Acquisition of Complement Resistance through Incorporation of CD55/Decay-Accelerating Factor into Viral Particles Bearing Baculovirus GP64[▽]

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A major obstacle to gene transduction by viral vectors is inactivation by human complement *in vivo*. One way to overcome this is to incorporate complement regulatory proteins, such as CD55/decay accelerating factor (DAF), into viral particles. Lentivirus vectors pseudotyped with the baculovirus envelope protein GP64 have been shown to acquire more potent resistance to serum inactivation and longer transgene expression than those pseudotyped with the vesicular stomatitis virus (VSV) envelope protein G. However, the molecular mechanisms underlying resistance to serum inactivation in pseudotype particles bearing the GP64 have not been precisely elucidated. In this study, we generated pseudotype and recombinant VSVs bearing the GP64. Recombinant VSVs generated in human cell lines exhibited the incorporation of human DAF in viral particles and were resistant to serum inactivation, whereas those generated in insect cells exhibited no incorporation of human DAF and were sensitive to complement inactivation. The GP64 and human DAF were detected on the detergent-resistant membrane and were coprecipitated by immunoprecipitation analysis. A pseudotype VSV bearing GP64 produced in human DAF knockdown cells reduced resistance to serum inactivation. In contrast, recombinant baculoviruses generated in insect cells expressing human DAF or carrying the human DAF gene exhibited resistance to complement inactivation. These results suggest that the incorporation of human DAF into viral particles by interacting with baculovirus GP64 is involved in the acquisition of resistance to serum inactivation.

Gene therapy is a potential treatment option for genetic diseases, malignant diseases, and other acquired diseases. To this end, safe and efficient gene transfer into specific target cells is a central requirement, and a variety of nonviral and viral vector systems have been developed (6, 44). Recombinant viruses can be used for efficient gene transfer. Retroviruses, adeno-associated viruses, and lentiviruses are able to integrate foreign genes into host genomes and are suitable for gene therapeutics by virtue of their permanent expression of the therapeutic genes, whereas adenoviruses, herpesviruses, and baculoviruses can transiently express foreign genes (6, 12, 44). Pseudotype particles bearing other viral envelope proteins have been developed to improve transduction efficiency and the safety of viral vectors, including retrovirus (4, 7), lentivirus (25), vesicular stomatitis virus (VSV) (29), and baculovirus (17, 42). Pseudotype retroviruses and lentiviruses bearing the baculovirus envelope protein GP64 of *Autographa californica* nucleopolyhedrosis virus (AcNPV) have been shown to exhibit efficient gene transduction into a wide variety of cells with a lower cytotoxicity compared to those bearing the VSV envelope protein G (VSVG), which is commonly used for pseudotyping (18, 32, 35, 36).

However, a drawback of gene transduction by viral vectors is

that human sera inactivate the vectors (11, 40). Complement is a major element of the innate immune response and serves to link innate and adaptive immunity (8). Complement activation can occur via classical, lectin, and alternative pathways (2, 8). All pathways invoke several responses, such as virus opsonization, virolysis, anaphylatoxin, and chemotaxis production, as well as others (2, 8). VSV and baculovirus are inactivated by human sera via the classical pathway (1, 11). Because complement activation also induces potential damage to host cells, the complement system is tightly regulated by the complement regulatory proteins (CRPs), including CD55/decay-accelerating factor (DAF), CD46/membrane cofactor protein (MCP), and CD59 (2, 8, 15). DAF and CD46 inhibit activation of C3/C5-converting enzymes, which regulate the activation of classical and alternative pathways, whereas CD59 regulates the assembly of the membrane attack complex (2, 8, 15).

Viral vectors can be manipulated to confer resistance to the complement inactivation. Human immunodeficiency virus (HIV) is known to develop resistance to human complement through the incorporation of DAF, CD46, and CD59 to the viral particles (22, 30, 31, 38). Moloney murine leukemia virus vectors produced in HT1080 cells are resistant to complement inactivation (5). Baculovirus and lentivirus vectors bearing DAF or the fusion protein between the functional domains of human DAF and the GP64 were resistant to complement inactivation (9, 13). It has been shown that lentivirus vectors pseudotyped with the GP64 are more resistant to inactivation in the sera of mice and rats (14, 32) and are capable of executing longer expression of

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the transgenes in nasal epithelia compared to those pseudotyped with the VSVG (35, 36). However, the precise mechanisms underlying the resistance to complement inactivation by pseudotyping of the GP64 is not known.

To clarify the molecular mechanisms underlying the resistance of the viral vectors pseudotyped with the GP64 to the complement inactivation, we produced pseudotype and recombinant VSVs bearing the GP64. The recombinant VSVs carrying the gp64 gene generated in human cells but not in insect cells exhibited incorporation of human DAF on the viral particles and were resistant to the complement inactivation. Furthermore, production of the gp64 pseudotype VSV in the DAF knockdown human cells impaired serum resistance, whereas production of the gp64 recombinant VSV in the CHO cell lines stably expressing human DAF and the recombinant baculoviruses in the insect cells stably expressing human DAF or encoding the DAF gene in the genome conferred resistance to the complement inactivation. These results suggest that DAF incorporation into viral particles bearing baculovirus GP64 confers resistance to serum inactivation.

MATERIALS AND METHODS

Plasmids. The cDNA encoding AcNPV gp64 gene was generated by PCR, cloned into pCAGGS/MCS-PM (26) and designated as pCAGgp64. The cDNA encoding human DAF was amplified by PCR, cloned into pcDNA3.1 (Life Technologies, Carlsbad, CA) and pIB/V5-His (Life Technologies), and designated as pcDNA3.1DAF and pIBDAF, respectively. The VSVG gene of pVSV-GLPLF, a plasmid encoding a full-length cDNA clone of VSV carrying the green fluorescent protein gene between M and L genes under the T7 RNA polymerase (41), was replaced with the gp64 gene by using the restriction sites of MluI and NheI, and the resulting plasmid was designated pVSVΔG-gp64. pCAGVSVG, the plasmid encoding the VSVG under the CAG promoter, was constructed as described previously (41). The targeting fragment for DAF knockdown (GATCCGAAGAGTTCTGCAATCGTACTCAAGAGATACGATTGCAGAACTCTCAATTTTGGAAA) was introduced into the BamHI and HindIII sites of pSilencer 2.1 U6 Hygro vector (Ambion, Austin, TX) as described previously (23) and designated pSilencer shDAF.

Cells. Sf9 and BmN cell lines derived from *Spodoptera frugiperda* and *Bombyx mori*, respectively, were grown in SF900-II medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) at 27°C. Huh7, 293T, HeLa, COS7, Vero, BHK, CHO, and NMuLi cell lines were cultured in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS. U937 and Raji cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS. CHO3B2A, a cell line constitutively expressing human DAF and CD59, and CHO parental control cells were maintained in F12-HAM medium (Sigma) supplemented with 10% FBS as described previously (24). To establish an insect cell line stably expressing human DAF, Sf9 cells were transfected with pIBDAF, and a drug-resistant clone, Sf9DAF, was selected by treatment with blasticidin (Life Technologies) at a final concentration of 50 μg/ml. To establish a human DAF knockdown cell line, 293T cells were transfected with pSilencer-shDAF and a control plasmid, pSilencer 2.1 U6 Hygro negative control (Ambion), and drug-resistant clones DAFKD293T and Cont293T were selected by treatment with hygromycin (Wako, Tokyo, Japan) at final concentrations of 100 μg/ml, respectively.

Antibodies. Anti-VSVΔG polyclonal antibody was prepared by immunization of goats with the purified VSVΔG, in which the G gene was deleted, as described previously (41). Anti-GP64 monoclonal antibodies (AcV1 and AcV5), anti-human DAF monoclonal antibody (H319), and anti-GAPDH (FL335) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human DAF monoclonal antibody (IA10) was prepared as described previously (16). Anti-VSVG polyclonal antibody (antibody 3326) was kindly provided by M. A. Whitt, University of Tennessee. Rabbit anti-VP39 polyclonal antibody was prepared by intradermal injection of the synthetic peptides of amino acid residues from 37 to 49 (SPDAYHDDGWFC) of AcNPV VP39 protein into a Japanese white rabbit purchased from Kitayama Laboratories (Nagano, Japan).

Sensitivity of viruses to sera. Blood was freshly drawn from animals and healthy volunteers who had provided informed consent. Sera were isolated by

FIG. 1. Schematic representation of the genome structures and the production of pseudotype and recombinant viruses. Luciferase and gp64 genes were inserted into the full-length cDNA clone of VSV in place of the G gene and designated as ΔG-Luc and ΔG-gp64, respectively. gp64pv and VSVpv were generated by infection of VSVΔG/Luc-*G into 293T cells transiently expressing GP64 and VSVG, respectively. gp64rv was generated in 293T or BmN cells by infection with VSVΔG/gp64-*G. AcCALuc carrying the luciferase gene under the control of the CAG promoter was generated in Sf9 cells.

centrifugation at $3,000 \times g$ for 10 min at 4°C. Human and guinea pig complement sera were obtained from Sigma. Pseudotype and recombinant viruses were incubated with human, guinea pig, rat, and mouse sera, which were pretreated with or without 56°C for 30 min at 37°C for 60 min. The residue infectivity was then determined.

Reverse genetics of VSV. Recombinant VSVs were generated as described previously (41), with minor modification. Briefly, BHK cells were grown to 90% confluence on 35-mm tissue culture plates and infected with a recombinant vaccinia virus encoding T7 RNA polymerase at a multiplicity of infection (MOI) of 5. After incubation at room temperature for 1 h, cells were transfected with 4 μg of mixed plasmids encoding each component of VSV proteins (pBS-N:pBS-P:pBS-L:pBS-G, 3:5:1:8), and 2 μg of pVSV-GLPLF, pVSVΔG-Luc, or pVSVΔG-gp64 plasmid (Fig. 1) by using TransIT-LT1 (Mirus, Madison, WI). After 48 h of incubation, supernatants were passed through a filter with a pore

size of 0.22 μm (Millex-GS; Millipore, Tokyo, Japan) to remove the vaccinia virus and were then inoculated into 293T cells already transfected with pCAGVSVG at 24 h. Recovery of the progeny virus was assessed by the appearance of cytopathic effects at 24 to 36 h postinfection. VSV and VSVG-complemented (*G) recombinant viruses lacking the VSVG gene but transiently bearing the VSVG for an efficient infection were stored at -80°C . The infectious titers of the recovered viruses were determined by a plaque assay.

Production of pseudotype and recombinant viruses. Pseudotype and recombinant VSVs and baculovirus were generated as described previously (41, 43). Figure 1 schematically represents the genome structures and the production of recombinant and pseudotype VSVs and recombinant baculovirus. To generate pseudotype VSV bearing GP64 (gp64pv) and VSVG (VSVpv), 293T cells were transfected with pCAGgp64 and pCAGVSVG, respectively, and infected with VSV $\Delta\text{G/Luc-*G}$ in which the VSVG gene was replaced with the luciferase gene and was pseudotyped with the VSVG at an MOI of 0.1. The virus was adsorbed for 2 h at 37°C and extensively washed four times with serum-free DMEM. After 24 h of incubation at 37°C with 10% FBS-DMEM, the culture supernatants were centrifuged to remove cell debris and stored at -80°C . To generate a recombinant VSV carrying the gp64 gene (gp64rv), 293T cells were infected with the VSV $\Delta\text{G/gp64-*G}$ at an MOI of 5 for 2 h at 37°C and then extensively washed four times with serum-free DMEM. The culture supernatants were collected and stored at -80°C at 24 h postinfection. AcDAF-CALuc was generated by insertion of the cDNAs of human DAF and luciferase under the control of the polyhedrin and CAG promoters, respectively. AcDAF-CALuc and AcCALuc (34) were infected with Sf9 cells at an MOI of 0.1, and the culture supernatants were collected and stored at -80°C at 4 days postinfection.

Characterization of the viruses. The pseudotype and recombinant viruses were purified as described previously (34, 42, 43) and examined by immunoblotting to determine the incorporation of the GP64 into the viral particles by using an anti-GP64 antibody, AcV5. To determine the specificity of infection, gp64pv was incubated with anti-GP64 monoclonal antibody (AcV1) for 1 h at room temperature and inoculated into 293T cells. After 1 h of adsorption, the cells were washed three times with DMEM containing 10% FBS, and residual infectivity was determined at 24 h postinfection by luciferase assay. The infectivity was expressed as a percentage of the values of those treated with an isotype control antibody. To examine the effects of oligosaccharide modification of the GP64 on resistance to inactivation by human sera, gp64rv was generated in 293T cells in the presence of 2 mM deoxymannojirimycin hydrochloride (DMJ; Toronto Research Chemicals, Inc., Downsview, Ontario, Canada), a Golgi mannosidase inhibitor. Purified particles were digested with endoglycosidase H (Endo H) or peptide-N-glycosidase F (PNGase F) (Boehringer Mannheim, Mannheim, Germany), according to a protocol provided by the manufacturer, and analyzed by immunoblotting. The gp64rv generated in insect (BmN) cells was used as particles bearing high-mannose-type glycosylation.

Titration of infectivity. Infectivities of the viruses were determined by luciferase and plaque-forming assays. The relative light unit (RLU) of luciferase was determined by the Bright-Glo luciferase assay system (Promega, Madison, WI) according to a protocol provided by the manufacturer. Huh7 cells infected with VSV or gp64rv were cultured at 37°C with 0.8% methylcellulose in 10% FBS-DMEM for 24 or 48 h, respectively, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, after which the plaques were visualized by 0.1% crystal violet staining. For AcCALuc, Sf9 cells were infected with 10-fold serial dilutions of the viruses for 2 h at 27°C and cultured at 27°C with 1% low-melting-point agarose in 10% FBS SF900-II for 4 days, and then the plaques were visualized by 0.01% neutral red staining. The infectious titers of the viruses were expressed as PFU.

Transfection, immunoblotting, and immunoprecipitation. Transfection and immunoprecipitation analyses were carried out as described previously (10). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Flotation assay. The flotation assay has been described previously (27). Briefly, 10 million transfected cells were washed with ice-cold PBS containing 5 mM EDTA and then harvested with a rubber policeman. Collected cells were suspended in 1 ml of TNE buffer (25 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, a protease inhibitor mix [Nacalai Tesque, Tokyo, Japan], and 5 mM EDTA) and then homogenized with a Dounce homogenizer or suspended with a 24-gauge needle. Each homogenate was incubated for 30 min on ice with 0.2% Triton X-100 (TX-100). The lysates were mixed with Optiprep (Sigma) to 2 ml of a final concentration of 35%. This mixture was overlaid with 1 ml of 30%, 1

ml of 25%, 1 ml of 20%, and 0.5 ml of 0% Optiprep and then centrifuged at 42,000 rpm and 4°C for 10 h in an SW50 rotor (Beckman Coulter, Fullerton, CA). Each fraction was collected as 0.45 ml from the top of the centrifuging tube. The fractions were examined by immunoblotting.

Statistical analysis. The results were expressed as the mean $\pm$ the standard deviation. The significance of differences in the means was determined by using the Student *t* test.

RESULTS

Characterization of pseudotype and recombinant VSVs bearing GP64. To determine the biological functions of GP64 in gene transduction into mammalian cells, we generated pseudotype and recombinant VSVs bearing GP64 in 293T cells. To determine the efficiency of gene transduction by the viruses, gp64pv, VSVpv, and AcCALuc were inoculated into various mammalian cell lines at an MOI of 50, and the luciferase activities were determined at 24 h postinfection. Although gp64pv and VSVpv exhibited higher gene transduction into a range of cell lines than AcCALuc, no clear difference in the cell tropism was observed between gp64pv and AcCALuc (Fig. 2A). To examine the growth profiles of the viruses, infectious titers of a recombinant VSV possessing the gp64 gene (gp64rv) and a wild-type VSV were determined in Huh7 cells by plaque assay. Compared to VSV, gp64rv produced smaller plaques (Fig. 2B) and exhibited lower levels of growth kinetics (Fig. 2C). To examine the incorporation of the GP64 into viral particles, purified viral particles were examined by immunoblotting (Fig. 2D). The amounts of VSV viral particles were normalized by those of the VSV N and P proteins. Although comparable amounts of the GP64 was detected in the particles of gp64rv and gp64pv, the amounts of the VSVG in the purified particles of VSV were slightly less than those of VSVpv. To determine the specificity of GP64-dependent infection, gp64pv, VSVpv, and AcCALuc were pretreated with AcV1 antibody, a neutralizing antibody of AcNPV infection. Residual infectivities were determined by luciferase assay and are expressed as percentages of infectivity compared to those not treated with antibody (Fig. 2E). Both gp64pv and AcCALuc were neutralized by AcV1 antibody, whereas VSVpv infection was resistant to the antibody. Although we do not know why gp64pv infection was more sensitive than AcCALuc infection to neutralization by the AcV1 antibody, other baculovirus envelope proteins such as Ac23 (20, 45) in the AcCALuc particles might be attributable to the resistance to neutralization, in addition to the difference in the posttranslational modification of the GP64 expressed in insect and mammalian cells.

Resistance of the viruses to inactivation by serum. To determine the role of the GP64 on resistance to inactivation by serum, gp64pv, VSVpv, and AcCALuc were incubated with a final concentration of 40% from human, guinea pig, mouse, and rat sera (Fig. 3A). The residual infectivities after incubation with untreated sera were determined by the luciferase activities and are expressed as percentages of infectivity compared to those incubated with heat-inactivated sera. Although gene transduction by AcCALuc was severely or completely impaired by treatment with fresh sera, gp64pv exhibited clear resistance to serum inactivation (Fig. 3A). We further examined the effects of commercially available human and guinea-pig complements on the infectivity of the viruses. Although gene transduction by VSVpv and AcCALuc was inhibited by

FIG. 2. Characteristics of gp64pv and gp64rv. (A) Efficiency of gene transduction into various cell lines by gp64pv, VSVpv, and AcCALuc. The viruses were inoculated into various mammalian cell lines at an MOI of 50, and the luciferase activities were determined at 24 h postinfection. Infectivities of the viruses were determined by relative luciferase units (RLU). (B) Focus formation of VSV and gp64rv. Huh7 cells were infected with serial 10-fold dilutions of VSV and gp64rv and stained with 0.1% of crystal violet at 24 and 48 h postinfection, respectively. (C) Growth kinetics of gp64rv and VSV. The viruses were inoculated into Huh7 cells, and infectious titers in the culture supernatants were determined by using a plaque-forming assay. (D) Immunoblot analyses of the purified particles. Incorporation of GP64 and VSVG into viral particles was examined by immunoblotting. VSV-N/P proteins were used for the standardization of VSV particles. (E) Neutralization of gene transduction by anti-GP64 monoclonal antibody (AcV1). gp64pv, VSVpv, and AcCALuc were incubated with the indicated dilutions of the antibody for 1 h at room temperature and inoculated into Huh7 cells. The residual infectivity was expressed as a percentage of that of the untreated controls. The results shown are from three independent assays, with error bars representing the standard deviations.

incubation with the complement sera, gp64pv exhibited resistance up to an 80% concentration of the sera (Fig. 3B). Next, to examine the effect of GP64 glycosylation on sensitivity to serum inactivation, we took advantage of the fact that gp64rv can be generated in both mammalian and insect cells, because VSV is capable of propagating not only in various mammalian cells but also in some insect cells. In the present study, we used BmN cells to generate gp64rv in insect cells, because the infectivity of VSV in this cell line was more efficient than that in Sf9 cells, which are generally used for the amplification of the AcCALuc baculovirus. The gp64rv generated in human 293T cells exhibited resistance to inactivation by human and guinea pig sera, whereas the gp64rv generated in insect BmN cells was

more sensitive to inactivation by the sera (Fig. 3C). These results suggest that the GP64 expressed in mammalian cells confers viral particles with the ability to resist to serum inactivation.

Glycosylation of GP64 exhibits no effect on resistance to serum inactivation. To further determine the effect of glycosylation of GP64 on resistance to serum inactivation, gp64rv was generated in 293T cells in the presence or absence of 2 mM DMJ, a Golgi mannosidase inhibitor. Purified particles were digested with Endo H or PNGase F and examined by immunoblotting. The GP64 in the purified gp64rv particles produced in 293T cells irrespective of DMJ treatment exhibited a single 60-kDa band, whereas two bands were detected in

FIG. 3. Resistance of the viruses to inactivation by serum. (A) gp64pv, VSVpv, and AcCALuc were incubated at 37°C for 60 min with a final concentration of 40% human, mouse, and rat sera treated with (Inactivated) or without (Untreated) at 56°C for 30 min and inoculated into Huh7 cells. Luciferase activities were determined at 24 h postinfection. (B) Sensitivity of gp64rv, VSVpv, and AcCALuc to sera from human and guinea-pig. The viruses were incubated with various concentrations of human and guinea-pig sera for 1 h at room temperature and inoculated into Huh7 cells. (C) Sensitivity of gp64rv produced in 293T cells (gp64rv/293T) or in BmN cells (gp64rv/BmN) to sera from humans and guinea pigs treated with (inactivated) or without (untreated) at 56°C for 30 min. Residual activities were expressed as the percent RLU of those obtained by treatment with the heat-inactivated sera. The results shown are from three independent assays, with error bars representing the standard deviations.

those in BmN cells, probably due to the difference in post-translational modifications of the glycoproteins. Although treatment with Endo H reduced the size of the GP64 in the gp64rv particles generated in 293T cells in the presence of DMJ and in BmN cells, those generated in 293T cells in the

FIG. 4. Effects of glycosylation of GP64 on resistance to serum inactivation. (A) The gp64rv particles generated in 293T cells in the presence or absence of a Golgi mannosidase inhibitor, DMJ, and those generated in BmN cells were treated with endoglycosidase H (lane H) or peptide-N-glycosidase F (lane F) and examined by immunoblotting with anti-GP64 monoclonal antibody (AcV5). Lane C, untreated control. (B) The gp64rv generated in the presence or absence of DMJ were incubated with human and guinea pig sera treated with (inactivated) or without (untreated) at 56°C for 30 min at a final concentration of 80% for 60 min at 37°C and inoculated into Huh7 cells. Luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with the error bars representing the standard deviations.

absence of DMJ exhibited more resistance. Treatment with PNGase F reduced the sizes of the GP64 in the gp64rv particles irrespective of the production conditions (Fig. 4A). These results suggest that GP64 in the gp64rv particles generated in 293T cells in the absence or presence of DMJ possesses complex- or hybrid-type and high-mannose-type glycans, respectively. Next, the gp64rv generated in 293T cells in the presence or absence of DMJ were incubated with human and guinea-pig sera at a final concentration of 80% for 60 min at 37°C and inoculated into Huh7 cells, and the luciferase activities were determined at 24 h postinfection. There was no significant difference in the resistance of the viruses to the inactivation by sera (Fig. 4B). These results suggest that glycosylation of the GP64 has no effect on the sensitivity of gp64rv to inactivation by sera.

Incorporation of DAF into viral particles generated in human cells. It has been reported that incorporation of the complement regulatory proteins such as CD55/DAF from host cells into viral particles contributes to the acquisition of resistance to the complement inactivation of human enveloped viruses, such as HIV, human T-cell leukemia virus type I, and human cytomegalovirus (2, 30, 37). To determine the incorporation of DAF into viral particles, purified particles of gp64rv and VSV generated in 293T cells, gp64rv generated in BmN cells, and AcCALuc generated in Sf9 cells were examined by immunoblotting with antibody to human DAF. A clear incorporation of human DAF was detected in the particles of gp64rv propagated in 293T cells, whereas a small amount of human DAF was observed in those of VSV propagated in 293T cells (Fig.

FIG. 5. Incorporation of DAF into viral particles and subcellular localization of GP64. (A) The purified particles of gp64rv and VSV generated in 293T cells, gp64rv generated in BmN cells, and AcCALuc generated in Sf9 cells were examined by immunoblotting with antibodies to human DAF, VSVΔG, GP64, VSVG, and VP39. (B) Subcellular localization of the GP64 analyzed by flotation assay. 293T cells transfected with pCAGgp64, lysed in 0.2% TX-100 TNE buffer at 2 days posttransfection, fractionated after Optiprep gradient centrifugation, and analyzed by immunoblotting with antibodies to GP64, human DAF, caveolin 1 and calreticulin. (C) Interaction of GP64 and human DAF. Lysates of 293T cells expressing GP64 and/or DAF were immunoprecipitated with anti-DAF antibody, and the precipitates were examined by immunoblotting with anti-GP64 antibody.

5A). No incorporation of human DAF was detected in the particles of gp64rv and AcCALuc generated in insect cells. These data are consistent with the serum resistance of gp64rv produced in 293T cells but not of that produced in BmN cells (Fig. 3C). The GP64 proteins incorporated into the viral particles generated in insect cells (gp64rv/BmN and AcCALuc)

were smaller than those in 293T cells (gp64rv/293T), reflecting the difference in posttranslational modifications of the protein, as seen in Fig. 4A. These results suggest that incorporation of human DAF into viral particles participates in the acquisition of resistance to serum inactivation of the VSVs bearing GP64 generated in mammalian cells.

Subcellular localization of the GP64 and interaction with human DAF. DAF is a glycosylphosphatidylinositol (GPI)-anchored protein, localized in lipid rafts, which are resistant to solubilization by TX-100 at 4°C, and isolated in a detergent-resistant membrane (DRM) fraction (19). We used a flotation assay to examine the subcellular localization of the GP64 in cells expressing the protein. 293T cells transfected with pCAGgp64, lysed in 0.2% TX-100 TNE buffer at 2 days post-transfection, fractionated after Optiprep gradient centrifugation, and analyzed by immunoblotting. The GP64 was fractionated in the DRM fraction together with human DAF and a marker protein of lipid rafts, caveolin 1, but not with calreticulin, a marker protein of endoplasmic reticulum, indicating that GP64 colocalized with GPI-anchored proteins including human DAF in the DRM domain of mammalian cells (Fig. 5B). To determine the interaction of the GP64 with human DAF, 293T cells were cotransfected with expression plasmids encoding gp64 and human DAF and precipitated with anti-human DAF monoclonal antibody (IA10), after which the immunoprecipitates were examined by immunoblotting with anti-GP64 monoclonal antibody (AcV5) (Fig. 5C). The interaction of human DAF and GP64 was detected in cells coexpressing both proteins. These results suggest that GP64 and human DAF are colocalized in the DRM domain and that the viral particles bearing the GP64 incorporate human DAF during the budding process.

Knockdown of DAF expression impairs the serum resistance of the viruses. To determine the role of human DAF incorporation into viral particles in resistance to complement inactivation, 293T cells were transfected with a plasmid encoding shRNA targeted to human DAF and a DAF knockdown cell line, DAFKD293T. The results showed a clear reduction of DAF expression (Fig. 6A, left). The incorporation of DAF into the gp64pv particles generated in the DAFKD293T cells was severely reduced compared to those generated in the control cells (Fig. 6A, right). The gp64pv generated in the DAFKD293T cells exhibited more sensitivity to the inactivation by the human serum than that generated in the control cells (Fig. 6B). These data suggest that the reduction of DAF incorporation into viral particles induces a loss of resistance to serum inactivation.

Incorporation of human DAF into VSV and baculovirus particles confers resistance to complement inactivation. To further determine the effect of human DAF incorporation into viral particles on resistance to serum inactivation, we used a CHO cell line constitutively expressing human DAF and human CD59 proteins (24). Immunoblotting analyses revealed the expression of human DAF in CHO3B2A cells but not in parental CHO cells and the incorporation of human DAF into gp64rv particles generated in CHO3B2A cells (gp64rv/3B2A) but not in parental CHO cells (gp64rv/CHO) (Fig. 7A, left). However, only a twofold increase in resistance to the human serum was observed in the gp64rv particles generated in CHO3B2A cells compared to those produced in the parental CHO cells, in spite of the abundant incorporation of human

FIG. 6. Knockdown of DAF expression impairs the serum resistance of the viruses. (A) Expression of human DAF in the control and DAFKD293T cell lines and incorporation of human DAF into the viral particles were determined by immunoblotting with antibodies to human DAF, VSVΔG, GP64, and actin. The amounts of proteins in cells and particles were normalized by those of actin and VSV-N/P protein, respectively. (B) Sensitivity of gp64pv generated in control cells (gp64pv/Cont) and in DAFKD293T cells (gp64pv/DAFKD) to human serum. The viruses were incubated at 37°C for 60 min with a final concentration of 80% of human sera treated with or without at 56°C for 30 min and inoculated into Huh7 cells. The luciferase activities were determined at 24 h postinfection. Residual activities were expressed as the percent RLU compared to those obtained by the treatment with the heat-inactivated sera. The results shown are from three independent assays, with error bars representing the standard deviations.

DAF into the viral particles (Fig. 7A, right). As previously indicated, it has been reported that recombinant baculovirus displaying gp64-human DAF fusion proteins in the viral particles exhibited resistance to complement inactivation (13). To examine the roles of human DAF incorporation into viral particles in resistance to complement inactivation, we established an insect cell line, Sf9DAF, stably expressing human DAF. The expression of human DAF in Sf9DAF cells but not in parental Sf9 cells, and the incorporation of human DAF into AcCALuc particles generated in Sf9DAF cells (AcCALuc/DAF) but not in those generated in parental Sf9 cells were detected by immunoblotting (Fig. 7B, left). AcCALuc/DAF exhibited more resistance to inactivation by human serum than AcCALuc (Fig. 7B, right). To further confirm the role of human DAF on resistance to human sera, we generated a recombinant baculovirus, AcDAF-CALuc, encoding human DAF gene under the control of the polyhedrin promoter to efficiently incorporate human DAF into the viral particles. Human DAF was detected in cells infected with AcDAF-CALuc and in the purified particles by immunoblotting (Fig. 7C, left). Consistent with the data in Fig. 7B, AcDAF-CALuc exhibited more resistance to the inactivation by human complement serum than did AcCALuc (Fig. 7C, right). Collectively, these results indicate that incorporation of human DAF into viral particles through interaction with GP64 confers on them resistance to serum inactivation.

DISCUSSION

Welsh et al. were the first to report that murine leukemia viruses and Moloney sarcoma virus pseudotyped with the envelope of gibbon ape leukemia virus or simian sarcoma-associated virus, were inactivated by fresh but not heat-inactivated human serum (46, 47). Inactivation of retrovirus vectors by human complement was shown to be controlled by the cell line used to produce the viral particles and by viral envelope determinants (39), and the retrovirus vectors produced in human

HT1080 cells were shown to be resistant to inactivation by human serum (5).

Baculovirus is an efficient vector not only for a high level of gene expression in insect cells but also for gene transfer into mammalian cells *in vitro* and *in vivo* (3, 11, 34). However, the complement system has been defined to represent a potent primary barrier to *in vivo* application of baculovirus vectors produced in insect cells (11). The recombinant baculovirus possessing a chimeric GP64 envelope protein fused with human DAF exhibited resistance to the complement-mediated inactivation (13). Furthermore, the recombinant baculoviruses bearing VSVG exhibited more resistance to complement inactivation by animal sera than did the unmodified baculovirus (42). In contrast, it is known that pseudotype lentiviruses bearing baculovirus GP64 exhibited more resistance to serum inactivation and a longer expression of the transgenes than those bearing the VSVG (35, 36). However, the molecular mechanisms underlying resistance to complement inactivation by pseudotypes bearing the GP64 has not been characterized yet. In the present study, we have shown that human DAF incorporation through interaction with the baculovirus GP64, but not the posttranslational modification of the GP64, is crucial for the acquisition of resistance to the serum inactivation of viral particles.

It was shown previously that lentivirus vectors pseudotyped with both GP64 and a chimeric GP64 fused with human DAF and the GP64 pseudotypes produced in DAF-overexpressing cells acquired resistance to complement inactivation (9). Furthermore, among the pseudotyped lentiviruses produced through an incorporation of each CRP, DAF-containing lentiviruses generated in the viral producer cells overexpressing a native form of DAF exhibited potent resistance to inactivation by human complement (33). However, our data indicated that pseudotype and recombinant VSVs bearing the GP64 produced in naive mammalian cells actively incorporate sufficiently large amounts of human DAF into viral particles to confer the particles with resistance to serum inactivation.

FIG. 7. Incorporation of human DAF into viral particles confers resistance to complement inactivation. (A) Expression of human DAF in CHO and CHO3B2A cell lines and incorporation of human DAF into gp64rv particles generated in the cells were determined by immunoblotting with antibodies to human DAF, VSVΔG, GP64, and actin (left). The amounts of proteins in cells and the gp64rv particles were normalized by those of actin and VSV-N/P protein, respectively. Resistance to the human serum of the gp64rv generated in the CHO cell lines (right). (B) Expression of human DAF in Sf9 and Sf9DAF cell lines and incorporation of human DAF into AcCALuc particles generated in the cell lines were determined by immunoblotting by antibodies to human DAF, VP39, GP64, and GAPDH (left). The amounts of proteins in cells and in the AcCALuc particles were normalized by those of GAPDH and VP39 protein, respectively. Resistance to the human serum of AcCALuc generated in the Sf9 cell lines (right). (C) Expression of human DAF in Sf9 cells infected with AcCALuc or AcDAF-CALuc and incorporation of human DAF into the viral particles were determined by immunoblotting by antibodies to human DAF, VP39, GP64, and GAPDH (left). The amounts of proteins in cells and the viral particles were normalized by those of GAPDH and VP39 protein, respectively. Resistance to the human serum of AcCALuc and AcDAF-CALuc generated in Sf9 cells (right). The viral particles were incubated at 37°C for 60 min with a final concentration of 80% of human sera treated with (inactivated) or without (untreated) at 56°C for 30 min and inoculated into Huh7 cells. Luciferase activities were determined at 24 h postinfection. Residual activities were expressed as the percent RLU compared to those obtained by the treatment with the heat-inactivated sera. The results shown are from three independent assays, with error bars representing the standard deviations. Asterisks indicate a significant difference versus control viruses (*, $P < 0.05$).

There was a loss of resistance in the gp64 recombinant VSV particles produced in the DAF knockdown cells and a gain of resistance in the recombinant baculoviruses produced in the insect cells stably expressing human DAF. These findings indicate that the interaction of the baculovirus GP64 and human DAF is involved in the acquisition of complement resistance to the viral particles.

The DAF is a GPI anchor protein and is localized in the lipid rafts. HIV enters cells by interacting with a major receptor molecule, CD4, which is localized in the lipid rafts. The virus then egresses from the plasma membrane via the lipid rafts that incorporate human DAF into the virus particles (21, 28). Flotation analyses revealed that both GP64 and human DAF fractionated in the lipid raft fractions, suggesting that the gp64 pseudotype particles bud from the plasma membrane, including lipid rafts bearing human DAF on the viral surface, through direct or indirect interaction with GP64, as immunoprecipitation analysis shows. In the present study, we do not examine the possibility that other CRPs participate in the acquisition of resistance to complement inactivation. The gp64 pseudotype particles generated in the human DAF knockdown 293T cells and the gp64 recombinant viruses produced in the CHO cell line stably expressing human DAF and CD59 proteins exhibited only a 50% reduction and increase compared to the controls, respectively. Although we do not yet know why the manipulation of human DAF in mammalian cells had only a marginal effect, other CRPs in 293T cells and some homologues in CHO cells might be involved in the acquisition of serum resistance. Further studies are needed to clarify the possibility that CRPs participate in the acquisition of resistance of viral particles to serum inactivation.

In addition, we have shown that human DAF expressed in insect cells was capable of conferring resistance to serum inactivation through an efficient incorporation of human DAF into AcNPV particles. To circumvent inactivation by human serum of baculovirus vectors for *in vivo* gene delivery, the Sf9DAF cell line stably expressing human DAF established in the present study should be a useful tool for producing serum-resistant baculoviruses in addition to constructing recombinant baculoviruses carrying the human DAF gene.

Efficient gene transduction into appropriate target cells is a crucial step in gene therapy, and a complement system is a critical barrier to *in vivo* gene delivery by viral vectors. In the present study, we have demonstrated that baculovirus GP64 is not only an envelope protein mediating virus entry through an endocytotic pathway but also a key molecule conferring resistance to serum inactivation through the recruitment of human DAF into viral particles. The acquisition of resistance to the complement by the incorporation of baculovirus GP64 represents a step in the development of novel viral vectors for improved gene therapy.

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