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Development of a Novel Preparation Method of *Recombinant* Proteoliposomes Using Baculovirus Gene Expression Systems

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We have developed a novel method for the preparation of '*recombinant* proteoliposomes'. Membrane proteins were expressed on budded virus (BV) envelopes using baculovirus gene expression systems, and proteoliposomes were prepared by fusion of these viruses with liposomes. First, plasmid DNA containing the gene for the thyroid-stimulating hormone receptor (TSHR) or the acetylcholine receptor α-subunit (AChRα) was co-transfected with wild type virus [*Autographa californica* nuclear polyhedrosis virus (AcNPV)] genomes into insect cells [*Spodoptera frugiperda* (Sf9)] to obtain recombinant viruses via homologous recombination. The recombinant viruses were again infected into Sf9 cells, and the resulting BVs were shown to express TSHR and AChRα. Next, the fusion behaviour of AcNPV-derived BVs and liposomes was examined via a fluorescence assay, and BVs were shown to fuse with phosphatidylserine-containing liposomes below pH 5.0, the pH at which fusion glycoprotein gp64 on the virus envelope becomes active. TSHR- or AChRα-expressed BVs were also shown to fuse with liposomes. Finally, TSHR- and AChRα-recombinant proteoliposomes were immobilized on enzyme-linked immunosorbent assay plates, and their reactivities were examined via a general immunoassay, which showed that the recombinant proteoliposomes were fully active. These results successfully demonstrate the development of a method based on a baculovirus gene expression system for the preparation of recombinant and functional proteoliposomes.

Key words: baculovirus, gene expression, membrane fusion, membrane receptor, proteoliposome.

Abbreviations: AChR, acetylcholine receptor; AChRα, acetylcholine receptor α-subunit; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Bt-PE, 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-biotinyl) phosphoethanolamine; BV, budded virus; ELISA, enzyme-linked immunosorbent assay; IFV, influenza virus; LUV, large unilamellar vesicle; MCS, multiple cloning site; MLV, multilamellar vesicle; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; R_{18}, octadecyl rhodamine B chloride; Sf9, *Spodoptera frugiperda*; TAE, Tris–Acetate-EDTA; TSHR, thyroid-stimulating hormone receptor.

Proteoliposomes are membrane protein-reconstituted lipid vesicles, and proteins can be reconstituted in the bilayer membranes of liposomes, facilitating studies of membrane protein functions (*1*). At present, proteoliposomes are usually prepared by extracting target proteins from cell membranes and reconstituting them into liposome membranes. Membrane proteins can be classified into extrinsic proteins, which bind to the surface of membranes, and intrinsic proteins, which are buried within membranes. Since most membrane proteins *in vivo* are intrinsic proteins, detergents or organic solvents are required for their solubilization. For this reason, proteoliposomes are prepared mainly by the detergent removal method (*2*), that is, by the removal of detergents from micelles containing lipids and membrane proteins. However, detergents are difficult to remove completely, and can lead to toxicity *in vivo*. Moreover, in the reconstituted membranes, protein orientation is random, and minor changes in protein conformation and activity may occur. Therefore, novel detergent-free methods of the proteoliposome preparation are required.

Baculovirus expression vectors enable specific post-translational modification and expression of large amounts of proteins (*3*). Recently, it has been reported that some foreign membrane proteins are expressed on budded virus (BV) envelopes derived from baculoviruses (*4–7*), and that the expression of the intrinsic envelope glycoprotein gp64 by BVs results in fusion with host cell membranes (*8*). Based on these observations, we speculated that proteoliposomes containing recombinant membrane proteins could be prepared without detergents, by fusing BVs containing target recombinant membrane

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proteins with liposomes, in a process similar to that of BV-cell fusion.

Here, we report a novel method of proteoliposome preparation, using baculovirus expression systems to produce recombinant proteoliposomes. First, the human thyroid-stimulating hormone receptor (TSHR) and the human nicotinic acetylcholine receptor α-subunit (AChRα), used as model membrane proteins, were expressed in BV envelopes using a baculovirus expression system. TSHRs have seven membrane-spanning domains and consist of two subunits, an α-subunit whose N-terminus is an extracellular domain, and a β-subunit with transmembrane and cytoplasmic domains (*9*). Acetylcholine receptors (AChRs) are pentameric glycoproteins. Each of their five subunits ($\alpha_2\beta\gamma\delta$) has four membrane-spanning domains, and the α-subunit has binding sites for acetylcholine and autoimmune anti-AChR antibodies (*10*). Second, the fusion behaviour of BVs and liposomes was examined. Finally, proteoliposomes were prepared, and the availability of recombinant proteoliposomes was examined by measuring the reactivities of the reconstituted TSHR and AChRα proteins.

MATERIALS AND METHODS

Materials—Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI) and 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-biotinyl) phosphoethanolamine (Bt-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Al, USA). *Spodoptera frugiperda* (Sf9) cells, derived from *Spodoptera frugiperda* ovary, were purchased from Invitrogen Corp. (Carlsbad, CA, USA) and cultured at 27°C with Sf-900 II Serum Free Medium (Invitrogen Corp.). Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) was obtained from BD Bioscience (San Jose, CA, USA). Rabbit anti-His-Tag antibody, goat anti-rabbit IgG (H + L chain) and goat anti-mouse IgG (H + L chain) were purchased from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan), and rabbit anti-TSHR (H-155) and mouse anti-human AChRα (MCA1327) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Serotec Co., Ltd. (Sapporo, Japan), respectively.

Preparation of TSHR Recombinant AcNPVs—First, TSHR coding regions were subcloned from human thyroid gland cDNA (Clontech Laboratories, Inc., Mountain View, CA, USA) by PCR using pfu polymerase. The forward primer (5′-AGTCGGATCCACCATGAGCCGGCG GACTTGCT-3′) contained a *Bam*HI restriction site (underlined) and the reverse primer (5′-TGTTCTCGAG CAAAACCGTTTGCATATACTCTT-3′) contained an *Xho*I restriction site (underlined). The resulting DNA fragment of ~2.3 kbp in length was cut with *Bam*HI and *Xho*I and inserted into the multiple cloning site (MCS) of pET-28a(+) (5.4 kbp; Novagen, Madison, WI, USA) to produce pET/TSHR (7.7 kbp). Six His-tags were added to the downstream region of the TSHR C-terminus in pET-28a(+) to allow confirmation of TSHR expression. Next, PCR was performed with a forward primer (5′-AGTCGG ATCCACCATGAGCCGGCGGACTTGCT-3′) containing a *Bam*HI restriction site and a reverse primer (5′-TTCGGA ATTCGTTAGCAGCCGGATCTCAGT-3′) containing an *Eco*RI restriction site. The resulting DNA fragment was cut with *Bam*HI and *Eco*RI and inserted into the MCS under a polyhedron promoter in pVL1393 (9.6 kbp; BD Bioscience) to produce pVL1393/TSHR with six His-tags (12 kbp). Using the calcium phosphate method, pVL1393/TSHR with six His-tags and linearized AcNPV–DNA (BD BaculoGold, BD Biosciences) were co-transfected to produce TSHR recombinant AcNPVs, which were purified by plaque assay.

Preparation of AChR Recombinant AcNPVs—As with the TSHR, AChRα coding regions were subcloned from a human skeletal muscle library (Clontech) by PCR using pfu polymerase. The forward primer (5′-GTAGCATATG GAGCCCTGGCCTCTCCT-3′) contained an *Nde*I restriction site and the reverse primer (5′-TTTCCTCGAGTCC TTGCTGATTTAATTCAATGAG-3′) contained an *Xho*I restriction site. The resulting DNA fragment of ~1.4 kbp was cut with *Nde*I and *Xho*I and inserted into the MCS of pET-30a(+) (5.4 kbp; Novagen) to produce pET/AChRα (6.8 kbp). Six His-tags were added to the downstream region of the AChRα C-terminus in pET-30a(+) to allow confirmation of AChRα expression. Next, PCR was performed with a forward primer (5′-CGGA ATTCGATATGGAGCCCTGGCCTCTC-3′) containing an *Eco*RI restriction site and a reverse primer (5′-GCTCTA GAGCTTTGTTAGCAGCCGGATC-3′) containing an *Xba*I restriction site. The resulting DNA fragment was cut with *Eco*RI and *Xba*I and inserted into the MCS under a polyhedron promoter in pVL1392 (9.6 kbp; BD Bioscience) to produce pVL1392/AChRα with six His-tags (11 kbp). Using Cellfectin reagent (Invitrogen Corp.), pVL1392/AChRα with six His-tags and AcNPV–DNA were co-transfected to produce AChRα recombinant AcNPVs, which were purified by plaque assay.

Purification of BV—AcNPV–BVs were harvested as follows: Sf9 cells were cultured in 10 culture flasks (75 cm^2) containing 12 ml of Sf-900 II Serum Free Medium. AcNPV–BV suspensions were added to each flask at a multiplicity of infection (MOI) of 1, incubated at 27°C for 72 h, and then placed at 4°C. After 120 h infection, culture supernatants were separated from cells and centrifuged at 1,000*g* for 5 min at 4°C. The resulting supernatants were ultracentrifuged at 100,000*g* for 60 min at 15°C in a Optima L-90K ultracentrifuge (Beckman-Coulter, Inc., Fullerton, CA, USA). Precipitates containing BV particles were resuspended in phosphate-buffered saline (PBS) (1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl, pH 6.2) and again ultracentrifuged at 40,000*g* for 30 min at 15°C with a stepwise sucrose gradient of 10%, 15%, 20%, 25% and 30% sucrose (w/v) in PBS (pH 6.2). The resulting two fractions containing AcNPV–BVs (see Results section) were collected, diluted with PBS (pH 6.2), and ultracentrifuged at 100,000*g* for 60 min at 15°C. Precipitates were suspended in PBS (pH 6.2).

Electron Microscopy—Electron microscopic observation of negative staining samples was carried out using a H-700HX electron microscope (Hitachi, Ltd., Tokyo, Japan).

Extraction and Electrophoresis of DNA—DNA extraction and agarose gel electrophoresis were performed as

described previously (*11*). Viral DNA was extracted from BV fractions with extraction buffer (10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.5% SDS), and proteinase K was added to the extracts to a final concentration of 1 mg/ml. After incubation at 37°C for 4 h, proteins were eliminated by phenol/chloroform treatment, DNA was precipitated by addition of ethanol, the resulting precipitates were dissolved in Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and the DNA concentration was determined by measuring the absorbance at 260 nm with a spectrophotometer. A one-tenth volume of loading buffer (10% SDS, 50% glycerol and 0.02% bromophenol blue) was added to the DNA samples, and the mixture was applied to a 0.8% agarose gel in Tris–Acetate-EDTA (TAE) buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0). Electrophoresis was performed for ~60 min at a constant voltage of 100 V. After electrophoresis, gels were stained in TAE buffer containing 0.5 μg/ml ethidium bromide, and stained bands were visualized with UV light using a UV Transilluminator (Funakoshi Co., Ltd., Tokyo, Japan).

SDS–PAGE—SDS–PAGE was performed according to the method described by Laemmli (*12*). Briefly, one volume of sample buffer (114 mM Tris–HCl, pH 6.8, 3.6% SDS, 25% glycerol, 9% β-mercaptoethanol and 0.02% bromophenol blue) was added to the protein samples, and the mixture was boiled for 5 min. Electrophoresis was performed using mini slab-gels consisting of a 12% separate gel and a 3.9% stacking gel, for ~60 min, at a constant current of 30 mA. Gels were stained using Bio-Safe Coomassie (Bio-Rad, Hercules, CA, USA) or a Silver Stain II Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Western blotting was performed at a constant voltage of 20 V for 90 min using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad), as described by Khyse-Andersen (*13*). Polyvinylidene difluoride membranes were reacted with an anti-His-Tag antibody and a peroxidase-conjugated goat anti-rabbit IgG (H + L chain) antibody. Blots were visualized using the immunoreaction enhancer solution Can Get Signal (Toyobo Co., Ltd., Osaka, Japan), and Immunostaining HRP-1000 (Konica Minolta Medical and Graphic, Inc., Tokyo, Japan).

Liposome Preparation—Large unilamellar vesicles (LUVs) were prepared in 10 mM Tris–HCl/10 mM NaCl (pH 7.5) by the reverse-phase evaporation method and filtered through polycarbonate membranes of 0.1 μm pore size (*14*). Multilamellar vesicles (MLVs) were prepared by vortex mixing of the lipid film in 10 mM Tris–HCl/10 mM NaCl (pH 7.5) at 30°C for 30 s. The vesicles were passed through polycarbonate membranes of 0.4 μm pore size and washed four to five times at 6,000*g* for 20 min at 4°C to remove small multilamellar and unilamellar vesicles. The liposome concentration was determined by measuring total lipid phosphorus by the method of Bartlett (*15*).

Membrane Fusion Assay—BVs labelled with octadecyl rhodamine B chloride (R_{18}) (Molecular Probes, Inc., Eugene, OR, USA) were prepared by adding the ethanolic R_{18} solution (*16*). Then, the fluorescence intensity of 1 μg R_{18}-labelled BVs was measured at 25°C for 1 min by adding unlabelled liposomes at an excitation wavelength of 560 nm and an emission wavelength of 580 nm in a F-4010 fluorescence spectrophotometer (Hitachi, Ltd.) equipped with a constant-temperature cell holder and stirrer, and polyoxyethylene lauryl ether of a final concentration of 1% was added to the mixture. The fusion rate (%) was calculated according to the following equation:

$$\text{Fusion rate (\%)} = 100 \times (F_s - F_0)/(F_t - F_0)$$

where F_s is the fluorescence intensity in 20 s after addition of liposomes, F_0 is the fluorescence intensity of R_{18}-labelled BVs and F_t is the fluorescence intensity after addition of polyoxyethylene lauryl ether.

Enzyme-linked Immunosorbent Assay—First, samples were added to streptavidin-coated microplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) and left at 4°C overnight. Next, the plates were blocked at 37°C for 2 h by addition of 1% gelatin, and incubated at 37°C for 1 h in the presence of anti-His-Tag, anti-TSHR or anti-AChRα primary antibodies and goat anti-rabbit IgG (H + L chain) or goat anti-mouse IgG (H + L chain) peroxidase-conjugated secondary antibodies. Finally, a colouring reagent containing *o*-phenylene diamine and H_2O_2 was added to the reaction mixture, and the absorbance at 490 nm was measured.

RESULTS

Expression of Recombinant TSHR and AChRα on BV Envelopes—First, we prepared recombinant AcNPV–BVs for fusion with liposomes to produce recombinant proteoliposomes. TSHR or AChRα recombinant AcNPVs, which contained the gene encoding human TSHR or AChRα, respectively, were prepared. After viral infection and a period of culture, the Sf9 cells were subjected to sucrose density-gradient centrifugation. Two fractions were found to contain BVs. Electron microscopic observation revealed that BVs in the upper fraction were somewhat disrupted (Fig. 1A), whereas those in the lower fraction were normal (Fig. 1B). Agarose gel electrophoresis showed that the lower fraction contained virus genomes, while the upper fraction did not (Fig. 1C). The fraction without virus genomes was used for further analysis.

Next, fractions were analysed by SDS–PAGE and western blotting. The fractions derived from TSHR recombinant AcNPV–BVs were found to contain a protein band that was not observed in wild type AcNPV–BVs (Fig. 2A and C). However, the position of this observed band was lower than that of the full length of TSHR (about 87 kDa), probably due to β-mercaptoethanol cleavage of the disulfide bonds linking the α- and β-subunits. Observation of the His-tagged β-subunit alone showed that it was ~50 kDa in size. SDS–PAGE and western blot analyses also showed that the fractions derived from AChRα recombinant AcNPV–BVs contained a protein band that was not observed in wild type AcNPV–BVs, its molecular weight being comparable with that of the AChRα (~50 kDa; Fig. 2B and D). These results indicate that target TSHRs and AChRα were expressed on recombinant AcNPV–BVs.

In the western blot analysis, multiple bands were detected in the Sf9 cell fractions (Fig. 2C and D),

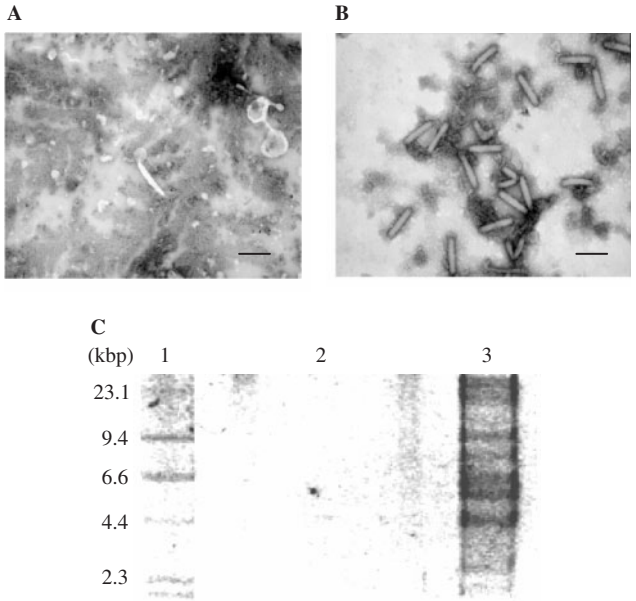

Fig. 1. **Electron micrographs (A and B) and agarose gel electrophoresis (C) of fractions obtained by sucrose gradient centrifugation of AcNPV-infected Sf9 cell-culture supernatants.** Cultured supernatants of Sf9 cells incubated for 72 h after infection of wild type AcNPV were subjected to sucrose-density gradient centrifugation at 40,000*g* for 30 min at 15°C, and their upper (**A**) and lower (**B**) fractions were observed using electron microscopy. Scale bars, 300 nm. DNA was extracted from the two fractions and subjected to agarose gel electrophoresis (**C**). Lane 1, DNA marker; lane 2, DNA extracted from the upper fractions; lane 3, DNA extracted from the lower fractions.

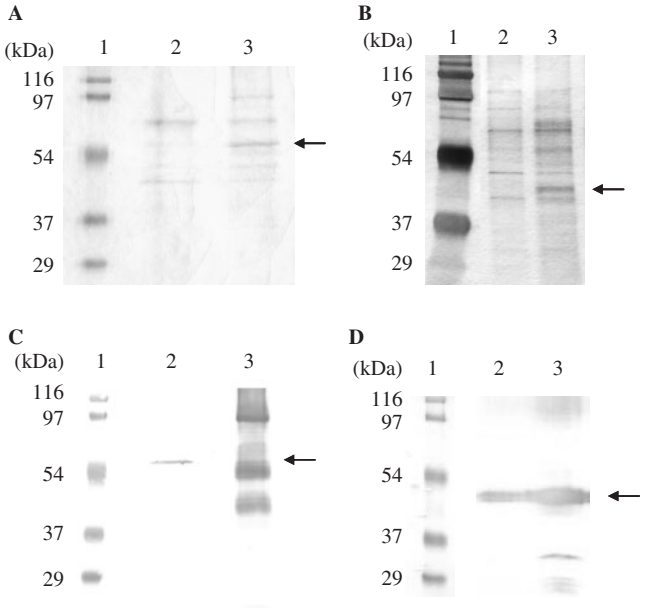

Fig. 2. **SDS–PAGE (A and B) and western blot (C and D) analyses of recombinant AcNPV–BVs.** BV fractions without DNA were obtained from TSHR and AChRα recombinant AcNPVs, and SDS–PAGE (**A** and **B**) and western blotting was performed using anti-His-Tag antibodies (**C** and **D**). Arrows indicate the locations of TSHR (**A** and **C**) and AChR (**B** and **D**). (**A**) Lane 1, protein marker; lane 2, wild type AcNPV–BV fraction without DNA; lane 3, TSHR recombinant AcNPV–BV fraction. (**B**) Lane 1, protein marker; lane 2, wild type AcNPV–BV fraction without DNA; lane 3, AChRα recombinant AcNPV–BV fraction. (**C**) Lane 1, protein marker; lane 2, TSHR recombinant AcNPV–BV fraction; lane 3, TSHR recombinant AcNPV-infected Sf9 cell. (**D**) Lane 1, protein marker; lane 2, AChRα recombinant AcNPV–BV fraction; lane 3, AChR recombinant AcNPV-infected Sf9 cell.

probably due to degradation of TSHR and AChRα and formation of aggregates. In contrast, only one band was detected in the virus fractions (Fig. 2C and D), showing that only the active form of the target recombinant membrane protein is expressed on the AcNPV–BV envelope (*6*, *7*).

Fusion between Wild Type BVs and Liposome Membranes—Recombinant proteoliposomes could be prepared by fusion between recombinant AcNPV–BVs and liposomes. However, fusion of AcNPV–BVs and liposomes has not been reported, even though AcNPV–BVs are able to fuse with host cells (*8*), and several envelope viruses such as the influenza virus (IFV) are able to fuse with liposomes (*17–19*). Therefore, fusion of wild type AcNPV–BVs with liposomes was examined using an R_{18}-dequenching assay, with R_{18}-labelled AcNPV–BVs. As wild type polyhedrin genes were replaced only by TSHR and AChRα genes in recombinant AcNPV–BVs, and as both the host cell infectivity and viral propagation were not altered by recombination, fusion activities of BVs from wild type and recombinant AcNPVs do not appear to differ. As shown in Fig. 3, when wild type AcNPV–BVs were combined with PC/PS (1:1) liposomes at pH 4.0, the fluorescence intensity increased, indicating that wild type AcNPV–BVs are able to fuse with liposomes.

It has been reported that fusion of AcNPV–BVs and host cells is mediated by the presence of gp64 on BV envelopes at low pH in endosomes (*8*). Therefore, we

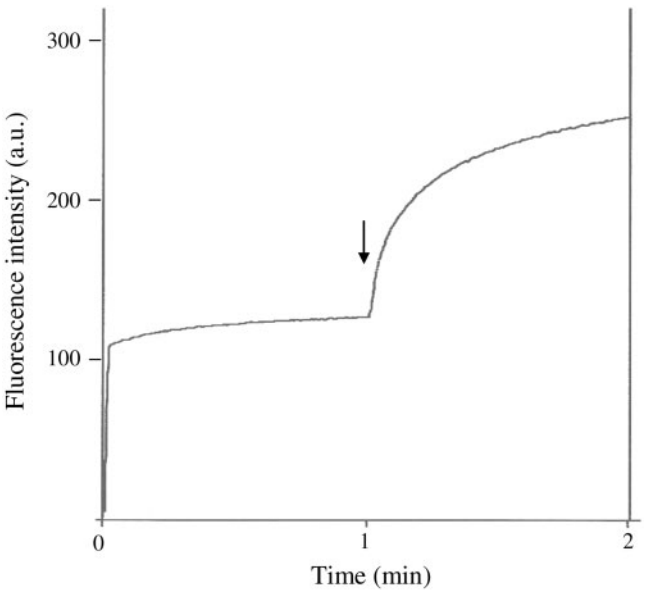

Fig. 3. **Time course of fusion between wild type AcNPV–BV and liposomes.** Changes in fluorescence intensity upon addition of liposomes to the R_{18}-labelled wild type AcNPV–BV solution (indicated by the arrow) were measured as described in the Materials and Methods section.

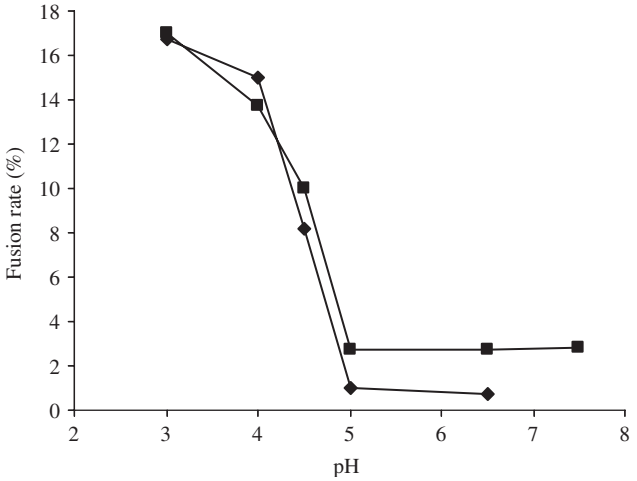

Fig. 4. **The pH dependence of fusion between wild type AcNPV–BV and liposomes.** Rates of fusion between R_{18}-labelled wild type AcNPV–BV and PC/PS (1:1) liposomes at concentrations of virus and liposomes of 1 μg/ml and 100 μM, respectively, at different pH values, were measured using an R_{18}-dequenching assay, as described in the Materials and Methods section. The fusion buffer consisted of 10 mM CH_3COOH–HCl and 10 mM NaCl at pH 3.0; 10 mM CH_3COOH–CH_3COONa and 10 mM NaCl at pH levels 4.0–5.0; 10 mM MES–NaOH and 10 mM NaCl at pH 6.5; and 10 mM Tris–HCl and 10 mM NaCl at pH 7.5. ■, Fusion rate with MLVs; ◆, fusion rate with LUVs.

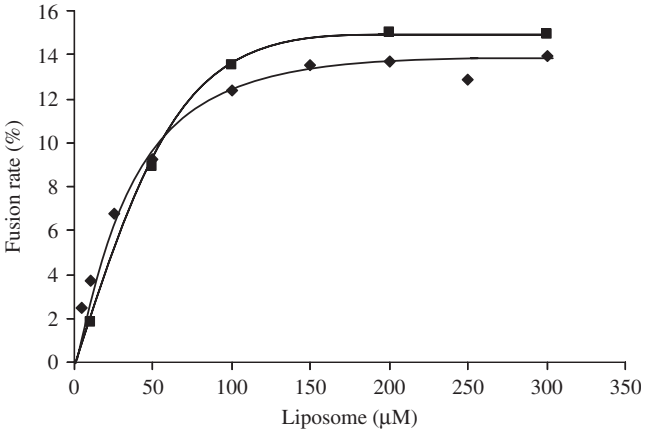

Fig. 5. **Dependence upon liposome concentration of fusion between wild type AcNPV–BV and liposomes.** Rates of fusion between R_{18}-labelled wild type AcNPV–BV and liposomes at pH 4.0, using a virus concentration of 1 μg/ml and different liposome concentrations, were measured using an R_{18}-dequenching assay, as described in the Materials and Methods section. ■, Fusion rate with MLVs; ◆, fusion rate with LUVs.

Table 1. **Dependence of fusion between wild-type AcNPV–BV and liposomes on lipid composition[a].**

Liposome	Lipid composition	Fusion rate (%)
LUV	PC/PS	13.7
	PC/PG	8.0
	PC/PA	4.2
	PC/PI	2.2
MLV	PC/PS	15.0
	PC/PG	12.2

[a]Rates of fusion between R_{18}-labelled wild type AcNPV–BV and liposomes at pH 4.0 and concentrations of virus and liposomes of 1 μg/ml and 100 μM, respectively, were measured by R_{18}-dequenching assay as described in Materials and Methods section.

examined whether gp64 also mediates the fusion of BVs and liposomes. Above pH 5.0, little fusion occurred, but when the pH was lowered below 5.0, BVs fused with PC/PS(1:1)-LUVs or -MLVs at high efficiency (Fig. 4), suggesting that gp64 is required for the fusion of AcNPV–BVs with liposomes.

In general, the fusion process involves close apposition of membranes (*20*). In other words, membrane fusion has multistep processes: processes that two adjacent membranes come in contact with each other, lipid molecules mix with each other, and new bilayer membranes are formed (*21*). Close apposition is the process that two adjacent membranes come in contact with each other. To examine whether AcNPV–BVs also fuse with liposomes via a process of close apposition, the fusion rate was measured at various concentrations of PC/PS(1:1)-LUV and -MLV, with the amount of BV held constant. As shown in Fig. 5, the fusion rates increased and reached a plateau at liposome concentrations of 100 μM or more, suggesting that close apposition actually occurs in the process of fusion.

AcNPV–BVs have been reported to bind to cell membranes by specifically recognizing PS (*22*). To examine whether PS is required for the fusion of AcNPV–BVs and liposomes, the fusion rates were measured for liposomes of various lipid compositions: PC/PS(1:1)-, PC/PG(1:1)-, PC/PA(1:1)- and PC/PI(1:1)-LUVs as well as PC/PS(1:1)- and PC/PG(1:1)-MLVs. The BV–liposome fusion rate was highest with PC/PS liposomes, lower with PC/PG liposomes and approached zero with PC/PA and PC/PI liposomes (Table 1), suggesting that AcNPV–BVs specifically bind to PS on liposomes. These results indicate that the fusion of AcNPV–BV with liposomes is dependent on the function of gp64 at low pH.

Preparation of Proteoliposomes by Fusion of Recombinant AcNPV-derived BVs with Liposomes—As mentioned above, TSHR and AChRα were expressed on BVs derived from TSHR and AChRα recombinant AcNPVs, and wild type AcNPV–BVs fused specifically with liposomes, suggesting that proteoliposomes reconstituted with TSHR or AChRα can be prepared via fusion of recombinant AcNPV–BVs with liposomes. To investigate this possibility further, TSHR-recombinant AcNPV–BVs and PC/PS(1:1)-MLVs were combined at pH 4.0 or 8.5, and incubated for 20 min. The mixture was then centrifuged at 6,000*g* for 20 min at 4°C to yield precipitates containing MLV and supernatants containing BV, and these were subjected to SDS–PAGE and western blot analyses using anti-His-Tag antibodies. As shown in Fig. 6A, protein bands in the supernatant fractions were detected more weakly when fusion occurred at pH 4.0, compared with pH 8.5, whereas proteins in the precipitate fraction were detected more strongly when fusion occurred at pH 4.0, compared with pH 8.5. As shown in Fig. 6B,

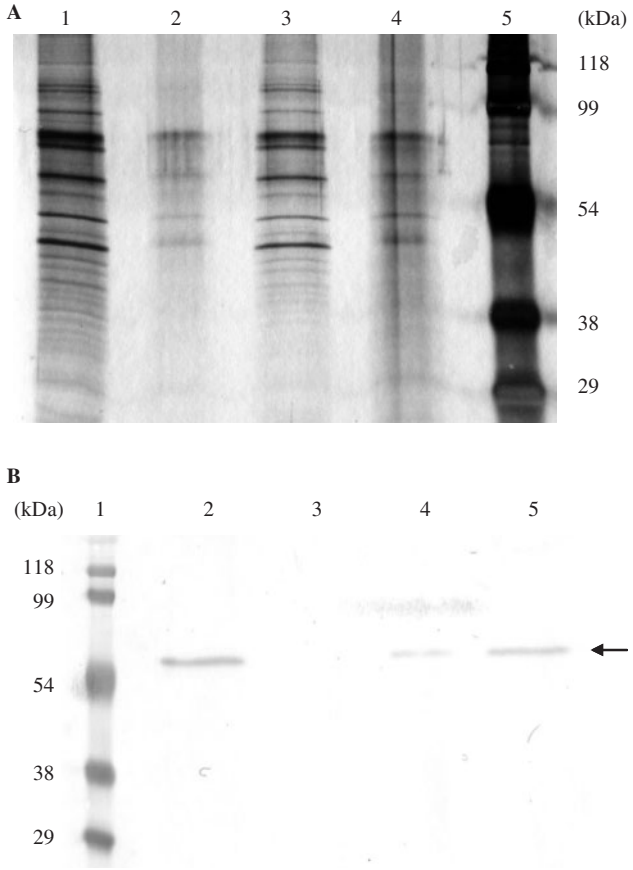

Fig. 6. **Characterization of proteoliposome samples by centrifugation.** TSHR-recombinant AcNPV–BV and PC/PS (1:1)-MLV were mixed at pH 4.0 or 8.5. After a 20 min incubation, the mixture was centrifuged at 6,000*g* for 20 min at 4°C, and the resulting precipitates and supernatants were subjected to SDS–PAGE (**A**) and western blot analysis using anti-His-Tag antibodies (**B**). (**A**) Lane 1, precipitates on fusion at pH 4.0; lane 2, supernatants on fusion pH 4.0; lane 3, precipitates on fusion at pH 8.5; lane 4, supernatants on fusion at pH 8.5; lane 5, protein marker. (**B**) Lane 1, protein marker; lane 2, precipitates on fusion at pH 4.0; lane 3, supernatants on fusion pH 4.0; lane 4, precipitates on fusion at pH 8.5; lane 5, supernatants on fusion at pH 8.5.

a protein band corresponding to TSHR in the supernatant fraction was barely detected when fusion occurred at pH 4.0, and was detected at significant levels when fusion occurred at pH 8.5. In contrast, the TSHR band in the precipitate fraction was detected more readily when fusion occurred at pH 4.0 than at pH 8.5, confirming that proteoliposomes can be prepared by fusion of recombinant AcNPV–BVs with liposomes.

Reactivity of Proteoliposome Preparations Examined by Enzyme-linked Immunosorbent Assay—To ascertain that recombinant proteoliposomes obtained by fusion between recombinant AcNPV–BVs and liposomes are active, the reactivities of the proteoliposome preparations were examined using enzyme-linked immunosorbent assay (ELISA). TSHR or AChRα recombinant AcNPVs were fused at pH 4.0 or 7.5 with MLVs composed of PC/PS/Bt-PE (1:1:0.066). The mixtures were centrifuged at 6,000*g* for 20 min at 4°C, and the resulting precipitates were applied to streptavidin-coated microplates and analysed by ELISA using anti-TSHR and anti-AChR antibodies. As shown in Table 2, protein reactivities were higher when fusion occurred at pH 4.0 than at pH 7.5, indicating that recombinant proteoliposomes are obtained by the fusion of recombinant AcNPV–BVs with liposomes at pH 4.0, and that the target membrane proteins TSHR and AChRα are functionally reconstituted into proteoliposomes.

Table 2. **Reactivity of recombinant proteoliposomes with specific antibody[a].**

Antibody	Liposome	pH	OD_{490}
Anti-TSHR	Proteoliposome	4.0	0.955
		7.5	0.569
	Liposome alone	4.0	0.497
Anti-AChR	Proteoliposome	4.0	0.257
		7.5	0.138
	Liposome alone	4.0	0.088

[a]TSHR or AChRα recombinant AcNPV–BV and PC/PS/Bt-PE (1:1:0.066)-MLV were mixed at pH 4.0 or 7.5, after incubation for 20 min at room temperature, their mixture was centrifuged at 6,000*g* for 20 min at 4°C, and the resulting precipitates were added to streptavidin-coated microplates and their reactivities were measured by ELISA with anti-TSHR or anti-AChR antibodies as described in Materials and Methods section. As a control, the same treatments were applied to PC/PS/Bt-PE (1:1:0.066)-MLV.

As seen in Table 2, the reactivities of the anti-TSHR antibody with liposomes were somewhat higher than those of the anti-AChRα antibody. This is attributable to non-specific binding of anti-TSHR antibodies to liposomes and ELISA plates.

DISCUSSION

We have developed a novel method for the detergent-free preparation of proteoliposomes, using baculovirus gene expression systems. At present, proteoliposomes have generally been prepared according to the detergent removal method (*2*), which involves the solubilization of membrane proteins and their reconstitution into liposome membranes. In the solubilization step, detergents or organic solvents are required to overcome the hydrophobic properties of membrane intrinsic proteins. However, it is difficult to completely remove these detergents from the proteoliposome preparations, and thus, proteoliposomes prepared by this method are unusable *in vivo* due to the toxicity of detergents towards living organisms. Moreover, the orientation of reconstituted proteins within the membrane is random, and changes in conformation and activity may occur. It has been reported that freeze-thaw is a suitable method only in the preparation of giant unilamellar proteoliposomes (*23*), but the detergent removal method is far more commonly used. Thus, to allow the *in vivo* use of proteoliposomes, and to preserve the integrity of membrane proteins within liposome membranes, novel, detergent-free methods for the preparation of proteoliposomes are required.

To obtain target membrane proteins for reconstitution into liposome membranes, gene expression systems such as the baculovirus expression system are useful, since these allow the expression of large amounts of proteins,

and specific post-translational modifications are possible (*3*). When recombinant proteins are expressed using baculovirus expression systems, they are expressed not only on cell membranes but also on BV envelopes released into the culture medium, and membrane proteins, which often become inactive on solubilization with detergents, are expressed as active forms on BV envelopes. For example, when the genes for G protein-coupled receptors (GPCRs), which are common drug targets, are recombined in baculoviruses and cells are infected, GPCRs are known to be expressed on BV envelopes with normal activity levels (*4*). Although, the normal expression of the leukotriene B_4 receptor (BLT1) on cell membranes is higher than that observed on BV envelopes, the binding of LTB_4 (leukotriene B_4) to BLT1 in BV envelopes occurs at a level approximately 10 times greater than that observed in cell membranes (*5*). SREBP-2 (sterol regulatory element-binding protein-2) and SCAP (SREBP cleavage-activating protein) membrane proteins, which are involved in the regulation of intracellular cholesterol, are expressed on BV envelopes in their active forms (*6*), and γ-secretase complexes, which are related to Alzheimer disease, are also expressed on BV envelopes with normal activity levels intact (*7*).

Enveloped viruses, such as IFV, feature a fusogenic protein, hemagglutinin, on their envelopes, and upon viral infection of cells, this protein induces fusion with cell membranes at low pH (*17*). IFV-infected cells fuse with liposomes at low pH in a similar manner (*18*), and IFV alone can also fuse with liposomes at low pH (*19*). Similarly, BVs fuse with cell membranes at low pH in a process mediated by the envelope membrane glycoprotein gp64 (*8*). Although it remains unknown whether BVs can fuse with liposomes, it is quite possible that this could result in the display of recombinant membrane proteins on liposome membranes.

Here, TSHRs or the AChRα were expressed on AcNPV envelopes using a baculovirus gene expression system, and protein expression was confirmed using SDS–PAGE and western blot analyses (Fig. 2), which indicated that TSHR and AChRα recombinant AcNPV–BVs can be obtained. Next, the fusion behaviour of wild type AcNPV–BVs and liposomes was examined using an R_{18}-dequenching assay, and wild type AcNPV–BVs were found to fuse with liposomes (Fig. 3). Fusion rates were higher below pH 5.0 and when liposomes contained PS rather than PG, PA or PI (Figs 4 and 5), indicating that BVs specifically bind to liposomal PS (*21*) and that fusion is induced by gp64, which is activated at low pH levels (*8*). When recombinant AcNPV–BVs were fused with liposomes at pH 4.0 and 8.5, TSHR was observed in the liposome fractions more strongly when fusion occurred at pH 4.0 than at pH 8.5 (Fig. 6), showing that proteoliposomes displaying recombinant membrane proteins can be prepared by the fusion of recombinant AcNPV–BVs with liposomes. The reactivities of TSHR and AChRα with anti-TSHR and anti-AChR antibodies, respectively, were higher when fusion occurred at pH 4.0 than at 7.5 (Table 2), suggesting that recombinant membrane protein-displaying proteoliposomes are fully active.

As described above, we prepared active recombinant proteoliposomes, but the following questions always arise. One question is whether the size of liposomes becomes larger and affects subsequent events because proteoliposomes are obtained by fusion between liposomes and BVs. However, it has been reported that the fusogenic protein hemagglutinin of IFV is activated at low pH but rapidly inactivated to lose membrane fusion competence (*24*), and it is likely that the behaviour of the fusogenic protein gp64 is similar to that of hemagglutinin. For fusion between liposomes induced by proteins and peptides, the fusion efficiency and the size of fused liposomes have been shown to decrease when the amount of liposomes exceeds that of bound peptides (*25*, *26*). In the present study, we prepared recombinant proteoliposomes at higher amounts of liposome than BV. Moreover, since BVs have a rod-shaped nucleocapsid with a size of 250–300 × 30–60 nm (*27*), and since liposomes used in this study had similar size of about 100 nm (LUV) and about 400 nm (MLV) in diameter, almost equal amounts of liposome and BV could participate in fusion. Thus, these facts suggest that the liposome after fusion is not always much larger.

Another question is whether intrinsic membrane proteins other than the target protein on the BV envelopes affect subsequent events, because in the preparation of recombinant proteoliposomes, they are not eliminated, although proteoliposomes prepared using the detergent removal method contain only the purified target protein. However, the effects of intrinsic membrane proteins can be clarified by using liposomes fused wild type AcNPV–BV and/or recombinant proteoliposomes prepared with a recombinant membrane protein other than the target protein as a control. Nevertheless, it is important to eliminate intrinsic membrane proteins from BVs. Gene targeting and RNA interference might be available, being a subject of future investigation.

In the present study, we report a novel method for the preparation of proteoliposomes, involving fusion of recombinant baculoviruses with liposomes. We named the proteoliposomes prepared using the baculovirus gene expression system '*recombinant* proteoliposomes'. Our method does not involve the use of detergents, and thus, recombinant proteoliposomes are extremely useful because their *in vivo* toxicity is low and because the orientation and conformation of the reconstituted membrane proteins is maintained. For instance, recombinant proteoliposomes could provide the cell-targeting properties to themselves in drug delivery and gene transfer more easily than conventional liposomes. In addition, recombinant proteoliposomes are available for immunoassay in diagnosis of autoimmune diseases possessing autoantibodies against membrane proteins, such as Graves' and Hashimoto's diseases and myasthenia gravis. At present, these diseases can be diagnosed directly only by competitive (indirect) assay, but recombinant proteoliposomes enable to diagnose directly these diseases using ELISA. Moreover, recombinant proteoliposomes are useful for detection and search of ligands, agonists or antagonists for membrane receptors, and in particular, those in the signal transduction systems. Thus, the availability of recombinant proteoliposomes seems quite high, because they are functionally active (Table 2). Further studies on these subjects are expected.

CONFLICT OF INTEREST

None declared.

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