Role of non-raft cholesterol in lymphocytic choriomeningitis virus infection via $\alpha$-dystroglycan

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Dystroglycan (DG) is an extracellular matrix receptor necessary for the development of metazoans from flies to humans and is also an entry route for various pathogens. Lymphocytic choriomeningitis virus (LCMV), a member of the family Arenaviridae, infects by binding to $\alpha$-DG. Here, the role of cholesterol lipid rafts in infection by LCMV via $\alpha$-DG was investigated. The cholesterol-sequestering drugs methyl-$\beta$-cyclodextrin (M/CD), filipin and nystatin inhibited the infectivity of LCMV selectively, but did not affect infection by vesicular stomatitis virus. Cholesterol loading after depletion with M/CD restored infectivity to control levels. DG was not found in lipid rafts identified with the raft marker ganglioside GM1. Treatment with M/CD, however, enhanced the solubility of DG. This may reflect the association of DG with cholesterol outside lipid rafts and suggests that association of DG with non-raft cholesterol is critical for infection by LCMV through $\alpha$-DG.

Dystroglycan (DG) is a widely expressed, cell-surface protein encoded by a single gene (Henry & Campbell, 1996). Post-translational processing of DG results in a highly glycosylated peripheral membrane protein, $\alpha$-DG, and transmembrane $\beta$-DG (Ibrahimov-Beskrovnaya et al., 1992). The $\alpha$ and $\beta$ subunits retain association by a non-covalent interaction mediated by residues 550-585 of the C-terminal region of $\alpha$-DG (Sciandra et al., 2001). In skeletal muscle, DG interacts with laminin (Douvillé et al., 1988; Ervasti & Campbell, 1993; Smallheiser & Schwartz, 1987), agrin (Gee et al., 1994), neurexin (Sugita et al., 2001), biglycan (Bowé et al., 2000) and other extracellular matrix components (Talts et al., 1999; Yamada et al., 1996). $\beta$-DG interacts directly with dystrophin (Chamberlain et al., 1997; Ervasti & Campbell, 1991, 1993) and utrophin (James et al., 2000), in addition to Grb2 and other proteins (Russo et al., 2000; Spence et al., 2004; Yang et al., 1995) implicated in intracellular signalling (Ilsley et al., 2002). Thus, DG is a structural link between the extracellular matrix and actin cytoskeleton and additionally mediates signalling required for survival (Langenbach & Rando, 2002; Li et al., 2002; Montanaro et al., 1999) and other cell functions (Matsumura et al., 1997; Moiseeva, 2001).

Interestingly, $\alpha$-DG acts as a receptor for the entry of Lymphocytic choriomeningitis virus (LCMV) and the bacterium Mycobacterium leprae (Rambukkana et al., 1998). LCMV is a prototypic member of the family Arenaviridae and infects through binding to $\alpha$-DG (Cao et al., 1998). LCMV is internalized into smooth-surfaced vesicles (Borrow & Oldstone, 1994), suggesting that binding of LCMV to $\alpha$-DG triggers signalling events leading to entry of the virus. Cells contain multiple pathways for endocytosis, including internalization of fluids and cell-surface molecules, as well as small and large microbes, the details of which are still being explored (Lakadamyali et al., 2004; Schwartz, 1995). Previous reports have shown that viruses can be endocytosed into clathrin-studded vesicles, as well as by clathrin-independent mechanisms (Pelkmans & Helenius, 2003). Lipid rafts are major sites for endocytosis into non-clathrin-coated invaginations called caveolae (Parton & Richards, 2003). Caveolae are regulated by the protein caveolin (Le et al., 2002) and can arise from lipid rafts (van Deurs et al., 2003). Caveolae and lipid rafts are cholesterol- and sphingolipid-rich microdomains in the plasma membrane (Brown & Rose, 1992) that are resistant to solubilization in cold, non-ionic detergents (Simons & Ikonen, 1997). Rafts have been implicated in a number of cellular functions such as signal transduction (Simons & Toomre, 2000), modulation of kinase activity (del Pozo et al., 2004; Palazzo et al., 2004; Young et al., 2003), cell migration (Manes et al., 1999; Pierini et al., 2003) and axonal guidance (Guirland et al., 2004). Lipid rafts are also known to be involved in the entry of toxins (Abrami et al., 2003; Montesano et al., 1982) and a number of infectious agents (Manes et al., 2003). The observation that LCMV enters by endocytosis into smooth-surfaced vesicles (Borrow & Oldstone, 1994) suggests the involvement of caveolae and/or lipid rafts. Recent data have implicated DG in the regulation of endocytosis (Zhan et al., 2005). However, membrane-anchored $\alpha$-DG lacking a cytoplasmic extension provided by $\beta$-DG is sufficient to trigger entry of LCMV (Kunz et al., 2003). Thus, the mode of entry of LCMV via membrane-anchored $\alpha$-DG is unclear, as are the signalling events leading to the entry of the virus. The
The aim of the present study was to investigate whether lipid rafts and cholesterol regulate LCMV entry and infectivity through α-DG.

First, we studied the effect of methyl-β-cyclodextrin (MβCD), which extracts plasma-membrane cholesterol and disrupts lipid rafts (Ilangumaran & Hoessli, 1998; Keller & Simons, 1998), on entry/infection of LCMV through DG. For these studies, wild-type mouse embryonic stem (ES) cells or ES cells null for DG (Côté et al., 1999) were infected with LCMV. Infection was detected with a mAb (VL4) (generously provided by Dr Pamela Ohashi, University of Toronto, Canada) to the LCMV nucleoprotein. LCMV was grown on BHK-21 cells and titres were determined on Vero cells (Battegay et al., 1991). Wild-type ES cells (DG+/+) and DG knockout cells (DG−/−) were seeded on gelatin-coated Thermanox cover slips (Nunc) in 24-well plates. Approximately 48 h later, cells were pre-treated with MβCD for 30 min, whilst control cultures were left untreated. Cells were washed twice with PBS and infected with LCMV (m.o.i. = 10−100) for 1−5 h at 37°C in a CO2 incubator. Cells were washed twice with PBS and overlaid with heavy medium [Dulbecco’s modified Eagle’s medium (DMEM) with 2% FBS and 1% methylcellulose] at 37°C in a CO2 incubator. Approximately 8–12 h post-infection, cells were washed twice with PBS, fixed with 4% paraformaldehyde and immunostained for viral nucleoprotein by using VL4 rat primary antibody and fluorescently labelled goat anti-rat secondary antibody. LCMV-positive cells were visualized by using a Zeiss epifluorescence microscope. Single infected cells were primarily found, with no indication of widespread infection or plaque formation. Infection of wild-type ES cells was 60–100-fold higher than DG-null cells (data not shown), confirming that DG is the dominant receptor for entry of LCMV into ES cells (Cao et al., 1998). Pre-treatment of wild-type ES cells with MβCD reduced infection of cells in a dose-dependent manner (Fig. 1a). There was no decrease in cell viability, as determined by trypan blue dye exclusion, after treatment of cells with the highest concentration of MβCD used in this study (data not shown). The residual entry of LCMV at the highest concentration of MβCD may reflect incomplete cholesterol depletion (Fig. 1d) and disruption of lipid rafts or a raft-independent pathway.

To investigate the inhibitory effect of MβCD, we used filipin and nystatin, two additional drugs that extract membrane cholesterol and disrupt lipid rafts. Treatment with either of these drugs inhibited infection by LCMV in a dose-dependent manner (Fig. 1b and c). This supported the results indicating that the inhibitory effect of MβCD was due to depletion of cholesterol. As cholesterol is a major membrane component, we wondered whether these drugs

![Figure 1](image-url)

**Fig. 1.** Membrane cholesterol is required for LCMV infection. (a−c) Wild-type ES (DG+/+) cells were pre-treated with MβCD (a), filipin (b) or nystatin (c) for 30 min or left untreated (controls). Cells were then infected with LCMV (●, m.o.i. = 10−100) or rVSV–GFP (○, m.o.i. = 0.5−1.0). Entry of LCMV was quantified by immunostaining. For rVSV–GFP infection, developing GFP-positive foci were counted. (d) ES (DG+/+) cells were treated with MβCD and total cholesterol was determined by using an Infinity assay kit (Thermo Electron Corp.) (Danthi & Chow, 2004). Each data point is the mean ± SD of at least two independent experiments. *P < 0.05 compared with the control.
were broadly disrupting cell function and rendering the cells non-permissive to viral endocytosis and infection. Therefore, we determined whether the effect of cholesterol depletion was specific to entry/infection of LCMV by monitoring endocytosis of vesicular stomatitis virus (VSV). Cells treated with MβCD (as above) were infected (m.o.i. = 0.5–1) with a replication-competent recombinant VSV expressing green fluorescent protein (rVSG–GFP) (kindly provided by John Bell, Ottawa Regional Cancer Centre, Canada) and GFP-positive developing foci were counted at 8–10 h post-infection. Cholesterol depletion by MβCD did not inhibit infection by rVSG–GFP (Fig. 1a–c). In fact, there was a consistent increase in infection by VSV after cholesterol depletion (Sánchez-San Martin et al., 2004). This could possibly be due to an increase in clathrin-mediated endocytosis. Nevertheless, this provided further support that inhibition of LCMV infectivity by cholesterol depletion was specific to LCMV and also was consistent with the notion that these drugs do not affect viral entry or replication etc. by grossly inhibiting cell function.

Next, we asked whether cholesterol recovery following MβCD treatment would reverse the inhibitory effects of MβCD. Cells were treated with MβCD (5 mM) for 30 min, washed twice with PBS, allowed to recover either in complete medium (DMEM with 10% FBS) or in complete medium supplemented with MβCD-conjugated cholesterol (Shigematsu et al., 2003) and then infected with LCMV. There was no significant recovery of infection when cells were allowed to recover in complete medium compared with cholesterol-depleted cells without recovery (Fig. 2). However, cells in medium supplemented with MβCD-conjugated cholesterol recovered within 30 min (the shortest time point tested) to levels equivalent to cells not treated with MβCD (Fig. 2). Together with previous results, these data suggested that cholesterol plays an important role in DG-mediated infection of cells by LCMV. As cholesterol depletion had no effect on the endocytosis/infection of VSV, we speculated that membrane cholesterol might be required at the entry step of LCMV by maintaining the DG receptor in cholesterol-rich microdomains on the cell surface.

On several occasions, it has been demonstrated biochemically that cell-surface proteins are either associated constitutively with rafts (Cottin et al., 2002; Drevot et al., 2002; Elortza et al., 2003; Popik et al., 2002) or recruited into lipid rafts after ligand binding (Lafont & Simons, 2001; Lang et al., 2002; Montixi et al., 1998; Pfeiffer et al., 2001). Lipid rafts are isolated easily by density-gradient centrifugation due to their high lipid content and resistance to detergent solubilization (Chamberlain, 2004; Doyle et al., 1998). To determine whether DG was a component of lipid rafts, wild-type ES cells were solubilized in extraction buffer [10 mM HEPES (pH 8.0), 150 mM NaCl, 0.5 mM CaCl2, 1 × protease inhibitor cocktail (Roche) containing 1:0 % Lubrol WX] for 30 min at 4 °C. This extract was mixed with an equal volume of 80 % sucrose in extraction buffer without detergent in a centrifuge tube. Solutions of 25 and 5 % sucrose in extraction buffer without detergent were layered on top of the extract and centrifuged at 275 000 g for 16 h. Fractions of approximately 1–0 ml were collected from the top of the gradient. The ganglioside GM1, a marker of lipid rafts (Dillon et al., 2000), was detected by dot-blot analysis of fractions using horseradish peroxidase (HRP)-labelled cholera toxin B (Sigma-Aldrich; Fig. 3a). Lubrol WX extraction resulted in enrichment of GM1 at the top of the gradient, consistent with the behaviour of lipid rafts in these gradients. Fractions containing lipid rafts as well as soluble fractions from the gradient were then concentrated, and equal volumes were subjected to SDS-PAGE and analysed by Western blotting using an affinity-purified β-DG antiserum raised against the last 15 aa (Zhan et al., 2005). As shown in Fig. 3(b), β-DG did not fractionate with GM1 in the density-gradient fractions, but was found exclusively in the soluble fraction, suggesting that it is not associated with lipid rafts. Similarly, α-DG was not found in the lipid rafts (Fig. 3c).

Next, we investigated whether β-DG is recruited into lipid rafts after binding of LCMV to α-DG. Wild-type ES cells were infected with LCMV (m.o.i. = 100) or were left untreated at 4 °C for 1–5 h. Cells were washed twice with ice-cold PBS and subjected to centrifugation as described above. There was no increase in the association of β-DG with lipid rafts, even after LCMV binding (data not shown). It is possible that DG, like several other proteins associated with lipid rafts, is solubilized by the non-ionic detergents used to isolate lipid rafts (Gustavsson et al., 1999; Simons & Toomre, 2000). It is also possible that binding of the virus increases movement of DG into lipid rafts, but even at the high m.o.i. that we used, the virus may occupy only a small fraction of the DG, making its shift into lipid rafts difficult to detect. Similar effects of cholesterol-depleting drugs have been

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**Fig. 2.** Cholesterol loading after MβCD treatment reverses the ability of LCMV to infect cells. Wild-type ES (DG+/+) cells were pretreated with MβCD or were left untreated for 30 min. After cholesterol depletion, cells were allowed to recover before LCMV infection as in Fig. 1. Entry of LCMV was quantified by immunostaining. Each data point is the mean ± SD of at least two independent experiments. *P < 0.05 compared with the MβCD-treated control.
Depletion of cell cholesterol alters the detergent solubility of non-raft-specific membrane proteins (Lambert et al., 2005). Indeed, the increase in β-DG signal in the Lubrol WX-soluble fraction after MβCD treatment (Fig. 3c) suggested that MβCD alters the lipid environment of DG. This was substantiated by treating wild-type ES cells with MβCD (5 mM) in serum-free DMEM for 15–90 min, followed by solubilization in Lubrol WX buffer. After centrifugation at 106 000 g for 30 min, supernatants were analysed for β-DG by Western blotting. As shown in Fig. 3(d and e), depletion of the cellular cholesterol by MβCD treatment resulted in increased solubility of β-DG with time. As these same conditions inhibit viral infection significantly, it is reasonable to speculate that cholesterol is critical for DG-mediated infectivity of LCMV. How can such a subtle effect of MβCD result in the significant inhibition of LCMV infection? One possibility is that disruption of DG–protein complexes in the cholesterol-rich microdomain results in loss of signal transduction for viral entry. Alternatively, cholesterol may form a significant fraction of the boundary lipids assembled with DG. Extraction of this layer of cholesterol would result in a DG pool that is non-functional for virus entry. Similar results have been reported for other receptors (von Tresckow et al., 2004). LCMV entry can be mediated by the extracellular domain of DG alone, raising the intriguing question of how downstream events are signalled intracellularly. Our data argue against the involvement of lipid rafts in this signalling pathway. Nevertheless, association of DG with non-raft cholesterol provides an environment crucial for proper signalling for LCMV infection. Delineating the mechanism of LCMV infection and the role of the effector molecules involved in signalling virus entry would provide important information that could be extended to other highly pathogenic members of this family of viruses. This could allow the design of therapeutics to interfere with the infection and spread of LCMV and other arenaviruses.

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