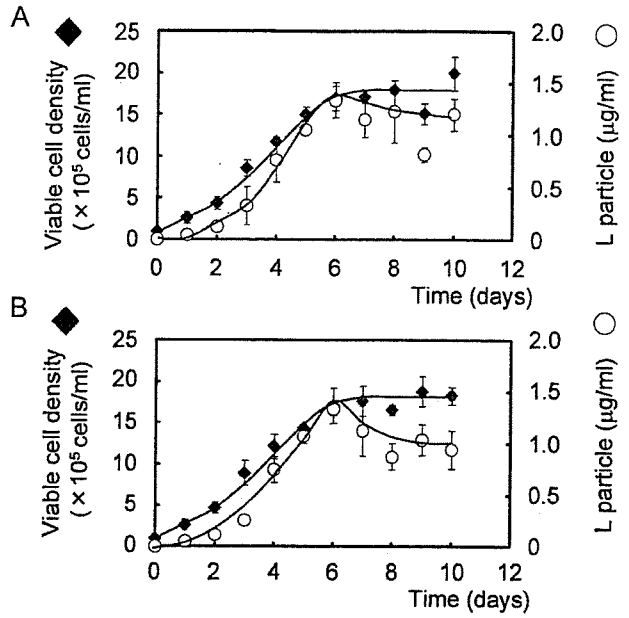

Fig. 4 Production of L particles in static culture of clone #38. Clone #38 was cultivated for 10 days with (**a**) and without G418 (**b**). The concentration of L particles in culture supernatant was determined using an IMx EIA kit. Viable cell density and L particle concentration are represented by *circles* and *diamonds*, respectively. The data points represent the average of three independent experiments

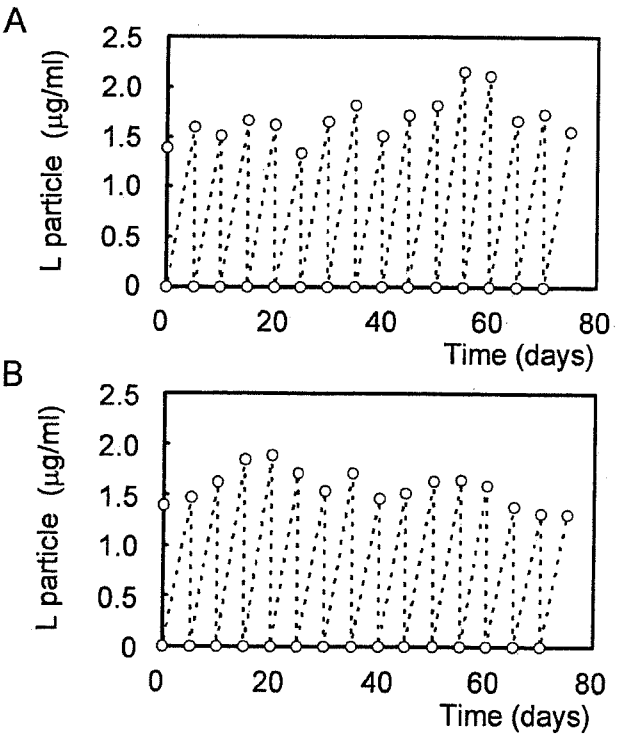

Fig. 5 Stability of L particle production in clone #38. The production stability of clone #38 was assessed every 5 days for 75 days with G418 (**a**) and without G418 (**b**)

Generation of stably transfected cell lines

High Five cells were cotransfected with pX-Mel and pBmA.neo as described in "Materials and methods." After antibiotic selection, 38 stably transfected clones were successfully obtained. Three days after the medium replacement in small static cultures, the concentration of L particles indicated the degree of heterogeneity of transformed cells (Fig. 3). The highest producer cell line (clone #38) was established by transfection at the weight ratio of 100:1 (pX:pBmA.neo). Therefore, clone #38 was used in the subsequent studies.

Production of L particle from clone #38 in static culture

Clone #38 was characterized in static batch cultures. The cells were seeded on a 12-well plate at a density of 1×10^5 cells in 1 ml of fresh medium in the presence or absence of antibiotic selective pressure (G418), and the concentration of L particles in culture supernatant was determined every day for 10 days using an IMx EIA kit. Clone #38 reached the maximum cell density of approximately 1.7×10^6 cells/ml and produced 1.3 μg/ml of L particles after 6 days of cultivation in the presence of G418 (Fig. 4a). A similar tendency was observed in the absence of G418 (Fig. 4b). These results indicated that clone #38 can produce L particles efficiently and should be subcultured to check the stability of clone #38 every 5 days at subconfluence (60–80%).

Stability of L particle production from clone #38

We confirmed the stability of clone #38, which represent high productivity of L particles in the presence or absence of antibiotic selective pressure in the long-term. The cells were subcultured every 5 days at an initial density of 1×10^5 cells/ml in a fresh medium and the culture medium was assayed for L particle production. The expression level of L particles did not change with time in the culture medium for 75 days in the presence or absence of 1.0 mg/ml G418 (Fig. 5). It is interesting to note that the average concentration of L particles reached approximately 1.7 μg/ml, which is higher than that in the static batch cultures (Fig. 4). Cell growth remained quite stable throughout the cultivation time. These results demonstrated that the stably transfected cell line showed an efficient production of L particles and the insect cell expression system was useful for vector production.

In vitro transfection of calcein with L particles

After purification by sulfate Cellulofine resin column chromatography, L particles were used for specific drug

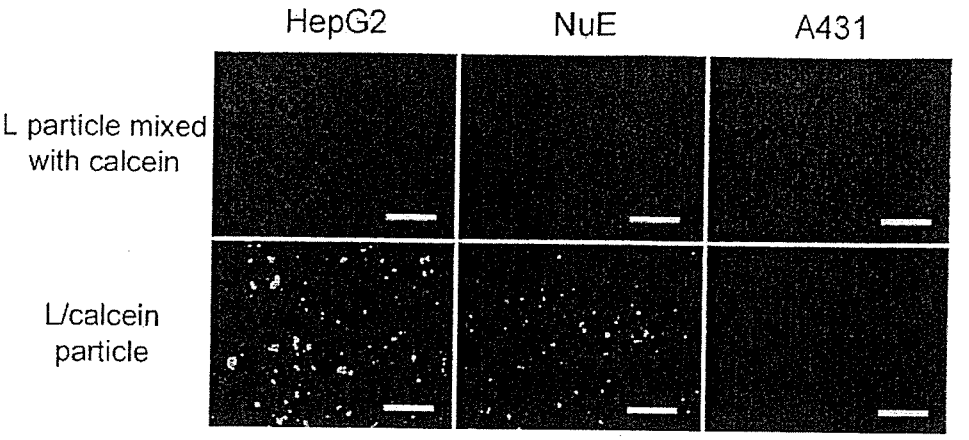

Fig. 6 Transfection of calcein with L particles. A mixture of L particles and calcein and calcein-carrying L particles (60 ng) were added to approximately 2×10^4 cells of HepG2, NuE, and A431. After 6 h of incubation at 37 °C, infection was evaluated by observing fluorescence under an LSM. *Scale bar*, 100 μm

delivery into hepatocytes. A fluorescent compound, calcein, was electroporated into L particles. The results showed that L particles can efficiently transfect the human hepatocarcinoma cell lines HepG2 and NuE (Fig. 6). On the other hand, fluorescence was not detected in the human epidermoid carcinoma cell line A431. Therefore, it was confirmed that L particles have specificity to hepatocyte and this result is consistent with the data for L particles obtained from yeast cells (Yamada et al. 2003).

Discussion

An insect cell expression system has several advantages in recombinant protein expression. The major advantage is a high level expression of secreted glycoproteins. Insect cell expression systems with HBsAg were demonstrated in previous reports. The expression of the S particle was investigated in the *Spodoptera frugiperda* Sf9 cell line using a baculovirus expression system (Lanford et al. 1989) and the stably transfected *Drosophila melanogaster* Schneider-2 (DS-2) cell line (Deml et al. 1999a,b). The coexpression of S and L proteins was achieved in the Sf9 cell line using a baculovirus expression system (Hofmann et al. 1995). However, an expression system with L particles consisting of only the L protein was not established to date except for yeast cell expression system.

We previously demonstrated that an L particle as a novel vector for the gene and drug delivery system can be prepared by overexpressing the L protein in *Saccharomyces cerevisiae* (Yamada et al. 2001; Kuroda et al. 1992). However, expressed L particles accumulate in the cytoplasm, therefore, complicated purification steps are required. Thus, we attempted to develop a stably transfected insect cell secretory expression system with L particles in this study.

To determine the host–vector system, two different systems were examined for transient expression using High Five cells, which are superior hosts for the production of recombinant proteins (Farrell et al. 1998; Keith et al. 1999). Moreover, the secretion signal peptide was fused to the L protein for the efficient production of L particles (Kuroda et al. 1992). These results indicated that the combination of pXINSECT-DEST38 and the melittin secretion signal peptide was suitable for the expression of L particles (Fig. 2). The difference in productivity between expression systems with various vectors might be attributed the nature of the promoter and the presence of homologous region 3 (HR3) and IE1 from *Bombyx mori* nuclear polyhedrosis virus in pXINSECT-DEST38 (Fig. 1a). HR3 is a viral transcriptional enhancer and it acts as a constitutive enhancer of the cytoplasmic actin promoter (Lu et al. 1997). IE1 is a viral transcriptional transactivator that stimulates L protein expression (Lu et al. 1996). Therefore, pXINSECT-DEST38 with the melittin secretion signal peptide represented high expression efficiency of L particles. The results of Western blot analysis suggested the homogeneous glycosylation of the L protein (Fig. 2b).

In this study, we successfully established the stably transfected insect cell lines for the production of L particles. The cotransfection of pX-Mel and pBmA.neo at the weight ratio of 100:1 (expression plasmid pX:antibiotic resistance plasmid pBmA.neo) produced higher L particle-expressing clones (Fig. 3). The concentration of expression vectors seemed to correlate with expression efficiency (Deml et al. 1999a). For practical applications, a long-term stability of expression of L particles is indispensable. Secretion stability was therefore continuously examined (Fig. 5). The result indicated that the expression level of L particles in clone #38 was stable for 75 days in the presence of G418 with an average concentration of approximately 1.7 μg/ml. The stable secretion of L particles for 75 days is considered to be sufficient for industrial applications. It is interesting to note that no significant difference in productivity was observed in the absence of G418. The nonrequirement of antibiotics will minimize the production cost of L particles.

The advantage of this stably transfected insect cell expression system is the secretory expression of L particles

in the serum-free medium. In the case of a mammalian cell culture system, the efficient production of L particles requires serum. On the other hand, in the case of an insect–baculovirus expression system, L particles have to be purified from cell lysates. Both systems required expensive and time-consuming purification steps to obtain pure L particles (Deml et al. 1999b). In contrast, the current system requires only chromatographic purification. Subsequently, the in vitro transfection experiment was carried out using L particles purified from an insect cell culture. It was demonstrated that L particles produced by insect cells can specifically deliver chemical compounds into human hepatocytes.

In conclusion, we established a stably transfected insect cell expression system with L particles for the first time by selecting the appropriate promoter and secretion signal peptide. L particles were efficiently secreted into the serum-free medium and facilitated an easy purification. Furthermore, it was demonstrated that L particles specifically targeted and successfully transfected human hepatocytes similarly to L particles obtained from yeast cells (Yamada et al. 2003). L particles from insect cells could be applied to specific gene and drug delivery systems, and in the preparation of HB vaccine. An insect cell expression system is an attractive tool for the production of L particles and biorecognition molecules displaying L particles for retargeting.

Acknowledgements This study was supported by grants-in-aid for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare.

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