INTRODUCTION

Many viral vectors have been developed for gene transfer in vitro, ex vivo, and in vivo. Retroviruses, adenoviruses, and adeno-associated viruses are currently used for gene delivery into mammalian hosts to test their application for gene therapy both in vitro and in vivo (Mulligan, 1993). These viral vectors are attractive because of their natural adaptation to mammalian hosts. However, their practical use may also raise serious concerns, for example, the emergence of replication-competent viruses, cytotoxicity, preexisting immune responses, and undesired viral gene expression.

The baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) expression system has been used for the expression of various foreign genes because of its high-level expression in insect cells. Its host specificity was originally thought to be restricted to cells derived from arthropods; however, it has been shown that AcNPV can infect mammalian cell lines (Hofmann et al., 1995; Boyce and Bucher, 1996), including hepatocytes and nonhepatocytes (Shoji et al., 1997), and stably maintain the expression cassette of the viral genome in the mammalian cells (Condreay et al., 1999). The advantages of the baculovirus vector are (i) a large capacity for the insertion of foreign DNAs, (ii) easy generation of recombinant viruses, and (iii) absence of the preexisting antibodies against baculovirus in animals. Recently, extension of the host range of the baculovirus and enhancement of foreign gene expression were further demonstrated by constructing a pseudotype virus possessing the vesicular stomatitis virus G glycoprotein (VSV-G) (Barsoum et al., 1997) and by using histone deacetylase inhibitor, which increased the transcription level (Condreay et al., 1999).

In general, viruses initiate infection in target cells by interacting with specific receptors on the cell surface. Those receptors are major determinants of viral tropism in tissues and species. Enveloped viruses recognize the specific receptors by their proteins, which are embedded in the viral outer membrane. Baculovirus that buds from the cell surface preferentially incorporates an envelope fusion glycoprotein, gp64, which is necessary for entry into host cells. Although recent studies revealed that baculovirus vectors, including pseudotype recombinants, efficiently infected mammalian cells and expressed foreign genes, the mode of entry of the baculovirus into the target mammalian cells remains unknown.

In this study, we have constructed a recombinant baculovirus that increases the concentration of gp64 on the virion surface. This recombinant baculovirus was capable of efficient transduction in many mammalian cells. Analyses of infection mechanisms of the baculovirus suggested the involvement of phospholipids in the entry of baculovirus into both insect and mammalian cells. This observation provides further insights into the effi-
cient transduction of mammalian cells and a better understanding of the virus–cell interaction of baculovirus at the molecular level.

RESULTS

Construction of recombinant baculoviruses. To generate recombinant baculoviruses, the baculovirus transfer vectors pAc64-CAluc, pAcVSVG-CAluc, pAcMHVS-CAluc, and pAcGFP-CAluc were constructed by inserting cDNAs encoding the gp64, VSVG, MHVS, or GFP gene under the control of the polyhedrin promoter in the opposite orientation. The gp64, VSVG, MHVS, or GFP gene would be activated by the polyhedrin promoter in insect cells, while the luciferase gene under the CAG promoter would be activated in mammalian cells. Nucleotide numbers of baculovirus DNA are shown on the vectors following the data reported previously by Ayres et al. (1994).

FIG. 1. (A) Construction of transfer vectors. Transfer vectors have the luciferase gene under the control of the CAG promoter and the gp64, VSVG, MHVS, or GFP gene under the control of the polyhedrin promoter in the opposite orientation. The gp64, VSVG, MHVS, or GFP gene would be activated by the polyhedrin promoter in insect cells, while the luciferase gene under the CAG promoter would be activated in mammalian cells. Nucleotide numbers of baculovirus DNA are shown on the vectors following the data reported previously by Ayres et al. (1994). (B) Schematic representation of the recombinant baculoviruses Ac64-CAluc, AcVSVG-CAluc, AcMHVS-CAluc, and AcGFP-CAluc.

FIG. 2. Incorporation of gp64, VSVG, or MHVS protein in baculovirus virions. The purified virions (5 × 10⁶ pfu) of Ac64-CAluc (lane 1), AcVSVG-CAluc (lane 2), AcMHVS-CAluc (lane 3), or AcGFP-CAluc (lane 4) were examined by Coomassie staining and Western blotting. Purified virions were subjected to 10% SDS–PAGE and immunostained with anti-gp64 antibody (top), anti-VSVG antibody (middle), or anti-MHVS antibody (bottom). The gp64:capid protein ratio was measured by NIH image on the bases of Coomassie staining and Western blotting data.
Susceptibility of various cell lines to the recombinant baculoviruses. In previous studies, we demonstrated that baculovirus vector can deliver and express foreign genes into not only hepatocytes but also other mammalian cells (Shoji et al., 1997). We then compared the efficiency of reporter gene expression by Ac64-CALuc, AcVSVG-CALuc, AcMHVS-CALuc, and AcGFP-CALuc in various mammalian cell lines. Figure 3 indicates the luciferase expression of various cell lines infected with the recombinants at a m.o.i. of 50. All of the cell lines infected with AcVSVG-CALuc and AcMHVS-CALuc exhibited 100- to 500-fold higher expression than did cell lines infected with AcGFP-CALuc. Cells infected with Ac64-CALuc also showed 5- to 100-fold higher expression than did cells infected with AcGFP-CALuc. Luciferase activity increased in a dose-dependent manner in mammalian cell lines infected with the recombinant viruses (data not shown). These results indicate that recombinant baculoviruses possessing an increased amount of gp64 or foreign envelope glycoproteins on the virions exhibit higher gene transduction efficiency than does the control baculovirus.

To confirm the roles of the gp64, VSVG, and MHVS envelope glycoproteins of the recombinant baculoviruses in facilitating the enhancement of infectivity, neutralization by a specific antibody against gp64, VSVG, and MHVS was examined in HepG2 cells (Fig. 4). The luciferase expression by Ac64-CALuc was inhibited by anti-gp64 antibody (Fig. 4A). Although treatment with anti-gp64 antibody was more efficient at inhibiting luciferase expression by AcMHVS-CALuc than by AcVSVG-CALuc, complete inhibition of luciferase expression by AcVSVG-CALuc or AcMHVS-CALuc required treatment with both of the anti-gp64 and anti-VSVG or anti-MHVS antibodies (Figs. 4B and 4C). These results indicate that the enhancement of reporter gene transduction by the recombinant baculoviruses in mammalian cells relies on the incorporation of the envelope glycoproteins gp64, VSVG, or MHVS onto the virions.

Effect of enzyme and chemical modification of cells on susceptibility of the recombinant baculoviruses. To examine the cell-surface determinants important for baculovirus infection, we compared the infectivities of Ac64-CALuc, AcVSVG-CALuc, and AcMHVS-CALuc against HepG2 cells treated with phospholipase C, Pronase, or sodium periodate. The gp64 is the major protein responsible for the initiation of the infection of baculoviruses, and the efficiency of gene transduction by Ac64-CALuc was higher than that of the control baculovirus AcGFP-CALuc as described above. Therefore, Ac64-CALuc was used for further experiments for characterization of the infection mechanisms of HepG2 cells. Phospholipase C and Pronase are known to digest phospholipids and proteins, respectively. Sodium periodate removes carbohydrates chains from glycoproteins. When cells were treated with phospholipase C, the infectivity of Ac64-CALuc and AcVSVG-CALuc was markedly reduced in a dose-dependent manner, while AcMHVS-CALuc was shown to be
more resistant to the treatment than were Ac64-CAluc and AcVSVG-CAluc (Fig. 5A). Treatment with Pronase or sodium periodate resulted in no reduction in luciferase expression of the cells infected with the recombinant baculoviruses. Although treatment of cells with Pronase at concentration of 5 μg/ml or more was sufficient to abolish the susceptibility of cells to pseudotype VSV possessing Ebola virus glycoprotein (Takada et al., 1997) or HCV envelope proteins (Matsuura et al., submitted), the infectivities of AcMHVS-CAluc were not changed with Pronase treatment. This might be explained by the fact that AcMHVS-CAluc can utilize gp64 during the infection of cells treated with Pronase. As shown in Fig. 4, anti-gp64 antibody showed higher neutralization activity than did anti-MHVS antibody in the infection with AcMHVS-CAluc, in contrast to the neutralization of infection with AcVSVG-CAluc in which anti-VSVG antibody showed higher activity than did anti-gp64 antibody.

To examine whether the treatment of insect cells with these reagents also affects the infectivity of baculoviruses, plaque assay was carried out in Sf9 cells treated with various chemicals (Fig. 5B). The reduction of plaque number of Ac64-CAluc was observed in cells treated with phospholipase C but not in those treated with Pronase or sodium periodate. Similar results were obtained by other recombinant baculoviruses (data not shown). Cytotoxic effect by the chemical treatments of mammalian and insect cells was determined by trypan blue staining. No serious cytotoxicity was observed after treatment with the chemicals on the range of a concentration used in these experiments (Figs. 5A and 5B). These results suggest that phospholipids on the cell surface play an important role in the infection of mammalian and insect cells by baculoviruses.

Effects of purified lipids on the infectivity of baculoviruses. To obtain additional information on the cell surface determinants important for baculovirus infection, we examined the effect of various purified lipids on the infectivity of Ac64-CAluc. AcVSVG-CAluc was utilized as a control for the experiments because the receptor of VSV was suggested to be some phospholipids, mainly plasma membrane PS (Schlegel et al., 1983). The purified lipids preparations (10 μM) of PA, PI, PS, PE, PG, PC, and SP prepared in PBS containing 50 mM n-octyl-β-D-glucopyranoside were incubated with the viruses, and the effect on luciferase expression in HepG2 cells was determined (Fig. 6). To examine the effect of the lipids’ solvent, the viruses were incubated with 50 mM n-octyl-β-D-glucopyranoside without lipid. PA (90%), PI (60%), and PS (43%) exhibited the most potent inhibiting activities on the infection of Ac64-CAluc. On the other hand, potent inhibiting activities on the infection of AcVSVG-CAluc were shown by the treatments with PA (80%), PG (67%), PE (50%), and PS (45%). The effects of PA and PI on the inhibition of infectivity of Ac64-CAluc were also exhibited in a dose-dependent manner (data not shown). These results indicate that phospholipids PA and PI might be involved in the initiation of baculovirus infection into mammalian cells.

Infectivities of the recombinant baculoviruses on mutant CHO cell lines. To confirm the effects of membrane phospholipids on the infection of baculoviruses, we examined the infectivity of Ac64-CAluc and AcVSVG-CAluc to the mutant CHO cell lines with altered membrane phospholipid composition (Fig. 7). In mutant 51.13, which is deficient in PI synthesis in growth medium lacking myo-inositol, we observed a 62% reduction of luciferase expression by Ac64-CAluc in the PI-depleted condition, whereas only a 23%
reduction was detected when PI-depleted cells were infected with Ac64-CALuc in the same condition. In mutant PSA-3, which is deficient in PS synthesis and requires exogenous PS for growth, we observed a 55% reduction of luciferase by Ac64-CALuc in the PS-depleted condition, whereas a 30% reduction in expression was detected by Ac64-CALuc. On the other hand, mutant CT58, which reduces PC synthesis at 40°C, showed no difference in susceptibility to Ac64-CALuc and AcVSVG-CALuc. The control cell line, CHO K1, exhibited no reduction in reporter gene expression irrespective of the culture conditions. These results are consistent with the data for inhibition obtained by the purified lipids shown in Fig. 6 and suggest that PI on the cell surface might play an important role in the infection with baculoviruses.

**DISCUSSION**

The infection of viruses into host cells is initiated by the binding of the virus to the cell surface and the entry
of the virus genome into cells. Binding is mediated by the viral binding proteins, which recognize a specific receptor molecule on the cell surface. Enveloped viruses use proteins embedded in the viral membrane for specific recognition of receptors such as protein molecules, carbohydrates, and lipids (Lentz, 1990). Specific receptor molecules have been identified, including a CD4 molecule (Dalgleish et al., 1984; Klatzmann et al., 1984) and chemokine receptor family as a co-receptor (Littman, 1998) for human immunodeficiency virus, sialyoligosaccharides for influenza virus (Paulson et al., 1979), and phosphatidylserine for VSV (Schlegel et al., 1983).

For a budded baculovirus, it has been shown that the major envelope fusion protein gp64 determines the viral receptor preference in the inhibition studies with an anti-gp64 monoclonal antibody and thus defines the viral host range or the efficiency of infectivity to the host (Hohmann and Faulkner, 1983; Volkman and Goldsmith, 1985). The biological characteristics of gp64 have been studied in detail in insect cells, and it has been found that gp64 is necessary and sufficient for low-pH-triggered membrane fusion activity (Blissard and Wenz, 1992; Jarvis and Garcia, 1994; Chernomordik et al., 1995; Monsma and Blissard, 1995; Oomens et al., 1995; Markovic et al., 1998; Plonsky et al., 1999). In truncation and substitution studies, gp64 was shown to be essential for the propagation of the budded virus infection through cell-to-cell transmission (Monsma et al., 1996) and important for efficient virion budding (Oomens and Blissard, 1999). Studies using electron microscopy and some inhibitors have suggested that for infection of mammalian cells with the budded baculovirus, viruses were internalized by an endosomal pathway (Volkman et al., 1983; Carbonell et al., 1985; Carbonell and Miller, 1987; Hofmann et al., 1995; Boyce and Bucher, 1996; Condreay et al., 1999). However, the precise mechanisms for the binding and entry of baculoviruses into mammalian cells and insect cells are not yet known.

**FIG. 6.** Inhibition of luciferase expression of the recombinant baculovirus by purified lipids. Ac64-CALuc or AcVSVG-CALuc was incubated with the concentration of 10 μM of purified lipids, and luciferase activity was examined in HepG2 cells after 24 h of incubation. As a control, the virus was treated with the same concentration of n-octyl-β-D-glucopyranoside solution including no lipids (non). PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SP, sphingomyelin. The results shown are the average of three independent assays with the error bars representing SD.

**FIG. 7.** Infectivities of the recombinant baculoviruses on mutant CHO cell lines. The mutant CHO cell lines S113 (A), PSA-3 (B), and CT58 (C) were cultured as described under Materials and Methods, and then these cells were infected with Ac64-CALuc or AcVSVG-CALuc and luciferase expression was examined after 24 h of incubation.
Recent studies suggest the possibility of using baculoviruses as vectors for gene therapy; therefore, identification of the host cellular receptor on mammalian cells will be important for further understanding of the host specificity of the budded baculovirus. To examine the role of the gp64 protein in infection of mammalian cells by baculovirus, we have constructed a recombinant baculovirus Ac64-CALuc possessing an extra gp64 gene under the control of the polyhedrin promoter. Ac64-CALuc was shown to have a 1.5 times higher amount of gp64 protein on the virion and to exhibit higher reporter gene expression in a broader range of mammalian cells than that of the control baculovirus AcGFP-CALuc. These results, in conjunction with the inhibition of expression by the specific antibody against gp64 protein, indicate that baculovirus gp64 envelope fusion protein recognizes some common molecules as a receptor on the surface of various mammalian cells. In this study, we utilized the very late polyhedrin promoter for expression of the gp64 or other foreign viral proteins. Therefore viruses budded in the early stage of infection may not be able to incorporate the proteins into virions. Application of the immediate early promoter for expression of the proteins might improve the efficiency of incorporation into the budded viruses.

Enzyme treatments of the host cells are commonly used to characterize the specific cellular receptors for many viruses. In baculoviruses, treatment of insect cells with proteases was shown to decrease virus binding (Wickham et al., 1992; Wang et al., 1997). In contrast, the infectivity of Ac64-CALuc was drastically decreased by the treatment of HepG2 cells with phospholipase C but not with Pronase or sodium periodate in this study. This might reflect the difference in assay systems or be due to the difference in enzymes or concentration used for treatment. In this study, we used pseudotype baculoviruses possessing VSVG or MHVS proteins on the virion as controls. VSVG and MHVS proteins were reported to recognize phospholipids and CEA-related proteins as receptors, respectively (Schlegel et al., 1983; Williams et al., 1991). Although the expression level of CEA-related proteins on a mammalian cell line was not examined, one of the MHV strains, JHM, which we used, was infectious to HepG2 cells (Koetters et al., 1999). Treatments of cells with a higher concentration of the chemicals caused excessive cell damage; however, the phospholipase C treatment used in this study did not cause any cell damage and AcMHVS-CALuc continued to be infectious to the treated cells. AcVSVSVCALuc was also shown to be sensitive to the treatment with phospholipase C as Ac64-CALuc. These enzyme and chemical modification analyses suggest that baculoviruses utilize phospholipids on the cell surface in the process of infection. Furthermore, the inhibition of viral infection by pretreatment with purified PA or PI and susceptibility of the viruses to the mutant CHO cell lines deficient in phospholipid synthesis suggest that these phospholipids play important roles in binding or penetration of baculoviruses into insect and mammalian cells.

The involvement of phospholipids in the binding or entry process of enveloped viruses has been reported. Semliki forest virus requires sphingolipids on the target membrane for fusion activity (Nieva et al., 1994). The binding and penetration of Ebola virus are activated by the presence of PI (Ruiz-Argueuillo et al., 1998). VSV preferentially binds to PS, PE, and PA (Schlegel et al., 1983) and also binds to PI, diposphatidylglycerol and gangliosides, suggesting that these substances interact electrostatically because of their common negative electric charges (Mastromarino et al., 1987). As for baculovirus, Chernomordik et al. indicated that biological membrane fusion mediated by the gp64 was modulated by some specific changes in the lipid composition of the cell membrane (Chernomordik et al., 1995). Although PA and PI inhibited the viral infection of HepG2 cells in this study, the major membrane phospholipids of HepG2 cells and insect cells were composed mainly by PC and PE (Koumanov et al., 1990; Marheineke et al., 1998). Both PA and PI are acidic lipids, which suggested ionic interactions between the baculovirus and the lipids. However, the infection of mammalian cells with baculovirus was not inhibited by preincubation with acidic glycosaminoglycans such as heparin or heparan sulfate (data not shown), suggesting that the inhibition of baculovirus infection by the purified lipids is not due to the ionic interactions. Further studies are needed to clarify the mechanisms of baculovirus and phospholipid interaction.

In previous studies, baculovirus AcNPV efficiently entered human hepatocytes and other mammalian cell lines (Hofmann et al., 1995; Boyce and Bucher, 1996; Shoji et al., 1997). Recently, it has been reported that a pseudotype baculovirus incorporating the VSVG protein extended the host range and increased the transduction efficiency in mammalian cells (Barsoum et al., 1997). The pseudotype retroviruses possessing VSVG protein have been shown to exhibit a broader host range and a higher transduction efficiency than that of the original retroviruses (Emin et al., 1991; Burns et al., 1993). This is because VSV was shown to binds to PS as a cellular receptor that is widely distributed on the plasma membrane and exhibits a broader host range. In the application of in vivo studies of baculoviruses for gene therapy, recent studies suggest that baculoviruses could not be utilized in vivo due to inactivation of the virus by the serum complement systems (Sandig et al., 1996; Hofmann and Strauss, 1998). In the case of retroviruses, pseudotype viruses with VSV-G were shown to be more resistant to the complement than were the normal retroviruses (Ory et al., 1996). Although Ac64-CALuc and AcVSVSVCALuc were shown to be more resistant to the human and animal complements in vitro than was the
control baculovirus, we have not succeeded in producing the expression of reporter gene in vivo (data not shown). Further studies in vivo for efficient transduction in animals are needed.

In this study, we were able to demonstrate that the infectivity of a baculovirus into mammalian cells can be enhanced by the incorporation of an extra amount of its own envelope protein, gp64. Thus, we have provided a direction for future research on cellular receptors and possible cofactors that affect baculovirus entry into mammalian or insect cells. Furthermore, if it was possible to complement the gp64 proteins with other foreign proteins, development of the targeting vectors for gene therapy and analyses of functions of other viral envelope proteins might become possible.

MATERIALS AND METHODS

Construction of plasmids. Recombinant baculoviruses were constructed by use of the transfer vectors pAcSurf-2, which has the gp64 gene under the polyhedrin promoter (provided by Dr. I. M. Jones) (Boublik et al., 1995), and pAcYM1 (Matsuura et al., 1987). pAcVSVG-G was constructed by excision of the cDNA of VSVG from pCA-VSVG (Takikawa et al., 2000) by digestion with EcoRI, was filled in with Klenow enzyme, and was cloned into the BamHI site of pAcYM1 after ligation of the BamHI linker. pAcMHVS was constructed by excision of the cDNA of MHVS from pTargetJHM-cl-2S (provided by Dr. F. Taguchi) (Taguchi et al., 1992) by digestion with XhoI and Smal, was filled in with Klenow enzyme, and was cloned into the Smal site of pAcYM1. pAcGFP was constructed by excision of the cDNA of GFP from pKS588 (provided by Dr. K. Sakai) by digestion with BamHI site of pAcYM1 after ligation of the BamHI linker. pAcGFP was constructed by excision of the cDNA of GFP from pKS588 (provided by Dr. K. Sakai) by digestion with BamHI site of pAcYM1 after ligation of the BamHI linker. To express foreign genes in mammalian cells, the Photinus pyralis luciferase gene was introduced into the baculovirus vectors under the control of the CAG promoter. To construct the transfer vectors pAc64-CAG, pAcVSVG-CAG, pAcMHVS-CAG, and pAcGFP-CAG, the CAG cassette was excised from pCAGGS (Niwa et al., 1991) by use of Sall and BamHI linker, was filled in with Klenow enzyme, and was inserted into the EcoRV site of pAcSurf-2, pAcVSVG, pAcMHVS, and pAcGFP in an opposite direction to the polyhedrin promoter. To generate pAc64-CAluc, pAcVSVG-CAluc, pAcMHVS-CAluc, and pAcGFP-CAluc, the luciferase gene was excised from a plasmid PG-CS (Toyo Ink Co. Ltd., Tokyo, Japan) with XhoI and XbaI, was filled in with Klenow enzyme, and was cloned into the BglII site of pAc64-CAG, pAcVSVG-CAG, pAcMHVS-CAG, and pAcGFP-CAG plasmid after addition of the BclI linker.

Cells and viruses. AcNPV and recombinant baculoviruses were grown and assayed in Spodoptera frugiperda (SF) 9 cells in TC100 medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.26% Bactotryptose broth (Difco, Detroit, MI), 100 μg of kanamycin per ml and 10% fetal bovine serum (FBS). The recombinant baculoviruses Ac64-CAluc, AcVSVG-CAluc, AcMHVS-CAluc, and AcGFP-CAluc were generated by homologous recombination of baculovirus DNA with pAc64-CAluc, pAcVSVG-CAluc, pAcMHVS-CAluc, and pAcGFP-CAluc, respectively. These recombinants were purified as follows; culture supernatants were harvested at 3 days after infection, and cell debris was removed by centrifugation at 6000 g for 15 min at 4°C. Virus was pelleted down by ultracentrifugation at 25,000 rpm in an SW28 rotor (Beckman, Palo Alto, CA) for 60 min and was resuspended in phosphate-buffered saline (PBS), was loaded on 10–60% (w/v) sucrose gradients, and was ultracentrifuged at 25,000 rpm in an SW41E rotor (Beckman) for 60 min. The virus band was collected and resuspended in PBS and was ultracentrifuged at 25,000 rpm in the SW41E rotor for 60 min. The virus pellet was resuspended in PBS, and infectious titers were determined by plaque assay (Shoji et al., 1997; Matsuura et al., 1987).

Various mammalian cell lines were used to examine gene expression by the recombinant baculoviruses, including human cell lines (HepG2, Huh7, FLC4, HeLa, and 293T), monkey kidney cell lines (CV-1), a porcine kidney cell line (CPK), hamster cell lines (BHK and CHO), a buffalo rat liver cell line (BRL3A), and a normal mouse liver cell line (NMuLi). These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Laboratories) containing 2 mM l-glutamine, penicillin (50 IU/ml), streptomycin (50 mg/ml), and 10% FBS. All of the cells, except FLC4, 293T, and CPK cells, were purchased from the Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). FLC4, 293T and CPK cells were provided by Drs. S. Nagamori, Y. Kitamura, and A. Fukushima, respectively. Mutant CHO cell lines with altered membrane phospholipid composition, 51.13 (Esko and Raetz, 1980a) and CT58 (Esko and Raetz, 1980b), were provided by Dr. J. D. Esko; isolation of PSA-3 was described previously (Kuge et al., 1986).

Expression of viral envelope protein. Expression of gp64, VSVG, MHVS, or GFP protein in insect cells infected with Ac64-CAluc, AcVSVG-CAluc, AcMHVS-CAluc, or AcGFP-CAluc was analyzed by Western blotting, immunofluorescence analysis, and fluorescence microscopic observation. The purified viruses were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were blotted to a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Anti-gp64 mouse monoclonal antibody, anti-VSV rabbit polyclonal antibody, and anti-MHVS mouse monoclonal antibody were used as the first antibody. Each protein was detected by using the alkaline phosphatase assay methods as described previously (Ruggieri et al., 1997). Immunofluorescence analysis was also carried out as described previously (Yap et al., 1997) except that...
the cells were fixed with 4% paraformaldehyde. Expression of GFP was examined by fluorescence microscopy.

**Luciferase assay.** Various mammalian cell lines were infected with the recombinant baculoviruses at m.o.i. of 50 and incubated for 24 h at 37°C. Luciferase activity was determined by the Pica Gene luciferase assay kit (Toyo Ink Co. Ltd.) according to the manufacturer's instructions. Cells (1 × 10⁵) were lysed with 100 μl of the cell lysis buffer LUC/PGC-50 (Toyo Ink Co. Ltd.). Twenty microliters of cleared cell lysate was incubated with 100 μl of a reaction mixture containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 20 mM Tricine, 1.07 mM (MgCO₃)₂Mg(OH)₂5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μM Coenzyme A, 470 μM luciferin, and 530 μM ATP. Relative light units (RLU) were measured by the use of a luminometer (Berthold, Wildbad, Germany). Bioluminescent reaction was catalyzed by adding 20 μl of each standard to 100 μl of reaction mixture.

**Reagents.** Phospholipase C (Bacillus cereus Grade I) was obtained from Boehringer Mannheim (Mannheim, Germany). Pronase, sodium periodate (NaIO₄), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidyserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and sphingomyelin (SP) were obtained from Sigma (St. Louis, MO).

Anti-VSVG monoclonal antibody was purchased from Lee BioMolecular Research Laboratories, Inc (San Diego, CA), and anti-VSVG monoclonal antibody was provided by Dr. M. A. Whitt. Anti-gp64 monoclonal antibodies, B12DS and AcV1, were provided by Drs. L. E. Volkman and P. Faulkner, respectively. Anti-MHV monoclonal antibodies, #3 (Kubo et al., 1993) and 10G (Routledge et al., 1991), were provided by Dr. F. Taguchi.

**Neutralization test.** To examine whether the antibody against gp64, VSVG, or MHVS would specifically block the expression by the recombinant baculoviruses, virus solution containing 10⁵ pfu of infectious virus in 50 μl of serum-free DMEM was incubated with various dilutions of the antibodies at 37°C for 60 min. The mixtures were then inoculated into HepG2 cells and were washed twice with medium after absorption for 60 min. After 24 h of incubation at 37°C, neutralization by the antibodies was determined by the reduction of luciferase expression.

**Chemical modification of cells.** HepG2 cells (1 × 10⁵) prepared in a 48-well plate were washed with serum-free DMEM three times and were incubated with 250 μl of the serum-free DMEM containing various concentrations of phospholipase C, Pronase, or sodium periodate at 37°C for 30, 20, and 60 min, respectively. After washing three times with DMEM containing 10% FBS, the cells were infected with Ac64-CAluc, AcVSVG-CAluc, or AcMHVS-CAluc at m.o.i. of 50. After absorption for 60 min at 37°C, the cells were washed three times with DMEM containing 10% FBS and were incubated for 24 h at 37°C. Then the cells were harvested, and luciferase activity was determined. Sf9 cells (1 × 10⁵) prepared in a 35-mm dish were treated with the serum-free TC100 containing various concentrations of these chemicals at 26°C for the same periods as the experiments with HepG2 cells. Reduction of infectivity of Ac64-CAluc (100 pfu) was determined by plaque assay. Viabilities of HepG2 and Sf9 cells treated with the reagents were determined by staining with trypan blue.

**Effects of pretreatment of the recombinant baculoviruses with purified lipids on expression.** Chloroform solutions of the purified lipids were dried up and solubilized in PBS containing 50 mM n-octyl-β-D-glucopyranoside (Sigma) at a concentration of 10 mg/ml. The lipid solutions were stored at 4°C and were diluted before use. Ac64-CAluc (2 × 10⁴ pfu) or AcVSVG-CAluc (2 × 10⁴ pfu) and various concentrations of the purified lipid solutions were incubated in 50 μl of PBS at room temperature for 90 min. The mixtures were inoculated into HepG2 cells (1 × 10⁵) in a 48-well plate and were incubated at 37°C for 1 h. After incubation, the solutions were completely removed, and the cells were washed twice and were incubated with 250 μl of DMEM containing 10% FBS. The cells were harvested after 24 h of incubation at 37°C, and luciferase activity was determined.

**Infectivities of the recombinant baculoviruses on the mutant CHO cell lines.** Mutant 5113, a myo-inositol auxotroph, reduces phosphatidylinositol synthesis under the condition of myo-inositol starvation (Esiko and Raetz, 1980a). Mutant PSA-3, a phosphatidyserine auxotroph, reduces phosphatidyserine content under the condition of phosphatidyserine starvation (Kuge et al., 1986). Mutant CT58 is a temperature-sensitive mutant that reduces phosphatidylcholine synthesis when cultured at a non-permissive temperature, 40°C (Esiko and Raetz, 1980b). These mutant cell lines and a parent cell line, CHO-K1, were cultured in a 48-well plate for 48 h under the permissive and non-permissive conditions as described previously (Esiko and Raetz, 1980a,b; Kuge et al., 1986). After 48 h of incubation, cells were washed with DMEM containing 10% FBS and were inoculated with Ac64-CAluc or AcVSVG-CAluc. Cells were washed twice with DMEM containing 10% FBS after 60 min absorption, and luciferase activity was determined after 24 h of incubation under the competent conditions for the phospholipid syntheses.

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