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Posttranslational Modification of α-Dystroglycan, the Cellular Receptor for Arenaviruses, by the Glycosyltransferase LARGE Is Critical for Virus Binding

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The receptor for lymphocytic choriomeningitis virus (LCMV), the human pathogenic Lassa fever virus (LFV), and clade C New World arenaviruses is α-dystroglycan (α-DG), a cell surface receptor for proteins of the extracellular matrix (ECM). Specific posttranslational modification of α-DG by the glycosyltransferase LARGE is critical for its function as an ECM receptor. In the present study, we show that LARGE-dependent modification is also crucial for α-DG's function as a cellular receptor for arenaviruses. Virus binding involves the mucin-type domain of α-DG and depends on modification by LARGE. A crucial role of the LARGE-dependent glycosylation of α-DG for virus binding is found for several isolates of LCMV, LFV, and the arenaviruses Mobala and Oliveros. Since the posttranslational modification by LARGE is crucial for α-DG recognition by both arenaviruses and the host-derived ligand laminin, it also influences competition between virus and laminin for α-DG. Hence, LARGE-dependent glycosylation of α-DG has important implications for the virus-host cell interaction and the pathogenesis of LFV in humans.

Arenaviruses merit significant attention both as powerful models to study viral pathogenesis and as important human pathogens (8, 41). Two groups of arenaviruses are presently recognized, the Old World arenaviruses, with lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LFV) as prototypes, and the New World arenaviruses. LCMV infection of its natural host, the mouse, provided novel concepts in immunology and virology that have been extended to other viruses, bacteria, and parasites (41). LFV is the causative agent of a severe hemorrhagic fever in humans with a mortality of approximately 15%. With over 200,000 infections per year and several thousand deaths, LFV represents a major threat for human health and a severe humanitarian problem (23, 38).

The bisegmented negative-strand genome of arenaviruses consists of two single-stranded RNA species, a larger segment encoding the virus polymerase (L) and a small zinc finger motif protein (Z), and a smaller segment encoding the virus nucleoprotein (NP) and glycoprotein (GP) precursor (GPC). GPC is processed into the peripheral GP1 and the transmembrane GP2. GP1 is implicated in receptor binding, and GP2 is structurally similar to the fusion-active, membrane-proximal portions of the GPs of other enveloped viruses.

α-Dystroglycan (α-DG) has been identified as the cellular receptor for LCMV, Lassa fever virus (LFV), and clade C New World arenaviruses (10, 52). Initially encoded as a single protein, DG is cleaved into α-DG, a peripheral protein, and β-DG, a membrane protein (28). α-DG is comprised of three distinct domains: an N-terminal globular domain, a central mucin-type domain, and a C-terminal globular domain. DG is expressed in most tissues (14) and plays a critical role in the cell-mediated assembly of basement membranes (26). At the extracellular site, α-DG undergoes high-affinity interactions with the extracellular matrix (ECM) proteins laminin, agrin, and perlecan (18, 19, 22, 55) and with neurexins (53). α-DG is noncovalently associated with β-DG. β-DG associates intracellularly with dystrophin and utrophin (31), which bind to the actin cytoskeleton, and with the signal transduction molecules grb2, MEK, extracellular signal-regulated kinase (ERK), and focal adhesion kinase (11, 51, 59).

In the host cell, α-DG is subject to a remarkably complex pattern of posttranslational modifications, including specific glycosylation, which is critical for its function as an ECM receptor (12, 39, 40). Known or putative glycosyltransferases implicated in α-DG glycosylation are targeted in a number of congenital muscular dystrophies (40). The phenotypes of these diseases are caused primarily by the aberrant glycosylation of α-DG and its loss of function as an ECM receptor. The genes involved in these disorders are fukutin in Fukuyama congenital muscular dystrophy (FCMD), the protein *O*-mannosyltransferase POMT1 in Walker-Warburg syndrome (4, 17, 37), the protein *O*-mannose β1,2-*N*-GlcNAc transferase POMGnT1 in muscle-eye-brain disease (MEB) (60), and the glycosyltransferase LARGE in human congenital muscular dystrophy 1D and the spontaneous myodystrophy mouse Large^{myd} (7, 24, 25, 35, 44). Similarities of the clinical and biochemical phenotypes of patients with these muscular dystrophies and the Large^{myd} mouse indicate that the same glycosylation pathways may be affected (27, 39). Among the glycosyltransferases implicated in α-DG modification, LARGE was found to play a key role in the biosynthesis of functional DG. Modification of the N-ter-

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minal part of the mucin-like domain of α-DG by LARGE is essential for its function as an ECM receptor (30). Recognition by LARGE involves α-DG's N-terminal domain, which is subsequently cleaved by a convertase-like activity. Further, LARGE can functionally bypass defects in other enzymes involved in the functional glycosylation of α-DG, such as fukutin, POMT1, and POMGnT1 (3).

The binding sites of the arenaviruses LCMV and LFV map to the N-terminal domain of α-DG and the N-terminal region of the mucin-type domain (32, 33), regions involved in LARGE-dependent glycosylation and proteolytic cleavage. A possible impact of the posttranslational modification of α-DG on virus binding affinity would be of interest, as studies on the pathogenesis of LCMV revealed a crucial role and direct association of α-DG binding affinity with tissue tropism and disease phenotype. LCMV isolates with a high binding affinity to α-DG efficiently target dendritic cells (DCs), a crucial population of antigen-presenting cells involved in the development of the innate and adaptive antiviral immune response resulting in a generalized immunosuppression and persistent infection (5, 48–50). In contrast, isolates with low α-DG binding affinity are unable to infect sufficient numbers of DCs to suppress the immune system. As a result, the acute viral infection is terminated and virus is cleared. A similar correlation between high α-DG binding affinity and disease was discovered in a different paradigm, myelin formation by Schwann cells, which express high levels of α-DG at their surfaces. LCMV isolates with high α-DG binding affinity but not low affinity binders efficiently target Schwann cells and render them incapable to form myelin (45). The receptor binding characteristics of immunosuppressive LCMV isolates are similar to those of the human pathogenic LFV, which also binds α-DG with an affinity in the low nanomolar range and depends on α-DG for infection of cells (10, 32).

The binding sites of LCMV and LFV on α-DG overlap with domains recognized by ECM proteins, resulting in a competition for α-DG binding (32, 33, 45). As a consequence, viruses with high binding affinity to α-DG but not low affinity binders are able to displace ECM ligands from α-DG, allowing the virus infection of specific α-DG-expressing target cells, resulting in a reproducible disease phenotype (33, 45, 48, 50).

The present study demonstrates that posttranslational modification by the glycosyltransferase LARGE is crucial for α-DG's function as a cellular receptor for arenaviruses. Virus binding involves the mucin-type domain of α-DG and critically depends on modification by LARGE. LARGE-dependent glycosylation is critical for the recognition of α-DG by several isolates of LCMV, LFV, and the arenaviruses Mobala and Oliveros. Modification of α-DG by LARGE further influences the ability of the virus to compete with the ECM protein laminin for receptor binding. Considering the crucial role of α-DG binding affinity for tissue tropism and viral pathogenesis, LARGE-dependent modification of α-DG has important implications for the virus-host interaction in vivo.

MATERIALS AND METHODS

Animals. We obtained homozygous myodystrophy LARGE^{myd} mice and control littermates LARGE$^{+/+}$ by mating heterozygous pairs provided by Jackson laboratories. Female and male C57BL/6 mice were obtained from the rodent-breeding colony at The University of Iowa and were bred and maintained under pathogen-free conditions.

Proteins and antibodies. α-DG was purified from mouse tissues as described previously (39). Mouse laminin-1 and fibronectin were from GIBCO-BRL (Gaithersburg, MD). Monoclonal antibodies (MAbs) 113 (anti-LCMVNP), 36.1, 33.1, and 83.6 (anti-LCMVGP) have been described previously (9, 58), as have MAb IIH6 anti-α-DG, anti-β-DG antibody AP83, and the anti-core protein α-DG antibody FPD (28, 39). Polyclonal rabbit anti-laminin-1 and mouse immunoglobulin M (IgM) isotype control antibody were from Sigma (St. Louis, MO). Rhodamine-X-conjugated anti-mouse IgM, biotinylated goat anti-mouse IgG, and mouse anti-human IgG Fc were from Jackson ImmunoResearch (West Grove, PA), and rabbit anti-influenza hemagglutinin (HA; Y11) was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies and streptavidin-HRP were from Pierce.

Construction of the DGFc5 mutants. The α-DG–Fc fusion proteins DGFc1 through -5 have been described previously (33). For the construction of the DGFc5 deletion mutants, rabbit DG cDNA fragments were amplified by PCR and inserted into the eukaryotic expression vector DGFc5 pcDNA3, resulting in the deletion mutants DGFc5ΔH30-R168, DGFc5182-H315, DGFc5ΔH30-A316, and DGFc5ΔH30-A312. For the construction of the noncleavable DGFc5 mutants, the amino acid changes R312K and R312A were introduced into DGFc5 pcDNA3. For the construction of the DGFc5 deletion mutants, the DG deletion mutants DGI (ΔH30-R168), DGD (ΔC182-H315), and DGE (ΔH30-A316) (33) were used. For the construction of DGFc5 (ΔH30-R168) pcDNA3, a 5′ KpnI-XhoI fragment containing the deletion ΔH30-R168 was excised from the mutant DGI (wild-type DG ΔH30-R168) and inserted into the construct DGFc5 pcDNA3, resulting in DGFc5 ΔH30-R168. To create DGFc5 (ΔC182-H315) pcDNA3, a 5′ KpnI-XhoI fragment containing the deletion ΔC182-H315 was excised from the mutant DGD (wild-type DG ΔC182-H315) and inserted into the construct DGFc5 pcDNA3, resulting in DGFc5 ΔC182-H315. For the construction of DGFc5 (ΔH30-H316) pDisplay, a 3′ XhoI-XhoI fragment was excised from the construct DGFc5 and inserted into the construct DGE (wild-type DG ΔH30-H316) pDisplay, resulting in DGFc5 ΔH30-H316 pDisplay. To create DGFc5 (ΔH30-H312) pcDNA3, for the deletion of amino acids 30 through 312, a ScaI restriction site was introduced into the cDNA of rabbit DG at position 256 by PCR amplification of the cDNA fragment FP-DGN1b, using rabbit DG cDNA (28) as a template. FP-DGN1b was cut with HindIII and ScaI and ligated to the PCR-amplified cDNA fragment NTDf-Eub, cut with XhoI, and inserted into DGFc5 pcDNA3.

For the construction of DGFc5R312A, the following PCR fragments were amplified and ligated into DGFc5 pcDNA3: DGC2f-CM1 cut with SacI and ScaI and CMf-Gnb cut with ScaI and XhoI. For the construction of DGFc5R312K, DGC2f-CMK was cut with SacI and CMf-Gnb was cut with ScaI and XhoI.

The following primer sequences were used: FP, 5′-CCCACTGCTTACTGG CTT-3′; DGN1b, 5′-GGGCCAAGTACTCTGAGCCACGGCCAC-3′; NTDf, 5′-CAGATCCATGCCACACCCACA-3′; Eub, 5′-GGCGTCCACCCTGTCGA TGTGGTT-3′; DGC2f, 5′-GCGTGCGCATGTGCTGCCGAGGAGCCG-3′; CM1, 5′-AATAGTACTATGGATCTGCGCTCGGATACGCTTGGG-3′; CMf, 5′-ATTAGTACTCCCACACCTGTCACTGCC-3′; and Gnb, 5′-CACGTACCC GGGAATGGTCACCGTTGC-3′.

Virus strains, purification, and quantification. The recombinant adenoviruses (AdV) Ad5/LARGE-enhanced green fluorescent protein (EGFP) and Ad5/EGFP have been described previously (3), as have the origin, passage, and characteristics of LCMV ARM53b, clone-13 (cl-13), WE54, and WE2.2 (2, 15, 56). LCMV TNPBL42 and PBL673, CD4-1, and CD8-4 have been described previously (48). Seed stocks of all viruses were prepared by growth in BHK-21 cells. Purified virus stocks were produced and titers determined as described previously (15).

LFV (Josiah), Mobala, Oliveros, and Guanarito were grown in Vero E6 cells in a BSL4 facility, polyethylene glycol precipitated, and γ-inactivated at the Centers for Disease Control and Prevention in Atlanta, GA, after the method described previously (16). Inactivation was verified by infection assay.

Recombinant vesicular stomatitis virus (VSV) pseudotyped with LFVGP or VSVGP was generated as described previously (32, 43). Briefly, HEK293T cells were transfected with pC-LFVGP, pC-VSVGP, or pC-EGFP using lipofectamine. Thirty-two hours posttransfection, cells were infected with VSVΔG* (54) at a multiplicity of infection (MOI) of 3 PFU/cell. After 1 h, cells were extensively washed, fresh medium was added, and cells were cultured for 20 h at 37°C in 5% CO_2. Supernatants were collected, cleared by centrifugation, and frozen at −70°C. Virus titers were determined by infection of HeLa cell monolayers as described previously (32).

Immunoblotting and VOPBA. Proteins were separated by gel electrophoresis and transferred to a nitrocellulose membrane. After blocking in 5% (wt/vol) skim

milk in phosphate-buffered saline (PBS), membranes were incubated with anti-human IgG Fc (1:1,000) and MAb IIH6 and antibodies, FTP, AP83, and Y11 (10 μg/ml) in 2% (wt/vol) skim milk–PBS for 12 h at 6°C. Secondary antibodies coupled to HRP were applied 1:5,000 in PBS–0.1% (wt/vol) Tween 20 for 1 h at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL substrate (Pierce). Laminin overlay assay (LOA) was performed as described previously (39). Virus overlay protein binding assay (VOPBA) with LCMV cl-13 and γ-inactivated LFV, Mobala, and Oliveros was performed as described previously (10). α-DG was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred to nitrocellulose and probed for virus binding using LCMV cl-13 and γ-inactivated LFV, Mobala, and Oliveros in concentrations of 1×10^7 PFU/ml. Bound virus was detected with monoclonal antibodies 33.6 and 83.6 (undiluted hybridoma supernatant) using ECL.

Binding of LCMV and laminin to α-DG–Fc fusion proteins. α-DG–Fc fusion proteins were expressed and purified as described previously (33). Production of DGFc5 in cells overexpressing LARGE was carried out as described previously (30).

Solid-phase laminin binding assays were performed as described in reference 39. For binding of virus to α-DG–Fc fusion proteins, 20 μg/ml purified proteins in PBS was immobilized in microtiter plates for 2 h and nonspecific binding blocked with 1% (wt/vol) bovine serum albumin (BSA)–PBS. LCMV cl-13 (10^7 PFU/ml) in 1% (wt/vol) BSA–PBS was applied for 12 h at 6°C. Bound virus was detected with MAb 83.6 (20 μg/ml) in 1% (wt/vol) BSA–PBS using an HRP-conjugated secondary antibody. ARM53b was applied at 10^8 PFU/ml and detected with MAb 83.6 (20 μg/ml), followed by biotinylated goat anti-mouse IgG (1:500) and HRP-conjugated streptavidin (1:500). For binding of α-DG–Fc fusion proteins to LCMV, purified viruses (10^7 PFU/ml) were immobilized in microtiter plates and incubated with the α-DG–Fc fusion proteins. Bound α-DG–Fc was detected with HRP-conjugated anti-human IgG Fc (1:1,000) or a combination of mouse anti-human IgG Fc (1:500) and HRP-conjugated goat anti-mouse IgG (1:500). Assays were developed with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate and the optical density at 405 nm recorded in an enzyme-linked immunosorbent assay (ELISA) reader. Background binding to BSA was subtracted. Competition of the binding of LCMV to DGFc5 with ECM proteins was performed as described previously (45).

Infection of mouse embryonic stem (ES) cells. Neutralization of LCMV infection in $DG^{+/-}$ with soluble α-DG was carried out as described previously (34). For blocking with MAb IIH6, 10^4 cells/well were plated in gelatin-pretreated 96-well plates. After 24 h, cells were blocked with MAb IIH6 or unrelated mouse IgM for 2 h at 4°C. Next, 200 PFU of LCMV was added for 45 min and infection assessed after 16 h by immunofluorescence staining for LCMVNP as described previously (50).

AdV-mediated gene transfer of wild-type DG and the DG deletion mutants DGΔH30-A316 was performed as described previously (33). After 48 h, the indicated PFU of LCMV or infectious units of VSV pseudotypes were added to AdV-transfected $DG^{-/-}$ ES cells, as well as untreated control $DG^{-/-}$ and $DG^{+/-}$ cells, and incubated for 1 h at 37°C. The viral particles were removed, cells washed twice with DMEM, and fresh medium added. After the time points indicated, infected cells were quantified by either immunofluorescence staining for LCMVNP or direct detection of EGFP by direct fluorescence microscopy in case of the VSV pseudotypes.

Overexpression of LARGE in Vero cells, immunocytochemistry, and laminin blocking. AdV-mediated gene transfer to Vero cells was performed as described previously (33). After 24 h, 10^4 cells were transferred to eight-well LabTek tissue culture chamber slides (Nunc) coated with 10 μg/ml laminin-1. After 24 h, cells were fixed with 2% (wt/vol) formaldehyde. The MAb IIH6 (10 μg/ml) was applied for 2 h at room temperature, and rhodamine-X conjugated anti-mouse IgM (1:200) for 1 h. EGFP was detected by direct immunofluorescence. Images were captured using a Zeiss Axiovert S100 microscope (Carl Zeiss Inc., Thornwood, NY) with a 20× objective and an AxioCam digital camera.

Blocking of LCMV infection with laminin-1 was carried out as described previously (33). Briefly, 10^4 Vero cells/well previously infected with Ad5/LARGE-EGFP or Ad5/EGFP were plated in laminin-coated LabTek slides. After 12 h, the culture medium was replaced with fresh medium containing 0, 10, 20, or 50 μg/ml laminin-1. After another 12 h, 200 PFU LCMV or VSV pseudotypes was added per well, which were then incubated for 45 min at 37°C. After 16 h, infected cells were determined by immunofluorescence staining for LCMVNP or direct fluorescence excitation in the case of the VSV pseudotypes.

RESULTS

The N-terminal region of the mucin-type domain of α-DG is necessary and sufficient for virus binding. During biosynthesis, the mucin-type domain of α-DG undergoes specific glycosylation and the N-terminal globular domain is subject to proteolytic cleavage (30). Since both regions of α-DG have been implicated in virus binding (33), we analyzed the role of these posttranslational modifications for α-DG's function as a receptor for LCMV.

First, we addressed the role of proteolytic cleavage of α-DG for virus binding. As observed with full-length DG, a soluble recombinant C-terminal fusion protein of α-DG with human IgG1 Fc is cleaved at the recognition site R309RIR312 by a furin-type convertase (Fig. 1A). α-DG–Fc fusion proteins containing the α-DG sequences 30 through 181 (DGFc1), 30 through 316 (DGFc2), 30 through 408 (DGFc3), and 30 through 485 (DGFc4) and full-length α-DG 30 through 653 (DGFc5) (Fig. 1A) were produced in HEK293T cells by transient expression. DGFc1 through -5, purified from cell lysates by protein A affinity chromatography, showed the expected molecular masses in Western blot analysis under reducing conditions (Fig. 1B). As reported previously (30), DGFc3 through -5, when purified from supernatant, showed significantly lower molecular masses than the forms isolated from cell lysates due to the proteolytic cleavage of amino acids 30 through 312 (Fig. 1B). These processed forms of DGFc3 through -5, but not DGFc1 and DGFc2, showed strong, specific binding to LCMV cl-13 in VOPBA and in a nondenaturing solid-phase binding assay (Fig. 1C and D). The virus binding fractions of DGFc3 through -5 showed higher apparent molecular masses than the core proteins detected by Western blot analysis (Fig. 1B and 1C), indicating preferred binding of the virus to specifically modified forms of the protein. Mutation of the cleavage site by the amino acid exchanges R312K and R312A resulted in reduced cleavage (Fig. 1E) but did not alter virus or laminin binding (Fig. 1E and F). Together, our data indicate that structures within amino acids 313 through 408 of the mucin-type domain of α-DG are necessary and sufficient for virus binding and that the virus/α-DG interaction does not depend on cleavage of the N-terminal domain.

Virus binding to α-DG depends on posttranslational modification by LARGE. Although amino acids 313 through 408 of the mucin-type domain of α-DG appear to be necessary and sufficient for virus binding, in earlier studies, deletion of the N-terminal domain of α-DG resulted in reduced virus binding (33). To address a possible indirect role of the N-terminal domain for virus binding, the following deletion mutants of the full-length α-DG–Fc fusion protein DGFc5 were made: DGFc5ΔH30-A168, DGFc5ΔA182-H315, DGFc5ΔH30-A312, and DGFc5ΔH30-A316 (Fig. 2A). When expressed in HEK293T cells and purified from culture supernatant, all DGFc5 variants, with the exception of DGFc5ΔA182-H315, which lacks the cleavage site 309RRIR312, showed similar molecular masses (Fig. 2B). Detection of the N-terminal HA epitope of DGFc5ΔH30-A316 demonstrated the integrity of its N terminus (Fig. 2C). When tested in overlay assays and in nondenaturing solid-phase binding assays, only DGFc5ΔH30-A168 and wild-type DGFc5 bound virus (Fig. 2D and E) and laminin (Fig. 2F and G). Since all mutants contain the complete

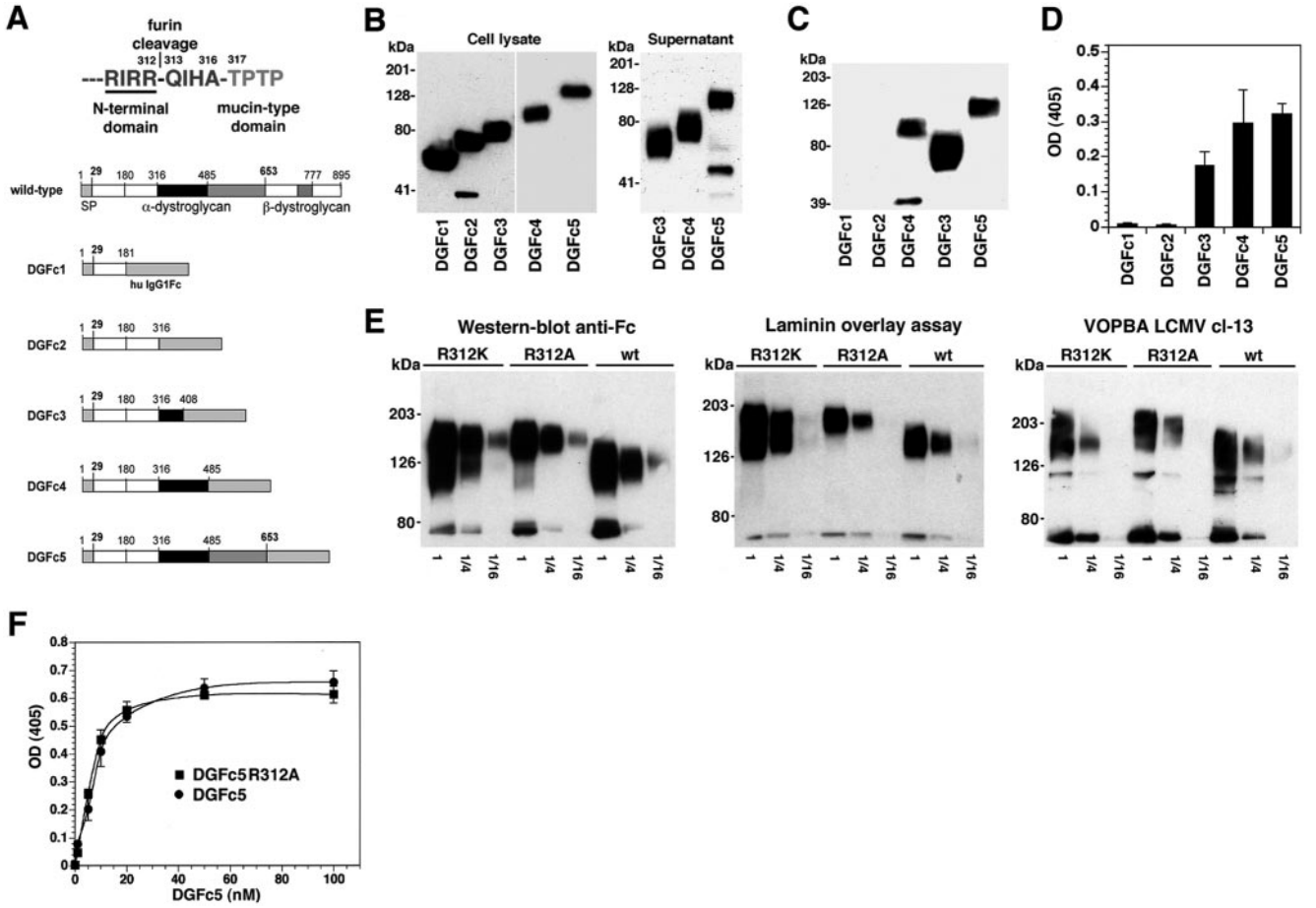

FIG. 1. The N-terminal region of the mucin-type domain of α-DG is necessary and sufficient for virus binding. (A) Furin recognition site of α-DG and schematic representation of the α-DG–Fc fusion proteins. The N-terminal domain (white), the mucin-type domain (black), and the C-terminal domain (gray) of α-DG, β-DG, and human IgG1Fc are indicated. SP, signal peptide. (B) Proteolytic processing of DGFc3 through -5. α-DG–Fc fusion proteins were purified from cell lysates or culture supernatant of transiently transfected HEK293T cells. Purified proteins were separated by SDS-PAGE under reducing conditions and analyzed by Western blot analysis using an anti-human IgG Fc antibody. Molecular masses are indicated to the left of the gels. (C) Virus binding to α-DG–Fc fusion proteins. Equal amounts of DGFc1 and -2 purified from cell lysates and DGFc3 through -5 purified from supernatants were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with 10^7 PFU/ml of LCMV cl-13. Bound virus was detected with anti-LCMVGP MAbs 33.1 and 36.1 and by ECL. (D) Solid-phase virus binding assay. Equal amounts of purified DGFc1 through -5 were immobilized in microtiter plates and incubated with 10^7 PFU/ml LCMV cl-13. Bound virus was detected with anti-LCMVGP2 MAb 83.6 and an HRP-conjugated secondary antibody using ABTS substrate. Optical density at 405 nm [OD (405)] was recorded in an ELISA reader and background binding to BSA subtracted (mean ± standard deviation; $n = 3$). (E) Binding of virus and laminin to DGFc mutants. Serial dilutions of equal amounts of purified DGFc5R312K, DGFc5R312A, and DGFc5 were subjected to Western blot analysis and VOPBA as described for panels B and C. For laminin overlay, 10 μg/ml EHS laminin-1 was used and bound laminin detected with a polyclonal anti-laminin-1 antibody using ECL. The prominent band at the bottom, close to the running front of the gel, was observed in some preparations of DGFc5 and may represent degradation products. wt, wild type. (F) Binding of DGFc5R312A and DGFc5 to virus. LCMV cl-13 was immobilized in microtiter plates and incubated with purified DGFc5R312A and DGFc5, which were detected with an HRP-conjugated anti-human Fc antibody in a color reaction using ABTS substrate (mean ± standard deviation; $n = 3$).

polypeptide core of the mucin-type domain, our data indicate a crucial role for the posttranslational modification of α-DG for virus binding.

The reduction in virus and laminin binding to α-DG after deletion of its N-terminal domain suggested the involvement of similar posttranslational modifications. Since modification of α-DG by the glycosyltransferase LARGE is essential for laminin binding and requires a recognition motif in α-DG's N-terminal domain, we addressed the role of LARGE-mediated modification of α-DG in virus binding. First, we tested the effect of LARGE overexpression on virus binding to α-DG. DGFc5 was transfected into HEK293T cells, which were subsequently infected with AdV vectors expressing either LARGE and EGFP in a bicistronic mRNA (AdV/LARGE-EGFP) or EGFP (AdV/EGFP) alone. Modified DGFc5 was then purified from culture supernatant. Consistent with previous studies (3, 30), LARGE overexpression resulted in a major increase in laminin binding affinity (Fig. 3A). A significant increase in virus binding affinity was observed with DGFc5 isolated from cells overexpressing LARGE (Fig. 3A). Quantitative analysis revealed half-maximal virus binding at 1.8 (± 0.6) nM for DGFc5 derived from cells overexpressing LARGE compared to 8.4 (± 1.6) nM for DGFc5 from EGFP controls (Fig. 3B).

LARGE overexpression increased laminin and virus binding

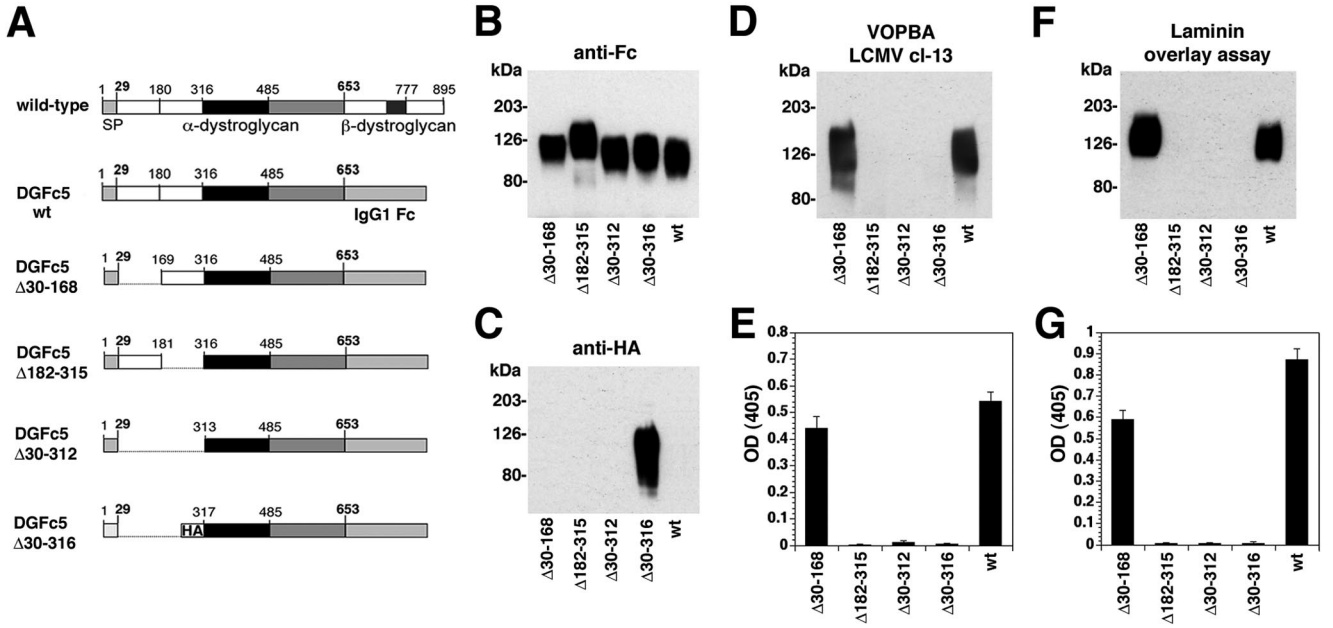

FIG. 2. Virus binding to α-DG depends on posttranslational modification. (A) Schematic representation of the DGFc5 deletion mutants. Domains of DG are depicted as described in the legend to Fig. 1. Equal amounts of DGFc5 deletion mutants and wild-type (wt) DGFc5 purified from the supernatants of transiently transfected HEK293T cells were subjected to Western blot analysis with an anti-Fc antibody (B) as described in the legend to Fig. 1B. The HA epitope present in DGFc5Δ30-316 was detected with the anti-HA antibody Y11 (C). VOPBA with LCMV cl-13 (D) and the solid-phase virus binding assay (E) were performed as described in the legend to Fig. 1C and 1D, respectively. OD (405), optical density at 405 nm. LOA (F) was done as described in the legend to Fig. 1F. For the solid-phase laminin binding assay (G) immobilized DGFc5 variants were incubated with 10 μg/ml laminin-1, which was then detected with an anti-laminin-1 antibody, using an HRP-conjugated secondary antibody (mean ± standard deviation; $n = 3$). SP, signal peptide.

to the deletion mutant DGFc5ΔH30-A168 and wild-type DGFc5 in a comparable manner, suggesting that amino acids 30 through 168 are dispensable for LARGE-dependent modification in this system (Fig. 3C). In earlier deletion studies with full-length DG in mouse ES cells, deletion of amino acids 30 through 168 significantly reduced virus and laminin binding and markedly impaired the function of DG as a virus or laminin receptor (30, 33), indicating differences in the recognition motifs for murine and human LARGE on α-DG.

To further demonstrate the involvement of the LARGE-dependent modification of α-DG in virus binding, we made use of the anti-α-DG MAb IIH6, whose epitope is sensitive to modification by LARGE (3). To test the ability of MAb IIH6 to block virus/α-DG binding, immobilized receptor was pretreated with increasing concentrations of MAb IIH6 prior to incubation with virus. Detection of receptor-bound virus revealed a specific, dose-dependent reduction of virus binding by MAb IIH6 (Fig. 4A). To test the ability of MAb IIH6 to block virus infection of cells, we used $DG^{-/-}$ ES cells and their parental $DG^{+/-}$ line (26). Infection in $DG^{+/-}$ ES cells was mediated by α-DG, as demonstrated by the lack of significant infection in $DG^{-/-}$ cells and the efficient blocking of virus infection with soluble α-DG (Fig. 4B). Preincubation of $DG^{+/-}$ ES cells with MAb IIH6 resulted in a specific, dose-dependent reduction of infection (Fig. 4C). In sum, our data indicate that virus binding to the mucin-type domain of α-DG critically depends on posttranslational modification by LARGE.

In humans and the mouse, tissue-specific expression of LARGE results in marked differences in functional α-DG glycosylation (44). To address the correlation between tissue-specific LARGE-dependent modification and virus binding affinity, α-DG was purified from skeletal muscle, kidney, brain, heart, liver, and lung by wheat germ agglutinin (WGA) affinity chromatography, a purification not affected by LARGE modification (39). Detection with an antibody to the α-DG polypeptide revealed the presence of α-DG in all organs with marked tissue-specific differences in the apparent molecular mass of the protein, most likely due to differential glycosylation (Fig. 5). Western blot analysis with MAb IIH6 revealed high levels of LARGE modification for α-DG derived from skeletal muscle, kidney, brain, and heart and markedly lower levels in liver and lung. The extent of LARGE-dependent modification was correlated with the binding affinity to laminin and virus (Fig. 5), indicating that modification of α-DG by LARGE is a critical determinant for α-DG's virus binding affinity in vivo.

In a complementary approach to confirm the role of the LARGE-dependent modification of α-DG for virus binding in vivo, we made use of the spontaneous myodystrophy mouse model $Large^{myd}$. $Large^{myd}$ mice carry a gene defect in LARGE (25), which results in the aberrant glycosylation of α-DG and loss of function as a laminin receptor associated with a muscular dystrophy phenotype (27, 39). We determined virus binding to α-DG isolated from the skeletal muscle and brain of homozygous $Large^{myd}$ mice and control littermates. Consistent with previous reports (39), we found high levels of underglycosylated α-DG in the brain and skeletal muscle of $Large^{myd}$ mice. As expected, α-DG from $Large^{myd}$ mice showed a complete loss of binding to laminin and the virus (Fig. 6). Together,

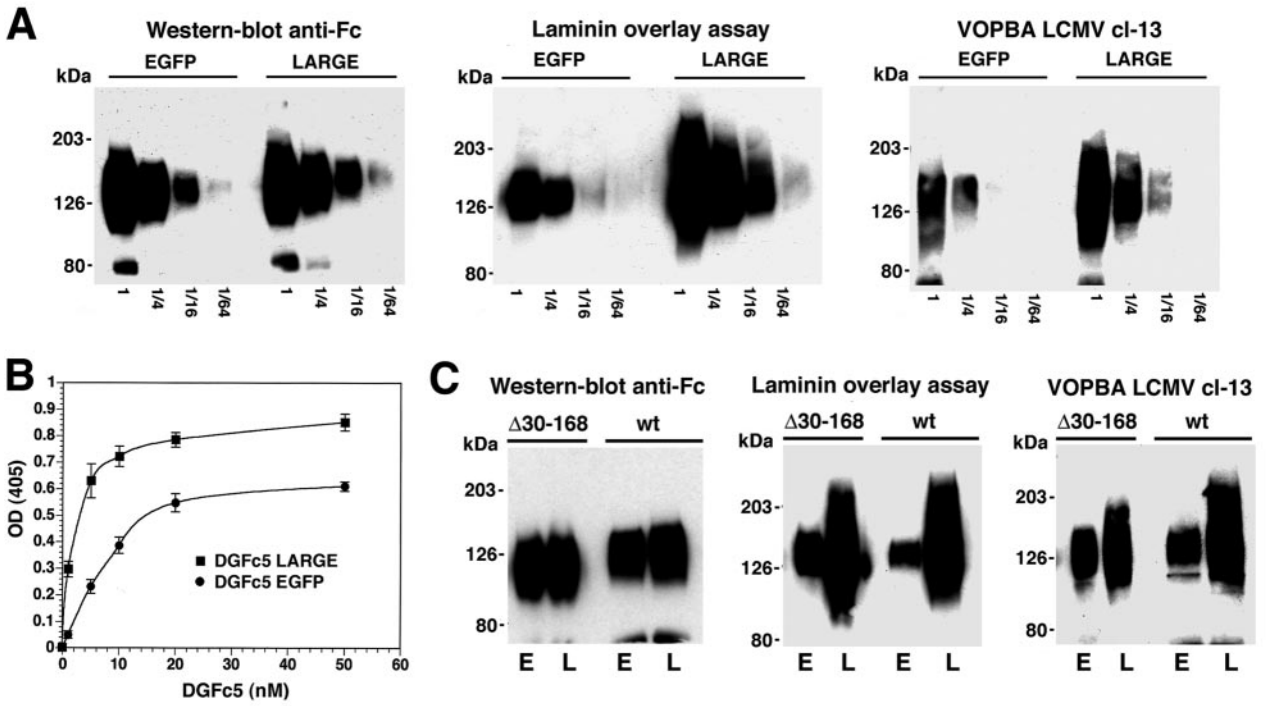

FIG. 3. Virus binding to α-DG increases after LARGE overexpression. (A) Binding of virus and laminin to DGFc5 from cells overexpressing LARGE. HEK293T cells transiently transfected with DGFc5 were infected with Ad5/LARGE-EGFP (LARGE) or Ad5/EGFP (EGFP). After 48 h, DGFc5 was purified from culture supernatants. Serial dilutions of equal amounts of DGFc5 were subjected to Western blot analysis using an anti-Fc antibody, LOA, and VOPBA as described in the legend to Fig. 1. (B) Binding of DGFc5 from cells overexpressing LARGE to virus. Immobilized LCMV cl-13 was incubated with the indicated concentrations of DGFc5 purified from cells overexpressing LARGE or EGFP controls and bound DGFc5 detected as described in the legend to Fig. 1F. OD (405), optical density at 405 nm. (C) DGFc5Δ30-168 is recognized by LARGE. Equal amounts of DGFc5Δ30-168 (Δ30-168) and wild-type DGFc5 (wt) purified from cells overexpressing LARGE (L) or EGFP controls (E) were subjected to Western blot analysis, LOA, and VOPBA as described for panel A.

our data indicate that virus binding to α-DG critically depends on posttranslational modification by LARGE.

LARGE-dependent glycosylation of α-DG is crucial for binding to several isolates of LCMV, LFV, and the arenaviruses Mobala and Oliveros. To address the role of the LARGE-dependent modification of α-DG for binding to different LCMV isolates, we first compared the prototypic isolates LCMV cl-13 (high affinity, immunosuppressive) and ARM53b (low affinity, nonimmunosuppressive). The GP of ARM53b differs from that of cl-13 by only a single amino acid exchange, F260L (46, 47). Yet, ARM53b binds at 2 to 3 logs less affinity to α-DG than does cl-13 (48). When tested for binding to the DGFc5 deletion mutants, both isolates showed reduced binding to DGFc5 variants not modified by LARGE (Fig. 7A).

To extend our findings, we included the immunosuppressive LCMV isolates WE54, TNPBL42, and PBL673 and the nonimmunosuppressive isolates CD4-1, CD8-4, and WE2.2, whose origins, disease phenotypes, and receptor binding characteristics are detailed in Table 1. Consistent with earlier studies (34, 48), we found high-affinity α-DG binding for all immunosuppressive isolates, low-affinity binding with the nonimmunosuppressive isolates ARM53b, CD4-1, and CD8-4, and no binding with WE2.2 (Fig. 7B). All α-DG binding isolates showed reduced binding to DGFc5Δ30-312, which lacks LARGE-dependent modification, when compared to wild-type DGFc5 (Fig. 7D). Enhanced modification of the receptor by LARGE significantly increased their binding affinity (Fig. 7C).

Within the arenavirus family, the Old World arenaviruses LCMV, LFV, and Mobala, as well as the clade C New World arenaviruses Oliveros and Latino, use α-DG as a receptor (10, 52). We therefore addressed the role of LARGE-dependent modification for α-DG binding of these viruses. Similar to LCMV, binding of LFV, Mobala, and Oliveros to DGFc5 was reduced after the deletion of α-DG's N-terminal domain that contains the LARGE recognition site (Fig. 8A and C). While LFV did not bind significantly to the unmodified receptor, Mobala and Oliveros showed detectable receptor binding in the absence of LARGE-dependent modification (Fig. 8A and C). α-DG binding of all viruses was significantly increased after LARGE overexpression (Fig. 8B and C). Consistent with previous studies, we did not detect α-DG binding with the clade B New World arenavirus Guanarito, which does not use α-DG as a receptor (10). Our results indicate that the LARGE-dependent glycosylation of α-DG is required for optimal binding to several isolates of LCMV and the arenaviruses LFV, Mobala, and Oliveros.

LARGE-dependent modification is crucial for α-DG's ability to mediate infection by several isolates of LCMV and recombinant VSV pseudotyped with LFVGP. When compared to their parental $DG^{+/-}$ ES line, $DG^{-/-}$ ES cells are less susceptible to infection with most LCMV isolates (10, 34, 50) and

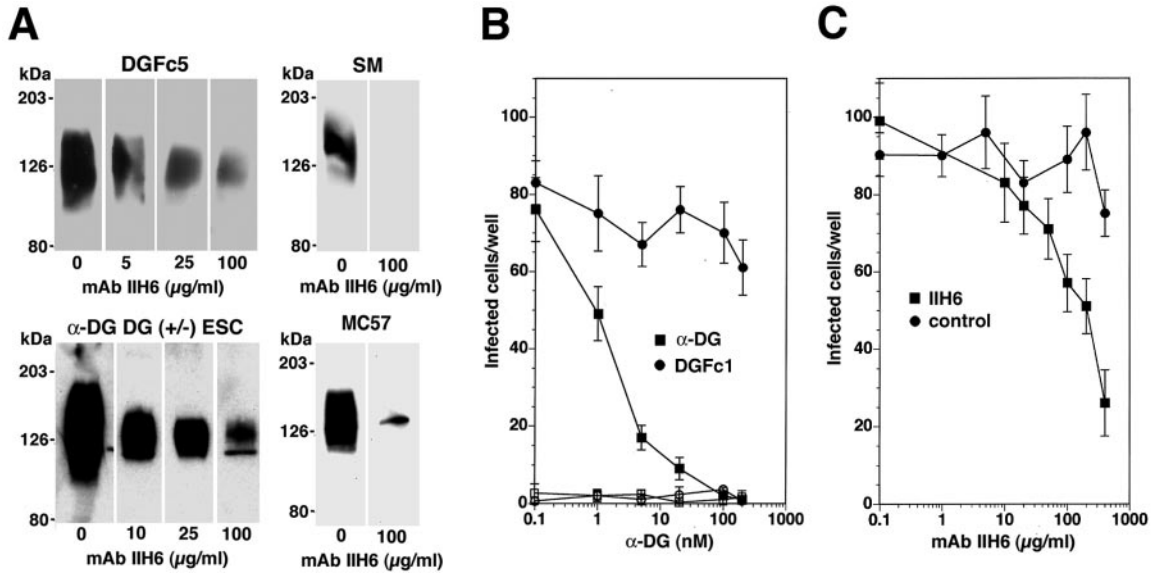

FIG. 4. Monoclonal antibody IIH6 blocks virus-receptor interaction. (A) Blocking of LCMV/α-DG binding by MAb IIH6. DGFc5, α-DG from $DG^{+/-}$ ES cells, MC57 cells (MC57), or rabbit skeletal muscle (SM) was immobilized on nitrocellulose and preincubated with the indicated concentrations of MAb IIH6. Blots were then incubated with 10^7 PFU/ml of LCMV cl-13 in the presence of the same concentrations of MAb IIH6. After several washes, bound virus was detected with anti-LCMVGP MAbs 33.1 and 36.1 and ECL. (B) Neutralization of LCMV infection with soluble α-DG. LCMV cl-13 (200 PFU) was incubated with the indicated concentrations of α-DG or DGFc1 for 1 h on ice and then added to either $DG^{+/-}$ ES cells (filled symbols) or $DG^{-/-}$ ES cells (open symbols). LCMV infection was assessed after 16 h by immunofluorescence staining for LCMVNP. (mean ± standard deviation; $n = 3$). (C) Blocking of infection with MAb IIH6. $DG^{+/-}$ ES cells were blocked with MAb IIH6 or an unrelated mouse IgM (control) for 2 h at 4°C. Next, 200 PFU of LCMV cl-13 was added for 45 min and infection assessed as described for panel B.

recombinant VSV pseudotyped with the glycoprotein of LFV (32). Previous studies from our laboratory demonstrated that infection of $DG^{-/-}$ ES cells with LCMV cl-13 or recombinant VSV pseudotyped with LFVGP can be reconstituted by the heterologous expression of recombinant wild-type DG, but not with a DG variant lacking the N-terminal domain of α-DG (32, 33). Since the N-terminal domain of α-DG is required for LARGE-dependent modification in mouse ES cells (30), this supports a crucial role for LARGE-dependent modification for α-DG's function as a cellular receptor for LCMV and LFV.

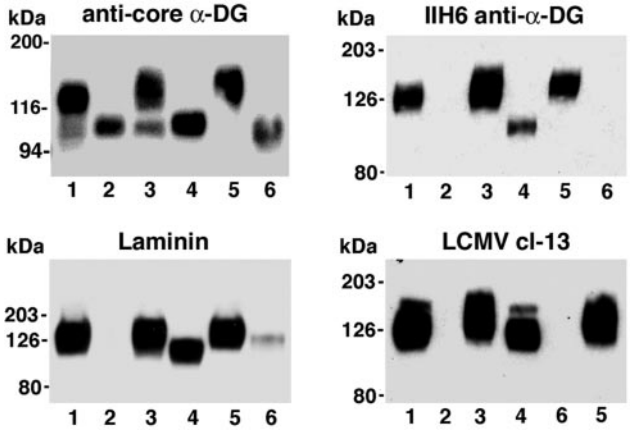

FIG. 5. Tissue-specific LARGE-dependent modification of α-DG correlates with virus binding affinity. Equal amounts of total tissue homogenates of adult mouse skeletal muscle (lane 1), liver (lane 2), kidney (lane 3), brain (lane 4), heart (lane 5), and lung (lane 6) were subjected to WGA affinity chromatography. Eluted glycoproteins were probed with an antibody recognizing the core protein of α-DG and MAb IIH6 in Western blot analysis. LOA and VOPBA were performed as described in the legend to Fig. 1. Molecular masses are indicated to the left of the gels.

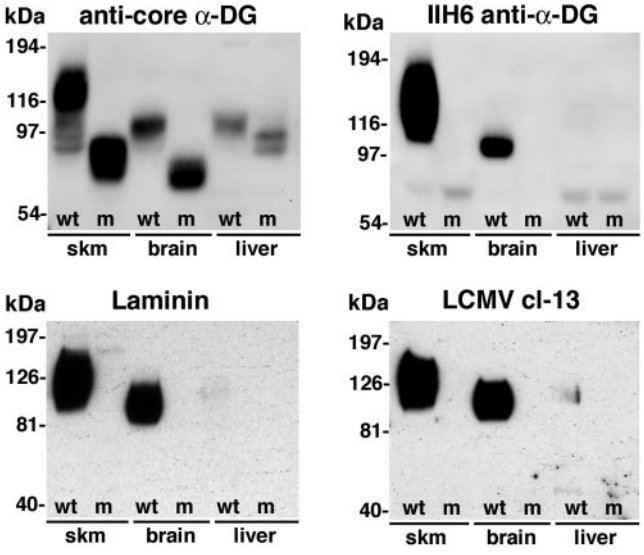

FIG. 6. α-DG derived from $LARGE^{myd}$ mice shows reduced binding to laminin and virus. α-DG was isolated from the skeletal muscle (skm), brain, and liver of homozygous $LARGE^{myd}$ mice (m) and control littermates (wt) by WGA affinity purification and was probed with the antibody FPD and MAb IIH6 in Western blot analysis, LOA, and VOPBA as described in the legend to Fig. 5.

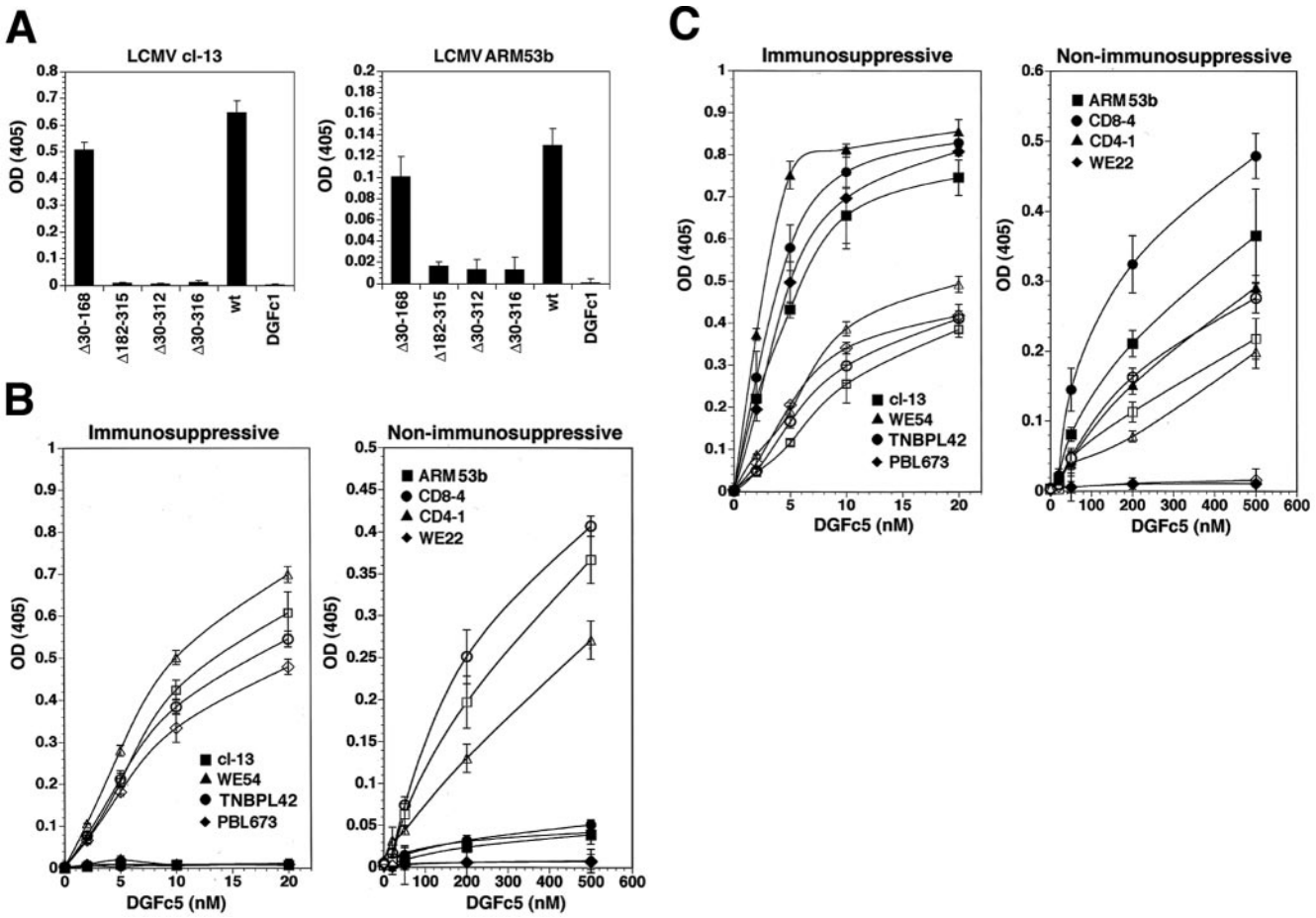

FIG. 7. LARGE-mediated modification of α-DG is critical for the recognition by different LCMV isolates. (A) Virus binding to DGFc5 deletion mutants. Immobilized DGFc5 variants were incubated with LCMV cl-13 (10^7 PFU/ml) and ARM53b (10^8 PFU/ml). For the detection of bound viruses, MAb 83.6 was combined with a biotinylated anti-mouse IgG secondary antibody and HRP-streptavidin (mean ± standard deviation; $n = 3$). OD (405), optical density at 405 nm. (B) Binding of DGFc5Δ30-312 and DGFc5 to LCMV isolates. Equal amounts of purified viruses were immobilized and incubated with the indicated concentrations of DGFc5Δ30-312 (filled symbols) and DGFc5 (open symbols). Bound DGFc5 was detected using a mouse anti-human Fc antibody, combined with a biotinylated anti-mouse IgG and HRP-streptavidin (mean ± standard deviation; $n = 3$). (C) Binding of DGFc5 from cells overexpressing LARGE to LCMV isolates. Immobilized viruses were incubated with DGFc5 from cells overexpressing LARGE (filled symbols) or EGFP (open symbols). Bound DGFc5 was detected as described for panel B (mean ± standard deviation; $n = 3$). wt, wild type.

To confirm and extend these studies, we infected $DG^{-/-}$ ES cells with AdV vectors containing wild-type DG or the DG deletion mutant DGΔ30-316 (Fig. 9A), which lacks the N-terminal domain of α-DG and is not recognized by LARGE. Within 48 h after AdV-mediated gene transfer, which resulted in >70% cells expressing recombinant protein, expression of recombinant DG protein was checked by the detection of β-DG in total cell lysates (Fig. 9B). The integrity of the α-DG part of the mutant DGΔ30-316 was verified by the detection of its N-terminal HA tag in a Western blot using an anti-HA antibody (Fig. 9B). As expected, only wild-type DG, but not DGΔ30-316, showed LARGE-dependent modification, as detected by Western blot analysis with MAb IIH6 (Fig. 9B). AdV-transfected $DG^{-/-}$ ES cells and $DG^{+/-}$ ES cells cultured in parallel were infected with the immunosuppressive LCMV isolates cl-13, WE54, TNPBL42, and PBL673 and the nonimmunosuppressive isolates ARM53b, CD4-1, CD8-4, and WE2.2. Infection was assessed after 16 h by detection of LCMVNP. In line with previous findings, the α-DG binding LCMV variants cl-13, WE54, TNPBL42, PBL673, ARM53b, CD4-1, and CD8-4 showed little infection of $DG^{-/-}$ ES cells after 16 h, indicating a strong dependence of infection on α-DG at this time point of infection (Fig. 9C). As previously reported (50), the nonimmunosuppressive variant WE2.2 did not depend on α-DG for infection. While transfection of $DG^{-/-}$ ES cells with wild-type DG restored infection with α-DG binding LCMV variants, no significant infection above background was observed in $DG^{-/-}$ ES cells reconstituted with the deletion mutant DGΔ30-316 (Fig. 9C).

As our data indicate a role for the LARGE-dependent glycosylation of α-DG for binding of the human pathogenic LFV (Fig. 8), we wanted to confirm the role of LARGE-dependent modification for α-DG's role as a LFV receptor. Since LFV is a BSL4 pathogen, the use of live virus in the laboratory is restricted. In order to study the LFV-receptor interaction in host cells, we incorporated LFVGP into the surface of recom-

TABLE 1. LCMV isolates used in this study[a]

Isolate	IS	P	Tropism	aa GP1260	α-DG binding
ARM53b	−	−	RP	F	+
CD4-1	−	−	RP	F	+
CD8-4	−	−	RP	F	+
cl-13	+	+	MZ/WP	L	+++
TNPBL42	+	+	MZ/WP	L	+++
PBL673	+	+	MZ/WP	L	+++
WE54	+	+	MZ/WP	L(S153)	+++
WE2.2	−	−	RP	L(F153)	−

[a] Variants CD4-1 and CD8-4 were isolated from CD4 and CD8 cells from perforin$^{-/-}$ mice after 24 days of infection with ARM53b (48). LCMV cl-13 is a spleen isolate of an adult BALB/WEH1 mouse infected persistently since birth with ARM53b (2). TNPBL42 and PBL673 were isolated from peripheral blood lymphocytes (PBLs) of tumor necrosis factor alpha knockout and perforin knockout mice persistently infected with LCMV ARM53b for 3 and 7 months, respectively (48). WE2.2 is derived from WE54 and differs from WE54 by the amino acid exchange S153F in GP1 (56). The phenotype of cl-13, TNPBL42, PBL673, and WE54 is as follows: they induce immunosuppression (IS, +), cause persistent infection (P, +), and show a tropism for the marginal zone (MZ) and the white pulp (WP) of the spleen. ARM53b, CD4-1, CD8-4, and WE2.2 are nonimmunosuppressive (IS, −), are cleared by the host immune system (P, −) and infect the red pulp (RP) of the spleen. The amino acid in position 260 of GP1 is indicated. WE54 and WE2.2 both contain an L at position 260 of GP1. There is a single amino acid difference at position 153 of GP1: WE54/WE2.2. S/F, relative binding affinity to α-DG (34, 48).

binant VSV in which the VSV G gene was replaced by a green fluorescent protein reporter gene (VSVΔG*) (32). Viral particles released from VSVΔG*-infected cells incorporated LFVGP, provided in *trans*, into their lipid membranes during budding. These pseudotyped VSVΔG* particles (VSVΔG*-LFVGP) acquire the receptor binding characteristics of LFV and depend on α-DG for infection of cells (32). As a control, we used recombinant VSV pseudotyped with the GP of VSV, which does not use α-DG as a receptor (VSVΔG*-VSVGP). As observed with the α-DG binding LCMV variants, only wild-type DG, but not DGΔ30-316, which lacks LARGE-dependent modification, was able to restore infection with VSVΔG*-LFVGP. As expected, VSVΔG*-VSVGP-infected all transfectants equally well. These results are consistent with our previous study that implicated α-DG's N-terminal domain in the binding of LFV (32).

Together, our data indicate a crucial role for LARGE-dependent glycosylation for α-DG's function as a receptor for α-DG binding LCMV isolates and LFV.

Modification of α-DG by LARGE influences the competition between virus and laminin for receptor binding. Previous studies demonstrated that the binding sites of LCMV and LFV on α-DG overlap with domains recognized by ECM proteins, resulting in a competition for α-DG binding (32, 33, 45). Since the posttranslational modification by LARGE is crucial for α-DG recognition by both arenaviruses and ECM proteins like laminin, it may influence competition between virus and ECM proteins for α-DG. To study the effect of the LARGE-dependent modification of α-DG on the competition of the virus with α-DG's ECM ligands, DGFc5, derived from cells overexpressing LARGE or from controls, was immobilized and preincubated with increasing concentrations of the prototypic α-DG binding ECM protein laminin-1 or fibronectin, which does not bind to α-DG. The complexes were then incubated with either the high-affinity binder LCMV cl-13 or the low-affinity binder ARM53b and bound virus detected. Laminin-1 but not fibronectin blocked the receptor binding of both isolates in a dose-dependent manner (Fig. 10). Enhanced LARGE modification significantly increased the receptor binding of cl-13, which was only partially blocked by laminin-1 (Fig. 10). In the case of ARM53b, increased LARGE modification resulted in significantly enhanced blocking of the virus/receptor interaction by laminin-1 (Fig. 10).

To address this issue at the cellular level, we chose Vero cells as an experimental system, as this cell type shows relatively low LARGE-dependent modification of α-DG and is susceptible to infection with arenaviruses. Overexpression of LARGE in Vero cells with AdV vectors for gene transfer resulted in a marked increase of membrane-associated immunofluorescence staining by MAb IIH6, indicating the enhanced glycosylation of α-DG at the cell surface (Fig. 11A). Consistently, α-DG isolated from cells overexpressing LARGE but not from controls showed increased binding affinity to laminin and LCMV cl-13 (Fig. 11B). Addition of soluble laminin-1 to cells overexpressing LARGE but not controls markedly reduced subsequent infection with LCMV ARM53b in a dose-dependent manner, while infection with cl-13 was not significantly altered (Fig. 11C).

To address the impact of the LARGE-dependent modification of cellular α-DG on the ability of LFV to compete with laminin for receptor binding and subsequent infection, we used the recombinant VSVΔG*-LFVGP pseudotypes described above. VSVΔG*-LFVGP and VSVΔG*-VSVGP, used as controls, efficiently infected Vero cells overexpressing LARGE (Fig. 11D). Addition of soluble laminin-1 to Vero cells overexpressing LARGE did not significantly reduce subsequent infection with VSVΔG*-LFVGP (Fig. 11D). This indicates the efficient competition of LFVGP with receptor-associated laminin, as was observed with LCMV cl-13.

DISCUSSION

The present study addressed the role of posttranslational modifications for α-DG's function as an arenavirus receptor and makes the following points. First, virus binding is mediated by the N-terminal part of the mucin-type domain of α-DG and critically depends on posttranslational modification by the glycosyltransferase LARGE. Second, LARGE-dependent modification is critical for optimal binding of α-DG to several isolates of LCMV, LFV, and the arenaviruses Mobala and Oliveros. Third, modification of α-DG by LARGE critically influences the ability of the virus to compete with the ECM protein laminin for receptor binding.

The interaction of a virus with its cellular receptor(s) is the first step for the virus-host cell relationship and thus provides a key determinant for both the tissue tropism and disease potential of a virus. The cellular receptor for LFV, LCMV, and clade C New World arenaviruses has been identified as α-DG (10, 52). In the host cell, α-DG is subject to a remarkably complex pattern of posttranslational modifications, including specific glycosylation, which appears critical for its function as an ECM receptor (40). Among the enzymes implicated in α-DG glycosylation, the glycosyltransferase LARGE was found to play a key role in DG biosynthesis (3, 7, 30). Modification of α-DG by LARGE occurs in the N-terminal part of the mucin-type domain and requires a recognition motif within

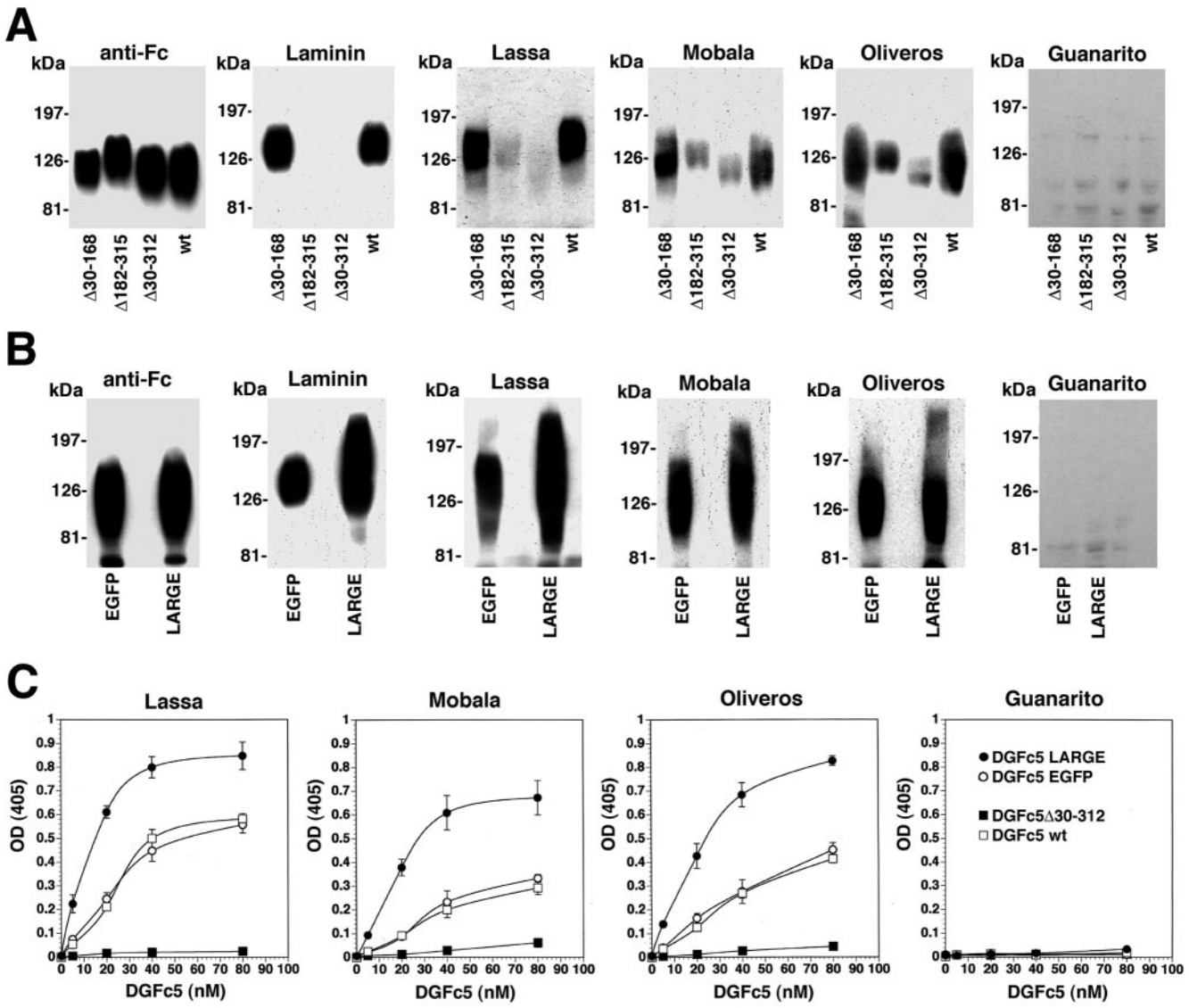

FIG. 8. Modification of α-DG by LARGE is involved in binding of LFV, Mobala, and Oliveros. Binding of LFV, Mobala, Oliveros, and Guanarito to DGFc5 deletion mutants (A) and DGFc5 derived from cells overexpressing LARGE or EGFP (B). Immobilized DGFc5 variants were incubated with γ-inactivated LFV, Mobala, Oliveros, and Guanarito (10^7 PFU/ml). Bound viruses were detected with MAbs 83.6 and 33.1 using an HRP-conjugated secondary antibody and ECL. Exposure times were 1 min for LFV, Mobala, and Oliveros and 10 min for Guanarito. LOA and Western blot analysis with anti-Fc antibody were performed as described in the legend to Fig. 3. (C) Binding of DGFc5 variants to immobilized viruses. Equal amounts of purified viruses were immobilized and incubated with the indicated concentrations of DGFc5Δ30-312, DGFc5, and DGFc5 from cells overexpressing EGFP (DGFc5 EGFP) or LARGE (DGFc5 LARGE). OD (405), optical density at 405 nm. Bound DGFc5 was detected as described in the legend to Fig. 7C (mean ± standard deviation; $n = 3$). wt, wild type.

the N-terminal globular domain, which is subsequently cleaved by a convertase-like activity.

Since mapping of the arenavirus binding site on α-DG implicated α-DG's N-terminal domain and the N-terminal region of the mucin-type domain in virus binding (32, 33), we addressed the impact of posttranslational modifications on virus binding. We found that N-terminal amino acids 313 through 408 of the mucin-type domain are necessary and sufficient for virus binding. Proteolytic cleavage of the N-terminal domain did not affect virus binding, indicating an indirect role of the N-terminal domain in the virus/α-DG interaction. Consistent with previous findings (33), deletion of the N-terminal domain of α-DG markedly reduced virus binding without affecting the integrity of the polypeptide core and the overall extent of glycosylation of the mucin-type domain. Thus, reduced virus binding was likely due to the absence of a specific posttranslational modification. The concomitant loss of laminin and virus binding after deletion of the N-terminal domain, which contains the LARGE recognition motif (30), suggested a key role of LARGE modification in virus binding. To test this hypothesis, we analyzed the effect of LARGE overexpression on the virus/α-DG interaction and found that enhanced mod-

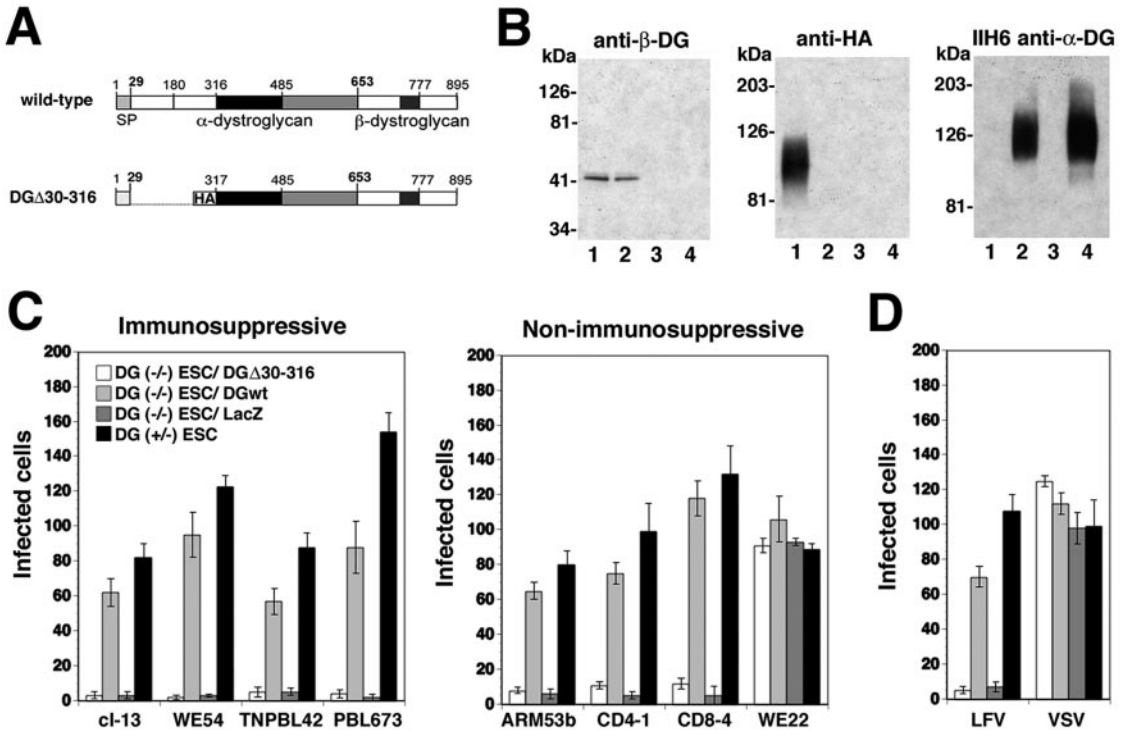

FIG. 9. LARGE-dependent modification is crucial for α-DG's ability to mediate infection by several isolates of LCMV and recombinant VSV pseudotyped with LFVGP. (A) Schematic representation of wild-type DG and the DG deletion mutant DGΔ30-316. The putative N-terminal subdomains (white), the mucin-type domain (black), and the C-terminal globular domain (gray) of α-DG are indicated. Amino acids 653 through 895 represent β-DG with the transmembrane domain (dark box). The influenza HA epitope in DGΔ30-316 is indicated. SP, signal peptide. (B) Expression of wild-type DG and DGΔ30-316. $DG^{-/-}$ ES cells were infected with AdV vectors containing DGΔ30-316 (lane 1), wild-type DG (lane 2), or a β-galactosidase reporter gene (LacZ) (lane 3) at an MOI of 10. Lane 4 represents the parental $DG^{+/-}$ ES cells in parallel. After 48 h, cells were lysed and total protein isolated, separated by SDS-PAGE, and transferred to nitrocellulose. Blots were probed with anti-β-DG polyclonal antibody AP83, anti-HA antibody Y11, and anti-α-DG MAb IIH6. Primary antibodies were detected with HRP-conjugated secondary antibodies and by ECL. (C and D) Reconstitution of virus infection in $DG^{-/-}$ ES cells by wild-type (wt) DG and DGΔ30-316. $DG^{-/-}$ ES cells were infected with AdV vectors containing DGΔ30-316 (white), wild-type DG (light gray), or LacZ (dark gray). As a positive control, $DG^{+/-}$ ES cells were cultured in parallel (black). After 48 h, cells were infected with 200 infectious units/well of either the LCMV variants indicated (C) or the VSV pseudotypes VSVΔG*-LFVGP (LFV) or VSVΔG*-VSVGP (VSV). Infection levels in panel C were assessed by immunofluorescence staining for LCMVNP as described in the legend to Fig. 4B. Data in panel D are for EGFP-positive cells per well (mean ± standard deviation; $n = 3$).

ification of α-DG by LARGE significantly increased virus binding. Further, the antibody IIH6, whose epitope is exquisitely sensitive to LARGE-dependent modification (30), blocked the binding of LCMV to α-DG in a dose-dependent manner.

In humans and the mouse, LARGE is expressed in a tissue-specific manner, resulting in differences in functional α-DG glycosylation (44). Examination of virus and laminin binding to α-DG isolated from different tissues of the mouse revealed a direct association between the extent of LARGE-dependent glycosylation of the receptor and the binding affinity to laminin and LCMV. A crucial role of LARGE glycosylation for virus binding to α-DG in vivo is further supported by the lack of virus binding to α-DG derived from homozygous $LARGE^{myd}$ mice, which carry a defect in their LARGE gene and are unable to express functionally glycosylated α-DG (25, 39).

The dependence of virus binding on glycosylation by LARGE is consistent with earlier findings that demonstrated a loss of virus binding after complete deglycosylation of the receptor (6, 10). An important role for glycosylation for the function of α-DG as an LCMV receptor comes independently from the observation that cells deficient in the biosynthesis of α-DG's *O*-mannosyl glycans are less susceptible to viral infection (29).

Studies with LCMV in the mouse demonstrated that persistent infections with arenaviruses allow the generation of viral variants that have distinct pathological phenotypes (1, 2, 20, 42, 48). The consistent correlation between high α-DG binding affinity and immunosuppressive disease potential of LCMV isolates (48, 50) pinpoints the virus-receptor interaction as a key determinant for viral pathogenesis. We therefore studied the role of LARGE-dependent modification for the receptor binding of a number of LCMV isolates with distinct receptor binding characteristics and disease phenotypes. To address the role of LARGE-dependent modification for the recognition of α-DG by LCMV isolates with high or low binding affinity, we examined the LCMV prototypic nonimmunosuppressive parental virus ARM53b, its immunosuppressive variants cl-13, TNPBL42, and PBL673, and its nonimmunosuppressive variants CD4-1 and CD8-4 (Table 1). In addition, we included the closely related LCMV isolates WE54 and WE2.2, which differ from each other by only a mutation, S153F, in GP1 but show

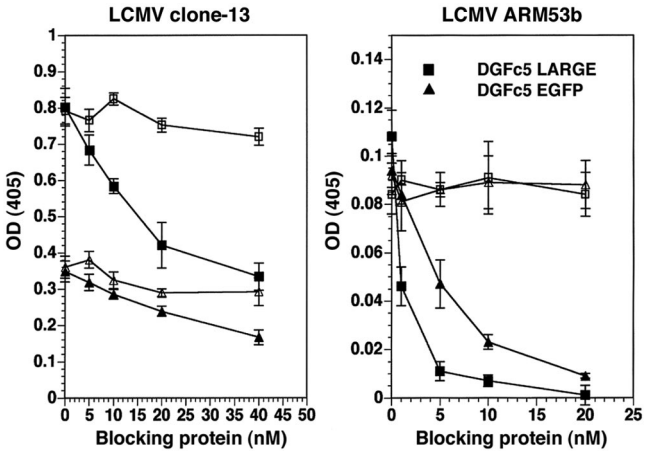

FIG. 10. Modification of α-DG by LARGE influences the competition between virus and laminin for receptor binding. Equal amounts of DGFc5 produced in cells overexpressing LARGE (DGFc5 LARGE) or EGFP (DGFc5 EGFP) were immobilized in microtiter plates and preincubated with the indicated concentrations of laminin-1 (filled symbols) or fibronectin (open symbols). LCMV cl-13 (10^7 PFU/ml) and ARM53b (10^8 PFU/ml) were added and bound virus detected as described in the legend to Fig. 7A (mean ± standard deviation; n = 3).OD (405), optical density at 405 nm.

strikingly different disease phenotypes and receptor binding characteristics (Table 1). While WE54 binds α-DG with high affinity and causes immunosuppression, WE2.2 does not use α-DG as a receptor and is efficiently cleared by the antiviral immune response (48, 50). Consistent with earlier studies (34, 50), we found high-affinity α-DG binding of all immunosuppressive isolates, low-affinity binding with the nonimmunosuppressive isolates ARM53b, CD4-1, and CD8-4, and no binding with WE2.2. Receptor recognition by all α-DG binding LCMV isolates depended on LARGE-dependent modification. An important role for the LARGE-dependent glycosylation of α-DG was also observed for binding to the human pathogenic LFV and the arenaviruses Mobala and Oliveros, indicating conservation in receptor recognition among arenaviruses. However, while binding of LFV strongly depended on the modification of α-DG by LARGE, Mobala and Oliveros showed significant binding to α-DG lacking the LARGE-dependent modification. These viruses may be able to bind to other glycans not related to LARGE and/or the polypeptide part of the receptor.

To confirm the role for LARGE-dependent modification of α-DG for its function as a cellular receptor for LCMV and

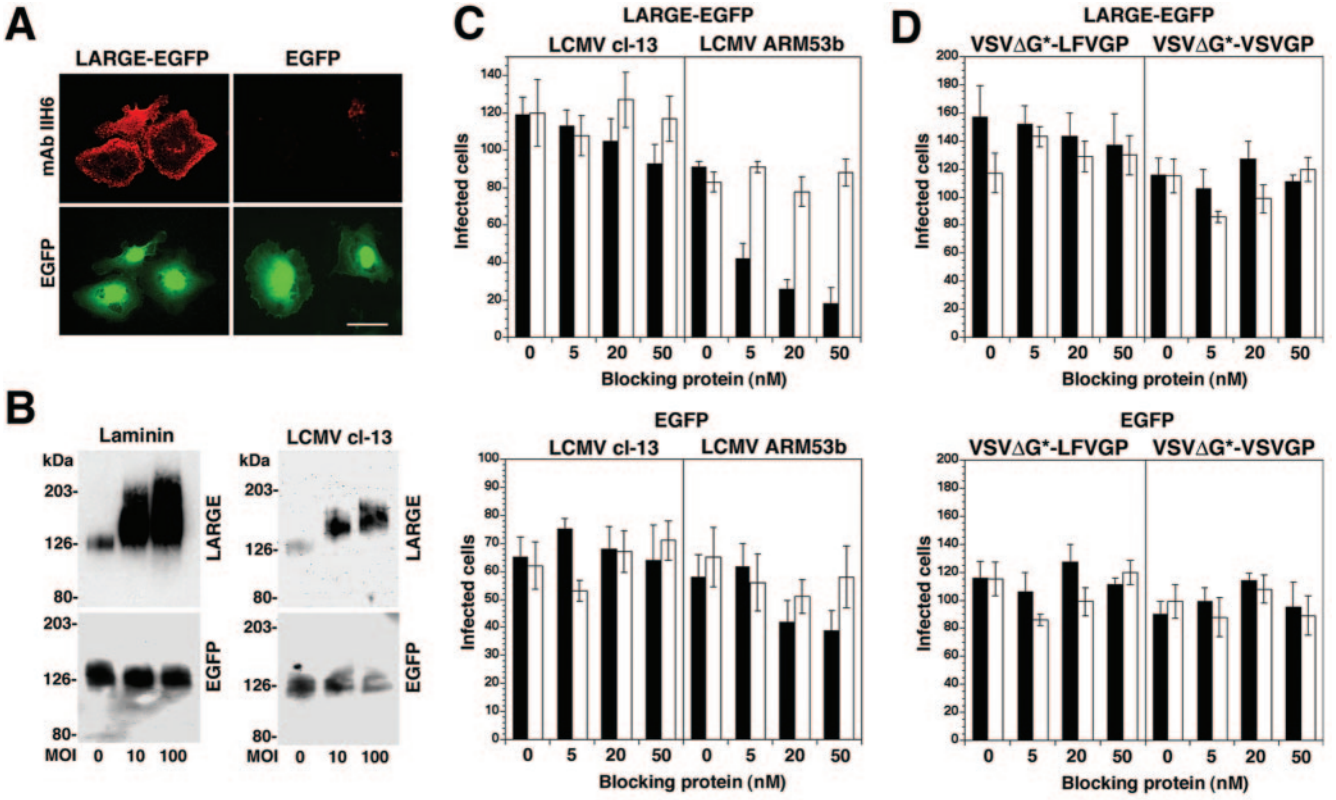

FIG. 11. Modification of α-DG by LARGE influences the ability of the virus to compete with cell-associated laminin. (A) Overexpression of LARGE in Vero cells. Vero cells were infected with Ad5/LARGE-EGFP and Ad5/EGFP (MOI = 10). After 24 h, cells were fixed and LARGE-modified α-DG was detected using MAb IIH6 and a rhodamine-X (red)-coupled anti-mouse IgM. EGFP was detected by direct fluorescence. Bar, 20 μm. (B) laminin and virus binding to α-DG. Vero cells were infected with Ad5/LARGE-EGFP and Ad5/EGFP at MOIs of 0, 10, and 100. After 48 h, cells were lysed and α-DG extracted and examined by LOA and VOPBA as described in the legend to Fig. 3. (C and D) Blocking of virus infection with laminin-1. Vero cells overexpressing LARGE or EGFP were cultivated on laminin in the absence (0) or presence of 5, 20, and 50 μg/ml soluble laminin-1 (black bars) or fibronectin (white bars) for 12 h. Cells were then infected with either 200 PFU LCMV (C) or VSVΔG* pseudotypes (D) for 16 h. Infection levels in panel C were assessed by immunofluorescence staining for LCMVNP as described in the legend to Fig. 4B. Data in panel D are for EGFP-positive cells per well (mean ± standard deviation; n = 3).

LFV, we performed reconstitution studies in α-DG-deficient $DG^{-/-}$ ES cells. When compared to their parental $DG^{+/-}$ ES line, $DG^{-/-}$ ES cells are less susceptible to infection with most LCMV isolates (10, 34, 50), as well as recombinant VSV pseudotyped with LFVGP (32). In line with the results of our binding studies, virus infection in $DG^{-/-}$ ES cells was reconstituted by the heterologous expression of wild-type DG but not with a DG variant lacking the N-terminal domain of α-DG (DGΔ30-316), which is not modified by LARGE. Previous studies demonstrated that DGΔ30-316 expressed in $DG^{-/-}$ ES cells is transported to the cell surface and is functional in the assembly of the DG-sarcoglycan complex (30, 33). Thus, the inability of DGΔ30-316 to restore virus infection in $DG^{-/-}$ ES cells is most likely due to its lack of functional glycosylation and not based on defects in expression and/or transport. These data confirm and extend our previous studies that implicated the N-terminal domain of α-DG in virus binding (32, 33). Together with our binding studies, they indicate a crucial role for LARGE-dependent glycosylation for α-DG's function as a cellular receptor for LCMV and LFV.

An important role of carbohydrate moieties for the recognition of cellular receptor proteins has also been reported for other viruses, like, e.g., influenza (13), Newcastle disease virus (21), and human immunodeficiency virus (57). This crucial role of posttranslational modification adds another level of complexity to the virus-receptor interaction and the virus-host interplay. Since the functional glycosylation of α-DG by LARGE is a highly specific type of modification that is crucial for the biological function of the receptor in the host cell (30), its critical role for virus binding makes it an important factor in the virus-host interaction. The impact of α-DG glycosylation on virus binding affinity is of particular interest considering the crucial role for α-DG binding affinity for the tissue tropism and the disease potential of the virus (5, 33, 45, 48, 50). Immunosuppressive LCMV isolates consistently bind α-DG with affinities in the low nanomolar range and generally depend on α-DG for infection of cells. At the structural level, high-affinity α-DG binding correlates with an aliphatic amino acid (L or I) at position 260 of GP1. Nonimmunosuppressive isolates bind with 2 to 3 logs less affinity to α-DG and generally contain an aromatic amino acid (F) at position 260 (48). The receptor binding characteristics of the immunosuppressive LCMV isolates are similar to those of the human pathogenic LFV, which also shows nanomolar binding affinities and dependence on α-DG for infection (32).

Based on their different receptor binding affinities, immunosuppressive LCMV isolates and LFV, but not nonimmunosuppressive LCMV isolates, are able to displace ECM ligands from α-DG, allowing them to infect specific target cells involved in their disease phenotypes (33, 45). Since glycosylation of α-DG by LARGE affects the binding of arenaviruses as well as ECM proteins, we studied the effect of LARGE-dependent modification on competition between virus and ECM proteins. Using in vitro competition binding assays, we were able to show that LARGE-dependent glycosylation of α-DG influences the ability of LCMV isolates to compete with laminin for receptor binding. To address this issue in the cellular context, we overexpressed LARGE in Vero cells, which show a relatively low endogenous level of LARGE activity. As previously observed with primary human fibroblasts (3), LARGE overexpression resulted in a dramatic increase of functional glycosylation of α-DG and strongly enhanced binding to laminin. Regardless of the level of LARGE-dependent glycosylation of α-DG, high-affinity binding viruses like LCMV cl-13 and LFV competed efficiently with receptor-associated laminin. In contrast, enhanced modification of α-DG by LARGE significantly reduced the ability of the low-affinity binder LCMV ARM53b to compete with laminin. These data suggest that the high receptor binding affinity of a virus is important for the infection of cell types with high LARGE activity which express α-DG forms with high levels of functional glycosylation that tightly associate with ECM ligands.

The crucial role of LARGE-dependent glycosylation for high-affinity α-DG binding by immunosuppressive LCMV isolates and LFV is consistent with overall similarities in their receptor binding characteristics (10, 32, 45, 52). How this conserved mechanism of receptor recognition relates to the pathogenesis of LFV in humans is currently unclear. However, a hallmark of fatal LFV infection in humans is immunosuppression, which results in a failure of the host's immune system to control the virus, leading to an unchecked viremia associated with hemorrhagic disease manifestations (23, 38). As shown earlier for LCMV, high α-DG binding affinity may also be crucial for the ability of LFV to target DCs and to cause immunosuppression. LFV is known to infect human DC in vitro and to alter the antigen presentation function of these cells (36).

We speculate that the crucial role of α-DG glycosylation for binding to human pathogenic arenaviruses like LFV may also in part explain the prevalence of genetic α-DG glycosylation defects in the human population. Although homozygotes suffer from severe muscular dystrophies, otherwise normal heterozygotes may have reduced mortality to arenavirus infection, resulting in a positive selection of these genetic traits.

In conclusion, we show that the interaction of arenaviruses with α-DG critically depends on the posttranslational modification of the receptor by the glycosyltransferase LARGE. The extent of the LARGE modification of α-DG is an important determinant for virus binding affinity and influences the ability of viruses to compete with ECM proteins for receptor binding. Considering the relevance of the receptor binding affinity for the infection of specific cell populations in vivo, the expression of LARGE and the consequent modification of α-DG is likely a key factor in viral pathogenesis.

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