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α-Dystroglycan can mediate arenavirus infection in the absence of β-dystroglycan

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Abstract

Dystroglycan (DG) is a highly versatile cell surface molecule that provides a molecular link between the extracellular matrix (ECM) and the actin-based cytoskeleton. Encoded by a single gene, DG is posttranslationally processed to form α-DG, a peripheral protein identified as the cellular receptor for lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LFV), and the membrane-spanning subunit β-DG. The link of β-DG to the actin-based cytoskeleton and its association with the cellular signal transduction network suggest that it may function as an essential cofactor for the activity of α-DG as a virus receptor. To address this issue, we constructed a deletion mutant lacking the cytoplasmic domain of β-DG and a C-terminal fusion between α-DG and the transmembrane domain of PDGF receptor. Both mutants were functional as virus receptors, indicating that β-DG does not act as a cofactor with α-DG for arenavirus binding and entry. These observations are in agreement with the fact that LCMV infection is independent from the structural integrity of the actin-based cytoskeleton and suggest that α-DG functions primarily in the attachment of arenaviruses to the cell surface.

Keywords: Lymphocytic choriomeningitis virus; Dystroglycan; Receptor

Introduction

Lymphocytic choriomeningitis virus (LCMV) is the prototypic member of the arenavirus family (Buchmeier et al., 2001). The bisegmented negative-strand genome of LCMV consists of two single-stranded RNA species. The larger segment named L encodes the virus polymerase (L) and a small zinc finger motif protein (Z), while the smaller RNA segment (S) encodes the virus nucleoprotein (NP) and glycoprotein precursor (GPC). GPC is processed into the peripheral glycoprotein GP1 and the transmembrane glycoprotein GP2 by the protease SKI-1/S1P (Lenz et al., 2001; Beyer et al., 2003; Kunz et al., 2003). GP1 is implicated in receptor binding (Parekh and Buchmeier, 1986, Borrow and Oldstone, 1992) and GP2 is structurally similar to the fusion active membrane proximal portions of the glycoproteins of other enveloped viruses (Gallaher et al., 2001). Upon receptor binding, arenavirus virions are internalized by uncoated vesicles and released into the cytoplasm by a pH-dependent membrane fusion step (Borrow and Oldstone, 1994; Di Simone et al., 1994; Di Simone and Buchmeier, 1995).

The cellular receptor for LCMV and the human pathogen Lassa fever virus (LFV) has been identified as α-dystroglycan (α-DG) (Cao et al., 1998). DG is a ubiquitously expressed, highly versatile cell surface receptor that provides a molecular link between the extracellular matrix (ECM) and the actin-based cytoskeleton and plays a critical role in cell-mediated assembly of basement membranes (reviewed by Henry and Campbell, 1999; Winder, 2001; Michele and Campbell, 2003). In vertebrates DG is encoded by a single gene (*DAG1*) and is posttranslationally processed to form α-DG, a peripheral protein, and the membrane protein β-DG (Ibraghimov-Beskrovnaya et al., 1992). An N-terminal signal peptide targets DG into the endoplasmatic retic-

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ulum. DG is then cleaved by an unidentified protease after amino acid 653 into α-DG and β-DG. α-DG is secreted and held at the extracellular face of the membrane via noncovalent associations with the extracellular portion of β-DG, which is mediated by residues 550–585 of the C-terminal region of α-DG (Sciandra et al., 2001). α-DG undergoes high-affinity interactions with ECM proteins containing LamG domains, such as laminin, agrin, neurexin, and perlecan and is noncovalently associated with the membrane spanning β-DG. Within the dystrophin glycoprotein complex of skeletal muscle, β-DG binds to dystrophin. In tissues outside of skeletal muscle and brain, full-length dystrophin is replaced by truncated dystrophin variants and the closely related molecule utrophin. Dystrophin and utrophin provide a physical link to the actin-based cytoskeleton and are associated with further cytoskeletal components, e.g., the syntrophins. Other molecules that bind the cytoplasmic tail of β-DG include the signal transduction molecule grb2 and the focal adhesion kinase FAK (Yang et al., 1995; Cavaldesi et al., 1999). The link of β-DG to the actin-based cytoskeleton and its association with the cellular signal transduction network suggest that it may participate as a cofactor for the receptor function of α-DG.

The present study addresses the role of β-DG in the function of α-DG as arenavirus receptor. Biochemical characterization of β-DG deletion mutants demonstrated that β-DG is not essential for biosynthesis and transport of α-DG. A fusion protein of α-DG with the transmembrane domain of platelet-derived growth factor (PDGF) receptor was functional as a receptor for LCMV, indicating that β-DG and its cytoplasmic binding partners are dispensable for the function of α-DG as a virus receptor. These findings suggest that α-DG functions primarily in the attachment of arenaviruses to the cell surface.

Results

β-DG is not essential for the biosynthesis and transport of α-DG

To study a possible role of β-DG for the biosynthesis, cell surface expression, and receptor function of α-DG, we made the following deletion mutants: DGΔC (Δ782–895), which lacks the cytoplasmic domain of β-DG, and α-DG-PDGF, a C-terminal fusion between α-DG and the transmembrane domain of PDGF receptor (Fig. 1A). The mutants were inserted into the E1 region of replication-deficient adenovirus (AdV) vectors. For initial characterization, DG (−/−) mouse embryonic stem (ES) cells were infected with the AdV vectors containing the β-DG deletion mutants and wild-type DG. Cells were solubilized 48 h after AdV infection, and total protein was isolated and subjected to Western blot analysis. α-DGPDGF was expressed at high level, as assessed by detection of the myc epitope (Fig. 1B). Clearly present were high levels of the β-DG part of wild-type DG. For the analysis of virus binding, DG (−/−) ES cells were infected with the AdV vectors containing the β-DG deletion mutants, wild-type DG, and green fluorescent protein (GFP). Cell lysates were prepared 48 h after transfection and subjected to affinity purification using the lectin jacalin that binds to the mucin-type glycans that are present on α-DG (Kunz et al., 2001). For the analysis of virus binding, jacalin-bound glycoproteins were subjected to virus overlay protein binding assay (VOPBA) with LCMV clone-13 (cl-13) (Fig. 1C). Strong binding of LCMV cl-13 to DGΔC, α-DGPDGF, and wild-type DG, but not to proteins isolated from GFP transfectants, indicated that β-DG is not essential for the biosynthesis of α-DG that is functional in virus binding. The very broad appearance of the processed α-DG in the mutants α-DGPDGF and DGΔC, as well as wild-type DG, suggests a similar, high degree of carbohydrate addition to all three DG variants.

Recent reports have demonstrated the release of a soluble form of α-DG from some cultured cell types such as RT4 schwannoma cells (Matsumura et al., 1997) and bovine aortic endothelial cells (Shimizu et al., 1999), as well as from CHO cells expressing heterologous DG (Holt et al., 2000), suggesting some instability of the α/β-DG complex in these cell types. We addressed the release of soluble α-DG from DG (−/−) ES cells transfected with AdV vectors containing the β-DG deletion mutants, wild-type DG, and green fluorescent protein (GFP). Forty-eight hours post-transfection, total cell lysates and conditioned medium were subjected to affinity precipitation with wheat germ agglutinin (WGA) agarose (Holt et al., 2000). WGA-bound glycoproteins were tested for the presence of α-DG by VOPBA with LCMV cl-13 and for the presence of β-DG by Western blot (Fig. 1D). Comparable amounts of α-DG were detected in WGA precipitates of lysates from cells expressing α-DG-PDGF, DGΔC, and wild-type DG. In contrast, α-DG was detected only in the conditioned culture medium of cells expressing DGΔC and wild-type DG but not in conditioned supernatants of cells expressing the fusion protein α-DGP-DGF. The shedding of α-DG from cells expressing DGΔC and wild-type DG is reminiscent of the situation in other cultured cells types and in cells expressing heterologous DG (Holt et al., 2000). The absence of α-DG from conditioned supernatants of cells expressing α-DGPDGF (Fig. 1D) and the presence of the myc-epitope in α-DGPDGF derived from total cell lysates (Fig. 1B) suggest that no significant cleavage occurs between the α-DG moiety and the myc epitope, which is localized N-terminal of the PDGF receptor transmembrane domain. As expected, β-DG was detected only in the cell lysate of cells transfected with wild-type DG. The additional β-DG-reactive band of 140–150 kDa in the Westen-blot of Fig. 1D most likely corresponds to the uncleaved DG precursor, recently described in cells over-expressing DG (Holt et al., 2000). Its absence in the VOBPA suggests incomplete modification of the precursor which reduces its virus binding affinity.

To assess the requirement of β-DG for transport of α-DG

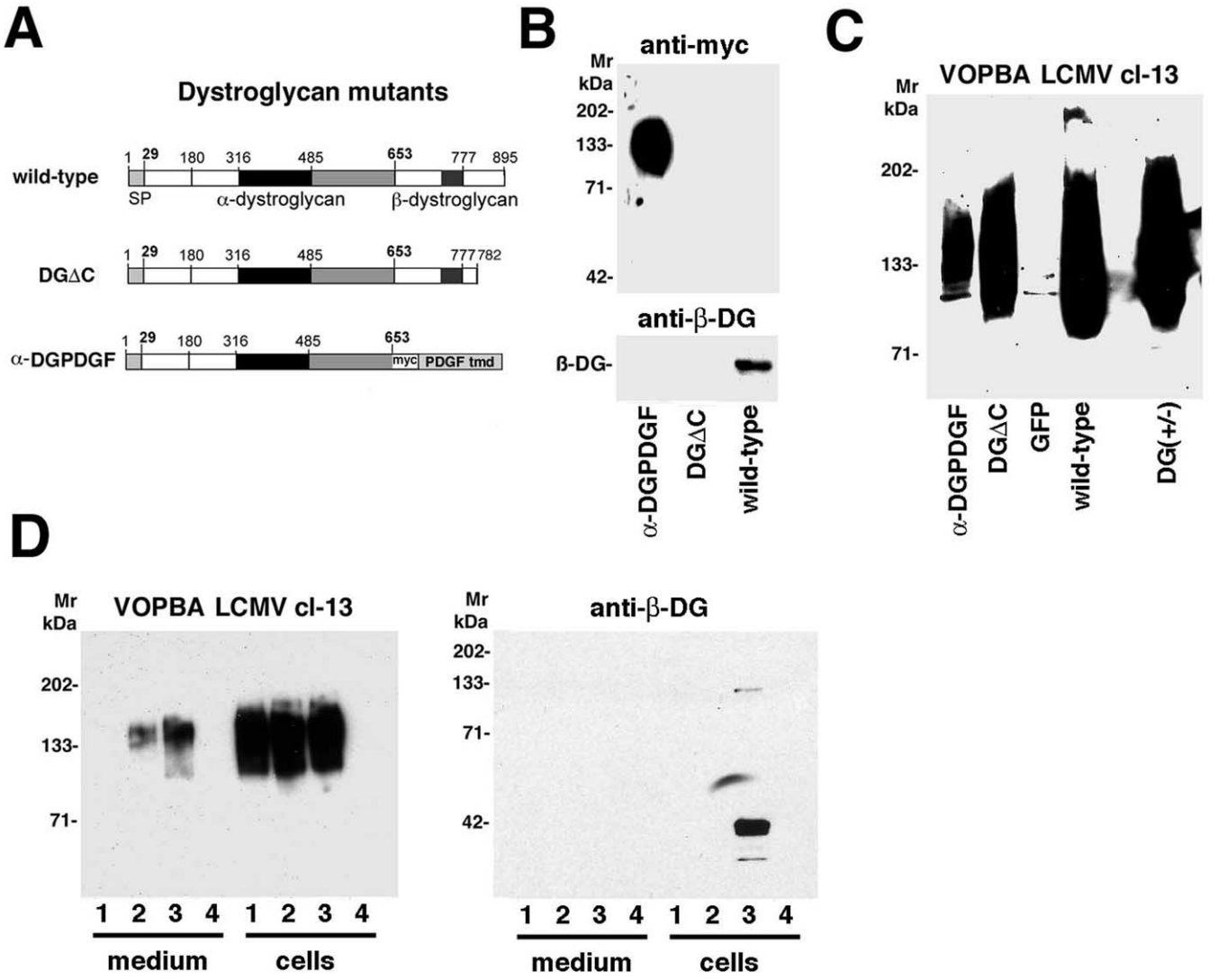

Fig. 1. Expression of β-DG deletion mutants in DG (−/−) ES cells. (A) Schematic representation of the β-DG deletion mutants. The putative N-terminal subdomains, amino acids 30–160 and 181–316 (white), the mucin-related central domain 317–485 (black), and the C-terminal globular domain 486–653 (gray) of α-DG are indicated. Amino acids 654–895 represent β-DG with the transmembrane domain (dark box). The myc epitope at the C-terminus of α-DG and the PDGF receptor transmembrane domain (PDGF tmd) in α-DG PDGF are indicated. (B) Expression of the DG deletion mutants. DG (−/−) ES cells were infected with AdV vectors containing the β-DG deletion mutants or wild-type DG. After 48 h, total protein was isolated, separated by SDS–PAGE, and transferred to nitrocellulose. The blots were probed with antibodies anti-myc (upper panel) and anti-β-DG (lower panel) using ECL for detection. Molecular masses are indicated in kilodaltons (kDa). (C) Binding of LCMV cl-13 to β-DG deletion mutants: DG (−/−) ES cells were infected with AdV containing the β-DG deletion mutants, wild-type DG, and GFP. As a positive control, DG (+/−) ES cells were used. After 48 h, cells were lysed and lysates subjected to jacalin affinity chromatography. Jacalin-bound glycoproteins were separated by SDS–PAGE, transferred to nitrocellulose, and subjected to VOPBA with 10^7 PFU/ml of LCMV cl-13. Bound virus was detected with mAb WE33 and WE36 against LCMVGP using ECL. (D) Detection of the secretion of α-DG into the cell culture medium; 48 h posttransfection, conditioned cell culture medium (medium) and detergent extracts (cells) of cells transfected with AdV vectors expressing α-DGPDGF (1), DGΔC (2), DG wild-type (3), and GFP (4) were subjected to WGA affinity chromatography. WGA-bound glycoproteins were eluted, separated by SDS–PAGE, blotted to nitrocellulose, and subjected to VOPBA with LCMV cl-13 or Western-blot analysis using an anti-β-DG antibody.

to the cell surface, we investigated the cellular distribution of α-DGPDGF expressed in COS1 cells by immunofluorescence microscopy. For comparison with an intracellular protein, cells were transfected with the NP of LCMV ARM53b. As an example for membrane glycoprotein, we used a 3′ transcriptional fusion of CD46 membrane protein with EGFP (Shestopalov and Bassnett, 1999). Forty hours posttransfection, cells were fixed using 2% (wt/vol) formaldehyde and 0.1% (wt/vol) glutaraldehyde to obtain complete immobilization of membrane proteins without permeabilization (Dubreuil et al., 1996). In nonpermeabilized cells, strong staining for α-DGPDGF was detected with an anti-myc antibody (Figs. 2a and e). Lack of permeabilization was verified by the absence of significant staining for the intracellular LCMVNP (data not shown). Upon treatment with 0.1% (wt/vol) saponin, which permeabilizes cell

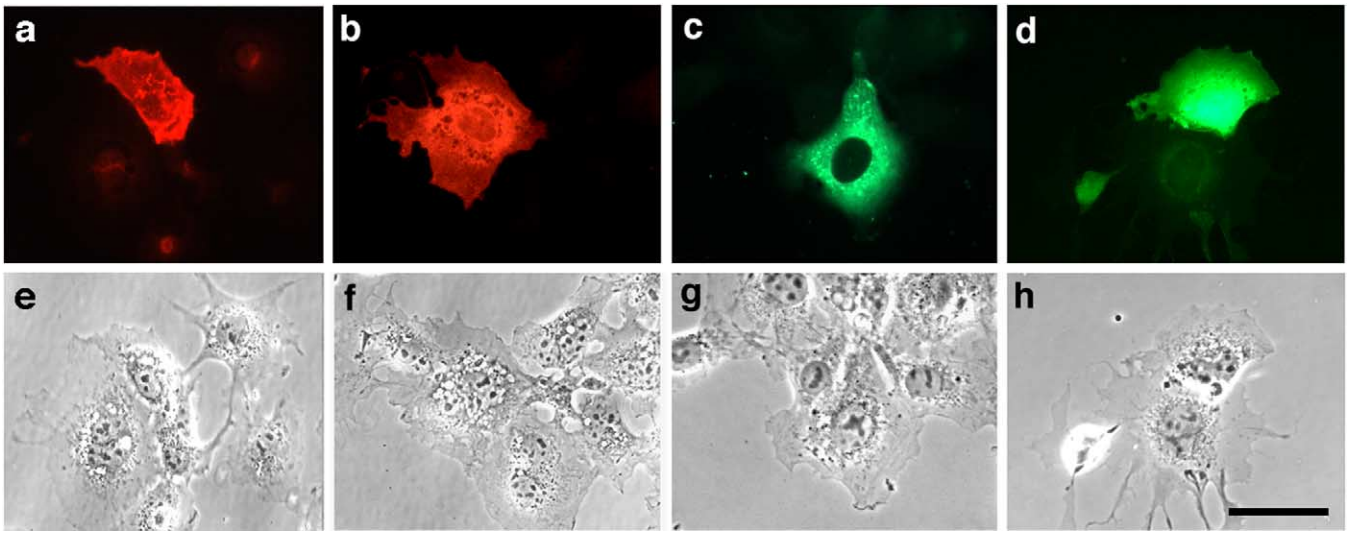

Fig. 2. Localization of α-DGPDGF in mammalian cells. COS1 cells were transiently transfected with α-DGPDGF (a, b, e, f), LCMVNP (c, g), and CD46-EGFP (d, h) using Lipofectamine; 24 h after transfection, cells were transferred to LabTek tissue culture chamber slides. After 24 h, cells were fixed and samples b/f and c/g permeabilized with saponin. α-DGPDGF was detected using an anti-myc antibody (a, b: fluorescence optics; e, f: phase optics). LCMVNP was detected with mAb 113 anti-LCMVNP (c: fluorescence optics; g: phase optics). Primary antibodies were detected with rhodamine-X (red)- or FITC (green)-coupled anti-mouse IgG secondary antibody. The CD46-EGFP fusion protein was detected by direct immunofluorescence excitation using the FITC channel (d: fluorescence optics; h: phase optics). Bar = 20 μm.

membranes but does not affect the overall cytoarchitecture, staining for α-DGPDGF (Fig. 2b, f) resembled that in nonpermeabilized cells (Figs. 2a and e), and, as expected, strong specific intracellular staining was observed for LCMVNP (Figs. 2c and g). The CD46-EGFP fusion protein, detected by direct fluorescence excitation, was found predominantly in the plasma membrane (Figs. 2d and h). The strong staining for α-DGPDGF in nonpermeabilized cells and the similarity of its cellular distribution with the membrane protein CD46-EGFP suggest efficient cell surface expression of the β-DG deletion mutant.

For a more quantitative assessment of cell surface expression of both β-DG mutants, the presence of recombinant proteins in the plasma membrane of transfected cells was determined by a cell surface biotinylation assay (Gonzalez-Dunia et al., 1997). DG (−/−) ES cells were infected with the AdV vectors carrying the β-DG deletion mutants and wild-type DG. Forty hours after AdV-mediated gene transfer, cell surface proteins were specifically labeled by the membrane-impermeable biotinylation reagent NHS-X-biotin. Biotin-labeled proteins were subsequently recovered by incubating total cell lysates with streptavidin–agarose. VOPBA analysis revealed similar amounts of α-DG exposed at the surface of cells transfected with DGΔC and wild-type DG but reduced cell surface expression of α-DG PDGF (Figs. 3A and B). The validity of this approach was shown by negligible biotin-labeling of the intracellular protein α-tubulin (Fig. 3A).

Together, these data indicate that the intracellular domain or β-DG is not required for biosynthesis and transport of α-DG. The lower cell surface expression level of α-DGPDGF when compared to wild-type DG is most likely not due to increased release of α-DG from α-DG PDGF-transfected cells, as no significant shedding of α-DG was observed (Fig. 1D), but may reflect impaired transport of α-DG PDGF or a reduced half-life in the membrane.

β-DG is dispensable for the LCMV receptor function of DG

Since the interaction of β-DG with the cytoskeleton or other binding partners may influence viral binding and/or internalization, we tested the β-DG deletion mutants for reconstitution of susceptibility to virus infection in nonpermissive cells. DG (−/−) ES cells were infected with the AdV vectors carrying the β-DG deletion mutants and wild-type DG. As negative controls we used the AdV containing the α-DG deletion mutant DGH (ΔH30-S485) which is deficient in binding to LCMV (Kunz et al., 2001) or a vector containing a β-galactosidase transgene (*Lac*Z). Forty-eight hours after AdV-mediated gene transfer, transgene expression was verified by VOBPA and Western blot analysis (Fig. 4A). Cells in parallel samples were infected with LCMV cl-13 48 h after infection with the AdV vectors. LCMV infection of cells was assessed after 12, 24, and 48 h by immunofluorescence staining for LCMVNP. Infection of DG (−/−) ES cells with AdV vectors carrying the mutants DGΔC, α-DGPDGF, and wild-type DG, but not DGH or *Lac*Z, made these cells susceptible to LCMV infection (Fig. 4B). These data indicate that β-DG is not essential for entry of LCMV while α-DG is. The reduced cell surface expression levels of α-DGPDGF when compared to wild-type DG, as detected by cell surface biotinylation assay (Fig. 3),

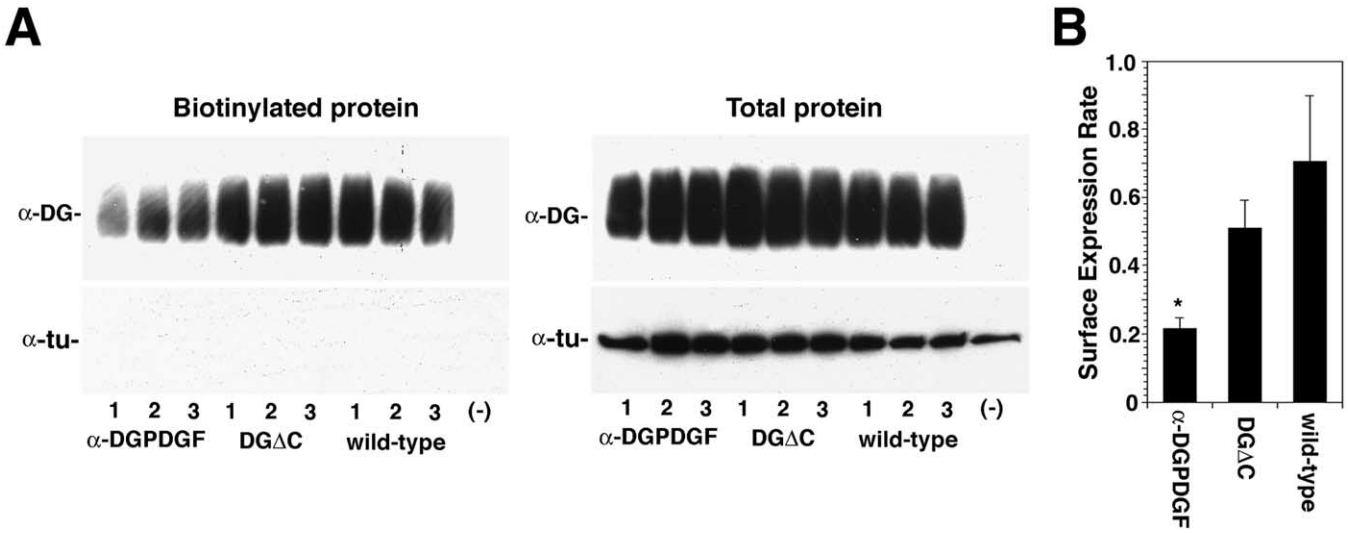

Fig. 3. Detection of β-DG deletion mutants at the cell surface. (A) Cell surface biotinylation assay: DG (−/−) ES cells were infected with AdV vectors containing the β-DG deletion mutants and wild-type DG (triplicates), and GFP (−). After 40 h, cell surface protein was labeled with biotin-X-NHS and the surface-labeled cells were subsequently lysed; 50% of the lysates were subjected to affinity precipitation with streptavidin–agarose. From the residual 50% of lysates total protein was isolated. Streptavidin–precipitated proteins (biotinylated protein) and total protein were separated by SDS–PAGE and transferred to nitrocellulose. Blots were subjected to VOPBA with LCMV cl-13 (upper panel) as described in Fig. 1C and analyzed by Western blot using mAb DM1A anti-α-tubulin (lower panel). Primary antibodies were detected with peroxidase-conjugated secondary antibodies using ECL for detection. Positions of α-DG and α-tubulin (α-tu) are indicated. (B) Quantitative analysis of cell surface expression of α-DG PDGF, DGΔC, DG wild-type by densitometry. X-ray films shown in (A) were scanned with a Storm 860 densitometer and the relative surface expression rates (SERs) were calculated as SER = $I_{\text{cell surface}}/I_{\text{total protein}}$, with $I_{\text{cell surface}}$ = intensity of the signal of the biotinylated protein and $I_{\text{total protein}}$ = intensity of the signal of the total protein, in arbitrary densitometric units ($n = 3 \pm$ SD). *Statistically significant differences.

has apparently no significant effect on susceptibility to virus infection.

Discussion

The present study shows that β-DG is not required for the biosynthesis and transport of α-DG and demonstrates that β-DG does not act as a cofactor with α-DG for arenavirus binding and entry.

The link of β-DG to the actin-based cytoskeleton and its association with the cellular signal transduction network (Henry and Campbell, 1999; Winder, 2001) suggest that it may function as an essential cofactor for the activity of α-DG as a virus receptor. To address this issue, we constructed a deletion mutant lacking the cytoplasmic domain of β-DG (DGΔC) and a C-terminal fusion between α-DG and the transmembrane domain of PDGF receptor (α-DG PDGF). Biochemical characterization of these mutants expressed in DG (−/−) ES cells revealed that their α-DG moieties were functional in virus binding and transport to the cell surface occurred. Quantitative assessment of the cell surface expression levels of the DG variants by cell surface biotinylation revealed a significant reduction in surface expression of α-DGPDGF when compared to wild type. Since no significant shedding of α-DG from cells expressing α-DGPDGF was observed, the reduced cell surface expression levels of α-DGPDGF is most likely not due to increased release of α-DG from α-DGPDGF-transfected cells. The reduced cell surface expression of α-DGPDGF may be either due to a reduced half-life in the plasma membrane and/or impaired transport to the cell surface when compared to wild-type DG.

The function of the β-DG deletion mutants as virus receptors was tested by their ability to reconstitute LCMV infection of DG (−/−) ES cells. Despite its lack of β-DG and its partially reduced cell surface expression level, α-DG PDGF was functional as a virus receptor, indicating that β-DG is dispensable and not required for the receptor function. Hence, association with neither the actin-based cytoskeleton nor with signal transduction molecules that interact with β-DG is needed for the virus receptor function of DG. These observations are in agreement with another report that LCMV infection was independent from the structural integrity of the actin-based cytoskeleton (Borrow and Oldstone, 1994). Taken together, these findings suggest that α-DG functions primarily in the attachment of arenaviruses to the cell surface. Binding of the viral GP to α-DG may be sufficient for the subsequent pH-induced conformational changes that result in the exposure of a fusion peptide-related structure of GP2. Alternatively, initial attachment of virions to target cells mediated by α-DG may be followed by the interaction of the viral GP with another molecule on the cell surface, which may then mediate internalization.

Last, although β-DG is not required for virus receptor

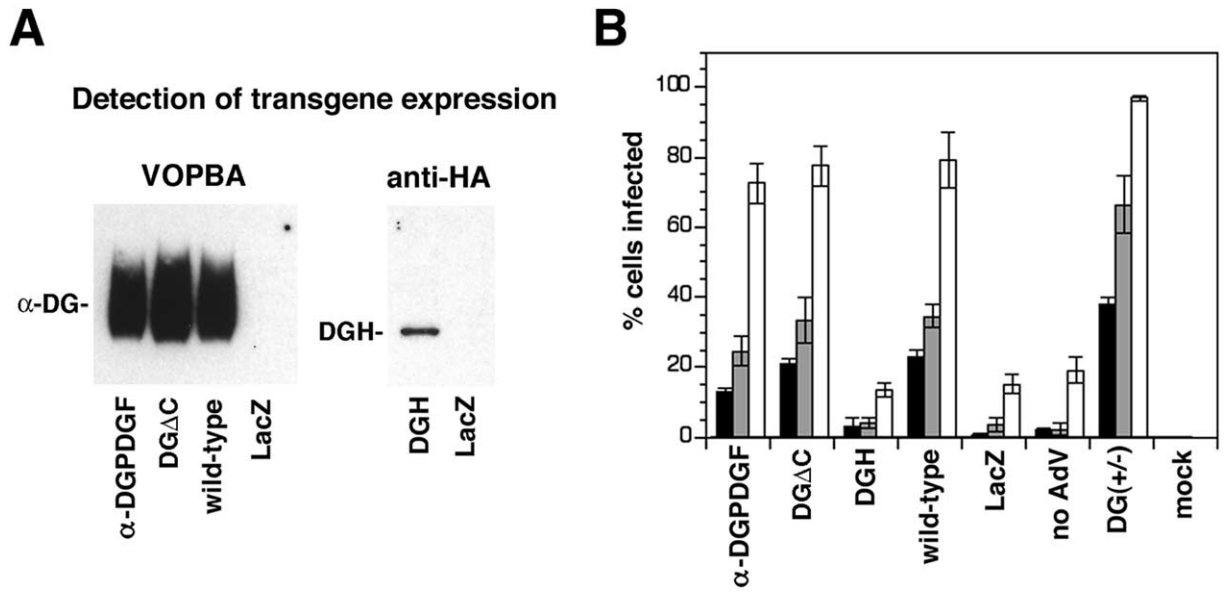

Fig. 4. Reconstitution of susceptibility to virus infection in DG (−/−) ES cells. (A) Verification of transgene expression. DG (−/−) ES cells were infected with AdV vectors containing wild-type, deletion mutants of β-DG, DGH, or a β-galactosidase reporter gene (*Lac*Z) at a MOI of 10. Forty-eight hours after AdV-mediated gene transfer, total cell protein was isolated and separated by SDS–PAGE. Total protein derived from cells expressing α-DG PDGF, DGΔC, DG wild-type, and *Lac*Z were probed by VOPBA with LCMV cl-13 (VOPBA) as described in Fig. 1. Expression of DGH was verified by Western blot (anti-HA). Total protein from cells transfected with DGH and *Lac*Z was separated by SDS–PAGE, blotted to nitrocellulose and probed with an anti-HA antibody using ECL for detection. (B) Reconstitution of susceptibility to virus infection. DG (−/−) ES cells infected with AdV vectors in parallel to the cells examined in (A) were infected with LCMV clone-13 (MOI = 3) 48 h after AdV-mediated gene transfer. Infection levels were assessed after 12 (black bars), 24 (gray bars), and 48 h (white bars) by detection of the presence of LCMVNP by using mAb 113 anti-LCMVNP and a FITC-labeled secondary antibody. For each mutant, 100 cells were analyzed and green fluorescing cells scored as positive ($n = 3$; ±SD). DG (+/−) ES cells were used as a positive control. Cell not infected with LCMV (mock) did not show any detectable LCMVNP staining above background.

function, it may still play a role in virus–host cell interaction. The association of β-DG with signal transduction molecules such as grb2 and FAK (Yang et al., 1995; Cavaldesi et al., 1999) suggests a role of DG in cellular signaling involved in differentiation, growth, and homeostasis. However, the exact role of DG-associated signaling in the host cell is currently not known and recent studies suggest a high degree of complexity of DG-mediated signaling, involving multiple pathways. For example, disruption of laminin–DG interactions in muscle cells resulted in a reduced phosphorylation of protein kinase B (AKT) and its downstream effector glycogen synthase kinase (GSK)-3β, suggesting a link between DG and the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway (Langenbach and Rando, 2002). Depletion of β-DG in an animal model for skeletal muscle atrophy led to decreased activities in the small GTPases H-Ras, Rac1, and Cdc42, supporting a link between DG and the GTPases (Chockalingam et al., 2002). It is conceivable that binding of LCMV virions to the surface of target cells may induce clustering of α/β-DG complexes, resulting in activation of β-DG-associated signal transduction molecules, a possibility we plan to investigate. Activation of cellular signal transduction pathways upon virus–receptor binding may influence target cell function and contribute to viral pathogenesis.

Materials and methods

Proteins and antibodies

Monoclonal antibody (mAb) to LCMV 113 (anti-LCMVNP) and mAb 83.6 anti-LCMVGP2, WE33 (anti-LCMVGP2), and WE36 (anti-LCMVGP1) have been described (Buchmeier et al., 1981; Weber and Buchmeier, 1988), as has polyclonal antibody AP83 against β-DG (Williamson et al., 1997). Polyclonal anti-influenza hemagglutinin epitope (HA) antibody Y11 and mAb 9E10 anti-myc epitope were purchased from St. Cruz Biotechnology (St. Cruz, CA). mAb DM1A anti-α-tubulin was purchased from Sigma. FITC- and rhodamine-X-conjugated anti-mouse IgG were from Jackson Immuno-Research Laboratory (West Grove, PA). HRP-conjugated anti-mouse and anti-rabbit IgG were from Pierce Chemical Co. (Rockford, IL).

Viruses and cells

LCMV clone-13 is a plaque-purified variant of ARM53b derived from spleen cells of an adult BALB/WEHI mouse persistently infected since birth with ARM53b (Ahmed et al., 1984). Seed stocks of all viruses were prepared by growth in BHK-21 cells. Purified virus stocks were pro-

duced and virus titers determined as described (Dutko and Oldstone, 1983; Ahmed et al., 1984).

DG (−/−) and DG (+/−) mouse ES cells were maintained as described (Smelt et al., 2001). Reconstitution of susceptibility to LCMV infection by AdV-mediated gene transfer was performed as described in Cao et al. (1998). AdV vector was applied in a multiplicity of infection (MOI) of 10 and cells were incubated for 48 h prior to infection with LCMV. LCMV was used in a MOI of 3 and infection determined after 12, 24, and 48 h. Infected cells were determined by immunofluorescence staining for LCMVNP (Smelt et al., 2001).

Construction of DG deletion mutants and generation of adenoviral vectors

For the construction of α-DGPDGF, the fragment Fdf-PD was amplified by PCR on the full-length rabbit DG cDNA (Ibraghimov-Beskrovnaya et al., 1992) cloned into the expression vector pDisplay (Invitrogen). Fdf-PD was digested with *Sac*I and *Sal*I and inserted into DG pDisplay. The mutant was verified by double-strand DNA sequencing. The construct DGΔC, containing the deletion Δ782–895 in β-DG, was kindly provided by Jane Lee and Michael Henry (University of Iowa): Fdf: 5′ACCACCGCCGGCGTGCCCCGCGGGGG-A3′; FD: 5′ATTGTCGACGCCGCGGGTGATGGTCTG3′.

The cDNA fragments encoding the β-DG deletion mutants were excised from the pDisplay-based expression constructs and inserted into the shuttle plasmid pAdV/RSV (Kunz et al., 2001). Replication-deficient AdV vectors were generated by in vivo recombination as described (Bett et al., 1994). Twofold plaque-purified isolates were amplified, purified by CsCl gradient centrifugation, and screened for protein expression by Western blot analysis.

Immunoblotting and VOPBA

Total protein and jacalin-bound glycoproteins (Kunz et al., 2001) as well as WGA-bound glycoproteins (Holt et al., 2000) were isolated from ES cells, separated by gel electrophoresis, and transferred to nitrocellulose. After blocking in 5% (wt/vol) skim milk powder in PBS, membranes were incubated with the primary antibody (polyclonal rabbit antibody AP83 against β-DG in a dilution of 1:500, polyclonal rabbit antibody Y11 anti-HA 1: 500, mAb 9E10 anti-myc: 1: 100, and mAb DM1A anti-α-tubulin 1: 1000) in 2% (wt/vol) skim milk powder, PBS for 12 h at 6°C. After several washes in 0.1% (wt/vol) Tween-20, PBS (PBST), the secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG coupled to peroxidase (Roche), were applied 1:5000 for 1 h at room temperature. Blots were developed using Super Signal West Pico enhanced chemiluminescence (ECL) substrate (Pierce) and signals were recorded on autoradiographic film (Kodak, Rochester, NY). VOPBA was performed as described (Cao et al., 1998) using 10^7 pfu/ml LCMV cl-13 purified by ultracentrifigation on a renografin gradient.

Biotinylation of cell surface proteins

For cell surface biotinylation assay, DG (−/−) mouse ES cells (4×10^5 cells/well) were plated in gelatin-pretreated six-well plates. After a 24-h incubation, AdV vectors were applied in a MOI of 10 and cells incubated for 40 h prior to cell surface labeling. Surface-specific biotinylation and isolation of biotinylated proteins were accomplished as described in Gonzalez-Dunia et al. (1997). Densitometric analysis was performed with a Storm 860 scanner using ImageQuant software.

Immunofluorescence staining

COS1 cells (2×10^5 per well of a six-well plate) were transfected with the expression constructs α-DGPDGF pDisplay, LCMVNP pcAGGS (Lee et al., 2000), and pFusN115 that contains a C-terminal transcriptional fusion of CD46 with EGFP (Shestopalow and Bassnett, 1999) using Lipofectamine (GIBCO BRL) resulting in 40–50% transfection efficiency. Cells were maintained for 24 h after transfection, washed, briefly trypsinized, and suspended in medium. After cell count in the presence of trypan blue, 5000 live cells were transferred to each well of poly-L-lysine-pretreated eight-well LabTek tissue culture glass chamber slides (Nunc). After 24 h, cells were fixed with 2% (wt/vol) formaldehyde and 0.1% (wt/vol) glutaraldehyde as described (Dubreuil et al., 1996). Cells were washed three times in PBS and blocked with 1% (vol/vol) FBS, PBS for 30 min. For permeabilization of cell membranes, 0.1% (wt/vol) saponin was added to all buffers and solutions. Primary antibodies mAb 9E10 anti-myc (1:50) and mAb 113 anti-LCMV NP (1:100) were applied for 2 h at room temperature. Secondary antibodies, anti-mouse IgG conjugated to FITC (1:100) or rhodamine-X (1:200), were applied in 1% (vol/vol) FBS, PBS for 1 h at room temperature. The CD46-EGFP fusion protein was detected by direct immunofluorescence excitation using the FITC channel. Images were captured using a Zeiss Axiovert S100 microscope (Carl Zeiss, Inc., Thornwood, NY) fitted with a 20× objective, an AxioCam digital camera, and an automated stage.

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