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Membrane localization of Junín virus glycoproteins requires cholesterol and cholesterol rich membranes

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ABSTRACT

Arenavirus morphogenesis and budding occurs at cellular plasma membrane; however, the nature of membrane assembly sites remains poorly understood. In this study we examined the effect of different cholesterol-lowering agents on Junín virus (JUNV) multiplication. We found that cholesterol cell depletion reduced JUNV glycoproteins (GPs) membrane expression and virus budding. Analysis of membrane protein insolubility in Triton X-100 suggested that JUNV GPs associate with cholesterol enriched membranes. Rafts dissociation conditions as warm detergent extraction and cholesterol removal by methyl-β-cyclodextrin compound showed to impair GPs cholesterol enriched membrane association. Analysis of GPs transfected cells showed similar results suggesting that membrane raft association is independent of other viral proteins.

1. Introduction

The *Arenaviridae* family is divided geographically and phylogenetically into the New World and the Old World complex. The former is subdivided into three different clades, of which clade B is of particular interest since it contains five of the seven significant human pathogens that cause severe hemorrhagic fever [1–3]. The high mortality rate and the lack of effective treatment raised a concern about these viruses [4]. Therefore, the complete knowledge of arenavirus multiplication mechanism would facilitate and improve design of new strategies to combat or avoid these dangerous microbial agents. Junín virus is the etiological agent of Argentine hemorrhagic fever, an endemo-epidemic disease affecting the population of the most fertile farming lands of Argentina [5]. Virions are enveloped and contain two segments of ambisense single-stranded RNA encoding the RNA-dependent RNA polymerase, the matrix protein Z, the nucleocapsid-associated protein NP and the glycoprotein precursor GPC. This precursor is processed by proteolytic cleavage into three spike component proteins: GP1 is the peripheral membrane glycoprotein involved in receptor binding, GP2 is the integral glycoprotein necessary for fusion and the signal peptide is required for efficient trafficking of GP1–GP2 complex to the plasma membrane and its subsequent fusion activity [6].

Enveloped viruses are able to use host cell structures and strategies to process and transport glycoproteins from their site of synthesis to a suitable localization in the plasma membrane. Among other cellular factors, cholesterol was shown to play vital roles in virus entry and release. Examples of viruses that require cholesterol to enter cells include hepatitis B virus [7], respiratory syncytial virus [8], Newcastle disease virus [9] and Rift Valley Fever Virus [10]. It was previously reported that entry of both Lassa virus and lymphocytic choriomeningitis virus, both members of the Old World arenaviruses, requires cholesterol [11,12]. In contrast, we have showed that JUNV entry is not affected by cholesterol depletion [13].

The role of cholesterol has also been studied at late steps of virus replication. It has been demonstrated that cholesterol-rich rafts membranes serve as sites for recruitment of several viral proteins, including influenza virus [14], human immunodeficiency virus type 1 [15] y measles virus [16]. Protein targeting in a limited area of the cell surface would improve and direct viral morphogenesis and budding from the plasma membrane serving as platforms for virus egress [17]. The aim of the present study is to define cholesterol involvement at final steps of Junín virus replication.

2. Materials and methods

2.1. Cells, viruses and plasmids

Vero (ATCC CCL81) and MDCK cells (ATCC CCL34) were cultured in Eagle's minimum essential medium supplemented with 50 μg/ml gentamycin and containing 10% heat-inactivated fetal bovine serum. The naturally attenuated JUNV IV4454 strain [18] was used in all experiments with live virus and propagated in Vero cells. The Influenza A/Texas/36/91 strain was kindly provided by V. Savy

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(ANLIS Dr. C.G. Malbrán, Argentina). Virus yields were determined by plaque formation (PFU) assay. Plasmid pcDNA3.1 (Invitrogen) containing the full sequence of GPC and C-terminal fused to myc tag allows detection of GP2 protein.

2.2. Reagents and antibodies

The compounds 20α-hydroxycholesterol (20-OHC), 25-dihydroxycholesterol (25-OHC), lovastatin (L), mevalonate (M), methyl-β-cyclodextrin (CD), and secondary antibodies conjugated to FITC were purchased from Sigma (USA). MAbs reactive against JUNV GPs (GB03-BE08, GD01-AG02 and OD01-AA09) reveal both; GP1 subunit of GPC and uncleaved GPC. MAb NA05-AG12 was used to reveal JUNV NP [19]. MAb reactive against influenza A virus hemagglutinin (HA) was provided by Dr. J. Cámara (Instituto Vanella, Argentina). Anti-caveolin-1 antibody was purchased from Santa Cruz (Biotechnology, Inc) and anti-myc MAbs from Millipore.

2.3. Assays to reduce cholesterol

Vero cells were infected with JUNV (MOI = 0.1) for 1 h at 37 °C and then incubated in medium containing 10–100 μM of oxysterols (25-OHC or 20-OHC), or cholesterol. In other experiments Vero cells were pre-incubated for 24 h in the presence or absence of 10 μM lovastatin and 0.125 mM mevalonate (L/M) [20]. Then, the cultures were infected, incubated the subsequent 24 h in the same mix of L/M and virus yield determined by PFU assay. When CD was used, previously to cultures fixation for membrane immunofluorescence, a short pulse of extraction with 10 mM CD was performed in PBS for 30 min at 37 °C. Viability of cell cultures under each treatment condition was determined by MTT assay. To determine released virus in L/M treated cells, supernatants of 24 h p.i., in the presence of the compounds L/M were concentrated (100 kDa molecular mass centrifugal membrane, Millipore) and the presence of JUNV NP protein revealed by western blot.

2.4. Cholesterol determination

Control and both L/M and L/M + CD treated cultures were analyzed for cholesterol content. Membrane cells were extracted by Bligh & Dyer method and resolved in thin layer chromatography (TLC). Cholesterol quantification was carried out according to Igal et al. [21]. Neutral lipid separation was performed with the following solvent system: hexane: ethyl ether: acetic acid, 80:20:2. Known amounts of pure lipid standard were seeded and run on the TLC plates in parallel to samples. An aliquot of 100 μM of 1,6-diphenyl-1,3,5-hexatriene was added to solvent systems to visualize the lipid spots on the plate under UV light. Fluorescent lipid spots from samples and standard were photographed and cholesterol mass was determined using a DS120 Kodak Image system.

2.5. Indirect immunofluorescence (IFI) assay

Cells grown on coverslips, and treated with compounds as detailed above, were fixed with methanol for 10 min at −20 °C, for cytoplasmic staining or with 4% of formaldehyde for 10 min at room temperature for membrane staining. To detect JUNV NP and GP proteins MAbs were used as described in Martinez et al. [13]. Samples were examined using an Olympus BX51 microscope and the average number of positive cells on each coverslip was calculated as the number of virus positive cells over the number of total cells of 20 optical fields chosen randomly. Background red colour cells correspond to Blue Evans staining.

2.6. Isolation of detergent-resistant membrane (DRM) fractions

A total of 10^7 cells were infected and DRM isolation was performed according to Manie et al. [16]. Briefly, infected monolayers were lysed in 0.6 ml of ice-cold TNE/TX-100 buffer (25 mM Tris–HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA containing 1% Triton X-100 (TX-100) plus protease inhibitors at 4 °C or 37 °C for 30 min. The ratio of lysis buffer volume to cell number was kept constant throughout the experiments. After TX-100 treatment, the lysate was made 40% with respect to sucrose. Then, complete lysate-sucrose mixture was sequentially overlaid with 2 ml of 30% sucrose and 1 ml of 4% sucrose prepared in TNE, and the mixture was centrifuged at 200,000×*g* for 20 h in a SW50.1 Ti rotor (Beckman). In some experiments volumes of sucrose was replaced by Optiprep (Sigma). The gradient was fractionated into 0.6 ml-fractions from the top to the bottom of the tube. Treatments with CD or specific antibodies cross-linking were performed before TNE/TX-100 extraction, for 30 min at 37 °C.

2.7. Immunoprecipitation and westernblot

Equal volumes (150 μl) of fractions were subjected to immunoprecipitation by incubating them with monoclonal antibodies directed against JUNV GPs Immunoprecipitates were washed, boiled and after SDS–polyacrylamide gel electrophoresis (SDS–PAGE), gel slabs were subjected to fluorography.

For western blot analysis, 20 μl of each gradient fraction was resolved in SDS–PAGE gels and proteins were transferred onto PVDF membranes, followed by incubation with specific MAbs. Enhanced chemiluminiscence system (Amershan–Pharmacia) was used to reveal protein bands.

3. Results

3.1. Effect of cholesterol-lowering agents on JUNV multiplication

To explore the role of cholesterol in JUNV multiplication we tested two oxysterols, 20-OHC and 25-OHC cholesterol, known to downregulate the synthesis of Hydroxy Methyl Glutaryl (HMG) CoA reductase, a key enzyme in the endogenous synthesis of cholesterol by cells [22]. The lack of toxicity of the compound was determined by MTT assay, under the same treatments conditions that were used in virus infected cells (Fig. 1A). When Vero cells were infected and treated with the oxysterols for 24 h, a dose-dependent inhibition was observed. Inhibition of virus infectivity reached values of 90% at the maximum concentration tested. In contrast, cholesterol treatment tested in parallel showed no effect. To address the effect of a more direct cholesterol-lowering agent on JUNV multiplication we used lovastatin, a competitive inhibitor of HGM CoA reductase activity [23]. For this purpose, we combined lovastatin (5–80 μM), which inhibit the "de novo" synthesis of cholesterol, with small amounts (0.125–0.250 mM) of mevalonate to allow the synthesis of non-sterols products [20]. Cells were treated for 48 h with different combinations of L/M and it was observed that in complete absence of mevalonate, cell viability decreased significantly even at lowest lovastatin concentrations (data not shown). Based on cell toxicity data, we chose the combination 10 M/0.125 mM of L/M, which showed 96% viability. Cells were treated, infected and then supernatants were collected. In these conditions we found an inhibition of 68% on JUNV yield (Fig. 1B). We have previously demonstrated that JUNV entry is not affected by cholesterol reduction/depletion [13]. Then, reduced yield of JUNV virions depicted in Fig. 1B were not due to impairment on virus cell entry. To address the question whether reduced infectivity in cholesterol depleted cells was due to low levels of total virus budding/

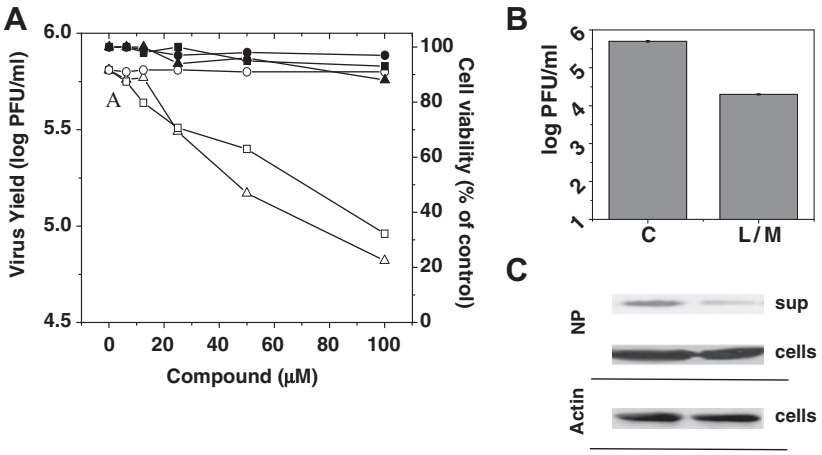

Fig. 1. Effect of cholesterol-lowering agents on JUNV production. (**A**) Infected Vero cells were treated with different concentrations of cholesterol (○) or cholesterol analogs: 20-OCH (Δ) and 25-OCH (□). Supernatant was collected at 24 h p.i. and virus titers were determined by PFU method. Cell viability was assessed by MTT assay (black symbols). Each point is the mean value of triplicate independent experiments where controls were incubated in the absence of compounds. (**B**) Vero cells were pre-incubated for 24 h in the presence or absence of L/M. Then, the cultures were infected and incubated the subsequent 24 h in the same mix of L/M. Untreated infected cultures (C) were used as controls. An aliquot of each supernatant was collected at 24 p.i. and virus yield determined by PFU assay. (**C**) The remaining supernatants (sup) and total cell lysates (cells) were subjected to western blot to detect viral nucleoprotein (NP) and actin protein. Representative photographs of 3 independent experiments are shown.

release or a reduced/defective infectivity of the released new particles after treatment, western blot was performed. JUNV NP levels were followed in both total cell lysates and supernatant samples to check viral protein synthesis and virus particle production respectively. While cholesterol depleted cells showed to produce normal amounts of intracellular NP, reduced levels of this protein in supernatants suggest the impairment of either virus budding or release under these conditions (Fig. 1C).

3.2. JUNV glycoprotein expression under cholesterol lowering conditions and cholesterol removal

To further characterize the effect of cholesterol depletion on JUNV production, intracellular viral proteins expression was analysed. Cells were grown on coverslips, infected and treated with L/M as in Fig. 1B, cell cholesterol mass was determined and cytoplasmic expression of both, NP and GP, was analysed by immunofluorescence (Fig 2). On treated cells; neither the number of positive cells nor the pattern of expression of these proteins was altered confirming that protein synthesis was not affected (Fig. 2a and d and b and e). Under this L/M treatment membrane cholesterol levels diminished up to 35% (Fig. 2B, right panel).

When immunofluorescence assays were performed to reveal membrane antigen we found a reduced JUNV glycoprotein expression on the surface of infected cells (Fig. 2f) comparing to control conditions (Fig. 2c). Quantification of number of membrane GP positive cells showed around 72% of inhibition (Fig. 2B) suggesting that reduced levels of cholesterol might be affecting membrane destination of GPs.

3.3. Cholesterol extraction efficiently removes JUNV glycoproteins from the cell membrane

In the previous experiments we used strategies that globally reduce cell cholesterol and they showed to specifically reduce GP expression on the membrane of infected cells. To further reduce cholesterol mass in membrane cells, we combined L/M treatment with a short pulse of CD, a cyclic sugar that efficiently and rapidly extracts cholesterol from the plasma membrane [24]. GP membrane localization was assessed by immunofluorescence and cholesterol mass determined as describe in methods. The data revealed that this treatment was the most effective in reducing overall cell cholesterol up to 70% (Fig. 2B, right panel). Also in this condition we found that JUNV glycoprotein plasma membrane expression was dramatically decreased (91%) as shown in Fig. 2g. These results suggested that cholesterol could be not only necessary during transport after glycoprotein synthesis but also that the localization of GP in the membrane could be cholesterol related.

3.4. Membrane localization. Glycoprotein insolubility in Triton X-100

The results described above led us to explore the contribution of cholesterol enriched membrane rafts on JUNV GPs localization. Infected Vero cells were extracted on ice with TX-100 1% and centrifugation in density gradient was performed to examine JUNV GP localization. In parallel, cells infected with influenza virus were processed to follow HA positive raft localization [14]. As it can be seen in Fig. 3A left panel, after cold TX-100 treatment HA was found predominantly on the top fractions of the gradient showing its characteristic raft protein behaviour or DRM association [25]. Likewise, JUNV GPs localized to the same DRM fractions. On the other hand viral nucleoprotein NP was found all along the fractions suggesting non raft association. When JUNV infected cells were TX-100 treated at warm temperature specific low dense distribution was lost in accordance with raft model prediction and HA behaviour (Fig. 3A, right panel).

3.5. Removal of cholesterol renders JUNV glycoproteins soluble

Our results indicated that JUNV GPs would associates with TX-100 insoluble membranes. DRM fractions were similar to those in which HA localized. Then, to address the prediction that cholesterol-depletion would disrupt rafts and render soluble JUNV GPs, we used CD prior to cold solubilisation [16]. Treatment of JUNV infected Vero cells with CD, caused a shift on JUNV GPs localization, now found in the bottom fractions (Fig. 3B, left panel). Removal of cholesterol dramatically affected cold insolubility of GPs as can be seen when comparing with Fig. 3A, left panel.

3.6. Stabilization of membrane domains by induced antibody-cross-linking

When cross-linked by specific antibodies, or multivalent ligands, a number of raft-associated proteins were observed to improve the interaction with DRMs [26]. We therefore tested whether cross-linking of JUNV GPs previous to TX-100 extraction

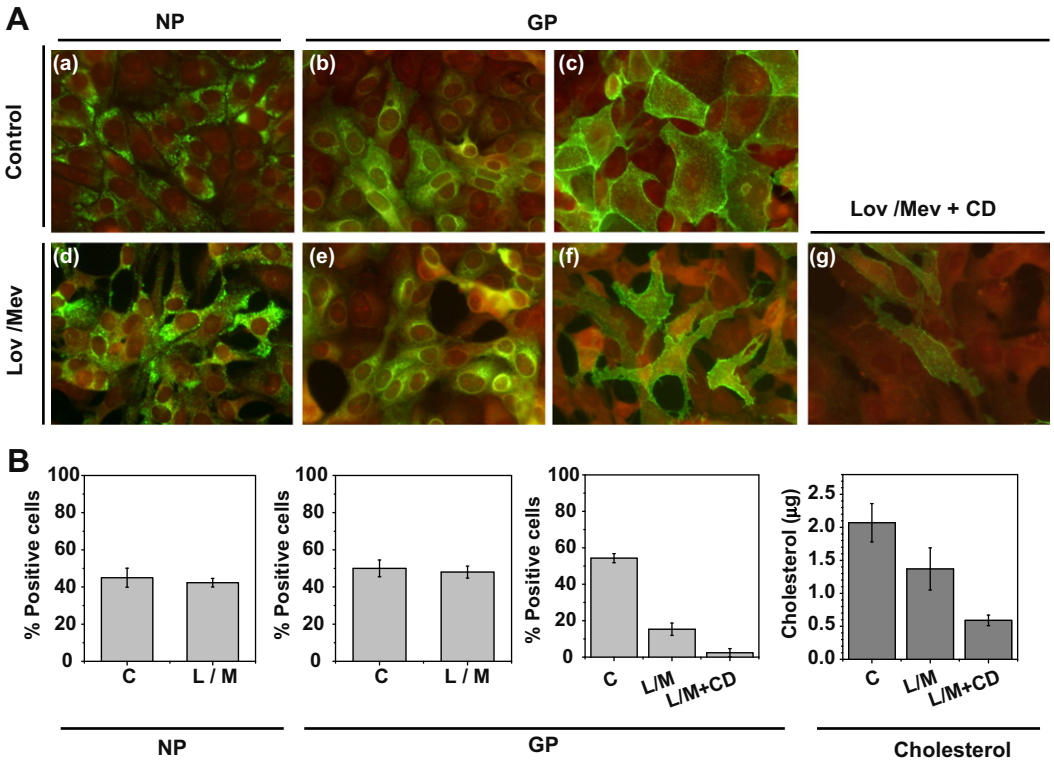

Fig. 2. Effect of diminished cholesterol cell levels over JUNV protein expression. (**A)** Vero cells pre-treated for 24 h in the presence of L/M were then infected with JUNV. After infection cells were incubated the subsequent 24 h in the same mix of L/M (d, e and f) and short pulsed with CD (g). Untreated infected cultures were used as controls (a, b and c). Cells were processed for cytoplasmic immunofluorescence of NP (a and d) and GPs (b and e) expression or membrane immunofluorescence of GPs surface distribution (c, f and g). (**B)** Quantification of positive fluorescent cells and cholesterol mass determination corresponding to each of the treatment showed in 2A.

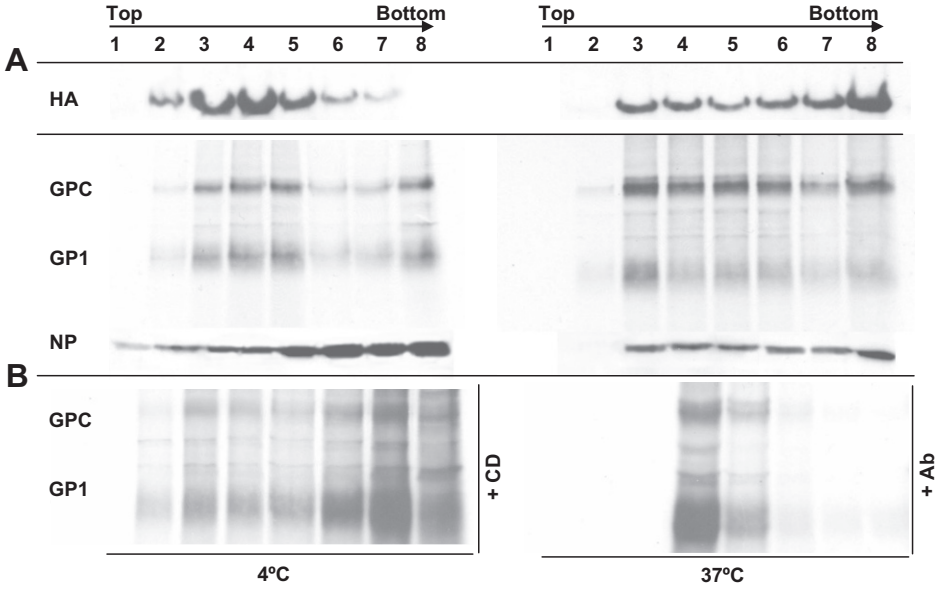

Fig. 3. JUNV glycoproteins are associated with DRM. (**A)** Infected monolayers were lysed in ice-cold TNE/TX-100 1% buffer at 4 °C (left panel) or 37 °C (right panel) for 30 min. After TX-100 treatment the lysates were subjected to sucrose gradient ultracentrifugation and fractions 1 to 8 were taken from the top of the gradient. Viral proteins were immunoprecipitated with MAbs against JUNV GPs or analyzed by western blot for Influenza HA or JUNV NP. (**B)** Cholesterol removal (+CD) or specific antibodies cross-linking (+Ab) were performed for 30 min before TNE/TX-100 extraction. Temperatures depicted correspond to temperatures at which detergent solubilisation was perform. Representative photographs of 3 independent experiments are shown.

had an effect on their association to insoluble membrane fractions. Fig. 3B right panel, shows top fraction localization of JUNV GPs when cross-linked cells were solubilised at 37 °C. As it can be seen the addition of antibodies improved the insolubility of GPs avoiding complete re-distribution in the gradient (compare with 3A right panel).

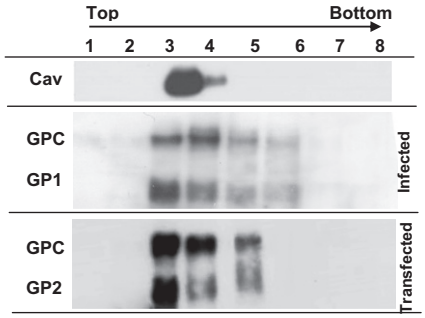

Fig. 4. JUNV GPs localization is independent of other viral components. Infected monolayers or transfected with JUNV GPs expression vector were lysed in ice-cold TNE/TX-100 1% buffer at 4 °C for 30 min. After TX-100 treatment the lysates were subjected to Optiprep gradient and ultracentrifugation and fractions 1 to 8 were taken from the top of the gradient as exactly defined for sucrose gradients. Viral proteins were revealed with MAbs against myc-tag. Caveolin-1 (cav) control protein is also shown to confirm DRM association and to make comparable different gradient experiments. Representative photographs of 3 independent experiments are shown.

3.7. JUNV GPs localization is independent of other viral components

Many reports have demonstrated the role of Z protein in forming virus like particles [27]. We tested whether the specific membrane localization of JUNV GPs was still an occurring phenomenon in the absence of any other viral component. Cells were transfected with a vector containing the full length sequence of JUNV glycoprotein precursor GPC and performed TX-100 extraction followed of Optiprep gradients as explained in material and methods. This construction additionally allows us to analyze GP2 presence by means of anti-myc antibodies. As it can been seen in Fig. 4 Optiprep gradient localization of GPs from infected cells or cells expressing viral GPC alone are similar to depicted in Fig. 3A. Moreover, GP2 localization revealed comparable distribution to GP1 seen in infected cells. Caveolin-1 control protein is also shown to confirm DRM association and to make comparable different experiment conditions.

4. Discussion

In the present work we studied the possible role of cholesterol during late stages of JUNV multiplication. Our data showed: (i) the cholesterol dependence of Junín viral particles production and membrane glycoprotein localization and (ii) that lipid raft microdomains could represent a cellular structure where JUNV GPs target and associate during the course of virus budding. Different cholesterol-lowering agents were used to address the cholesterol dependence of JUNV multiplication. Supernatants after treatment of cells with L/M showed reduced amounts of viral nucleoprotein suggesting the impairment of virus budding or release in cholesterol-depleted conditions. Although the exact composition of JUNV envelope is unknown, when CD activity over virus particles for 1 h at 37 °C was tested the residual infectivity decreased around 90% compared to untreated virions (data not shown). This data suggest the requirement of cholesterol presence in mature JUNV particles.

Schlie et al. [28] showed that Lassa virus glycoprotein does not distribute in cholesterol-rich membrane areas of the cell. Similar conclusions, although with different experimental approaches, were published by Agnihothram et al. [29]. Using Candid#1, the vaccinal strain of JUNV, and electron microscopy analysis they showed that glycoproteins cluster into discrete microdomains different from lipids rafts.

In this work we made use of TX-100 insolubility property to study membrane cell localization of GPs. This biochemical approach remains as an useful tool to explore the presence of a particular protein in DRMs and provides preliminary inference of their affinity for ordered rafts domains. Changes in the DRM association under different conditions, like temperature and cholesterol concentration, also add proofs of a raft environment [30]. Our experiments showed the insolubility of JUNV GPs during cold TX-100 extraction evidenced by flotation of the proteins on top DRM fractions of gradients. This pattern was abolished by cholesterol depletion and warm temperature demonstrating that both conditions destabilized JUNV GPs association to DRM, rendering soluble the glycoproteins (Fig. 3). Harder et al. [31] have demonstrated that cross-linking has an effect on raft microdomains improving their stability and protecting them against TX-100 warm solubilisation. In accordance, our results showed the improvement of the association of JUNV glycoproteins to DRM fractions at 37 °C, in the presence of specific antibodies.

JUNV GP1conforms the peripheral globular head of the spike and it is tightly attached to the lipid membrane by means of GP2 membrane-spanning domain [6]. According to that, the presence of GP1and GP2 in DRM fractions represents the presence of the JUNV spike in the membrane of cells. We showed that removal of cholesterol with CD impaired the localization of GPs in the membrane (Fig. 2g) and also modified DRM distribution (Fig. 3B). Together with the evidence that cholesterol depleted cells failed to produce normal amounts of virus particles we consider that this membrane lipid would play a role in virus budding or release by means of raft microdomain platforms. Finally the specific membrane distribution of the spike proteins was preserved in the absence of any other viral component (Fig. 4).

Glycoproteins usually determine the site of budding within the cell membrane and, when are present, also viral matrix proteins can play an important role on membrane targeting of glycoproteins and ribonucleoprotein complexes. Our results suggest that JUNV GPs localization into these platforms seems to be independent of other viral proteins, although others have reported different [29]. At present is unknown whether JUNV strains differences could account for the observed disparity in results. Functional role of these microdomains in the process of viral assembly and budding still remains to be dissected.

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