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A novel method for viral display of ER membrane proteins on budded baculovirus

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Abstract

The baculovirus expression system has been used to express large quantities of various proteins, including membrane receptors. Here, we reveal a novel property of this expression system to be that certain membrane proteins can be displayed on the budded virus itself. We introduced the genes encoding sterol regulatory element-binding protein-2 (SREBP-2) or SREBP cleavage-activating protein (SCAP), important integral membrane proteins of the endoplasmic reticulum (ER) and/or the Golgi apparatus related to cellular cholesterol regulation, into a baculovirus vector. When insect cells were infected with SREBP-2 or SCAP recombinant viruses, it was found that these ER membrane proteins appeared on the budded baculovirus in addition to the host cell membrane fraction. Compared to proteins expressed on the cell membrane, membrane proteins displayed on virus exhibited both less aggregation and less degradation upon immunoblotting. Using this viral displayed SCAP as the screening antigen, we then generated a new monoclonal antibody specific against SCAP, which was useful for immunological localization studies. This system, which takes advantage of the viral display of membrane proteins, should prove to be a powerful additional tool for postgenomic protein analysis.

Keywords: SREBP; SCAP; Endoplasmic reticulum; Sf9; Baculovirus; Budded virus; Monoclonal antibody

Membrane proteins are one of the major targets for drug discovery in the postgenomic era [1]. The integral membrane proteins play a central role in biological systems as diverse as energy production, intracellular signal transduction, and intercellular communication. For example, the G-protein-coupled receptors (GPCRs), which exist on the plasma membrane, constitute a family of membrane receptors which contribute to many cellular effects through the signal transduction triggered by their binding of extracellular ligands [2].

In another vein, SREBP-2 and SCAP are ER membrane proteins which are thought to play an important role in cellular cholesterol homeostasis [3,4]. SREBP-2 exists on the ER membrane in the form of precursor proteins and complexes with SCAP. SCAP, a polytopic membrane protein, plays a dual role as an escort protein and as a sterol sensor. In sterol-depleted cells, SCAP escorts SREBP-2 to the Golgi apparatus where two sequential proteolytic events release the NH_2-terminal mature form of SREBP-2 into the cytoplasm [4–6]. Then, the mature SREBP-2 enters the nucleus and enhances the transcription of genes encoding enzymes which regulate cholesterol synthesis.

To investigate membrane proteins by biochemical analysis, it is important to exogenously express sufficient amounts of the target functional membrane proteins. Various expression systems have been tried in this quest

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for sufficient expression, but to date a suitable system for the expression of membrane proteins has not been established because of their hydrophobic nature [7–9]. Among the systems tried, baculovirus and insect cells have been commonly used for high quantity expression for a number of different proteins, not only soluble proteins but also membrane proteins as well [10–12]. The baculovirus expression system has several advantages, including posttranslational modifications such as fatty acid acylation and phosphorylation, which do not normally occur in *Escherichia coli*. It may be that the yeast cell wall interferes with the recovery of the expressed protein on a large scale and the insect cell is easier to handle. However, it is also reported that a drawback of high-level expression is that as the immature protein is expressed, it causes massive protein aggregation or even degradation in some cases [10–12].

Loisel et al. [13] have reported that functional β2-adrenergic receptors were recoverable from extracellular virus particles when *Spodoptera Frugiperda* (Sf9) cells were infected with β2-adrenergic receptor recombinant baculovirus. Masuda et al. [14] showed that a trimeric G-protein and BLT1, a leukotriene B4 receptor, could be functionally reconstituted on the budded virus by co-infection of each with recombinant virus in Sf9 cells. Thus, the membrane receptors, which reside on the plasma membrane, were functionally displayed on the budded form of the baculovirus.

Here, we show that the ER membrane proteins are also expressed on budded virus in addition to the Sf9 cells. In contrast to proteins expressing in Sf9 cells, viral displayed proteins were less aggregated and less degraded. And as mentioned already, this suggests an application to screening for specific monoclonal antibody generation using this viral display system.

Materials and methods

Constructs. To construct the baculovirus vectors expressing human SREBP-2 or SCAP (full-length or amino acids 799–958), the following steps were used. pBlueBac3-BP2 was prepared; first, the 5′ half fragment of SREBP-2 was prepared by digesting a plasmid, in which a linker containing a *Spe*I site was added just before the initiation codon of SREBP-2 (1–481) in pOPI3BP2 [15], with *Spe*I and *Bcl*I. Secondarily, the 3′ half fragment was prepared by digesting a plasmid containing 151–1141 of SREBP-2 with *Bcl*I and *Cpo*I. The fragments were cloned into pBlueBac3 (Invitrogen) in which *Cpo*I site was introduced between *Bam*HI and *Hin*dIII sites. pBlueBacHis2-SCAP or pBlueBacHis2-SCAP-C was generated by cloning an *Eco*RI/*Hin*dIII or *Xho*I fragment based on KIAA0199 (a gift from the Kazusa DNA research institute) into pBlueBacHis2 (Invitrogen).

Production and purification of recombinant virus and Sf9 cell culture. Sf9 cells were cultured in Grace's Insect Media supplemented (Gibco-BRL) with 10% fetal bovine serum (FBS, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco-BRL) at 27 °C. Large scale culturing was performed with the addition of 0.001% pluronic F-68 (Gibco-BRL). The generation of recombinant viruses was performed according to the manufacturer's method (Bac-N-Blue Transfecton Kit, Invitrogen). Briefly, cells were transfected with Bac-N-Blue DNA and 4 μg of recombinant transfer vector. Recombinant virus was isolated by successive rounds of plaque assay. After the generation of high titer stock, the viral titer was also determined by plaque assay.

Immunoblotting for expression level of SREBP-2. Sf9 cells (0.83×10^6 cells/6 well dish) were infected with the recombinant virus at a "multiplicity of infection" (MOI) level of 5. Sf9 cells were scraped and centrifuged at 800*g* for 10 min after culturing for 24, 48, or 72 h. Then, a pellet was obtained as the cell fraction and a culture supernatant was obtained. The cell fraction was suspended in 100 μl/well of isotonic buffer (PBS, phosphate buffered saline, containing 0.1% Triton X-100, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μg/ml PMSF, phenylmethylsulfonyl fluoride). After vortexing at 4 °C for 30 min and centrifugation at 1000*g* for 10 min, 20 μl of 5× SDS sample buffer was added to 80 μl of the supernatant, followed by heat treatment at 95 °C for 10 min. The culture supernatant (80 μl) was added to 5× SDS sample buffer (20 μl), and the mixture was subjected to heat treatment. Those samples were applied to 8% SDS–PAGE and transferred to a nitrocellulose membrane (Highbond ECL, Amersham). After blocking with Block Ace for 30 min, the membrane was immunoblotted with monoclonal antibody IgG-1C6 (anti-human SREBP-2 antibody, ATCC No. CRL-2224). Immunoreactive proteins were detected by SuperSignal West Dura (Pierce).

Preparation of budded virus and sucrose density gradient centrifugation. Sf9 cells (5×10^8 cells/500 ml) were infected with SREBP-2 recombinant virus at a MOI of 5 and cultured for 48 h. The cells were removed by centrifugation at 800*g* for 10 min, and then the supernatant was ultra-centrifuged at 40,000*g* for 20 min. The precipitate was suspended in 4 ml of a TE buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA). The suspension was overlaid onto a 25–56% linear sucrose density gradient and centrifuged at 100,000*g* for 90 min in a SW28 rotor. Fractions (1.5 ml) were collected from the top of the gradient, and the distribution of SREBP-2 was examined by immunoblotting with IgG-1C6 and SDS–PAGE with Coomassie brilliant blue staining.

Production of monoclonal anti-human SCAP antibody. Sf9 cells (5×10^8 cells/500 ml) were infected with SCAP-C recombinant virus at a MOI of 5 and cultured for 48 h. The cells were harvested and lysed by sonication, and then the lysate was centrifuged at 10,000*g* for 30 min. The supernatant was applied to a Ni–NTA column (QIAGEN) and the recombinant proteins were purified as an antigen by standard method. Six-week-old female BALB/c mice were immunized three times. Primary ELISA screenings were performed by standard method using purified SCAP-C as the capture antigen. To check the reactivity to full-length SCAP, SCAP (full-length) expressing viruses underwent SDS–PAGE and were transferred to nitrocellulose membranes. Each culture supernatant of positive clones on ELISA was probed to a strip of the transferred membrane.

Preparation of microsomal membranes from several cell lines or rat tissues. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma)/5% FBS/penicillin (100 U/ml) and streptomycin (100 μg/ml). CaCo-2 cells were grown in DMEM/5% FBS/1% non-essential amino acids (Gibco-BRL)/penicillin (100 U/ml) and streptomycin (100 μg/ml). CHO cells were maintained in Ham-F12 (Sigma)/5% FBS/penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured at 37 °C in a 5% CO_2. The cells were suspended in buffer A (10 mM Hepes–KOH at pH 7.4, 0.25 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 5 μg/ml aprotinin, pepstatin A, leupeptin, 2 mM PMSF, and 25 μg/ml *N*-acetyl–Leu–Leu–norleucinal), passed through a 27 G needle 10 times, and centrifuged at 1000*g* for 10 min at 4 °C. The 1000*g* supernatant was ultra-centrifuged at 100,000*g* for 1 h to prepare the microsome fraction and the following pellet was resuspended in SDS sample buffer.

Twelve-week-old Wistar rats were used for all experiments. Liver and testis tissues were homogenized in buffer A with a Teflon homogenizer, and the microsome fraction was prepared by the above-mentioned sequential centrifugation.

Immunohistochemistry. Immunohistochemistry was performed as described [16]. Briefly, rat tissues were fixed in 10% formalin, dehydrated with alcohol, and embedded in paraffin. Tissues were sectioned in 4 μm thick samples. After blocking of endogenous biotin, tissues were incubated for 2 h at room temperature with a monoclonal antibody against SCAP (K7623) which was diluted in 1% BSA/PBS to a final concentration of 10 μg/ml. After several washes with PBS, the sections were incubated with anti-mouse IgG for 1 h and were stained with an avidin–biotin kit according to the manufacturer's protocol (Vectastain Elite ABC kit, Vector, CA). The sections were then counterstained with hematoxylin.

Results

Expression of SREBP-2 on budded virus

By using IgG-1C6, a SREBP-2 specific antibody, the expression level of human SREBP-2 in Sf9 cells was determined via immunoblotting (Fig. 1). After infection with SREBP-2 recombinant virus, SREBP-2 was detectably expressed at 24 h in Sf9 cells. In addition, we detected an intense anti-SREBP-2 reactive band in culture supernatant after 48 h.

To clarify whether this band was derived from cell debris or not, we fractionated the culture supernatant from the 48-h infection timepoint by serial centrifugations. The culture supernatant was centrifuged at 800*g* for 10 min to separate cell debris as a pellet. Then, the supernatant was ultra-centrifuged at 40,000*g* for 20 min. The anti-SREBP-2 reactive band was recovered in the supernatant of the 800*g* centrifuged sample and also recovered in the pellet fraction of a 40,000*g* centrifuged sample (Fig. 2). These results suggest that the SREBP-2 present in the culture supernatant is not debris such as dead cells, but rather is derived from membrane or extracellular virus. To exclude the possibility that it was a contaminant of the cell membrane, the pellet fraction was fractionated by sucrose density gradient centrifugation. As a result, SREBP-2 was observed in the same fraction as that of the virus envelope protein gp64, a finding which was confirmed by SDS–PAGE with Coomassie brilliant blue staining (Fig. 3). This result indicates that the SREBP-2 observed in the ultra-centrifuged pellet fraction is not debris of cell membrane debris, but rather is SREBP-2 expressed on budded virus.

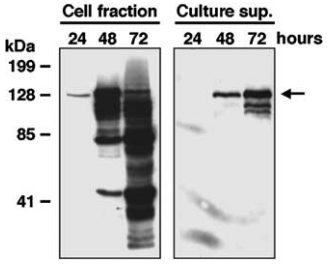

Fig. 1. Expression of SREBP-2 in Sf9 cells and culture supernatants. Sf9 cells were infected at MOI 5, and cultured for 24, 48, or 72 h. The cell fraction and culture supernatant were prepared as described in Materials and methods. Aliquots were separated by SDS–PAGE (8%) and were subjected to immunoblotting with anti-SREBP-2 (IgG-1C6). The arrow indicates SREBP-2.

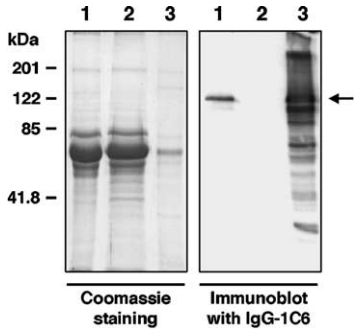

Fig. 2. Centrifugal separation of SREBP-2 expressed in culture supernatant. The culture supernatant from 48 h of infection was fractionated by serial centrifugation. Aliquots were separated by SDS–PAGE (8%) and were subjected to Coomassie brilliant blue staining or immunoblotting with IgG-1C6. Lane 1, 800*g* 10 min supernatant; lane 2, 40,000*g* 20 min supernatant; and lane 3, 40,000*g* 20 min pellet. Arrow indicates SREBP-2.

Expression of SCAP on budded virus

We checked for the expression of human SCAP, which has eight membrane-spanning domains, on budded virus. Similar to SREBP-2, SCAP was also displayed on budded virus after 48-h infection (Fig. 4). In the immunoblotting with anti-His-tag antibody, we noticed that SCAP expressed in the Sf9 membrane largely consisted of high molecular weight immunoreactive bands, presumably aggregates of SCAP. SCAP in the

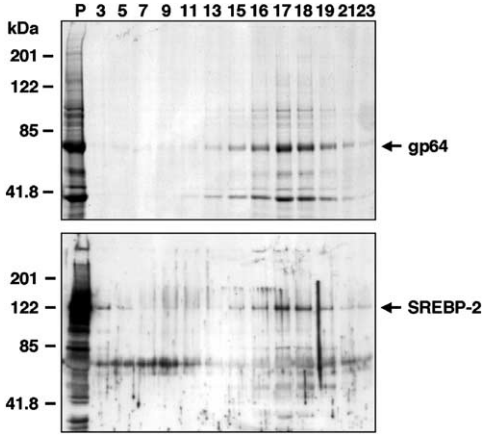

Fig. 3. Sucrose density gradient centrifugation of the pellet fraction of the 40,000*g* centrifuged sample. Aliquots of the gradient fraction were analyzed by Coomassie brilliant blue staining (above panel) and immunoblotting with IgG-1C6 (bottom panel). *P* denotes the pellet fraction of the 40,000*g* centrifuged sample. Fraction numbers are denoted at the top of the panel. Arrows indicate gp64 and SREBP-2, respectively.

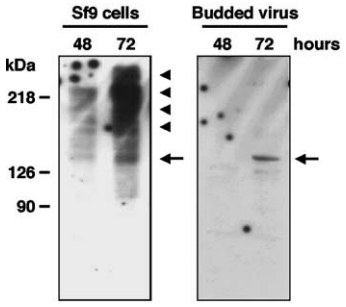

Fig. 4. Expression of SCAP on budded virus. Sf9 cells were infected at MOI 5 and cultured for 48 or 72 h. The cell fraction and viral fraction were prepared by the same method as in Fig. 2, and immunoblotting was performed with an anti-His-tag antibody (Qiagen). The arrow indicates SCAP and the arrowheads indicate high molecular weight immunoreactive proteins, respectively.

budded virus fraction appeared as a single major band with the proper or expected molecular weight.

Application of baculovirus-expressed SCAP as a screening tool for antibody generation

We next evaluated whether proteins expressed on budded virus could be used as a screening tool for antibody generation. SCAP is a polytopic membrane protein which has 8 transmembrane domains in its amino-terminal region. The carboxyl terminal region of SCAP contains WD repeats for interaction with SREBP and projects into the cytoplasm [17]. Therefore, we constructed a baculovirus expression vector which included SCAP carboxyl terminal sequences (799–958 amino acids) with his-tag at the amino terminus. After infection with recombinant virus at a MOI of 5, Sf9 cells were incubated for 48 h and cell lysates were recovered. Recombinant proteins were purified with a Ni–NTA column and were then immunized in mice.

After selection with ELISA using purified SCAP-C as the capture antigen, we performed an immunoblotting screening with SCAP (full-length) expressed virus as described in Materials and methods. As shown in Fig. 5A, it was found that several clones had raised antibodies which recognized full-length SCAP expressed on budded virus.

To determine whether the generated antibody recognized intrinsic SCAP, cell extracts from HepG2, CaCo-2, and CHO cells were immunoblotted with the purified antibody (K7601). Total microsomal fractions prepared from the liver and testis of rat were also tested (Fig. 5B). The monoclonal antibody against human SCAP (K7601) recognized one main band which had the expected molecular size of SCAP in HepG2 and CaCo-2 cells. K7601 also cross-reacted, with a major single band with the expected molecular weight for SCAP, in the liver and testis of rat as well as in CHO cells (Fig. 5B).

Immunohistochemical localization study in rat tissues

Using the anti-SCAP antibody, an immunohistochemical study was performed with rat tissues. Among the tissues tested, the testis and adrenal gland were positively stained. In the testis, the cytoplasm of the interstitial cells, known as Leydig cells, were powerfully stained. In the adrenal gland, the cells in the zona fasciculata and zona reticularis were positively stained. The positive staining of the capsule is considered to be a nonspecific staining frequently seen at the edge of the specimen. These results suggest that SCAP localizes abundantly in the cytoplasm of steroid hormone generating tissues (Fig. 6).

Discussion

A baculovirus system in Sf9 cells enables high-level expression by means of a potent promoter of a baculoviral polyhedrin gene. This system has many advanta-

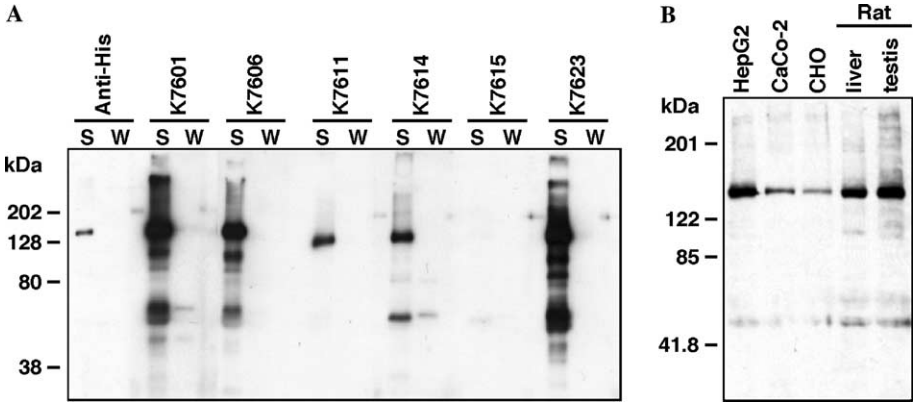

Fig. 5. Screening and specificity of monoclonal antibody against human SCAP. (A) The SCAP (full-length) recombinant virus (S) and wild-type virus (W) were immunoblotted with the culture supernatants of positive clones by ELISA, or with an anti-His-tag antibody as a positive control. (B) Specificity of K7601 was analyzed using HepG2, CaCo-2, and CHO cells, and rat tissues (liver and testis). The microsome fractions (30 μg/lane) were subjected to immunoblotting.

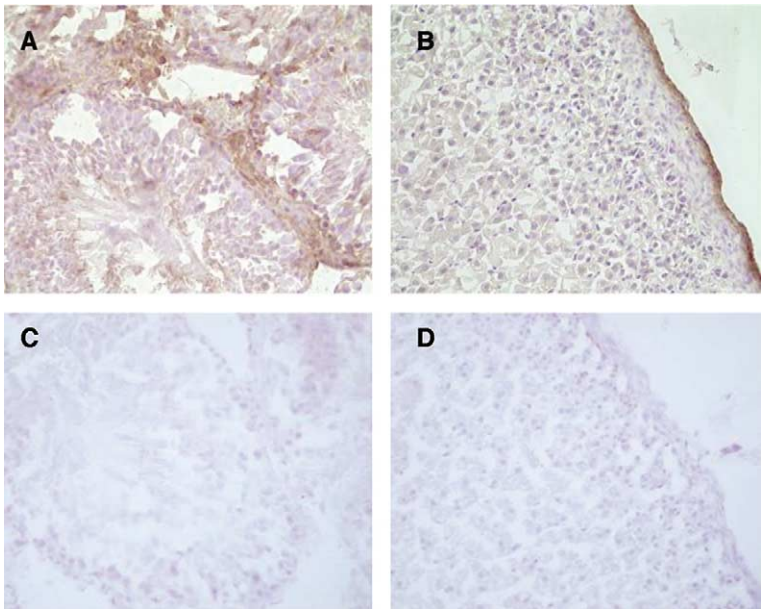

Fig. 6. Immunohistochemical localization of SCAP in rat tissues. Twelve-week-old Wistar rats were treated with an anti-SCAP antibody (K7623). SCAP was localized especially to Leydig cells in testis (A), and zona fasciculata, as well as the zona reticularis cells of adrenal gland (B). (C) and (D) Negative controls, staining without the primary antibody for (A) and (B), respectively.

ges over that of *E. coli*, such that the expressed proteins do not easily aggregate, and do undergo the posttranslational modification which is necessary for protein function.

About 48 h after infection, baculovirus assembles into a budded form and buds on the outside of the insect cells. Utilizing this characteristic, it has been shown that foreign protein is displayed on the viral surface by fusing with gp64, which is a viral structural transmembrane protein [18–21]. Bouvier et al. [22] have reported that a certain seven-transmembrane domain type receptor is expressed not only on the cell membrane but also on budded virus. They reported that the receptors recovered from the viral envelope are functional in comparison to the non-functioning receptors recovered from the cell surface, ostensibly because the majority of receptors on the virus have undergone sufficient posttranslational modification [13,22]. We found in this study that integral membrane proteins distributed on ER or Golgi apparatus were also expressed on budded virus. The membrane proteins displayed on budded virus exhibited either less degradation (Fig. 2) or less aggregation (Fig. 4). These observations imply that these ER membrane proteins are expressed properly on the viral membrane, as in the case of the other membrane receptors.

The precursors of SREBP-2 and SCAP are considered to form heterodimer complexes and reside in the ER in mammalian cells [3]. When the cellular sterol level decreases, SCAP escorts SREBP-2 to the Golgi apparatus where SREBP-2 is successively cleaved by two processing enzymes [5]. After liberation of mature SREBP-2, SCAP returns to the ER [6]. Although it can not be excluded that SREBP-2 and SCAP are inserted in the plasma membrane in Sf9 cells, our observations suggest the possibility that the baculovirus utilizes the ER or Golgi membrane as its envelope, at least for most part, rather than the plasma membrane.

An application toward the generation of monoclonal antibodies was also evident. In general, monoclonal antibodies against membrane proteins are difficult to produce because of certain conformational characteristics. In this study, we used the budded virus as a screening antigen in immunoblotting, so that a specific monoclonal antibody was obtained which could be used not only in immunoblotting (Fig. 5) but also in immunohistochemistry (Fig. 6). It is believed that the conformation of the expressed protein on the budded virus is similar to that of endogenous protein. Therefore an antibody screened by this method would be useful for immunohistochemistry analysis.

By immunoblotting, this anti-SCAP antibody was shown to recognize one major band, which had a molecular weight appropriate for SCAP (Fig. 5). Using this anti-SCAP monoclonal antibody, the immunohistochemical localization of SCAP was studied in various rat tissues. Among the samples tested, interstitial cells of Leydig in the testis and zona fasciculata, as well as the zona reticularis cells of the adrenal gland, which are known to be a major steroidogenic cells, were strongly stained (Fig. 6). On the other hand, the liver was only weakly positive (data not shown). Those observations are compatible with the data on tissue distribution of SCAP mRNA obtained by DNA microarray analysis (available at http://www.lsbm.org/db/index.html).

This study demonstrates that membrane proteins embedded in the membrane of ER or Golgi apparatus are displayed on budded virus when those proteins are expressed using a baculovirus expression system. It was further demonstrated that a system utilizing the baculovirus display system of membrane proteins is a highly useful tool for monoclonal antibody screening.

Acknowledgments

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